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Effects of Modifications of Residues in Position 3 of Dynorphin A(1−11)-NH_2 on κ Receptor Selectivity and Potency

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Tyrosine[1] and phenylalanine[4] in dynorphin A (Dyn A) have been reported to be important residues for opioid agonist activity and for potency at κ receptors. The glycine residues in the 2 and 3 positions of dynorphin A may affect the relative orientation of the aromatic rings in positions 1 and 4, but their flexibility precludes careful analysis. To examine these effects on dynorphin A, we previously have synthesized the linear analogues [D-Ala[3]]Dyn A(1−11)-NH_2 (**2**) and [Ala[3]]Dyn A(1−11)-NH_2 (**3**) and reported their biological activities. Analogues **2** and **3** displayed affinities for the central κ opioid receptor (IC_{50} = 0.76 and 1.1 nM, respectively) similar to that of Dyn A(1−11)-NH_2 (**1**) (IC_{50} = 0.58 nM) and greatly enhanced selectivities for κ vs μ and κ vs δ receptors (IC_{50} ratios of 350 and 1300 for **2**, and 190 and 660 for **3**, respectively). These results suggest that the structure and lipophilicity of the amino acid present in position 3 of Dyn A(1−11)-NH_2 as well as the conformational changes they induce in the message sequence of dynorphin have important effects on potency and selectivity for κ opioid receptors. To further investigate structure−activity relationships involving the residue at the 3 position of Dyn A(1−11)-NH_2, a series of Dyn A analogues with aromatic, charged, and aliphatic side chain substitutions at the 3 position was designed, synthesized, and evaluated for their affinities for κ, μ, and δ opioid receptors. It was found that analogues with lipophilic amino acids at the 3 position of Dyn A(1−11)-NH_2 generally displayed higher affinity but similar selectivities for the κ receptor than analogues with charged residues at the same position. It is suggested that the structural, configurational, and steric/lipophilic effects of amino acids at position 3 of Dyn A(1−11)-NH_2 may play an important role in potency and selectivity for the κ receptor.

Introduction

Dynorphin A (Dyn A(1−17)), a heptadecapeptide, H-Tyr-Gly-Gly-Phe-Leu[5]-Arg-Arg-Ile-Arg-Pro[10]-Lys-Leu-Lys-Trp-Asp[15]-Asn-Gln-OH, was first isolated from porcine pituitary and recognized as a potent opioid agonist.[2−4] It is now well established that there are at least three types of opioid receptors, namely μ (mu), δ (delta), and κ (kappa).[5−7] However, the physiological and pharmacological roles of these receptors still need clarification, thus requiring the design and synthesis of highly potent and selective ligands. Research in this area has expanded rapidly in the past decade, with considerable effort devoted to the development of δ and μ opioid receptor selective peptide ligands primarily based on enkephalin.[8−11] The potential of targeting the κ opioid receptor as an effector for analgesia has yet to be explored in detail.[12] Previous structure−activity studies have shown that the truncated derivative, dynorphin A(1−11)-NH_2, retains the high binding potency of dynorphin A at the κ receptor. Thus we and others have primarily used Dyn A(1−11)-NH_2 as a template to examine the structure−activity relationships of dynorphin.[13,14] Since Tyr[1] and Phe[4] are reported to be important for opioid agonist activity, the effects of the glycine residues in positions 2 and 3 of Dyn A on the relative orientation of the two aromatic rings may be biologically important.[13] To assess these possible effects, we previously synthesized [D-Ala[3]]Dyn A(1−11)-NH_2 and [Ala[3]]Dyn A(1−11)-NH_2 and evaluated for their bioactivities. Interestingly, both analogues were found to be very potent for κ receptors and very selective for κ vs μ and κ vs δ receptors.[15] These results suggest that substitution of lipophilic residues and/or certain D-amino acids at position 3 of Dyn A(1−11)-NH_2 may lead to novel Dyn A analogues that exhibit enhanced selectivities for κ receptors, while retaining strong affinities. Therefore, we designed and synthesized a series of linear Dyn A(1−11)-NH_2 analogues with various substitutions at the 3 position and evaluated their binding affinity, selectivity, and bioactivity for κ receptors.

Results and Discussion

Opioid Receptor Binding Affinities and Selectivities in the Guinea Pig Brain (GPB). The peptide analogues of dynorphin A(1−11)-NH_2 (Chart 1) were evaluated for their receptor binding affinities at κ, δ, and μ receptors by measuring the inhibition of binding of [^{3}H]U-69,593 (*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzo[*b*]furan-4-acetamide) (κ), [^{3}H]-cyclo[D-Pen[2], *p*-Cl-Phe[4], D-Pen[5]]enkephalin (δ), and [^{3}H]DAMGO ([D-Ala[2], MePhe[4], Glyol[5]]enkephalin) (μ) to opioid receptors in guinea pig brain (GPB) homogenates (Table 1).[16]

In the GPB binding assay, substitution of Gly[3] with D- and L-alanine leads to analogues **2** and **3** that displayed affinities at the central κ opioid receptor (IC_{50} = 0.76 and 1.1 nM, respectively), similar to that of Dyn A(1−11)-NH_2 **1** (IC_{50} = 0.58 nM), and greatly enhanced selectivities for κ vs μ and κ vs δ receptors (IC_{50} ratios of 350 and 1300 for **2**, and 190 and 660 for **3** respectively, compared to 17 and 44 for **1**), due to poor

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Table 1. Opioid Receptor Binding Affinities and Selectivities of Various Dyn A Analogues in Guinea Pig Brain Homogenate

	IC_{50} (nM)[a]			selectivity	
analogue of Dyn A(1−11)-NH_2	κ	μ	δ	μ/κ	δ/κ
1, Dyn A(1−11)-NH_2	0.58 ± 0.03	9.9 ± 2.0	25.5 ± 3.4	17	44
2, [D-Ala^3]	0.76 ± 0.28	260 ± 57	1000 ± 422	350	1300
3, [Ala^3]	1.1 ± 0.35	210 ± 40	730 ± 4.5	190	660
4, [D-Ile^3]	55.0 ± 2.1	1030 ± 14	14000 ± 150	19	260
5, [D-Phe^3]	5.90 ± 0.77	86 ± 13	740 ± 190	15	125
6, [Phe^3]	0.89 ± 0.31	27.1 ± 7.2	280 ± 41	30	315
7, [D-Tyr^3]	23.1 ± 4.6	650 ± 180	5600 ± 570	28	240
8, [D-Lys^3]	380 ± 81	1900 ± 220	16000 ± 2300	5	42
9, [Lys^3]	240 ± 36	7400 ± 1400	30%/80000 nM	31	>330
10, [D-Arg^3]	230 ± 69	5100 ± 1300	>60000	22	>260
11, [D-Glu^3]	1100 ± 210	2500 ± 230	13000 ± 3800	2.3	12
12, [Glu^3]	150 ± 20	6600 ± 1300	3040 ± 560	44	20
13, [(2*S*,3*S*)-β-MePhe^3]	121 ± 16	1350 ± 510	1520 ± 76	11	13
14, [(2*S*,3*R*)-β-MePhe^3]	6.0 ± 1.2	112 ± 8	670 ± 51	20	112
15, [(2*S*,2*S*)-β-Me-2′,6′-Me_2-Phe^3]	113 ± 6	2800 ± 510	4490 ± 405	25	40
16, [(2*S*,3*R*)-β-Me-2′,6′-Me_2-Phe^3]	127 ± 27	2350 ± 100	2830 ± 145	19	22
17, [Ile^3]	40 ± 3.8	860 ± 58	nt	22	−
18, [Tyr^3]	8.9 ± 0.55	290 ± 80	>10000	33	>1100

[a] The radioligands used were [^{3}H]U-69,593 (κ receptor), [^{3}H]DAMGO (μ receptor), and [^{3}H]cyclo[D-Pen^2, *p*-Cl-Phe^4, D-Pen^5]enkephalin (δ receptor); nt = not tested.

Chart 1. Structures of Dynorphin A Analogues Synthesized

1. Dyn A(1-11)-NH_2

2. [D-Ala^3] Dyn A(1-11)-NH_2

3. [Ala^3] Dyn A(1-11)-NH_2

4. [D-Ile^3] Dyn A(1-11)-NH_2

5. [D-Phe^3] Dyn A(1-11)-NH_2

6. [Phe^3] Dyn A(1-11)-NH_2

7. [D-Tyr^3] Dyn A(1-11)-NH_2

8. [D-Lys^3] Dyn A[1-11)-NH_2

9. [Lys^3] Dyn A(1-11)-NH_2

10. [D-Arg^3] Dyn A(1-11)-NH_2

11. [D-Glu^3] Dyn A(1-11)-NH_2

12. [Glu^3] Dyn A(1-11)-NH_2

13. [(2S, 3S)-β-MePhe^3]Dyn A(1-11)-NH_2

14. [(2S, 3R)-β-MePhe^3]Dyn A(1-11)-NH_2

15. [(2S, 3S)-β-Me-2', 6'-Me_2-Phe^3]Dyn A(1-11)-NH_2

16. [(2S, 3R)-β-Me-2', 6'-Me_2-Phe^3]Dyn A(1-11)-NH_2

17. [Ile^3] Dyn A(1-11)-NH_2

18. [Tyr^3] Dyn A(1-11)-NH_2

affinities for μ and δ receptors (Table 1). Analogue **2** is one of the most κ receptor selective dynorphin-like peptides reported and can be compared to [*N*-benzyl-Tyr^1, D-Pro^{10}] Dyn A(1−13)-NH_2, a peptide that has been reported to have the highest selectivity for the central κ vs μ and δ receptors ($\kappa/\mu/\delta$ K_i ratio = 1/1070/6080).[17] The binding affinity obtained for analogue **3** differs somewhat from one previously published for [Ala^3]Dyn A(1−13).[18] No satisfactory explanation can be given for this discrepancy except that this latter study used a 1−13 analogue, a different radioligand, and much shorter incubation time in the binding assay (30 vs 180 min). The fact that a higher κ receptor selectivity can be observed with both analogues **2** and **3**, by incorporating the two alanine enantiomers, suggests that an important factor could be related more to the increase in lipophilicity than to a specific orientation of the methyl group of alanine. Another possibility is that replacing an α-helix breaking residue like glycine could increase the α-helical content of the message segment of dynorphin A, which has been postulated to be important for κ-site selectivity.[19,20] To further examine these effects, Dyn A analogues with various substitution at position 3 were synthesized. Substitution with the bulky lipophilic D-Ile lead to the [D-Ile^3]Dyn A(1−11)-NH_2 (**4**) analogue which displays moderate affinity (IC_{50} = 54.8 nM) for the κ receptor and enhanced selectivity for κ vs δ receptors (IC_{50} ratio = 258), compared to **1**. These results suggest that the topographical effect of the β-methyl group of D-Ile or the increased lipophilicity may be biologically important, and similar results with the L-Ile^3 analogue **17** (Table 1) are consistent with this. Further modification with aromatic amino acid led to [D-Phe^3]Dyn A(1−11)-NH_2 (**5**). It displayed good affinity (IC_{50} = 5.91 nM) for the κ receptor, but is only selective for κ vs δ receptors (IC_{50} ratio is 125). Interestingly, the related [Phe^3]Dyn A(1−11)-NH_2 analogue (**6**) displayed high affinity (IC_{50} = 0.89 nM) for the κ receptor and is quite selective for κ vs μ and κ vs δ receptors (IC_{50} ratios are 31 and 319, respectively). These results demostrate that the configuration and the structure of residues at the 3 position of Dyn A can have important effects on the potency and selectivity for the κ receptor. Substitution with D-Tyr and L-Tyr in position 3 was designed to examine the usefulness of the phenolic hydroxyl function. The [D-Tyr^3]Dyn A(1−11)-NH_2 analogue (**7**) displayed a moderate potency (IC_{50} = 23.1 nM) and enhanced selectivities for κ vs μ and κ vs δ receptors (28 and 240, respectively), whereas the [L-Tyr^3]Dyn A(1−11)-NH_2 analogue (**18**) was slightly more potent (IC_{50} = 8.9 nM) and selective (33 and >1100, respectively). To further explore the importance of other functional groups at position 3 of Dyn A, D-Lys, L-Lys, D-Arg, D-Glu, and L-Glu were incorporated into Dyn A to yield analogues **8**, **9**, **10**, **11**, and **12**. Though some of these showed increased selectivities for κ vs μ and/or κ vs δ receptors, all of them displayed poor affinities for the κ receptor (IC_{50} ranged from 150 to 1100 nM). It is concluded that Dyn A(1−11)-NH_2 analogues with lipophilic residues at position 3 (compounds **1**−**7**, **17**, **18**) are more potent and/or selective for the κ receptor than analogues with charged residues at the same

Table 2. Bioassays with the Smooth-Muscle Tissue of the Guinea Pig Ileum and Central (GPB) vs Peripheral (GPI) Nervous System Selectivities at κ Opioid Receptors of Dyn A(1−11)-NH_2 Analogues

analogue of Dyn A(1−11)-NH_2	IC_{50} (nM) GPI	shift[a]	ratio of IC_{50} GPI/GPB
1, Dyn A(1−11)-NH_2	1.0 ± 0.3	ns	1.9
2, [D-Ala^3]	8.1 ± 2.3	ns	11
3, [Ala^3]	1.7 ± 0.2	ns	1.5
4, [D-Ile^3]	5.7% at 60 μM	nt	>1100
5, [D-Phe^3]	370 ± 61	nt	62
6, [Phe^3]	1.1 ± 0.1	ns	1.2
7, [D-Tyr^3]	41.7% at 60 μM	nt	>2600
8, [D-Lys^3]	21000 ± 9300	nt	55
9, [Lys^3]	15.6% at 30 μM	nt	>120
10, [D-Arg^3]	14.3% at 60 μM	nt	>260
11, [D-Glu^3]	5700 ± 950	nt	5
12, [Glu^3]	48% at 30 μM	nt	>200
13, [(2*S*,3*S*)-β-MePhe^3]	2190 ± 720	nt	18
14, [(2*S*,3*R*)-β-MePhe^3]	2800 ± 735	nt	467
15, [(2*S*,3*S*)-β-Me-2′,6′-Me_2-Phe^3]	40800 ± 7400	nt	360
16, [(2*S*3*R*)-β-Me-2′,6′-Me_2-Phe^3]	2800 ± 192	nt	22
17, [Ile^3]	460 ± 180	nt	12
18, [Tyr^3]	220 ± 60	ns	25

[a] nt = not tested; ns = no significant shift observed with 1000 nM of CTAP used as a μ antagonist.

position (**8**−**12**). The interesting activity profile of [Phe^3]-Dyn A(1−11)-NH_2 (**6**, Table 1) suggests that the incorporation of local side chain conformational constraints such as β-substituted phenylalanine derivatives into this analogue should provide further insights into the stereochemical and topographical requirements for high potency and selectivity for the κ receptor. Thus, several constrained amino acids were synthesized[21,22] and incorporated into Dyn A to yield analogues **13**−**16**. They displayed lower affinities (IC_{50} values range from 6.0 to 127 nM) and selectivities for the κ receptor than the related [Phe^3]Dyn A(1−11)-NH_2 analogue. Of all the analogues with constrained amino acids, [(2*S*,3*R*)-β-Me-Phe^3]Dyn A(1−11)-NH_2 (**14**) displayed the highest affinity for the κ receptor (IC_{50} value is 6.0 nM) and is as selective as the [D-Phe^3]Dyn A(1−11)-NH_2 for κ vs μ and κ vs δ receptors (IC_{50} ratios are 20 and 112, respectively). The (2*S*,3*S*)-β-Me-Phe^3 analogue **13** displayed poor potencies for all three receptors and decreased selectivities for κ vs μ and κ vs δ receptors. Analogues **15** and **16** with further 2′,6′-dimethyl substitution in the aromatic ring displayed slightly increased selectivity for κ vs μ receptors (IC_{50} ratios are 25 and 19, respectively), but decreased selectivity for κ vs δ receptors (IC_{50} ratios are 40 and 22, respectively).

Biological Activity in the Guinea Pig Ileum (GPI). The κ (and μ) opioid activities of these peptides were measured by their ability to inhibit the electrically evoked contraction of the guinea pig ileum (GPI) (Table 2),[14−16] and in the case of potent analogues the effect of the μ opioid receptor antagonist CTAP on the IC_{50} value.[23]

The results obtained in the guinea pig ileum (GPI) bioassay show that, as no shift in activity can be observed upon addition of the μ antagonist CTAP, analogues **2** and **3** interact only or specifically with the peripheral κ receptor. It should be noted that all of the analogues appear to be more potent for the central (GPB) than peripheral (GPI) κ opioid receptors, though it is important to point out that the central assays are binding assays whereas the GPI assays are bioassays, and differences in efficacy and other factors could account in part for the differences. It also was found that Dyn A analogues with lipophilic residues at position 3 are more potent than analogues with charged residues at this position at the peripheral κ receptor. Though [D-Ala^3]Dyn A(1−11)-NH_2 and [L-Ala^3]Dyn A(1−11)-NH_2 are still potent at these opioid receptors, they display somewhat lower potencies than the standard **1** (IC_{50} = 8.1 and 1.7 nM for **2** and **3** vs 1.1 nM for **1**). Interestingly, the [Phe^3]Dyn A(1−11)-NH_2 analogue (**6**) is the most potent (IC_{50} = 1.1 nM) of all of the analogues at the peripheral κ receptor, but the related [D-Phe^3]-Dyn A(1−11)-NH_2 (**5**) displayed weak potency (IC_{50} = 370 nM). Interestingly, [D-Ala^3]Dyn A(1−11)-NH_2 was less potent than [L-Ala^3]Dyn A(1−11)-NH_2 (IC_{50} = 8.1 and 1.7 nM, respectively) at the peripheral κ receptor. These results are opposite those measured for binding at the central κ receptor. These results suggest that the sterochemical requirements for high potency and selectivity may be different for the central and peripheral κ receptors. Analogues [D-Ile^3]Dyn A(1−11)-NH_2 (**4**) and [D-Tyr^3]Dyn A(1−11)-NH_2 (**7**), which are potent at the central κ receptor, displayed poor potencies at the peripheral κ receptor, making them very selective for the central vs peripheral κ receptors (Table 2), but interestingly this was not the case for the L-Ile^3 and L-Tyr^3 analogues **17** and **18** (Table 2).

Conclusions

We have found that substitution of Gly^3 with D- and L-alanine in Dyn A(1−11)-NH_2 (analogues **2** and **3**) resulted in a dramatic increase in selectivity for κ receptors, when compared to Dyn A(1−11)-NH_2 itself.[15] This increase may be due to (1) an increase in lipophilicity of the peptide, (2) a possible enhancement of the α-helical content of the message segment of this peptide, (3) a more favorable spatial arrangement of the relative positions of the aromatic residues, and (4) a combination of these different effects. Both analogues are highly interesting and provide us with new lead compounds for further enhancement of potency and selectivity. Nonetheless, since relatively little had been reported previously on the structure−activity relationships involving amino acid residues at position 3 of Dyn A,[18,19] we have substituted Gly^3 with a variety of amino acids in Dyn A(1−11)-NH_2 to examine their structure−activity relationships. Substitution of Gly^3 with Phe^3 resulted in an enhanced selectivities for κ vs μ and κ vs δ receptors relative to dynorphin A(1−11)-NH_2, while substitution of Gly^3 with D-Phe, D-Tyr, and D-Ile only resulted in modestly increased selectivity for κ vs δ receptors. These results suggest that the configurational and/or structural effects of residue at the 3 position of Dyn A played a key role on the κ receptor selectivity and potency. The greatly decreased binding affinity for the κ receptor, caused by the introduction of charged residues in position 3 of Dyn A, suggests that the chemical properties of residues at the 3 position of Dyn A also is important for the potency and/or selectivity at the κ receptor. The high potency and excellent selectivity of [D-Ala^3]Dyn A(1−11)-NH_2 suggests that for further design of novel peptides with high potency, selectivity, and stability at the κ receptor, the 3 position of Dyn A is a promising site for incorporation of global conformational constraints such as a disulfide bridge

or a lactam bridge. The interesting results for [Phe^3]-Dyn A(1−11)-NH_2 and for analogues with β-substituted phenylalanine derivatives also demostrate that the incorporation of a local conformationally constrained amino acids into Dyn A analogues provide further insights into the stereochemical and topographical requirements for high potency and selectivity at the κ receptor. These substitutions should form the basis for further conformational constraints in Dyn A consistent with potent and selective κ receptor binding. Such studies are in progress.

Experiment Section

General Methods for Peptide Synthesis and Purification. Syntheses of dynorphin A analogues **1**−**12**, **17**, and **18** were performed by the solid phase method either manually or utilizing an automated synthesizer (Applied Biosystems Inc. Model 431 A), a Boc/benzyl strategy, and a *p*-methylbenzhydrylamine (*p*-MBHA) resin (Advanced Chem Tech, Louisville, KY), as previously described for the synthesis of other Dyn A analogues.[14−16] Analogues **13**−**16** were synthesized manually, using BOP reagent for the coupling of amino acid residues to the growing peptide-resin. Side chain protected N^{α}-*tert*-butyloxycarbonyl (Boc)-protected amino acids were purchased from Bachem (Torrance, CA), whereas the other unprotected amino acids were converted to their N^{α}-*tert*-butyloxycarbonyl derivatives with di-*tert*-butyl dicarbonate (Bachem California, Torrance, CA) following literature procedures. Side-chain protecting groups used were 2,4-dichlorobenzyloxycarbonyl for Lys, tolylsulfonyl for Arg, 2,6-dichlorobenzyl for Tyr, and benzyl for Glu. Unusual amino acids, (2*S*,3*S*)-β-methylphenylalanine, (2*S*,3*R*)-β-methylphenylalanine, (2*S*,3*S*)-β-Me-2′,6′-Me_2-phenylalanine, and (2*S*,3*R*)-β-Me-2′,6′-Me_2-phenylalanine, were synthesized by methods developed in our laboratory.[21,24] The synthesized analogues were purified by RP-HPLC and characterized by FAB-mass spectrometry and amino acid analysis. The purity of the synthetic peptides was assessed by TLC (single spot in four different solvent systems, ninhydrin detection) and HPLC (one single peak, UV detection at 280 and 225 nm, using two different linear gradients). The structure assignment was corroborated by the results of the amino acid analysis and mass spectrometry, and the purity of the products was characterized by analytical HPLC and TLC (Supporting Information).

Thin-layer chromatography of synthetic peptides was performed on silica gel plates (0.25 mm, Analtech, Newark, DE) with the solvent systems given in the Supporting Information. Peptides were detected with ninhydrin reagent. HPLC was carried out by using of a binary pump (Perkin-Elmer LC 250) equipped with a UV/vis detector (Perkin-Elmer LC 90 UV model) and intergrator (Perkin-Elmer LCI 100 model). The solvent system used for analytical HPLC was a binary system composed of water containing 0.1% of TFA (pH 2.0) and acetonitrile as the organic modifier and solvent programs using the following linear gradients: (1) 10−90% acetonitrile over 40 min and (2) 10−50% over 30 min with flow rates of 2 mL/min for both cases. The column used for analytical chromatography had dimensions of 4.5 × 250 mm (Vydac, 10 μm particle size, C-18). HPLC on a semipreparative scale was performed with a reverse phase column (Vydac, 10 × 250 mm, 10 μm particle size, C-18) employing the solvent system (1) above with a flow rate of 5 mL/min. Mass spectra (fast-atom bombardment, low-resolution full-scan, glycerol matrix) were performed by the Center for Mass Spectrometry, University of Arizona, Tucson, AZ. Hydrolysis of the peptides was performed in 4 N methanesulfonic acid (0.2% 3-(2-aminoethyl)-indole) at 110 °C for 24 h, and amino acids were analyzed with an automatic analyzer (Beckman Instruments, Model 7300). These results are reported in Supporting Information.

Solid Phase Peptide Synthesis of Protected Dyn A (1−11)-NH_2-Resin. *p*-Methylbenzhydrylamine resin (0.98 g, 0.5 equiv) was linked with N^{α}-Boc-N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)lysine via its *N*-hydroxybenzotriazole (HOBt) active ester. The following N^{α}-Boc amino acids were sequentially coupled to the growing peptide chain: N^{α}-Boc-Pro, N^{α}-Boc-N^{g}-tosyl-Arg, N^{α}-Boc-Ile, N^{α}-Boc-N^{g}-tosyl-Arg, N^{α}-Boc-N^{g}-tosyl-Arg, N^{α}-Boc-Leu, N^{α}-Boc-Phe, N^{α}-Boc-Gly, N^{α}-Boc-Gly, N^{α}-Boc-*O*-(2,4-dichlorobenzyl)-Tyr. All the N^{α}-Boc amino acids (4 equiv) were coupled to the growing peptide chain by using diisopropylcarbodiimide (DIC, 2.5 equiv) and *N*-hydroxybenzotriazole (HOBt, 2.5 equiv) in *N*-methyl-2-pyrrolidinone (NMP), and the coupling reaction time was 1 h. Trifluoroacetic acid (TFA) was used to remove the Boc group. Diisopropylethylamine (DIEA) was used as a base, and dichloromethane (DCM) and NMP were used as solvents for washing. After deprotection of the last N^{α}-Boc group, the peptide-resin was dried *in vacuo* to yield the protected Dyn A(1−11)-NH_2-resin.

Cleavage of the Peptide Dyn A(1−11)-NH_2 (1) from the Support. The protected peptide-resin was treated with liquid anhydrous hydrofluoric acid (HF) (10 mL/g of resin) in the presence of cresol (10% w/v) for 1 h at 0 °C. After removal of the HF *in vacuo*, the residue was washed three times with anhydrous ether and extracted with 30% aqueous acetic acid (3 × 50 mL). The acetic acid solutions was then evaporated and lyophilized to give the white residue. The crude peptide was purified by semipreparative reverse phase HPLC under the mentioned condition to yield a white powder after lyophilization. The purity of the the product was characterized by analytical HPLC and TLC. The structure assignment was corroborated by the results of the amino acid analysis and mass spectrometry.

Synthesis of Peptide Analogues 2−12, 17, and 18. The crude peptides **2**−**12** were synthesized and purified in a manner similar to that employed for compound **1**. After purification, the final product was obtained as a white powder. The purity of the the product was characterized by analytical HPLC and TLC. The structure assignment was corroborated by the results of the amino acid analysis and mass spectrometry.

Synthesis of Peptide Analogues 13−16. The crude peptides were synthesized and purified in a manner similar to that employed for compound **1**, except that each of the unusual amino acid N^{α}-Boc-β-MePhe derivatives (1.1 equiv) was added to the growing peptide chain using BOP reagent (2 equiv) and DIEA (4 equiv) in NMP as coupling reagents. The purity of the the product was characterized by analytical HPLC and TLC. The structure assignment was corroborated by the results of the amino acid analysis and mass spectrometry.

Synthesis of Peptide Analogues 17 and 18. The peptide-resins were synthesized on an Applied Biosystems 433A automated peptide synthesizer using FastMoc synthesis chemistry on a 0.1 mmol scale. The peptides were cleaved from the resin with 95% TFA/2.5% ethanedithiol/2.5% water and then purified in a manner similar to that employed for compound **1**. The purity of the the product was characterized by analytical HPLC and TLC. The structure assignment was corroborated by the results of the amino acid analysis and mass spectrometry.

Radioligand Binding Assay. Radioligand displacement binding assays were performed using membranes prepared from whole brains taken from adult male guinea pigs (200−400 g) obtained from SASCO using methods previously reported in detail.[14] The radioligands used were [^{3}H]cyclo[D-Pen^2, *p*-Cl-Phe^4, D-Pen^5]enkephlin (δ receptor) at a concentration of 0.75 nM, [^{3}H]DAMGO (μ receptor) at a concentration of 1.0 nM, and [^{3}H]U-69,593 (κ receptor) at a concentration of 1.5 nM (all obtained from New England Nuclear, Boston, MA).

Binding data was analyzed by 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. Data best fitted by a one-site model were analyzed using the logistic equation. Data obtained at least three independent measurements are presented as the arithmetic mean ± SEM.

***In Vitro* GPI Bioassay.** Electrically induced smooth muscle contraction of strips of guinea pig ileum longitudinal muscle−myenteric plexus were used for the bioassay following methods previously reported in detail elsewhere. IC_{50} values,

relative potency estimates, and their associated standard errors were determined by fitting the mean data to the Hill equation by a computerized nonlinear least-square method.

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Supporting Information Available: Tables containing the HPLC solvents and R_f values, HPLC solvents and k' values, FAB-MS data, and amino acid analysis data for all new compounds (3 pages). Ordering information is given on any current masthead page.

References

(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC−IUB Commission on Nomenclature (*J. Biol. Chem*. **1972**, *247*, 977−983). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations: Dyn A, dynorphin A; GPB, guinea pig brain; GPI, guinea pig ileum; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; CTAP, cyclo[D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2].

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