[L-Ala³]DPDPE: A New Enkephalin Analog with a Unique Opioid Receptor Activity Profile. Further Evidence of δ-Opioid Receptor Multiplicity

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Received December 17, 1993•

To investigate δ-opioid receptor topography near the 3-position of [D-Pen²,D-Pen⁵]enkephalin (DPDPE), a series of small-group 3-position analogs of DPDPE have been synthesized and assayed for binding potencies and *in vitro* biological activities. L-Amino acid substitutions at this position are highly favored over D-amino acid substitutions, with the smallest, [L-Ala³]DPDPE (DPADPE), being the most favored in the series investigated. [L-Ala³]DPDPE is nearly as δ-potent and more δ-selective in both rat brain binding (18 nM vs [³H][*p*-ClPhe⁴]DPDPE and $\mu/\delta = 610$) and peripheral bioassays (12 nM in the MVD and GPI/MVD = 4500) when compared to DPDPE (8.5 nM, $\mu/\delta = 73$ and 4.1 nM, GPI/MVD = 1800, respectively). Whereas DPDPE is a potent analgesic when given icv, [L-Ala³]DPDPE is only a weak analgesic. However, [L-Ala³]DPDPE has been found to antagonize DPDPE, but not Deltorphin II, in a moderately potent ($pA_2 = 5.7$) and selective fashion *in vivo*. Thus, [L-Ala³]DPDPE is a fairly potent agonist at peripheral δ receptors and is a moderately potent (mixed) antagonist of δ_1 receptors in the brain. It appears that [L-Ala³]DPDPE does not interact in any significant manner with δ_2 or μ receptors in the brain.

Introduction[1]

The isolation of the enkephalins,[2] endogenous pentapeptides specific for opioid receptors in mammalian brain, has led to the preparation of many tetra-, penta-, and hexapeptide analogs.[3,4] Of these, one of the more selective and potent compounds is [D-Pen²,D-Pen⁵]enkephalin (DPDPE).[5] DPDPE has a high selectivity for δ-opioid receptors relative to the other currently accepted μ and κ types of opioid receptors.

Evidence from binding and bioassay studies has increasingly shown that subtypes of these receptors may exist. Thus, DPDPE is thought to bind preferentially to the δ_1-opioid receptor and the deltorphins to bind selectively to the δ_2-opioid receptor.[6] Furthermore, recent cloning of some of the receptors tends to confirm the heterogeneity of opioid receptor types.[7-10] Related studies have shown that the mammalian brain contains δ_1, δ_2, and possibility μ-δ complexed opioid receptors.[6,11-18]

One of the goals of our research is to prepare ligands that are highly selective for the μ, δ, or κ receptor types and subtypes in order to identify the specific physiological and pharmacological properties of the various receptors. Definitive elucidation of the specific topography of the receptors and receptor subtypes still awaits careful structure-activity studies and especially biophysical studies including X-ray crystallography and detailed nuclear magnetic resonance spectroscopy. Meanwhile, the "rational" design of drug candidates that are specific for opioid receptor types and subtypes depends on a combination of *in vitro* bioassays and binding studies, *in vivo* assays, and conformational and computational studies that provide insight, inspiration, and ideas (models) as to which new analogs might lead to increased selectivity and potency.

A previous study had shown that [Aib³]DPDPE, in comparison to DPDPE, had somewhat reduced but quite favorable δ-opioid receptor selectivity and affinity.[19] To determine if one or the other of the α-methyl groups (*pro-R* of *pro-S*) of the [Aib³] residue was sterically hindering binding of the molecule to δ-receptors, both isomers of [Ala³]DPDPE have been prepared and assayed.

Another study[20] based on modeling of DPDPE and Deltorphin I concluded that the side chain of an L-amino acid substituted into the 3-position of DPDPE might fit into a pocket in the δ receptor. To explore further this possibility, both isomers of [Abu³]DPDPE have been synthesized and their effects in bioassays and brain binding studied. In addition, the D-isomer of [Ser³]DPDPE has been prepared and studied for comparison with the previously reported properties of [L-Ser³]DPDPE.[20] Also, to investigate further the effect of 3-position substitutions in DPDPE, [AC₅C³]DPDPE has been prepared and assayed.

We report here on the unusual structure/activity relationships of these compounds and new insights the results provide regarding δ-opioid receptor requirements.

Table 1. Inhibitory Potencies and Selectivities of DPDPE Analogs in GPI and MVD Bioassays

compound	IC_{50}(nM) ± SEM GPI	MVD	ratio (μ/δ)
DPDPE	7300 ± 1700	4.1 ± 0.5	1800
[L-Ala³]DPDPE	54000 ± 3000	12 ± 1.6	4500
[D-Ala³]DPDPE	33000 ± 820	570 ± 130	58
[L-Ser³]DPDPE[a]	39000 ± 7200	250 ± 33	160
[D-Ser³]DPDPE	3% inh at 100 μM	1300 ± 250	>79
[L-Abu³]DPDPE	4900 ± 350	85 ± 13	57
[D-Abu³]DPDPE	30000 ± 2700	1950 ± 200	15
[AC₅C³]DPDPE	860 ± 32	370 ± 160	2.3

[a] Data taken from ref 20.

Results

The results of binding and bioassay studies for the six new 3-position-substituted analogs of DPDPE are shown in Tables 1 and 2. DPDPE and the previously reported [L-Ser³]DPDPE are included for comparison. These

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• Abstract published in *Advance ACS Abstracts*, April 1, 1994.

0022-2623/94/1837-1572$04.50/0

Table 2. Binding Affinities and Selectivities of DPDPE Analogs in Competition with [3H]CTOP and [3H][p-ClPhe4]DPDPE Receptor Binding in Rat Brain Homogenate

compound	IC50(nM) ± SEM CTOP[a]	p-Cl-DPDPE[b]	ratio (μ/δ)
DPDPE	620 ± 280	8.5 ± 1.5	73
[L-Ala3]DPDPE	11000 ± 100	18 ± 0.4	610
[D-Ala3]DPDPE	>35000	540 ± 60	>65
[L-Ser3]DPDPE[c]	>22500	42 ± 3	>536
[D-Ser3]DPDPE	>50000	760 ± 110	>65
[L-Abu3]DPDPE	12000 ± 940	66 ± 3.0	180
[D-Abu3]DPDPE	>50000	2500 ± 200	>20
[AC5C3]DPDPE	1800 ± 50	310 ± 40	5.8

[a] Measured against [3H]CTOP, a highly μ-selective ligand.[27] [b] Measured against [3H][p-ClPhe4]DPDPE, a highly δ-selective ligand.[26] [c] Data taken from ref 20.

results show that an (S)-methyl group in position 3 ([L-Ala3]DPDPE) is tolerated fairly well in the δ receptor binding site, whereas an (R)-methyl group ([D-Ala3]-DPDPE) is not, presumably because of steric hindrance to binding. The diminished binding of the other analogs reported in Table 2 also is attributed to steric effects.

In all three cases where stereoisomers are compared, the L-residue is better tolerated at δ receptors than is the D-residue at the 3-position of DPDPE in both binding and *in vitro* bioassays. Moreover, the results of Tables 1 and 2 show that nothing larger than a methyl group is well tolerated at this position. Thus, the three D-amino acid substituted analogs all have MVD potencies of greater than 500 nM, and in the cases of the [D-Ser3] and [D-Ala3] analogs, greater than 1000 nM (Table 1). As a result, all three D-substituted analogs appear to have low GPI/MVD selectivities of less than 100.

The situation is similar for the brain binding affinities of the three D-substituted analogs, where they all have δ_1 receptor affinities greater than 500 nM, and selectivities apparently less than 100.

In previous studies,[19] [Aib3]DPDPE had IC_{50} values of 5340 and 25.3 nM at brain μ and δ receptors, respectively, and a selectivity ratio of 211, whereas [AC5C3]DPDPE shows brain binding affinities of 1760 nM at μ receptors and 300 nM at δ receptors and a selectivity ratio of 5.6 (Table 2). Whereas [Aib3]DPDPE had an MVD potency of 16.2 nM,[19] [AC5C3]DPDPE's was 370 nM, and it has a low selectivity ratio of 2.3 (Table 1).

The most potent and selective compound in the series is [L-Ala3]DPDPE(DPADPE), with an IC_{50} of 12 nM in the MVD and 54 000 nM in the GPI for a selectivity ratio (μ/δ) of 4500 in the periphery (Table 1), making it more selective than DPDPE (Table 1). In the binding assays, [L-Ala3]DPDPE is again more selective than DPDPE but not as δ-potent (Table 2). The μ/δ selectivity of [L-Ala3]-DPDPE of 610 is about 6 times greater than that of DPDPE (Table 1).

The *in vitro* and *in vivo* studies of [L-Ala3]DPDPE show it to have a unique δ-opioid activity profile. [L-Ala3]-DPDPE is a fairly potent and highly selective (4500-fold) agonist in GPI/MVD studies (Table 1) without demonstrable antagonist effects in the MVD against the highly potent and selective [D-Pen2,L-Pen5,Phe6]enkephalin ([Phe6]DPLPE, Figure 1).[21] Also, [L-Ala3]DPDPE is about 600-fold μ/δ selective vs. about 100-fold for DPDPE. By contrast, *in vivo* antinociception assays of [L-Ala3]-DPDPE show that it is essentially inactive in the 55 °C tail-flick test (Figure 2) and only weakly active in the 50 °C tail-flick and 55 °C hot-plate tests (Figure 3). A weaker agonist can give a positive result in the 50 °C tail-flick and 55 °C hot-plate tests when it would not do so in the 55 °C tail-flick test.

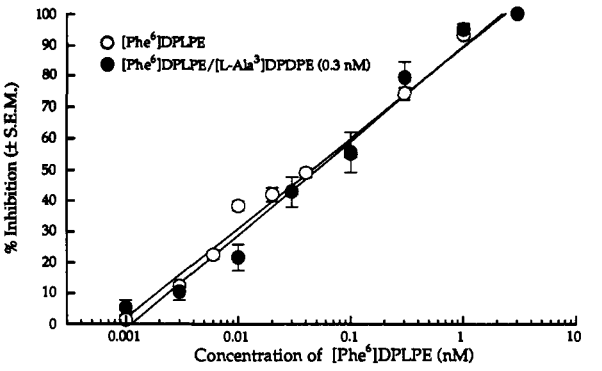

Figure 1. Effect of [L-Ala3]DPDPE on the dose–response curve of Tyr-D-Pen-Gly-Phe-Cys-Phe-OH [Phe6]DPLCE in the mouse vas deferens.

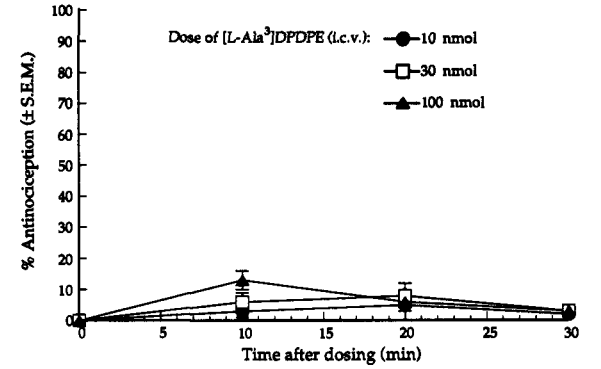

Figure 2. Time course of [L-Ala3]DPDPE-mediated antinociception in the mouse 55 °C tail-flick test.

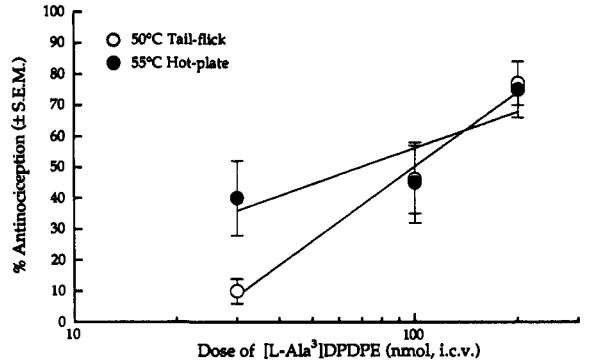

Figure 3. Effect of [L-Ala3]DPDPE in the mouse 50 °C tail-flick and 55 °C hot-plate tests.

Surprisingly, [L-Ala3]DPDPE is thus a potent agonist at peripheral δ receptors, but only a very weak agonist upon central icv administration, requiring a large dose (200 nmol) to achieve 75% antinociception. To verify that the lack of agonism of [L-Ala3]DPDPE was not due to enzymatic degradation, the stability of the compound in both rat serum and rat brain homogenate was assessed. Over a 240-min time course of incubation, neither brain nor blood caused any breakdown of [L-Ala3]DPDPE (Figure 4). We, therefore, undertook an investigation of the potential antagonist effects of [L-Ala3]DPDPE upon central administration.

Interestingly, [L-Ala3]DPDPE at a dose of 30 nmol does not antagonize Deltorphin II mediated antinociception at the δ_2 receptor (B in Figure 5), yet at the same dose it

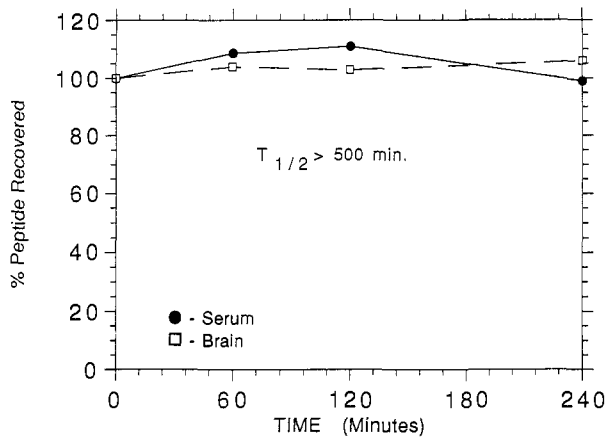

Figure 4. [L-Ala³]DPDPE incubated with rat serum and brain homogenate.

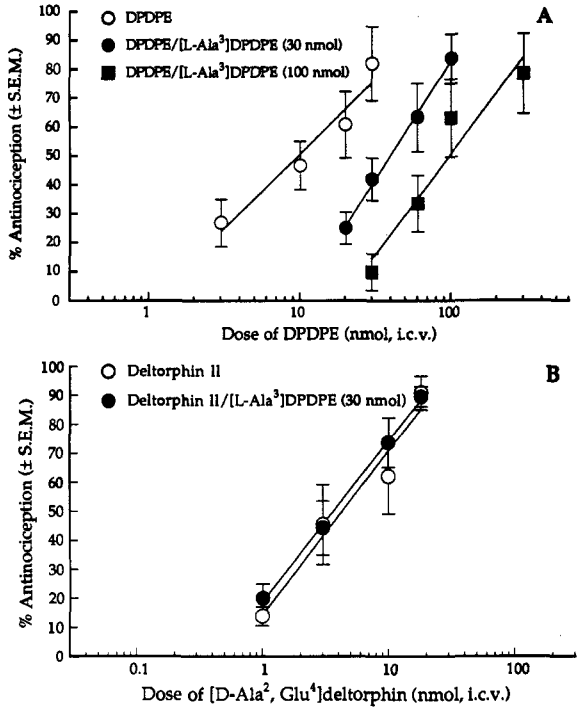

Figure 5. (A) Antagonism of DPDPE-mediated antinociception by [L-Ala³]DPDPE in the mouse 55 °C tail-flick test. (B) Lack of antagonism of Deltorphin II-mediated antinociception by [L-Ala³]DPDPE in the mouse 55 °C tail-flick test.

moderately potently antagonizes DPDPE-mediated analgesia at the δ_1 receptor subtype (A in Figure 5). The pA_2 value for the antagonism of DPDPE by [L-Ala³]DPDPE was estimated to be 5.7. No antagonism of morphine-induced analgesia at μ brain receptors was demonstrated at 100 nmol of DPADPE (Figure 6).

Conclusions

The results of a previous study[19] showing that [Aib³]-DPDPE had favorable δ-opioid receptor interactions led us to investigate the effects of other small-group 3-position substitutions in DPDPE. Of the methyl, ethyl, and hydroxymethyl groups substituted for a hydrogen in the Gly³ residue of DPDPE, only the (*S*)-methyl group was well tolerated at central and peripheral δ receptors. [L-Ala³]DPDPE is a potent δ agonist in the periphery as measured by the MVD bioassay, with no antagonist effects. [L-Ala³]DPDPE has high selectivity in the periphery for δ over μ receptors, as seen in the ratio of GPI/MVD IC_{50}

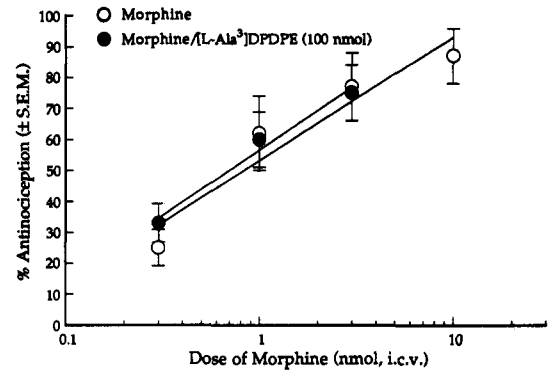

Figure 6. Lack of antagonism of morphine-mediated antinociception by [L-Ala³]DPDPE in the mouse 55 °C tail-flick test.

values. In brain binding assays, [L-Ala³]DPDPE shows a marked affinity and selectivity for central δ_1 over μ receptors, when it is measured against radiolabeled CTOP and [*p*-ClPhe⁴]DPDPE. It is thus surprising that central administration (icv) of [L-Ala³]DPDPE produces weak analgesia. When [L-Ala³]DPDPE was tested *in vivo* as an antagonist at central δ_1, δ_2, and μ receptors, it was found to be a moderately potent antagonist of DPDPE(δ_1)- but not Deltorphin II(δ_2)- or morphine(μ)-induced antinociception. In summary, DPADPE distinguishes between peripheral δ receptors, where it is a potent pure agonist with no antagonist effects; central δ_1 receptors, where it is a moderately potent antagonist and weak agonist; and central δ_2 receptors, where it has no effect.

Experimental Section

General Methods for Peptide Synthesis. With the exception of [AC_5C^3]DPDPE (see below), all of the peptides were synthesized in a stepwise fashion via the solid-phase method. 1% cross-linked, chloromethylated polystyrene resin (0.6–0.8 mmol/g) and D-Pen(*S*-*p*MeBzl) were purchased from Peptides International (Louisville, KY). N^{α}-Boc-protected Phe, Tyr, L-Ala, D-Ala, L-Ser(O-Bzl), and D-Ser(O-Bzl) were purchased from Bachem (Torrance, CA). L-Abu and D-Abu were purchased from Aldrich.

The cesium salt of Boc-D-Pen(*S*-*p*MeBzl) was prepared and added to the resin in DMF solution. Substitution levels of 0.47 and 0.53 mmol of Boc-D-Pen(*S*-*p*MeBzl) per gram of resin were obtained. Diisopropylcarbodiimide (DIPCDI) was used as the coupling reagent, and 3 equiv of amino acid and 2.4 equiv of DIPCDI and HOBT were used.

TFA–DCM–anisole (48/50/2, v/v) was used to deprotect the Boc protecting groups, and the peptides were cleaved from the resin using anhydrous HF with *p*-cresol/*p*-thiocresol (1/1 wt/wt).[22]

Before oxidation, the crude linear disulfhydryl peptides were purified by one pass through a preparative, C-18 HPLC column (Vydac 2181P152050), 5 cm × 25 cm fitted with a 5 cm × 10 cm precolumn handpacked with IMPAQ C18-bonded RG10205, using Rainin HPXL pumps and a UVD detector at 230 and 280 nM. The solvent system was acetonitrile and 0.1% TFA in water (buffer) and the gradient used was 0–25% organic component in 5 min, followed by 25–65% organic component in 40 min, all at a flow rate of 40 mL/min. The peptides eluted in about 30 min, near 50% organic/50% buffer. The fraction containing the peptide was concentrated *in vacuo* to remove the acetonitrile and lyophilized. Generally, the linear peptides were obtained in greater than 90% purity.

General Method of Oxidation/Cyclization. The peptides were oxidized by the syringe pump method developed in this laboratory.[23] The linear peptide (300–500 mg) was dissolved in 40 mL of 50% H_2O/25% acetonitrile/25% methanol, and nitrogen gas was passed through the solution for 20 min. Five milliliters of saturated ammonium acetate solution were added, and the pH was taken to 8.5 with NH_4OH. The peptide solution was then added at room temperature via syringe pump to a stirred

Table 3. Analytical Properties of 3-Position Analogs of DPDPE

compound	TLC[a] R_f values				HPLC[b]		FAB-MS		$[\alpha]^{23}_D$ (deg) (c 0.33, 30% HOAc)
	I	II	III	IV	V	VI	calcd	found	
[L-Ala^3]DPDPE	0.66	0.72	0.52	0.46	0.98	0.20	659	659	+114
[D-Ala^3]DPDPE	0.70	0.71	0.54	0.42	1.35	0.20	659	659	-48
[D-Ser^3]DPDPE	0.69	0.75	0.42	0.14	1.34	0.27	675	675	-48
[L-Abu^3]DPDPE	0.79	0.77	0.52	0.53	3.33	0.33	673	673	+124
[D-Abu^3]DPDPE	0.78	0.72	0.57	0.50	1.93	0.29	673	673	-67
[AC_5C^3]DPDPE	0.71	0.73	0.57	0.52	2.31	0.25	699	699	+87

[a] Sigma T-6145 0.25-mm analytical silica gel plates with fluorescent indicator were used. Solvent systems are as follows: I, 1-butanol/acetic acid/water (4:1:1); II, 1-butanol/acetic acid/pyridine/water (13:2:12:10); III, 2-propanol/ammonia/water (9:1:1); IV, acetic acid/ethyl acetate (1:2). [b] Capacity factor (k') for the following systems: Vadac 218TP104 C-18 reversed-phase column (25 × 0.45 cm) with 0.1% trifluoroacetic acid/CH_3CN (75/25 v/v) at a flow rate of 1.0 mL/min (V) and with 0.1% trifluoroacetic acid/CH_3CN (55/45 v/v) at a flow rate of 1.0 mL/min (VI), both monitored at λ = 230, 254, and 280 nm.

oxidant solution. The oxidant solution was prepared as follows: 2 equiv of potassium ferricyanide were dissolved in 400 mL of H_2O/200 mL of acetonitrile/200 mL of methanol. To this solution was added 100 mL of saturated ammonium acetate, and the pH was then taken to 8.5 with NH_4OH. The peptide solution was added at such a rate that approximately 10 mg of peptide was delivered per hour per liter of the oxidant.

After the addition of peptide was complete, the reaction mixture was stirred for an additional 5–6 h and then taken to pH 3.5 with glacial acetic acid. Amberlite IRA-68 (Cl^- form) was added to remove the iron ions and the solution stirred for 20 min and then filtered. The solution was concentrated using a rotary evaporator at 30 °C and then lyophilized. The material thus obtained was dissolved in glacial acetic acid, filtered to remove inorganic salts, and relyophilized.

The crude cyclic peptides were purified by preparative HPLC on the system described above, using a gradient of 100% buffer for 20 min, then 0–20% acetonitrile in 5 min, followed by 20–60% acetonitrile in 40 min, all at 40 mL/min. Again, the peptides eluted near 50% organic/50% buffer. The purity of the cyclic peptides was checked by analytical HPLC (C-18 column, Vydac 218TP104, 4.6 mm × 25 cm), using a Hewlett-Packard 1090 with detection at 230, 254, and 280 nm and by TLC in four solvent systems in silica gel with detection by UV light, iodine vapors, and ninhydrin. The amino acid analyses were done at the University of Arizona Biotechnology Core Facility using an Applied Biosystem Model 420A Amino Acid Analyzer with automatic hydrolysis (vaporphase at 160 °C for 1 h 40 min using 6 N HCl) or with prior hydrolysis (at 110 °C for 24 h using 6 N HCl) and precolumn phenylthiocarbamoyl-amino acid (PTA-AA) analysis (D-Pen could not be reliably quantitated). FAB-MS spectra were obtained from the College of Pharmacy at the University of Arizona. The analytical data of the compounds synthesized in this paper are given in Table III.

[L-Ala^3]DPDPE (H-Tyr-D-Pen-Ala-Phe-D-Pen-OH). The title compound was obtained by stepwise elongation of the peptide-resin by the method outlined above starting from 2 g of N^α-Boc-D-Pen(*S*-*p*MeBzl)-resin (substitution level 0.47 mmol/g). The following amino acids were added to the growing peptide chain: Boc-Phe, Boc-Ala, Boc-D-Pen(*S*-*p*MeBzl), Boc-Tyr. After the last amino acid was coupled, the Boc group was removed by TFA and the resin was washed several times with DCM and dried by passing N_2 over it; yielded, 2.7 g. The peptide-resin was mixed with 2.7 mL of a 1:1 mixture of *p*-cresol/*p*-thiocresol,[22] and approximately 25 mL of HF was added. The mixture was stirred at 0 °C for 1 h, after which the HF was removed *in vacuo*. Dry ether, 50 mL, was added and the mixture stirred for 20 min. The mixture was filtered to separate out the resin and precipitated peptide. These were then washed four times with 50-mL portions of ether and dried by passing nitrogen. The peptide was removed from the resin by extraction with glacial acetic acid (4 × 25 mL). The acetic acid solutions were pooled and lyophilized; yield, 553 mg. The crude linear peptide was partially purified as described above, and 504 mg was obtained. This material was oxidized by the method outlined above. The crude cyclic peptide was purified on preparative HPLC to give 280 mg of the title compound of greater than 99% purity. Amino acid analysis: Tyr 1.00 (1.0), Ala 0.99 (1.0), Phe 1.05 (1.0).

[D-Ala^3]DPDPE (H-Tyr-D-Pen-D-Ala-Phe-D-Pen-OH). This peptide was synthesized in the same manner as described above, starting from 2 g of N^α-Boc-D-Pen(*S*-*p*MeBzl)-resin (substitution level 0.47 mmol/g); yield of peptide-resin, 2.81 g; yield of crude disulfhydryl peptide, 565 mg; yield of partially purified disulfhydryl peptide, 342 mg; yield of pure cyclic peptide, 180 mg, >99% purity. Amino acid analysis: Tyr 0.97 (1.0), Ala 1.00 (1.0), Phe 1.00 (1.0). The other analytical data are found in Table 3.

[D-Ser^3]DPDPE (H-Tyr-D-Pen-D-Ser-Phe-D-Pen-OH). The peptide was prepared as above starting from 2.5 g of N^α-Boc-D-Pen(*S*-*p*MeBzl)-resin (substitution level 0.53 mmol/g); yield of peptide-resin, 3.44 g; yield of crude disulfhydryl peptide, 722 mg; yield of partially purified disulfhydryl peptide, 324 mg; yield of purified cyclic peptide, 125 mg, >99% pure and 114 mg, >93% pure. Amino pure analysis: Tyr 1.00 (1.0), Ser 0.90 (1.0), Phe 1.03 (1.0). Additional analytical data are found in Table 3.

[L-Abu^3]DPDPE (H-Tyr-D-Pen-Abu-Phe-D-Pen-OH). The title compound was synthesized as described above from 2.5 g of N^α-Boc-D-Pen(*S*-pMeBzl)-resin (substituted level 0.53 mmol/g); yield of peptide-resin, 3.05 g; yield of crude disulfhydryl peptide, 1.07 g; yield of partially purified disulfhydryl peptide, 400 mg; yield of pure cyclic peptide, 226 mg, >99% pure. Amino acid analysis: Tyr 0.90 (1.0), Abu 0.90 (1.0), Phe 1.2 (1.0). Additional analytical data are given in Table 3.

[D-Abu^3]DPDPE (H-Tyr-D-Pen-D-Abu-Phe-D-Pen-OH). The title compound was synthesized in the same manner as the analogs described above, starting from 2.5 g of N^α-Boc-D-Pen-(*S*-pMeBzl)-resin (substitution level 0.53 mmol/g); yield of peptide-resin, 3.14 g; yield of crude disulfhydryl peptide, 730 mg; yield of partially purified disulfhydryl peptide, 475 mg; yield of pure cyclic peptide, 212 mg, >99% pure. Amino acid analysis: Tyr 0.81 (1.0), Abu 1.00 (1.0), Phe 1.39 (1.0). Additional analytical data appear in Table 3.

[AC_5C^3]DPDPE (H-Tyr-D-Pen-AC_5C-Phe-D-Pen-OH). It was anticipated that sequential stepwise synthesis of the title compound would give problems similar to those encountered in the synthesis of [Aib^3]DPDPE.[19] Thus, Boc-D-Pen(*S*-*p*MeBzl)-AC_5-C-OH was prepared by solution coupling of Boc-D-Pen(*S*-*p*MeBzl)-OH to AC_5C-OEt followed by hydrolysis of the ethyl ester and was coupled to Phe-D-Pen(*S*-*p*MeBzl)-resin in the usual manner. The rest of the synthesis of the title compound was performed as described above, starting from 4.0 g of Boc-D-Pen-(*S*-*p*MeBzl)-resin (substitution level 0.47 mmole/g); yield of peptide-resin, 4.9 g; yield of partially purified disufhydryl peptide, 350 mg; yield of pure cyclic disulfide peptide, 164 mg >99% pure. Amino acid analysis: Tyr 0.95 (1.0), AC_5C 0.92 (1.0), Phe 1.1 (1.0). Additional analytical data are given in Table 3.

GPI and MVD Bioassays. Electrically induced smooth muscle contraction of mouse vas deferens and strips of guinea pig ileum longitudal muscle-myenteric plexus were used as a bioassay.[24] Tissues came from male ICR mice weighing 25–40 g and from male Hartley guinea pigs weighing 250–500 g. The tissues were tied to 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 of 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. The test for potential antagonism was performed by adding the compound to the bath 2 min before beginning the follow up dose–response curve. 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.[25]

Radioligand Binding Methods. Membranes were prepared from whole brains taken from adult male Sprague–Dawley rats (250–300 g) obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN). Following decapitation, the brain was removed, dissected, and homogenized at 0 °C in 20 volumes of 50 mM Tris-HCl (Sigma, St. Louis, MO) buffer adjusted to pH 7.4 using a Teflon-glass homogenizer. The membrane fraction obtained by centrifugation at 48000*g* for 15 min at 4 °C was resuspended in 20 volumes of fresh Tris buffer and incubated at 25 °C for 30 min to dissociate any receptor-bound endogeneous opioid peptides. The incubated homogenate was centrifuged again as described and the final pellet resuspended in 20 volumes of fresh Tris-HCl buffer.

Radioligand binding inhibition assay samples were prepared in a pH 7.4 assay buffer consisting of 50 mM Tris-HCl, 1.0 mg/mL bovine serum albumin, 30 μM bestatin, 50 μg/mL bacitracin, 10 μM captopril, and 0.1 mM phenylmethanesulfonyl fluoride, all from Sigma (St. Louis, MO), except bestatin which was obtained from Peptides International (Louisville, KY). The radioligands used were [^{3}H][D-Pen^2,*p*-Cl-Phe^4,D-Pen^5]enkephalin[26] at a concentration of 0.75 nM and [^{3}H]CTOP[27] (New England Nuclear, Boston, MA) at a concentration of 0.5 nM. Peptide analogues were dissolved in assay buffer (or prior to that in DMSO or methanol according to the chemists' recommendations and not to exceed 5% of the total volume) 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 (Sigma, St. Louis, MO). The final volume of the assay samples was 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) (0.5% w/v, Sigma, St. Louis, MO) treated GF/B glass fiber filter strips (Brandel, Gaithersburg, MD). The filters 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 7–10 mL of cocktail (EcoLite (+), ICN Biomedicals, Inc.) to each vial and allowing the sample to equilibrate over 8 h at 4 °C.

Binding data were analyzed by a nonlinear least-square regression analysis program named Inplot 4.03 (GraphPad, San Diego, CA). Statistical comparisons between one- and two-site fits were made using the *F*-ratio test using a *p* value of 0.05 as the cutoff for significance.[28] Data best fitted by a one site model were reanalyzed using the logistic equation.[29] Data obtained from independent measurements are presented as the arithmetic mean ± SEM.

***In Vivo* Assay Methods: Subjects.** Male ICR mice weighing 20–30 g were used throughout these studies. They were housed in groups of four in Plexiglas boxes, maintained in a light- and temperature-controlled environment, with food and water available *ad libitum* until antinociceptive testing. All testing was performed in accordance with the recommendations and policies of the International Association for the Study of Pain (IASP) and National Institutes of Health (NIH) and the University of Arizona Guidelines for the care and use of laboratory animals.

Antinociceptive Assay. Antinociception was assessed in mice using the warm water tail flick assay and the hot-plate assay. In the tail-flick assay, tails were dipped in 50 °C or 55 °C water, and the latency to a rapid flick was recorded with the baseline cutoff and maximal possible latencies set at 5 and 15 s, respectively. Percent antinociception was calculated according to the following formula: 100 × (test latency − control latency)/(15 s − control latency). In the hot-plate assay, mice were placed on a 55 °C surface and the mean time to lick the back paws or escape jump was recorded. Percent antinociception was calculated similar to the tail-flick test with the maximum cutoff latencies being 20 (baseline) and 60 sec (test). In each case, at least two assays ran in duplicate were used for each assay.

Drug Administration. The compounds DPDPE, [L-Ala^3]-DPDPE and Deltorphin II were dissolved in 20% dimethyl sulfoxide (DMSO). Morphine was dissolved in distilled water. Intracerebralventricular (icv) administration was performed using the methods of Haley and McCormick[30] as modified by Porreca et al.[31] Briefly, mice were lightly anesthetized with ether, and a small incision was made in the scalp. A Hamilton microsyringe was inserted to a depth of 3 mm at a point 2 mm caudal and 2 mm lateral from bregma. Compounds were injected in a 5-μL volume for all icv administrations. In studies assessing the effects of [L-Ala^3]DPDPE on DPDPE-, Deltorphin II-, and morphine-mediated antinociception, compounds were coadministered icv. Antinociception was assessed at the time of peak drug effects (10 min).

Statistics. Data are presented as the means ± the standard errors of the mean for groups of 10 mice. Regression lines, ED_{50} and AD_{50} values and their 95% confidence limits were calculated using individual data points with the computer program of Tallarida and Murray.[32]

Blood and Brain Stability. To investigate the effect of membrane-associated enzymes on [L-Ala^3]DPDPE, a modified method of Davis and Culling-Berglund[33] for isolating membrane-associated peptidases was used. Briefly, brain (minus cerebellum) was weighed and added to ice-cold 1 mM Tris-HCl buffer (pH 7.4) to make a 2% (w/v) homogenate. Following homogenation, the homogenate was put on ice to facilitate lysing of cells. After 30 min, half a volume of cold 50 mM Tris-HCl buffer (pH 7.4) was added, and the homogenate was centrifuged at 49000*g* for 45 min. The supernatant was discarded and the pellet resuspended in 50 mM Tris-HCl buffer (2% w/v) and again centrifuged (49000*g*, 45 min). The supernatant was decanted and the pellet resuspended in an appropriate volume of 50 mM Tris-HCl to yield a 15% (w/v) homogenate. The resuspended pellet was used for the *in vitro* peptide incubations and for Folin-Lowry protein determinations, performed to ensure consistency of preparation.

Time-course metabolism was accomplished by incubating 100 μM [L-Ala^3]DPDPE with the twice-washed membrane-associated enzyme brain preparation or collected serum at 37 °C for 0–240 min. Following incubation of peptide an equal volume of acetonitrile was added to each sample to precipitate proteins and stop enzymatic activity. Samples were then centrifuged at 13000*g* on a Beckman Microfuge 11 for 15 min (Beckman Instruments, Berkeley, CA). The supernatant was collected, and equal volume of sterile water was added, and the sample was frozen at −40 °C until HPLC analysis.

All peptide incubation samples were analyzed using a Series 410 HPLC gradient system (Perkin-Elmer, Norwalk, CT), Model 710B WISP autoinjector (Waters Assoc., Milford, MA), Model 441 absorbance detector (210 nm; Waters Assoc.), and a Model 3396A integrator (Hewlett-Packard, Palo Alto, CA). Samples were eluted from a Vydac 218TP54-ODS 5-μm column (Separations Group, Hesperia, CA) with a linear gradient of acetonitrile (10–40% in 30 min) versus 0.1 M NaH_2PO_4 buffer, pH 2.4 at 1.5 mL/min, 37 °C.

Acknowledgment. This research was supported by a grant from the National Institute of Drug Abuse DA 06284.

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(1) Symbols and abbreviations are in accordance with the recommendation of the IUPAC–IUB Commission on Nomenclature (*J. Biol. Chem.* 1972, *247*, 977–983). The optically active amino acids are L-chirality unless otherwise noted. Other abbreviations included: Abu, α-aminobutyric acid; ACN, acetonitrile; AC_5C,

1-aminocyclopentanecarboxylic acid; Aib, α-aminoisobutyric acid; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 DIPCDI, diisopropylcarbodiimide; DPDPE, [D-Pen2,D-Pen5]enkephalin; HOBt, 1-hydroxybenzotriazole; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; Pen, penicillamine; *p*MeBzl, *p*-methylbenzyl; SEM, standard error of the measurement; TFA, trifluoroacetic acid; DCM, dichloromethane.

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