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Potent Morphiceptin Analogs: Structure Activity Relationships and Morphine-Like Activities[1]

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ABSTRACT

Morphiceptin (Tyr-Pro-Phe-Pro-NH_2), a nonenkephalin peptide, is an opioid agonist highly selective for *mu* opiate receptors. Chemical modification of Tyr-Pro-Phe-Pro-NH_2 was carried out by substituting structurally related amino acids at residues 2, 3 and 4. The morphiceptin analogs synthesized were then examined for receptor binding activities using ^{125}I-labeled FK 33,824 (Tyr-D-Ala-Gly-NMePhe-Met(O)-ol) as the *mu*-ligand and ^{125}I-labeled D-Ala^2,D-Leu^5-enkephalin as the *delta*-ligand, and for inhibitory activities on electrically evoked smooth muscle contraction of mouse vas deferens and isolated myenteric plexus-longitudinal muscle strips of guinea-pig ileum. All of these analogs showed virtually no activity at *delta* opiate receptor binding sites. Methylation of the nitrogen atom of phenylalanine and the substitution at the C-terminal of L-proline by D-proline produced potent *mu*-agonists, the prototype analog being Tyr-Pro-NMePhe-D-Pro-NH_2 (PL017). The IC_{50} values of morphiceptin and its analogs for *mu* receptor binding were correlated to the ED_{50} values in the guinea-pig ileum assay, suggesting that the ileum effects were mediated by *mu* receptor interactions. A similar correlation between *mu* receptor binding activity and the ED_{50} values in the mouse vas deferens assay suggested that morphiceptin and its analogs also acted on *mu* receptors in this tissue. This idea is supported by the observation that naloxone has a high pA_2 value of 8.71 against PL017 in mouse vas deferens. In *in vivo* studies, PL017 administered centrally into the rostral portion of the 4th ventricle produced long-lasting, naloxone-reversible analgesia in rats. The analgesic activity of PL017 is comparable to that reported for other potent enkephalin analogs. PL017, infused centrally in rats with osmotic minipumps, produced physical dependence. The overall results show that chemical modification of peptides not related to the enkephalins can result in compounds with powerful morphine-like properties.

The isolation and identification of β-casomorphin from the milk protein, β-casein, first showed that peptides with opiate-like activity may be present in the diet (Henschen *et al.*, 1979). Recently, other exogenous opioids from food sources, called exorphins, have been described (Ziroudrou *et al.*, 1979; Schusdziarra *et al.*, 1981; Hazum *et al.*, 1981). The heptapeptide β-casomorphin and its fragments have been examined for opiate-like activity, and it has been shown that the N-terminal tetrapeptide and pentapeptide have substantial *in vivo* morphine-like properties (Wei *et al.*, 1980; Brantl *et al.*, 1981). The amidated tetrapeptide fragment, Tyr-Pro-Phe-Pro-NH_2, is of particular interest because it is a potent and selective ligand for the opiate *mu* receptor in binding studies on brain membranes and in isolated tissue studies (Chang *et al.*, 1981b). Because of this receptor selectivity, this peptide has been named morphiceptin. Morphiceptin produces transient bradycardia by i.v. administration (Wei *et al.*, 1980) and elicits analgesia after i.c.v. injection (Brantl *et al.*, 1981; Chang *et al.*, 1982). Studies with a series of morphiceptin and β-casomorphin-related peptide fragments suggest the correlation of the analgesia with their *mu* receptor binding potency (Chang *et al.*, 1982). The availability of such *mu* receptor specific ligand has also facilitated the classification of opiate receptor subtypes (Chang *et al.*, 1981a).

In this report, we describe the properties of a number of morphiceptin analogs. Morphiceptin analogs were synthesized in order to gain insight into the structural requirements for activity and to determine if analogs more potent than morphiceptin could be obtained. Similar studies have been reported in detail for the enkephalin analogs (see review by Morley, 1980). A second objective was to determine if morphiceptin analogs, like morphiceptin, maintained their selectivities for the *mu* receptor. Several potent analogs were obtained and *in vivo*

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ABBREVIATIONS: DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; Bzl, benzyl; DMF, dimethylformamide; HPLC, high-pressure liquid chromatography; DADLE, [D-Ala^2-D-Leu^5]enkephalin; MVD, mouse vas deferens, Thz, thiazolidine-4-carboxylic acid; Thz(O), thiazolidine-4-carboxylic acid sulfoxide.

activities of one of these analogs, Tyr-Pro-NMePhe-D-Pro-NH_2 (PL017), were described.

Materials and Methods

Solid-phase synthesis of morphiceptin analogs. Morphiceptin analogs were synthesized by solid-phase method (Merrifield, 1963). For synthesis of the C-terminal amide peptides (table 1, compounds 1, 2, 3, 5, 6, 9, 10, 12, 13, 14, 15, 16, 17 and 19), the benzhydrylamine resin (capacity, 0.5 mEq/g of resin) was used as the solid-support. The corresponding C-terminal Boc-amino acid (3 M excess) was attached to the resin using DCC as the coupling reagent and the coupling was monitored by the ninhydrin test. For synthesis of Tyr-Pro-NMePhe-D-Pro, hydroxymethyl resin was used as the solid-support and Boc-D-Pro was coupled to the resin with the DCC and 4,4-dimethylaminopyridine method and the unreacted hydroxyl group was blocked by benzoylation. Further synthesis was performed manually with a Peninsula Peptider. The N-terminal protecting group was removed by 33% TFA in CH_2Cl_2 containing 1% indole. The resin was then neutralized with excess triethylamine (Et_3N) and the free base was coupled with next protected amino acid using DCC as the coupling reagent.

The side-chain phenol group of tyrosine was blocked by 2-bromobenzyloxylcarbonyl group and the hydroxyl group of hydroxylproline was protected by a Bzl group. The final protected peptide resin was washed well with 33% TFA in CH_2Cl_2, CH_2Cl_2 and MeOH, respectively, and was dried *in vacuo* overnight. Cleavage of the peptide from the resin was accomplished with HF (10 ml of HF per g of peptide resin) at 0°C for 1 hr in the presence of distilled anisole (5 ml of anisole per g of peptide resin). The HF was evaporated *in vacuo* and the resin was rinsed well with anhydrous ether. The peptide was extracted by 10% HOAc solution and filtered. The filtrate was lyophilized to give the crude peptide. The peptides were purified by counter-current distribution using *n*-BuOH-HOAc-H_2O (4:1:5) as partition solvents.

Synthesis of Tyr-Pro-NMePhe-D-Pro-NH-NH_2. Boc-Tyr(2,6-DiCl-Bzl)-Pro-NMePhe-D-Pro-resin (4.3 mM) was treated with anhydrous hydrazine (10 ml) in distilled DMF (60 ml) at room temperature for 48 hr. After filtration and evaporation *in vacuo* to dryness, the residue was purified by counter-current distribution using MeOH-H_2O-$CHCl_3$-CCl_4 (37:10:26:27 v/v) as partition solvent to afford pure Boc-Tyr(2,6-DiCl-Bzl)-Pro-NMePhe-D-Pro-NH-NH_2. This protected peptide (270 mg) was cleaved by HF at 0°C for 1 hr in the presence of distilled anisole to give Tyr-Pro-NMePhe-D-Pro-NH-NH_2.

Solution method synthesis of Tyr-Pro-NMePhe-D-Pro-ol. Boc-Tyr(2,6-DiCl-Bzl)-O-N-succinimide was condensed with proline in dry DMF and Et_3N to afford Boc-Tyr(2,6-DiCl-Bzl)-Pro, which was coupled with TFA·NMePhe-D-Pro-ol using DCC/1-hydroxybenzotriazole in DMF and Et_3N to give Boc-Tyr(2,6-DiCl-Bzl)-Pro-NMePhe-D-Pro-ol. Synthesis of TFA·NMePhe-D-Pro-ol was carried out by the coupling of Boc-NMePhe with Pro-ol using DCC/1-hydroxybenzotriazole as coupling reagent and the resulting Boc-NMePhe-D-Pro-ol was deprotected by TFA treatment. The protected tetrapeptide, Boc-Tyr(2,6-DiCl-Bzl)-Pro-NMePhe-D-Pro-ol was cleaved by HF at 0°C for 1 hr in the presence of distilled anisole. The crude peptide was purified by a G-25 partition chromatography column using *n*-BuOH-HOAc-H_2O (4:1:5, upper layer) to afford pure Tyr-Pro-NMePhe-D-Pro-ol.

Synthesis of Tyr-Pro-Phe-Thz(O)-NH_2 and Tyr-Thz(O)-NMePhe-D-Pro-NH_2. Compounds 4 and 18 (table 1) were obtained by treatment of peptides 3 and 14, respectively, with glacial HOAc and H_2O_2.

Characterization of the peptides. Peptides were characterized by thin-layer chromatography, electrophoresis, amino-acid analysis and HPLC. Four thin-layer chromatographic systems, *n*-BuOH-HOAc-H_2O (4:1:5, upper layer), *n*-BuOH-pyridine-HOAc-H_2O (15:10:3:12), *n*-BuOH-HOAc-EtOAc-H_2O (1:1:1:1) with silica gel plate (20 × 20 cm) or *n*-BuOH-HOAc-H_2O (4:1:5, upper layer) with CM cellulose plate (20 × 20 cm) were employed. Fifty-micrograms of sample were loaded on the plate and the spot was visualized by ninhydrin or pauling reagent spray. The results were expressed as relative mobility using morphiceptin as internal standard and are summarized in table 1. Electrophoresis was run with a 100-μg sample in Whatman 3 MM paper in pyridine-acetate buffer (pH 6.4); 2000 V for 90 min. All peptides were migrating toward the cathode and morphiceptin was used as internal standard. The results were expressed as relative mobility to morphiceptin and are shown in table 1. Amino acid analysis was performed by hydrolysis of the peptide with 6 N HCl at 110°C for 24 hr. The hydrolysate was loaded on a Beckman Amino Acid Analyzer model 119CL. The result showed the correct ratio of corresponding amino acids (not shown).

TABLE 1

Physical chemical properties of morphiceptin analogs

Abbreviations: TLC, thin-layer chromatography; Pip, pipecolinic acid; ΔPro, 3,4-dehydroproline; Hyp, 4-hydroxyproline.

Peptide	TLC				Electrophoresis
	R_f[a]	R_f[b]	R_f[c]	R_f[d]	
1. Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	1.00	1.00	1.00	1.00	1.00
2. Tyr-Pro-Phe-D-Pro-NH_2 (PL032)	1.02	0.94	0.98	0.94	1.01
3. Tyr-Pro-Phe-Thz-NH_2	1.09	0.99	1.02	0.98	1.02
4. Tyr-Pro-Phe-Thz(O)-NH_2	0.85	0.89	0.91	0.80	0.98
5. Tyr-Pro-Phe-D-Leu-NH_2	1.26	0.99	1.08	1.10	0.96
6. Tyr-Pro-NMePhe-D-Pro-NH_2 (PL017)	0.85	0.90	0.93	1.00	1.05
7. Tyr-Pro-NMePhe-D-Pro	0.89	0.76	0.91	1.07	0.15
8. Tyr-Pro-NMePhe-D-Pro-ol	1.02	0.94	0.97	1.05	1.06
9. Tyr-Pro-NMePhe-NH_2	1.40	0.98	1.08	1.06	1.09
10. Tyr-Pro-NMePhe-Pro-NH_2	0.85	0.89	0.91	0.88	0.99
11. Tyr-Pro-NMePhe-D-Pro-NH-NH_2	1.64	1.10	1.18	1.20	
12. Tyr-Pro-NMePhe-D-Pro-Gly-NH_2 (PL030)	0.79	0.87	0.88	0.86	0.95
13. Tyr-Pro-NMePhe-Gly-D-Pro-NH_2	0.94	0.93	0.95	0.87	1.02
14. Tyr-Thz-NMePhe-D-Pro-NH_2	1.06	0.99	1.01	0.98	0.97
15. Tyr-Pip-NMePhe-D-Pro-NH_2	0.98	0.95	0.97	0.97	0.93
16. Tyr-ΔPro-NMePhe-D-Pro-NH_2	0.89	0.89	0.94	0.91	1.02
17. Tyr-Hyp-NMePhe-D-Pro-NH_2	0.94	0.91	0.96	0.83	0.99
18. Tyr-Thz(O)-NMePhe-D-Pro-NH_2	0.89	0.95	0.92	0.87	0.97
19. Tyr-Pro-Gly-Phe-Pro-NH_2	0.85	0.90	0.90	0.90	0.93

[a] Silica gel plate (20 × 20 cm), *n*-BuOH-HOAc-H_2O, 4:1:5 upper layer.
[b] Silica gel plate (20 × 20 cm), *n*-BuOH-pyridine-HOAc-H_2O, 15:10:3:12.
[c] Silica gel plate (20 × 20 cm), *n*-BuOH-HOAc-EtOAc-H_2O, 1:1:1:1.
[d] CM cellulose plate (20 × 20 cm), *n*-BuOH-HOAc-H_2O, 4:1:5 upper layer.

HPLC was carried in C_{18}-reverse phase column with a linear gradient of acetonitrile (0–60%) in 50 mM NaH_2PO_4 for 60 min at a flow rate of 1 ml/min. The purity of the peptides is 98% or greater. A representative profile of HPLC was shown in figure 1 for morphiceptin and [D-Pro^4]morphiceptin.

Other materials. [D-Ala^2,D-Leu^5]enkephalin was a gift from Dr. S. Wilkinson (Wellcome Research Laboratories, Beckenham, England) and naloxone HCl was from Endo Laboratories (Garden City, NY). Fk 33,824 and NMe-FK 33,824 were gifts from Dr. D. Romer, Sandoz Pharmaceuticals, (Basel, Switzerland).

Receptor binding studies. Opiate receptor binding assays were performed with rat brain membrane preparations as previously described (Chang *et al.*, 1979). Briefly, the crude brain membrane preparations were prepared by the method of differential centrifugation in isotonic sucrose solution. Whole brain (without cerebellum) from male Sprague-Dawley rats (150–200 g) was homogenized in 0.32 M sucrose with a Polytron PT-20 for 30 sec at a setting of 3.5. The homogenates were centrifuged at 6000 × *g* for 15 min to remove the nuclei and mitochondria. The supernatant obtained from two such centrifugations was combined and centrifuged at 40,000 × *g* for 30 min. The pellets were resuspended in 5 mM Tris-HCl and allowed to swell for 30 min. The synaptosomes thus obtained were disrupted with a polytron homogenizer and centrifuged at 6000 × *g* for 15 min to remove the released mitochondria. The supernatant was centrifuged at 40,000 × *g* for 30 min. The pellets were then resuspended in 50 mM Tris·HCl buffer and incubated with 0.1 M NaCl for 1 hr at 24°C to dissociate the endogenous bound opioids. The final membrane pellets were washed twice and suspended in the same buffer for binding assays.

Binding assays were performed at 24°C for 60 min using a rapid filtration method (Chang and Cuatrecasas, 1979). The protein concentration was about 0.5 to 1 mg/ml. For the competition curves, the concentration of labeled ligands was 0.3 nM for the ^{125}I-labeled *mu*-agonist, FK 33,824 [Tyr-D-Ala-Gly-NMe-Phe-Met(O)ol] and *delta*-agonist, DADLE. The total incubation volume was 0.25 ml. Nonspecific binding was determined in the presence of 1 μM DADLE. The binding reaction was stopped by rapidly filtering through GF/C glass filters and the filters were washed twice with 10 ml of ice-cold Tris·HCl buffer under vacuum. All assays were performed in duplicate and the variability of the duplicates was usually less than 10% of the mean. ^{125}I-labeled DADLE and FK 33,824 were prepared as described previously (Chang *et al.*, 1979).

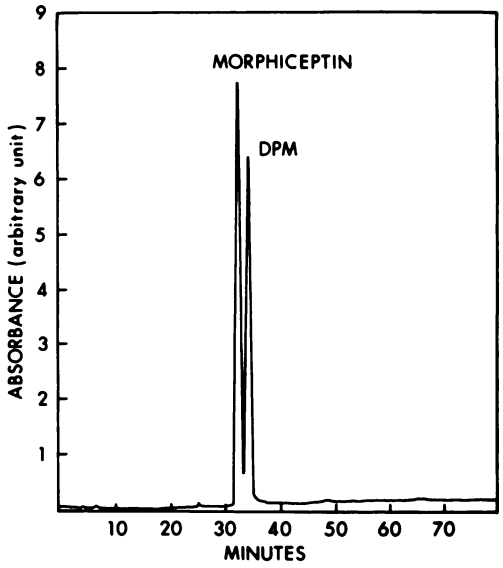

Fig. 1. HPLC profile of morphiceptin and [D-Pro^4]morphiceptin (DPM). Morphiceptin (20 μg) and DPM were applied to a C_{18}-μBondapack reverse phase column and eluted with a linear gradient of acetonitrile (0–60%) in 50 mM NaH_2PO_4 buffer for 60 min at a flow rate of 1 ml/min.

Isolated Tissues

MVD. Mice (Hartley-Sprague-Dawley, Indianapolis, IN), weighing between 25 and 30 g, were sacrificed by a blow to the head. Vasa deferens were removed and suspended in organ bath chambers containing a modified Krebs' buffer of the following composition (millimolar): NaCl 117.5; KCl, 4.75; $CaCl_2$, 2.6; KH_2PO_4, 1.20; $NaHCO_3$, 24.5; and glucose, 11. The buffer was saturated with 95% O_2-5% CO_2 and kept at 37°C. The vasa deferens were suspended between platinum electrodes and attached to a force transducer with 500 mg of tension. Tissues were stimulated every 10 sec with 400 msec pulse trains of 10 Hz, 1.0 msec duration at supramaximal voltage. ED_{50} values (the concentration which suppresses the contraction by 50%) were determined by regression analysis of concentration-response curves.

Guinea-pig ilea. Female guinea pigs (Hartley strain, Murphy Laboratories, Plainfield, IN) were sacrificed by CO_2 asphyxiation. Myenteric plexus-longitudinal muscle strips were prepared as described by Killian *et al.* (1981). Strips were suspended with 1 g of tension in Krebs' buffer similar to that described for the MVD with the exception of the presence of $MgSO_4$ (1.20 mM). Tissues were stimulated with 0.1 Hz pulses of 0.5 msec duration at supramaximal voltage. ED_{50} values were determined as described above.

For both preparations results were normalized to correct for variability. The ED_{50} value for a given experiment was multiplied by the ratio of [mean morphine ED_{50} for all assays/morphine ED_{50} of that experiment] (Kosterlitz *et al.*, 1980).

In Vivo Studies

All *in vivo* experiments were conducted on male Sprague-Dawley rats weighing 250 to 375 g. For analgesic assays, 5 μl of peptide solutions, dissolved in distilled water, were injected into the rostral portion of the 4th ventricle using methods described previously (Chang *et al.*, 1982). Analgesic activity was assessed by the method of Janssen *et al.* (1963) which was based on the inhibition of the tail-withdrawal response of rats to warm water (54–56°C) applied to the caudal 5-cm segment of the tail. The tail-withdrawal latencies were measured with a stopwatch at 8, 16 and 24 min after injection. Preinjection latencies (mean ± S.D.) were 1.8 ± 0.47 sec and, after injection of distilled water, were 3.6 ± 0.85, 3.5 ± 0.91 and 2.9 ± 0.9 sec at 8, 16 and 24 min, respectively (n = 15). None of the control latencies exceeded 5 sec. As suggested by Janssen *et al.* (1963), a latency of ≥ 6 sec was used as a quantal index of response with a cutoff time of 15 sec.

The median effective dose (ED_{50}) for each peptide was estimated according to the method of Dixon (1980) with three series of trials and N ≥ 4 animals per trial. The reported ED_{50} is thus the mean of three values. This statistical approach for estimation of the ED_{50} may be somewhat less precise than procedures utilizing a larger number of animals but was selected because of practical considerations.

The ability of PL017 to induce physical dependence was measured by infusion of this peptide into the junction of the aqueduct and 4th ventricle spaces with osmotic minipumps, using procedures described previously (Wei, 1981). PL017 was infused at 1.23 μl/hr for 70 to 72 hr and then the animals were weighed, placed in 1-gallon glass jars and injected with naloxone hydrochloride (4 mg/kg i.p.). The number of escape attempts from the jar was counted for 15 min and utilized as an index of withdrawal intensity.

Results

Structure-activity studies. The structure of morphiceptin was modified by substitutions on the fourth, third and second amino acid residues. The analogs were then tested in *mu* and *delta* receptor binding assays and in the guinea-pig ileum and MVD assays (table 2).

Modification of the fourth amino acid, L-*proline.* Morphiceptin and all related analogs showed virtually no competitive activity against DADLE for the *delta* receptor site. Replace-

TABLE 2

Structure-activities relationships of morphiceptin analogs

The results are expressed as mean ± S.E.M. of number of experiments (number in parentheses), NT, not determined. The values without S.E.M. are the mean of two determinations.

Peptide	*Mu* Binding Site[a] (IC_{50})	G. P. Ileum[b] (ED_{50})	*Delta* Binding Site[c] (IC_{50})	MVD[b] (ED_{50})
	nM ± S.E.M.	*nM* ± S.E.M.	*nM*	*nM* ± S.E.M.
1. Tyr-Pro-Phe-Pro-NH_2 (Morphiceptin)	63 ± 23 (3)	318 ± 71 (7)	30,000	4,800 ± 154 (3)
2. Tyr-Pro-Phe-D-Pro-NH_2 (PL032)	4.3 ± 2.2 (4)	81 ± 6(7)	20,000	195 ± 14 (4)
3. Tyr-Pro-Phe-Thz-NH_2	12 ± 3.8 (3)	206 ± 19 (7)	10,000	929 ± 187 (5)
4. Tyr-Pro-Phe-Thz(O)-NH_2	40	NT	NT	NT
5. Tyr-Pro-Phe-D-Leu-NH_2	43 ± 17 (3)	2,790 ± 430 (5)	15,000	10,000 ± 1050 (5)
6. Tyr-Pro-NMePhe-D-Pro-NH_2 (PL017)	5.5 ± 0.5 (2)	34 ± 7(3)	10,000	240 ± 48 (10)
7. Tyr-Pro-NMePhe-D-Pro	186 ± 7(3)	255 ± 26 (9)	20,000	2,700 ± 470 (7)
8. Tyr-Pro-NMePhe-D-Pro-ol	5	NT	20,000	NT
9. Tyr-Pro-NMePhe-NH_2	1,500	>10,000	80,000	>10,000
10. Tyr-Pro-NMePhe-Pro-NH_2	37 ± 3(3)	225 ± 21 (6)	10,000	2,300 ± 300 (5)
11. Tyr-Pro-NMePhe-D-Pro-$NHNH_2$	2,000	NT	20,000	NT
12. Tyr-Pro-NMePhe-D-Pro-Gly-NH_2 (PL030)	8.7 ± 1.2 (3)	31 ± 4(7)	20,000	303 ± 49 (5)
13. Tyr-Pro-NMe-Phe-Gly-D-Pro-NH_2	400	4,170 ± 370 (3)	40,000	13,730 ± 1530 (3)
14. Tyr-Thz-NMePhe-D-Pro-NH_2	25 ± 9(4)	61 ± 8(5)	8,000	480 ± 79 (4)
15. Tyr-Pip-NMePhe-D-Pro-NH_2	16 ± 5(3)	NT	30,000	NT
16. Tyr-ΔPro-NMePhe-D-Pro-NH_2	21 ± 2(3)	NT	30,000	NT
17. Tyr-Hyp-NMePhe-D-Pro-NH_2	800	NT	40,000	NT
18. Tyr-Thz(O)-NMePhe-D-Pro-NH_2	80	NT	>10,000	NT
19. Tyr-Pro-Gly-NMePhe-D-Pro-NH_2	10,000	NT	>10,000	NT
Morphine	0.4 ± 0.2 (3)	134 ± 13 (23)	35 ± 5(3)	1300 ± 290 (18)
DADLE	4 ± 0.5 (3)	26 ± 6(5)	1.6 ± 0.2 (3)	0.62 ± 0.8 (13)

[a] IC_{50} values were determined by the concentration which decreases the binding of ^{125}I-FK 33,824 (0.3 nM) by 50%.
[b] Medium effective dose (ED_{50}) in inhibiting the electrically evoked smooth muscle contraction.
[c] IC_{50} values were determined by the concentration which decreased the binding of ^{125}I-DADLE by 50%. All peptides had IC_{50} values greater than 10 μM.

ment of L-proline with various cyclic analogs, such as Thz, Thz(O) and D-proline as well as D-Leu yielded peptides with greater activity in both the *mu* receptor binding assay and in the guinea-pig ileum and MVD assays. The D-proline substitution was the most effective (Compound 2, PL032). This analog, relative to morphiceptin, is about 15 times more active in *mu* receptor binding, 4 times more active in the ileum and 20 times more active in the vas deferens (table 2).

Modification of the third amino acid, L-phenylalanine. The addition of a methyl group to the nitrogen of phenylalanine in [D-Pro[4]]-morphiceptin (PL032) produced an analog (Compound 6, PL017) with comparable *mu* receptor binding activity and slightly greater activity in the ileum assay. At present, among the morphiceptin analogs that have been synthesized, PL017 is the most active analog in the *in vitro* assays. The PL017 structure was, therefore, used as the parent model for subsequent changes.

Removal of the amide group on PL017 attenuates activity but activity is retained when the amide is changed to the carbinol (alcohol group on the carboxy terminal). Removal of the fourth amino acid or replacement of the amide with a hydrazine group markedly reduces activity. Activity is retained when glycineamide is added as a fifth residue on the carboxy terminus (Compound 12, PL030) but not if glycine is inserted between the third and fourth residue.

Modification of the second amino acid residue, L-proline. We have previously reported that replacement of L-proline with D-amino acids in position 2 produces analogs with little bioactivity (Chang *et al.*, 1981b). In PL017, L-proline in the second position can be replaced by Thz, pipecolinic acid, 3,4-dehydroproline or Thz(O) and activity is retained. Substitution of the second amino acid with 4-hydroxyproline or the insertion of a glycine between 2-L-proline and 3-N-Methylphenylalanine, however, substantially decreases activities.

Comparison of the relative activities of morphiceptin analogs in bioassays. Morphiceptin and most of its analogs inhibit the electrically stimulated smooth muscle contractions of the guinea-pig ileum. The inhibitory potency in the ileum is correlated to the *mu* receptor binding affinity ($r = 0.76$) (fig. 2). The correlation is stronger ($r = 0.84$) if Compound 5, with D-leu-NH_2 as the fourth residue, is not included in the regression analysis (fig. 2, dash line). These results provide further evidence that *mu* receptor binding sites in brain membranes are similar to sites that mediate the inhibitory effects of opiates on the ileum.

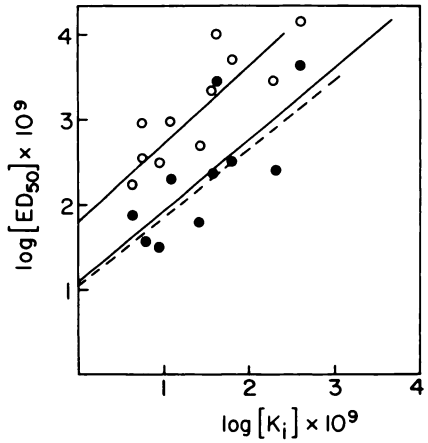

Fig. 2. Activity correlation between *mu* opiate receptor binding and isolated tissue studies. The log ED_{50} values of morphiceptin analogs in myenteric-longitudinal muscle strip of guinea-pig ileum (●) or MVD (○) were plotted against their log IC_{50} values in *mu* receptor binding assays. The regression analysis lines were calculated and yielded line of $y = 0.85X + 1.08$ ($r = 0.76$, $P < .01$) for guinea-pig ileum and $y = 0.91X + 1.80$ ($r = 0.87$, $P < .01$) for MVD. Some peptides which deviate from the regression lines may have different metabolic stabilities in tissues.

Although morphiceptin and its analogs show little competitive binding with the *delta*-ligand, DADLE, they have, surprisingly, quite potent activity in suppressing the contractions of the MVD, a putative *delta* receptor system. The inhibitory potencies of morphiceptin and its analogs are highly correlated to the *mu* receptor binding affinities ($r = 0.87$). The possibility that *mu* receptors may mediate morphiceptin effects in the vas deferens was further explored by measuring the antagonist potency of naloxone. Naloxone competitively antagonizes the inhibitory effects of PL017 in the vas deferens. Naloxone produces a parallel shift in the dose-response curve of PL017 and morphine sulfate. Naloxone is less effective against DADLE, a typical delta-agonist (fig. 3). Schild plots (Arunlakshana and Schild, 1959) yield pA_2 values (mean ± S.D.) of naloxone of 8.71 ± 0.06, 8.59 ± 0.05 and 7.56 ± 0.07 for PL017, morphine and DADLE, respectively (fig. 3).

***In vivo* effects of morphiceptin analogs.** The analgesic activities of morphiceptin, two of its analogs (PL017 and PL030), three enkephalin analogs and morphine sulfate are shown in table 3. All peptides produced analgesia and catalepsy which were reversible by injections of naloxone hydrochloride (1–4 mg/kg i.p.). It is apparent that the morphiceptin analogs, PL017 and PL030, can have activity at subnanomolar doses. The time course for the analgesic actions of PL017 is shown in figure 4. At lower doses, the time of maximum effect by this route of administration was approximately 15 to 30 min after injection. With larger doses, for example, 0.9 nmol produced analgesia lasting as long as 4 hr. Like other opioid peptide agonists, sustained administration of PL017 induced a dependent state which was revealed by naloxone-precipitated withdrawal (fig. 5). The dose of PL017 required to produce an average of 20 escape attempts was approximately 15 nmol/animal delivered over a 70- to 72-hr period. Under the same experimental conditions, the doses of FK 33,824 and morphine sulfate required to produce a comparable degree of dependence are 0.33 and 49 nmol/animal, respectively (Wei, 1981).

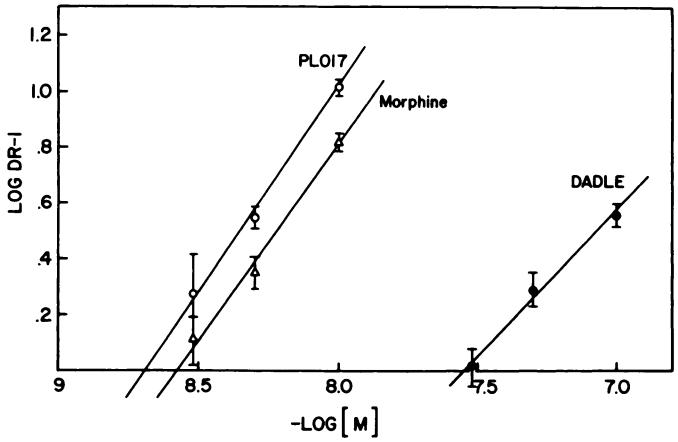

Fig. 3. Schild plots of naloxone antagonizing PL017 (Tyr-Pro-NMePhe-D-Pro-NH_2) (○), morphine sulfate (△) and DADLE (●) in MVD assay. Three doses of naloxone were employed for each agonist. The results are mean ± S.E.M. of four to six separate experiments. pA_2 values of 8.71 ± 0.06, 8.59 ± 0.05 and 7.56 ± 0.07 were obtained for PL017, morphine and DADLE, respectively.

TABLE 3

Analgesic activities of morphiceptin and enkephalin analogs

Chemical	ED_{50}
	nmol/rat ± S.E.M.
Tyr-D-Ala-Gly-NMe·Phe-Met(O)-ol (FK 33,824)	0.04 ± 0.009
NMe·Tyr-D-Ala-Gly-NMe·Phe-Met(O)-ol	0.02 ± 0.007
Try-Pro-NMe·Phe-D-Pro-NH_2 (PL017)	0.23 ± 0.009
Tyr-Pro-NMe·Phe-D-Pro-Gly-NH_2 (PL030)	0.45 ± 0.19
Tyr-D-Ala-Gly-Phe-D-Leu (BW 180C)	1.11 ± 0.21
Tyr-Pro-Phe-Pro-NH_2 (Morphiceptin)	5.5 ± 2.5
Morphine sulfate	8.4 ± 1.4

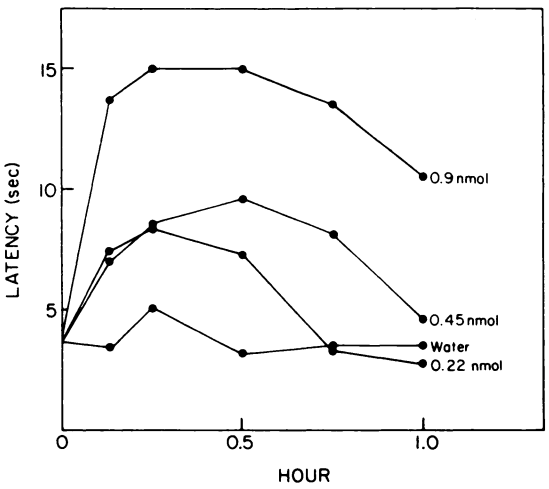

Fig. 4. Time course of the analgesic action of PL017 (Tyr-Pro-NMePhe-D-Pro-NH_2) administered i.c.v. in nanomoles per rat. Water or PL017 was applied as indicated. $N = 4$ to 6 per group.

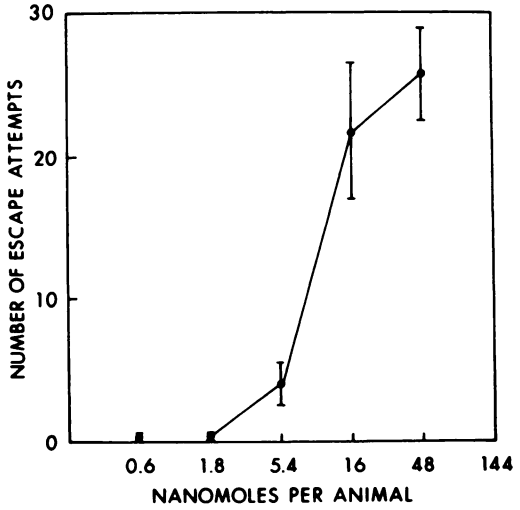

Fig. 5. Physical dependence on PL017 (Tyr-Pro-NMePhe-D-Pro-NH_2). The escape behavior of the rats was determined by challenging with naloxone (4 mg/kg i.p.) after 70 to 72 hr of osmotic minipump drug infusion into the i.c.v. space. $N = 4$ to 10 per group. The results are expressed as average number of escape attempts ± S.E.M.

Discussion

These results confirm earlier observations that morphiceptin has opiate-like properties in binding assays, in isolated tissue preparations and in *in vivo* pharmacology. The structure of morphiceptin is such that activity is retained or enhanced when the second amino acid is an unsubstituted cyclic amino acid of the L-configuration, when a methyl group is added to the nitrogen atom of the third amino acid, phenylalanine, or when a L- or D-configuration of cyclic amino acids or hydrophobic amino acids are substituted for the fourth amino acid, L-proline.

Three morphiceptin analogs with enhanced activities in binding and in the ileum were obtained: Tyr-Pro-Phe-D-Pro-NH_2 (PL032), Tyr-Pro-NMePhe-D-Pro-NH_2 (PL017) and Tyr-Pro-NMePhe-D-Pro-Gly-NH_2 (PL030). These analogs may be useful for further studies on the pharmacology of opioid peptides.

Morphiceptin analogs, like morphiceptin, retain a high selectivity for binding to the *mu* receptor subtype. This conclusion is inferred from the strong correlation between binding affinity and activity in the ileum, and the virtual absence of affinity for the *delta* receptor site. Surprisingly, morphiceptin analogs have significant activity in the MVD, a typical *delta* receptor system (Lord *et al.*, 1977), although the morphiceptin analogs are 5- to 10-fold less active in the vas deferens than in the ileum. The inhibitory activities in the vas deferens are correlated to the *mu* receptor binding potencies, suggesting the presence of *mu* receptors in the vas deferens. This idea is supported by the pA_2 values of naloxone against PL017, morphine and DADLE in the vas deferens. The naloxone pA_2 value of 8.71 against PL017 in the vas deferens is typical for naloxone against such *mu* agonists as normorphine and sulfentanyl in *mu* receptor subtype systems. Other investigators (Schulz *et al.*, 1980) have also demonstrated the presence of *mu* receptors in the MVD.

The lower potency of morphiceptin analogs in MVD *vs.* that in guinea-pig ileum may indicate a lower intrinsic activity of *mu* receptors in MVD or an existence of subtypes of *mu* receptors, such as *mu*-1 and *mu*-2 receptor as suggested by Pasternak *et al.* (1980). While *mu*-1 receptor may correspond to the *mu* opiate receptor in guinea-pig ileum, *mu*-2 receptor may correspond to the *mu* receptor in MVD.

PL017, the prototype morphiceptin analog, produces analgesia and physical dependence as would be predicted from its *in vitro* properties. The analgesic activity of PL017 is greater than that of morphine and morphiceptin but approximately 5 to 10 times less than that of the most potent enkephalin analogs that have been synthesized (Morley, 1980, Romer *et al.*, 1977) and are shown to have a high affinity for *mu* receptor sites (*i.e.*, FK 33,824) (Chang *et al.*, 1979; Kosterlitz *et al.*, 1980). The realization that exorphins can have powerful pharmacologic activity may be of interest in light of a recent report that dietary sources of exorphins may produce effects *in vivo* (Schusdziarra, 1981).

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References

ARUNLAKSHANA, O. AND SCHILD, H. O.: Some quantitative uses of drug antagonists. Br. J. Pharmacol. **14:** 48–59, 1959.

BRANTL, V., TESCHEMACHER, H., BLÄSIG, J., HENSCHEN, A. AND LOTTSPEICH, F.: Opioid activities of β-casomorphins. Life Sci. **28:** 1903–1909, 1981.

CHANG, K.-J., COOPER, B. R., HAZUM, E. AND CUATRECASAS, P.: Multiple opiate receptors: Different regional distribution in the brain and differential binding of opiates and opioid peptides. Mol. Pharmacol. **16:** 91–104, 1979.

CHANG, K.-J. AND CUATRECASAS, P.: Multiple opiate receptors: Enkephalins and morphine bind to receptors of different specificity. J. Biol. Chem. **254:** 2610–2618, 1979.

CHANG, K.-J., CUATRECASAS, P., WEI, E. T. AND CHANG, J.-K.: Analgesic activity of intracerebroventricular administration of morphiceptin and β-casomorphins: Correlation with the morphine (μ) receptor binding affinity. Life Sci. **30:** 1547–1551, 1982.

CHANG, K.-J., HAZUM, E. AND CUATRECASAS, P.: Novel opiate binding sites selective for benzomorphan drugs. Proc. Natl. Acad. Sci. U.S.A. **78:** 4141–4145, 1981a.

CHANG, K.-J., KILLIAN, A., HAZUM, E., CUATRECASAS, P. AND CHANG, J.-K.: Morphiceptin (Tyr-Pro-Phe-Pro-NH_2): A potent and specific agonist for morphine (μ) receptors. Science (Wash. DC) **212:** 75–77, 1981b.

DIXON, W. J.: Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. **20:** 441–462, 1980.

HAZUM, E., SABATKA, J. J., CHANG, K.-J., BRENT, D. A., FINDLAY, J. W. AND CUATRECASAS P.: Morphine in cow and human milk: Could dietary morphine constitute a ligand for specific morphine (μ) receptors? Science (Wash. DC) **213:** 1010–1012, 1981.

HENSCHEN, A., LOTTSPEICH, F., BRANTL, V. AND TESCHEMACHER, H.: Novel opioid peptides derived from casein (β-casomorphins). II. Structure of active components from bovine casein peptone. Hoppe-Seyler's Z. Physiol. Chem. **360:** 1217–1224, 1979.

JANSSEN, P. A. J., NIEMEGEERS, C. J. E. AND DONY, J. G. H.: The inhibitory effect of fentanyl and other morphine-like analgesics on the warm water induced tail withdrawal reflex in rats. Arzneimittel-Forsch. **13:** 502–507, 1963.

KILLIAN, A., SCHUSTER, C. R. AND WEINER, B. H.: Effect of antimorphine antibodies on an *in vitro* correlate of antagonist-precipitated opiate abstinence. J. Pharmacol. Exp. Ther. **217:** 820–827, 1981.

KOSTERLITZ, H. W., LORD, J. A. H., PATERSON, S. J. AND WATERFIELD, A. A.: Effects of changes in the structure of enkephalins and of narcotic analgesic drugs on their interactions with μ- and δ-receptors. Br. J. Pharmacol. **68:** 333–342, 1980.

LORD, J. A. H., WATERFIELD, A. A., HUGHES, J. AND KOSTERLITZ, H. W.: Endogenous opioid peptides: Multiple agonists and receptors. Nature (Lond.) **267:** 495–499, 1977.

MERRIFIELD, R. B.: Solid phase peptide synthesis. J. Am. Chem. Soc. **85:** 2149–2154, 1963.

MORLEY, J. S.: Structure-activity relationships of enkephalin-like peptides. Annu. Rev. Pharmacol. Toxicol. **20:** 81–110, 1980.

PASTERNAK, G. W., CHILDERS, S.R. AND SNYDER, S. H.: Opiate analgesia: Evidence for mediation by a subpopulation of opiate receptors. Science (Wash. DC) **208:** 514–516, 1980.

ROMER, D., BUESCHER, H. H., HILL, R. C., PLESS, J., BAUER, W., CARDINAUX, F., CLOSSE, A., HAUSER, D. AND HUGUNIN, R. A.: Synthetic enkephalin analogue with prolonged parental and oral analgesic activity. Science (Wash. DC) **268:** 547–549, 1977.

SCHULZ, R., WÜSTER, M., KRENSS, H. AND HERZ, A.: Selective development of tolerance without dependence in multiple opiate receptors of mouse vas deferens. Nature (Lond.) **285:** 242–243, 1980.

SCHUSDZIARRA, V., HENRICHS, I., HOLLAND, A., KLIER, M. AND PFEIFFER, E. F.: Evidence for an effect of exorphins on plasma insulin and glucagon level in dogs. Diabetes **30:** 362–364, 1981.

WEI, E. T.: Enkephalin analogs and physical dependence. J. Pharmacol. Exp. Ther. **216:** 12–18, 1981.

WEI, E. T., LEE, A. AND CHANG, J.-K.: Cardiovascular effects of peptides related to the enkephalins and β-casomorphin. Life Sci. **26:** 1517–1522, 1980.

ZIOUDROU, C., STREATY, R. A. AND KLEE, W. A.: Opioid peptides derived from food protein: These studies describe peptides with opioid activity (exorphins) in pepsin hydrolyzates of wheat gluten and α casein. J.Biol. Chem. **254:** 2446–2449, 1979.

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