Cyclic Enkephalin Analogues with Exceptional Potency and Selectivity for δ-Opioid Receptors[1]

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Superpotent and highly δ-opioid receptor selective cyclic peptides of the general formula H-Tyr-c[D-Pen-Gly-Phe(*p*-X)-Pen]-Phe-OH (where X = hydrogen or halogen) have been synthesized. In the binding assays the most selective and most potent compound is the *p*-bromophenylalanine-4 analogue (IC_{50} value = 0.19 nM, selectivity ratio = 21 000 for δ vs μ). In the GPI and MVD bioassays the most selective and most potent analogue is the *p*-fluoro-substituted analogue Tyr-[D-Pen-Gly-Phe(*p*-F)-Pen]-Phe-OH. In the MVD assay it has an exceptionally low IC_{50} value of 0.016 nM and a δ vs μ selectivity ratio of 45 000.

Introduction

Enkephalins are endogenous peptides with opioid activity.[2] They modulate many physiological functions such as spinal and supraspinal analgesia (antinociception), gastrointestinal motility, hypothermia, respiratory function, constipation, and many others.[3] Enkephalins interact with opioid receptors localized on the cell surface. Nowadays, it is generally accepted that there are at least three types of opioid receptors denoted as mu (μ), delta (δ), and kappa (κ), and much has been done to develop peptide ligands specific for these receptors.[4−8]

Recently, examples of all three of these receptors have been cloned.[9−17] All three receptors share the same feature—they are large proteins with seven transmembrane domains, belonging to the G-protein-coupled receptor family. Thus they reveal considerable sequence homology.[16] Based on pharmacological and physiological studies, there are many suggestions that there are subclasses of these receptors, i.e., δ_1, δ_2, μ_1, μ_2, and at least three κ subtypes,[17−27] though apparently no subtypes have been cloned to date.

The physiological roles of this receptor diversity are not yet fully understood and are a matter of intense investigation. The μ receptors prefer morphine-like drugs and are blocked by naloxone. It has been suggested that morphine-induced analgesia might be mediated by μ_1 receptors, whereas the excitation of the μ_2 receptors might be involved in respiratory depression, a serious side effect in the clinical use of morphine.[28,29] The κ receptors are recognized by ketocyclazocine and related drugs and presumably have their endogenous peptide counterparts in Dynorphin,[30] but these potent endogenous peptides are not highly selective. The δ-opioid receptors have a higher affinity for enkephalin and are relatively weakly blocked by naloxone.

In all cases, the stimulation of opioid receptors produces analgesia (antinociception), generally by the reduction of central autonomic and endocrine responses to a pain stimulus. However, the use of μ opiates has several disadvantages such as constipation, respiratory depression, dysphoria, increasing tolerance, and addiction. Since the question of physiological roles of this receptor diversity is still to be answered, the search for highly potent and selective opioid ligands has continued to help address this issue. Highly selective and very potent opioid ligands, both agonists and antagonists, should enable the precise description of how these particular receptors act in the human body and how they interact. It has been suggested recently that the binding sites of δ-opioid agonist and antagonist are different.[31,32] The possibility of "switching on−off" of particular receptor type or subtype should lead to a better understanding of the interactions that lead to desired pharmacological effects as well as to undesirable side effects. This approach will lead to clinically useful drugs for pain relief in long-term therapy and/or for the replacement of μ opiates (such as morphine) in high-dose treatment at terminal stages of cancer.

During the past 15 years considerable progress has been made in the development of potent and selective peptide ligands for each type of opioid receptor.[4−6] One of the most δ selective ligands is cyclic[D-Pen[2],D-Pen[5]]-enkephalin (DPDPE) synthesized in our laboratory.[34] The combination of cyclization via a disulfide bridge and the stereoelectronic properties of Pen residues has enabled these peptides to adapt a biologically active conformation that greatly prefers δ-opioid receptors rather than μ or κ receptors. In binding studies DPDPE showed favorable binding properties toward δ receptor (selectivity ratio was over 200), and in bioassays based on electrically induced smooth muscle contraction of mouse vas deferens (MVD assay) and guinea pig ileum longitudinal muscle-myenteric plexus tissue (GPI assay), DPDPE was found to be over 2000 times more potent at δ receptors than at μ receptors.[33,34] Introduction of *p*-halogen-substituted phenylalanine in place of the Phe[4] residue led to further improvement in the biological potencies and selectivities of DPDPE,[35] and the *p*-chloro-substituted analogue had better antinociceptive properties than DPDPE in the hot-plate test after icv administration.[36]

In a previous report we have shown that cyclic hexapeptides with general formula H-Tyr-c[D-Pen-Gly-Phe(*p*-X)-Cys]-Phe-OH (where X = hydrogen or halogen) are very potent ligands especially for peripheral δ-opioid receptors.[37] The rationale for the Phe[6] residue was that it could increase lipophilicity for crossing the blood−

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Table 1. Binding Affinities and Selectivities of Peptides Tested in Competition Experiments vs [^{3}H]CTOP (μ Receptors) and vs [^{3}H][*p*-Cl-Phe^4]DPDPE (δ Receptors) in Rat Brain (IC_{50} Values Given with SSE)

peptide	IC_{50} (nM) δ	IC_{50} (nM) μ	ratio[a]
1, H-Tyr-c[D-Pen-Gly-Phe-Pen]-Phe-OH, [Phe^6]DPLPE	4.0 ± 0.9	11400 ± 2600	2800
2, H-Tyr-c[D-Pen-Gly-Phe(*p*-F)-Pen]-Phe-OH	0.43 ± 0.08	1650 ± 210	3800
3, H-Tyr-c[D-Pen-Gly-Phe(*p*-Cl)-Pen]-Phe-OH	0.40 ± 0.08	1700 ± 210	4200
4, H-Tyr-c[D-Pen-Gly-Phe(*p*-Br)-Pen]-Phe-OH	0.20 ± 0.08	4200 ± 60	21000
5, H-Tyr-c[D-Pen-Gly-Phe(*p*-I)-Pen]-Phe-OH	0.47 ± 0.07	7300 ± 2200	15500
6, H-Tyr-c[D-Pen-Gly-Phe-Cys]-Phe-OH, HBP2	1.4 ± 0.2	280 ± 75	200
H-Tyr-c[D-Pen-Gly-Phe-Pen]-OH, DPLPE[b]	10.0	3700	370

[a] $IC_{50}(\mu)/IC_{50}(\delta)$. [b] Data taken from ref 34.

brain barrier. The most potent compound in that series was [Phe^6]DPLCE (H-Tyr-c[D-Pen-Gly-Phe-Cys]-Phe-OH, HBP2). In the MVD assay it was found to have exceptional potency within an IC_{50} value of 0.016 nM.

We report here on the compounds with the general formula H-Tyr-c[D-Pen-Gly-Phe(*p*-X)-Pen]-Phe-OH (where X = hydrogen or halogen) and their biological properties. These compounds appear to be the most potent and selective δ-opioid enkephalins reported to date.

Results and Discussion

The peptides were synthesized by the solid-phase method of peptide synthesis following methods previously used for this class of enkephalin analogues. The protected peptides were cleaved from the resin, and the side chain protecting groups were removed by published methods.[38] The linear peptides (with free −SH groups) were oxidized to cyclic peptides by $K_3[Fe(CN)_6]$ in 0.05 M ammonium acetate buffer at pH 8.5 as described previously[37,39] using a recently developed method[39] to obtain high yields of the monomer. The structures of the new analogues are given in Table 1.

The results of radioreceptor binding assays using rat brain membrane homogenate preparations are summarized in Table 1 using [^{3}H][*p*-Cl-Phe^4]DPDPE, a highly δ selective ligand,[40] as the δ receptor ligand. Competition with [^{3}H]CTOP, a highly μ selective ligand,[41] was used to evaluate μ-opioid receptor binding. All five of the new analogues (**1**−**5**), DPLPE (H-Tyr-c[D-Pen-Gly-Phe-Pen]-OH), a rigid enkephalin analogue which previously was shown to display good binding and selectivity properties,[33,36] and [Phe^6]DPLPE, a hexapeptide which showed improved δ-opioid receptor binding,[11] are shown in Table 1. Compared to [Phe^6]DPLCE (**6**; Table 1), the L-Pen^5 analogue [Phe^6]DPLPE (**1**; Table 1) was found to have similar binding potency at δ receptors (IC_{50} values are 1.4 and 4.0 nM, respectively), but it was about a 40 times less potent binder at μ receptors (IC_{50} values are 280 and 11 400 nM, respectively). As a result, the hexapeptide analogue [Phe^6]DPLPE (**1**) is a very selective ligand with a selectivity ratio of about 2800 for the δ- vs μ-opioid receptor. In comparison with the parent cyclic enkephalin analogue H-Tyr-c[D-Pen-Gly-Phe-Pen]-OH (DPLPE; Table 1), analogue **1** is both more potent (IC_{50} = 4.0 vs 10.0 nM) and more selective (2800 vs 370). The large decrease of affinity toward μ receptors for **1** clearly indicates the necessity of having additional methyl groups in the amino acid at position 5 of the peptide chain in order to get analogues of higher selectivity for δ vs μ receptors. These methyl groups apparently possess steric properties that do not allow the Pen^5-containing analogue to adapt a conformation that is very favorable for interactions with central μ receptors. Thus, one may conclude that the steric effects caused by the introduction of two methyl groups in position 5 play much more important roles in the improvement of the δ receptor selectivity (by diminishing μ affinity) than the introduction of an additional Phe moiety at the C-terminus does to improve δ receptor potency. Introduction of halogens at the para position in Phe^4 gave dramatic improvements in binding potency and selectivity for δ-opioid receptors in the rat brain. All the halogen-containing peptides (**2**−**5**) are more selective in binding studies than the unsubstituted analogue **1**, and even more so than DPLPE. The introduction of the halogens has increased the binding to δ receptors about 10-fold (in the case of the bromo analogue **4** about 20-fold). In the binding assay there is no simple relationship between the IC_{50} values and the lipophilicity factor of the substituent or its molecular weight.

Among previously reported L-Cys^5 compounds with the formula Tyr-c[D-Pen-Gly-Phe(*p*-X)-Cys]-Phe-OH,[37] where X was hydrogen or halogen, the lowest IC_{50} value vs tritiated CTOP (μ receptor) was observed for the chloro-substituted analogue (X = Cl). As for binding at μ receptors for the L-Pen^5 analogues, the *p*-halogen-substituted Phe^4 analogues **2**−**5** (Table 1) generally showed increased binding affinity (about 1.6−7-fold) relative to the unsubstituted [Phe^6]DPLPE (**1**). Although those compounds (**2**−**5**) have slightly higher affinity for central μ-opioid receptors than **1**, they have even higher affinity for central δ receptors, and thus the substitution by halogen gave analogues that are much more δ-opioid receptor selective. The binding selectivity, as measured by the selectivity ratio, was the best in the case of the bromo-substituted analogue **4,** with a ratio of about 21 000. Among enkephalin analogues including cyclic enkephalin analogues, this selectivity ratio is among the best of which we are aware and essentially renders compounds **4** and **5** to be specific for δ-opioid receptors under most experimental conditions.

The new compounds display very interesting biological properties as well as in vitro bioassays (see Table 2). In the MVD preparations, a tissue enriched in peripheral δ-opioid receptors, all the peptides expressed high potency. DPLPE was previously shown[33] to be a potent and selective δ-opioid ligand. Its IC_{50} values in the MVD and GPI (a tissue containing predominantly μ receptors) were 2.5 and 2720 nM, respectively, giving a selectivity ratio of 1100[33] (Table 2). The addition of an additional Phe at the C-terminus to give [Phe^6]-DPLPE (**1**) changed the IC_{50} value in the MVD assay from 2.5 to 1.89 nM and led to a moderately increased GPI value from 2700 to about 5800. The selectivity ratio

Table 2. Inhibitory Potencies and Selectivities of Tyr-c[D-Pen-Gly-Phe(*p*-X)-Pen]-Phe Analogues in Bioassay Tests (MVD for δ Receptors and GPI for μ Receptors, IC_{50} Values with SSE)

peptide	IC_{50} (nM) MVD (δ)	GPI (μ)	ratio[a]
1, H-Tyr-c[D-Pen-Gly-Phe-Pen]-Phe-OH, [Phe[6]]DPLPE	1.89 ± 0.072	5800 ± 1300	3100
2, H-Tyr-c[D-Pen-Gly-Phe(*p*-F)-Pen]-Phe-OH, HBP51	0.016 ± 0.005	740 ± 100	45000
3, H-Tyr-c[D-Pen-Gly-Phe(*p*-Cl)-Pen]-Phe-OH	0.17 ± 0.021	420 ± 130	2500
4, H-Tyr-c[D-Pen-Gly-Phe(*p*-Br)-Pen]-Phe-OH	0.18 ± 0.03	3400 ± 320	19000
5, H-Tyr-c[D-Pen-Gly-Phe(*p*-I)-Pen]-Phe-OH	0.80 ± 0.055	8700 ± 510	11000
6, H-Tyr-c[D-Pen-Gly-Phe-Cys]-Phe-OH, [Phe[6]]DPLCE	0.016 ± 0.0026	83 ± 11	5100
H-Tyr-c[D-Pen-Gly-Phe-Pen]-OH, DPLPE[b]	2.5	2700	1100

[a] $IC_{50}(\mu)/IC_{50}(\delta)$. [b] Data taken from ref 34.

also increased from 1100 to 3100. In comparison with the binding study, the effect on potency in the bioassays caused by the addition of Phe[6] is less. However, direct comparison of the Phe[6] peptide **6**, a Cys[5]-containing analogue, with [Phe[6]]DPLCE (**1**), a peptide with an L-Pen[5] substitution, shows again that the two additional β-methyl groups in position 5 have a substantial effect on the bioactivity. The Pen[5]-containing peptide [Phe[6]]-DPLPE (**1**) is much less potent, in both the MVD and GPI bioassays, than the Cys[5]-containing peptide **6**. However, the effect of the methyl groups on the δ potency is much stronger in the case of peripheral receptors than at central receptors. The IC_{50} values changed from 0.016 to 1.89 nM in the MVD assay but only from 1.4 to 4.0 nM in the binding assay for HBP2 and **1**, respectively. The effect of two β-methyl groups in position 5 for interaction of the cyclic enkephalin analogues with peripheral μ receptors resulted in tremendous diversity of potency (from 83 nM for [*p*-Cl-Phe[4]]DPLCE)[37] in the GPI test to an IC_{50} of 8700 nM for [*p*-I-Phe]DPLPE (**5**; Table 2). Nonetheless [Phe[6]]-DPLCE (**6**) is slightly more selective and much more potent than **1** at peripheral δ receptors. However, the introduction of *p*-halogens in the Phe[4] residue resulted in further differences. As reported previously for compounds such as [*p*-X-Phe[4]]DPDPE,[9] the introduction of a halogen-substituted phenylalanine generally increased potency in the MVD test. The exceptions were in the [Phe[6]]DPLCE series,[37] where para halogenation of Phe[4] led to analogues that were less potent. In the present series of compounds (Table 2), halogenation led to increased potency in the MVD assay, thus providing extremely potent and selective analogues such as [*p*-F-Phe[4],Phe[6]]DPLPE (**2**; Table 2). At the same time, para halogenation of Phe[4] led to increased potency of the compounds **2**−**5** toward μ receptors in the GPI assay with the exception of the *p*-iodo analogue **5** (Table 2), which is less potent in the GPI assay than the unsubstituted analogue **1**. However, as in the binding study, the influence of two β-methyl groups of Pen[5] and of *p*-halogen is not additive, and thus the hexapeptides **2**−**5** are both highly potent and exceptionally δ receptor selective. The most potent and the most selective analogue is [*p*-F-Phe[4],Phe[6]]DPLPE (**2**; Table 2). This compound has extraordinary high potency at peripheral δ receptors, as high as **6** (HBP2; Table 2), but due to its relatively poor potency at peripheral μ receptors, it is exceptionally selective too. Interestingly the *p*-bromo peptide **4**, though 10 times less potent than **2** at the δ receptor assay and 5-fold less potent in the GPI assay, still is exceptionally selective (Table 2). Halogenation has an additional important effect on biological properties, as it was shown earlier that [*p*-Cl-Phe[4]]DPDPE can cross the blood−brain barrier much more readily than DPDPE.[42] It will be interesting to determine how well the current analogues cross the blood−brain barrier.

Finally it is interesting to note that whereas all of the para-halogenated Phe[4] analogues (**2**−**5**) are much better binders and much more δ-opioid receptor selective in the binding assays than the nonhalogenated Phe[4] analogue **1**, and even more so than the lead compound **6** which has a L-Cys residue (Table 1), a somewhat different pattern emerges in the bioassay studies (Table 2). Generally the binding assays and bioassays give comparable results.[4] On the basis of the results obtained when one compares μ and δ binding studies with the GPI and MVD bioassay results, analogues **3**−**5** thus behave quite normally in that their in vitro bioactivities in the MVD and GPI (and therefore selectivities too) more or less parallel what one observes in the binding assay in terms of binding affinities vs the δ ligand and μ ligand, respectively (Table 1). However the *p*-F-Phe[4], Pen[5] analogue **2**, as well as the Phe[4],Cys[5] analogue **6**, has extraordinary potency (16 pM in each case) in the MVD assay (Table 2) and hence becomes extraordinary more potent than anticipated based on the binding studies. Whether this is a result of greatly enhanced efficacy[43] or some other effect may be observed is not clear, but needs further investigation, especially since the effects are so great.

Experimental Section

General Methods of Peptide Synthesis. The peptides in this report were synthesized by the solid-phase method following procedures used in our laboratory.[35,38] The protected amino acids and chloromethylated polystyrene (cross-linked with 1% divinylbenzene, 1 m equiv/g) were purchased from Bachem (Torrance, CA). D-Pen(S-*p*-MeBzl) was obtained from Peptides International (Louisville, KY). The C-terminal phenylalanine was attached to the resin by Gisin's method[44] (Cs salt in DMF for 18 h at 50 °C), and substitution levels of 0.77−0.84 mequiv/g were achieved. The N^{α}-Boc protecting group was removed using a TFA−DCM-anisole mixture (48/50/2, v/v). The protected amino acids were coupled using diisopropylcarbodiimide (DICI) as coupling reagent. The amino acids and DICI were used in 3-fold excess. To diminish the cost, the para-substituted phenylalanine residues were coupled to the growing peptide chain by means of BOP in the presence of *N*-hydroxybenzotriazole (HOBT) used in 1.2 excess (see details below). The peptides were removed from resin and the protecting groups removed at the same time using HF(l) containing 10% anisole. The sulfhydryl peptides were oxidized without purification. The sulfhydryl peptides were obtained with purities of 90% or more by HPLC.

The sulfhydryl peptides were oxidized by methods described previously.[37,39] Briefly, the peptide was dissolved in methanol (300−400 mg in 50 mL) and added from a syringe pump to a well-stirred aqueous solution of oxidant (1−1.5 L). The oxidizing agent was prepared by dissolving potassium ferri-

Table 3. Analytical Properties of Peptides with General Formula H-Tyr-c[D-Pen-Gly-Phe(*p*-X)-Pen]-Phe-OH

peptide (substituent)	TLC[a] R_f values				HPLC[b]		FAB-MS	
	I	II	III	IV	red[c]	ox[d]	calcd	obsd
1 (H)	0.80	0.86	0.76	0.86	22.82	23.84	792	793
2 (F)	0.78	0.79	0.74	0.83	27.08	26.06	810	811
3 (Cl)	0.81	0.83	0.76	0.81	27.91	26.99	827	828
4 (Br)	0.81	0.82	0.77	0.81	28.40	27.76	872	873[e]
5 (I)	0.82	0.83	0.77	0.79	29.70	28.33	919	919

[a] Silica gel plates (Analtech), 0.25 mm, solvent path 8 cm. Eluents used are as follows: I, *n*-butyl alcohol−acetic acid−water, 4:1:1; II, *n*-butyl alcohol−acetic acid−pyridine−water, 13:3:12:10; III, isopropyl alcohol−ammonia−water, 3:1:1; IV, *n*-butyl alcohol−acetic acid−ethyl acetate−water, 1:1:1:1. [b] Retention times (min) for the following system: Hewlett-Packard 1090, column C-18 (Vydac 218TP104), 4.6 mm × 25 cm, buffer A 0.1% TFA in acetonitrile, buffer B 0.1% TFA in water, gradient 0.50% A in 30 min, flow rate 1.0 mL/min, simultaneous detection at 225, 254, and 280 nm. [c] Retention times (min) of nonoxidized linear peptides. [d] Retention times (min) of pure oxidized (cyclic) peptides. [e] The peak P + 2 for Br isotope was observed.

cyanide ($K_3Fe(CN)_6$) in 4-fold excess in 0.05 M ammonium acetate buffer, pH 8.5. The use of buffer allowed easy maintenance of the slightly basic conditions, and further control of pH was unnecessary. The addition rate was calculated to be approximately 10 mg of peptide/h/L of oxidant. In this way the formation of peptide oligomers was diminished greatly.

After addition of the peptide was completed, the reaction mixture was stirred for an additional 5−6 h and acidified to pH 4.5 with glacial acetic acid. The excess ferro and ferricyanide ions were removed by ion-exchange resin Amberlite IRA-45 (Cl^- form). The resin was filtered off and the solution concentrated under diminished pressure at temperature below 40 °C and lyophilized. The lyophilized powder was dissolved again in acetic acid, filtered to remove inorganic salts, and relyophilized.

The crude cyclic peptides were purified by preparative HPLC on an ODS column (Vydac 218TP152050), 5 × 25 cm, using a Rainin HPLX instrument with detection at 220 and 254 nm. The pure fractions were pooled and lyophilized. The purity of the peptides was checked by analytical HPLC (ODS column, 4.6 × 25 cm, Vydac 218TP104) using a Hewlett-Packard 1090 instrument (detection at 220, 254, 280 nm) with 0.1% aqueous TFA and a 0−50% acetonitrile gradient over 30 min. The chromatograms were analyzed by a computer program provided by the manufacturer (Hewlett-Packard), and the peptides were shown to be >98% pure. TLC was performed in four solvent systems on silica gel and visualized by ninhydrin and iodine vapors; all peptides showed single spots (Table 3). The amino acid analyses were performed at the University of Arizona Biotechnology Core Facility. The system used was a dedicated Applied Biosystem model 420A amino acid analyzer with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min using 6 N HCl) and precolumn phenylthiocarbamoyl-amino acid (PTA-AA) analysis (D-Pen was not detected). FAB-MS spectra were in agreement with the amino acid sequence and the composition of each analogue. The analytical data of the compounds synthesized in this paper are given in Table 3.

c[D-Pen²,Pen⁵,Phe⁶]enkephalin (Tyr-D-Pen-Gly-Phe-Pen-Phe, 1). This peptide was obtained by the stepwise synthesis method outlined above starting from 1.5 g of Boc-Phe resin, substitution level 0.96 mequiv/g. The following amino acids were coupled to the resin: Boc-Pen(*p*-MeBzl), Boc-Phe, Boc-Gly, Boc-D-Pen(*p*-MeBzl), and Boc-Tyr(2,6-Cl_2Bzl). After the peptide was assembled the N^α-Boc group was removed by TFA, the resin was washed several times with DCM, and dried overnight under diminished pressure over KOH; yield was 2.32 g. The peptide resin was mixed with 2.5 mL of a mixture of 1:1 cresol and *p*-thiocresol, and then approximately 25 mL of liquid HF was added. The reaction mixture was stirred for 1 h at 0 °C, and then the HF was distilled off in vacuo. The scavengers were removed by washing the residue three times with dry ether, and the resin with precipitated peptide was dried in a vacuum desiccator. The peptide was extracted three times with acetic acid, and the acetic solutions were pooled and lyophilized; yield of crude peptide was 678 mg. The crude peptide was cyclized as described above. The peptide was then purified by preparative HPLC: 15 min of 0.1% aqueous TFA and then a gradient of 0−60% acetonitrile in 0.1% TFA in 120 min. The main fractions were pooled, concentrated on a rotary evaporator, and lyophilized; yield was 78 mg. The purity was assessed to be at least 98%, and an additional 39 mg was obtained with a purity of 92−95%. Amino acid analysis: Tyr, 0.92 (1.0); Gly, 1.09 (1.0); Phe, 1.89 (2.0). The other analytical results are given in Table 3.

c[D-Pen²,Phe(*p*-F)⁴,Pen⁵,Phe⁶]enkephalin (Tyr-D-Pen-Gly-Phe(*p*-F)-Pen-Phe, 2, HBP51). This analogue was synthesized by similar methods as those described above (from 2 g of resin, 0.84 mequiv/g) except that the Boc-Phe(*p*-F) (1.2 equiv) was coupled to the resin using BOP (1.2 equiv), HOBT (1.2 equiv), and DIPEA (4 equiv) in *N*-methylpyrrolidinone. The yields were 3.46 g of peptide resin, 1.16 g of crude peptide, and 93 mg of pure peptide. Amino acid analysis: Tyr, 0.90 (1.0); Gly, 1.01 (1.0); Phe(*p*-F), 0.94 (1.0); Phe, 1.0 (1.0).

c[D-Pen²,Phe(*p*-Cl)⁴,Pen⁵,Phe⁶]enkephalin (H-Tyr-D-Pen-Gly-Phe(*p*-Cl)-Pen-Phe-OH, 3). This compound was synthesized by the method described above starting from 2 g of resin (substitution level 0.84 mequiv/g). The Boc-Phe(*p*-Cl) was attached using BOP as outlined above for **2**. Yields were 3.48 g of peptide resin, 1.14 g of crude peptide, and 81 mg of the pure peptide. Amino acid analysis: Tyr, 1.0 (1.0); Gly, 1.11 (1.0); Phe(*p*-Cl), 1.01 (1.0); Phe, 1.05 (1.0). The other analytical results are given in Table 3.

c[D-Pen²,Phe(*p*-Br)⁴,Pen⁵,Phe⁶]enkephalin (H-Tyr-D-Pen-Gly-Phe(*p*-Br)-Pen-Phe-OH, 4). The title compound was synthesized starting from 2 g of resin (substitution level 0.78 mequiv/g) applying the above methods and yielded 3.70 g of peptide resin, 678 mg of crude peptide, and 82 mg of pure peptide. Amino acid analysis: Tyr, 0.91 (1.0); Gly, 1.09 (1.0); Phe(*p*-Br), detected but could not be calculated; Phe, 1.09 (1.0). The other analytical results are given in Table 3.

c[D-Pen²,Phe(*p*-I)⁴,Pen⁵,Phe⁶]enkephalin (H-Tyr-D-Pen-Gly-Phe(*p*-I)-Pen-Phe-OH, 5). This analogue was synthesized in the same manner from 2 g of resin (0.78 mequiv/g). Yields were 3.79 g of peptide resin, 1.10 g of crude peptide, and 53 mg of pure peptide. Amino acid analysis: Tyr, 0.89 (1.0); Gly, 1.11 (1.0); Phe, 1.0 (1.0). The other analytical results are given in Table 3.

c[D-Pen²,Cys⁵,Phe⁶]enkephalin (HBP2): synthesized as described previously.[37]

Radioligand Binding Methods. Membranes were prepared from whole (less cerebellum) brain taken from adult male Sprague−Dawley rats (250−300 g) obtained from Harlan Sprague−Dawley, Inc. Following decapitation, the brain was removed, dissected, and homogenized at 0 °C in 20 volumes of 50 nM Tris-HCl buffer adjusted to pH 7.4 using a Teflon glass homogenizer. The membrane fraction was obtained by centrifugation at 48000*g* for 15 min at 4 °C, then resuspended in 20 volumes of fresh Tris buffer, and incubated at 25 °C for 30 min to dissociate any receptor-bound endogenous opioid peptides. The incubated homogenate was centrifuged again and the final pellet resuspended in 20 volumes of fresh Tris buffer. Radioligand-binding inhibition assay samples were prepared in a buffer consisting of 50 mM Tris-HC1, 1.0 mg/mL bovine serum albumin, 30 μM bestatin, 50 μg/mL bacitracin, 10 μM captopril, and 0.1 mM toluenesulfonyl fluoride, pH 7.4. The radioligands used were [^{3}H]c[D-Pen²,*p*-Cl-Phe⁴,D-Pen⁵]-enkephalin[41] at a concentration of 0.75 nM and [^{3}H]CTOP[42] (New England Nuclear, Boston, MA) at a concentration of 0.5 nM. Peptide analogues were dissolved in assay buffer prior to each experiment and added to duplicate assay tubes at 10 concentrations over an 800-fold range. Control (total) binding

was measured in the absence of any inhibitor, while nonspecific binding was measured in the presence of 10 μM naltrexone. The final volumes of the assay samples were 1.0 mL of which 10% consisted of the membrane preparation in 0.1 mL, of Tris-HCl buffer. Incubations were performed at 25 °C for 3 h, after which the samples were filtered through poly(ethylenimine)-treated GF/B glass fiber filter strips. The filtrates were washed three times with 4.0 mL of ice-cold normal saline before transfer to scintillation vials. The filtrate radioactivity was measured after adding 10 mL of cocktail consisting of 16 g of Crystal Fluor (West Chemical, San Diego, CA) in 1.0 L of Triton X-100 and 2.0 L of toluene to each vial and allowing the samples to equilibrate over 8 h at 4 °C. The data were analyzed by using nonlinear least-squares regression analysis on the Apple II Plus Computer. Programs were generously written by Susan Yamamura. Each assay was repeated at least three times in duplicate for an $n = 6$ or more.

GPI and MVD Bioassays. Electrically induced smooth muscle contractions of mouse vas deferens (MVD) and strips of guinea pig ileum (GPI) longitudinal muscle-myenteric plexus were used for these bioassays. Tissues came from male ICR mice weighing 25−40 g (MVD) and from male Hartley guinea pigs weighing 250−500 g (GPI). The tissues were tied to a gold chain with suture silk, suspended in 20-mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (magnesium free for the MVD), and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length, previously determined to be 1 g tension (0.5 g for MVD), and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum wire electrodes at 0.1 Hz, 0.4-ms pulses (2.0-ms pulses for MVD), and supramaximal voltage. Drugs were added to the baths in 14−60-μL volumes. The agonists remained in contact with the tissue for 3 min before the addition of the next cumulative doses until maximum inhibition was reached. Percent inhibition was calculated by using the average contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the dose of the agonist. IC_{50} values represent the mean of not less than four tissues. IC_{50} estimates, relative potency estimates, and their associated standard errors were determined by fitting the mean data to the Hill equation by using a computerized nonlinear least-squares method. Each assay was repeated at least three times in duplicate for an $n = 6$ or more.

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC−IUB Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977−989). The optically active amino acids are of L-chirality unless otherwise noted. Other abbreviations: DPLCE, [D-Pen[2],L-Cys[5]]enkephalin; DPDPE, [D-Pen[2],D-Pen[5]]-enkephalin; DPLPE, [D-Pen[2],L-Pen[5]]enkephalin; N^α-Boc, N^α-*tert*-butyloxycarbonyl; BOP, benzotriazolyltris(dimethylamino)phosphonium hexafluorophosphate; DICI, diisopropylcarbodiimide; DCM, dichloromethane; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; Pen, penicillamine; HOBT, hydroxybenzotriazole; TFA, trifluoroacetic acid.

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