[Pro^3]Dyn A(1−11)-NH$_2$: A Dynorphin Analogue with High Selectivity for the κ Opioid Receptor[1]

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A proline scan at positions 2 and 3 of the opioid peptide dynorphin A(1−11)-NH$_2$ led to the discovery of the analogue [Pro^3]Dyn A(1−11)-NH$_2$. This analogue possesses high affinity and selectivity for the κ opioid receptor ($K_i(\kappa)$ = 2.7 nM, K_i ratio $\kappa/\mu/\delta$ = 1/2110/3260). The gain in selectivity is achieved through an overall reduction of opioid receptor affinity which is most pronounced at μ and δ receptors. The Pro^3 analogue exhibits antagonist properties. Despite its high κ affinity, [Pro^3]Dyn A(1−11)-NH$_2$ is a relatively weak antagonist in both the [^{35}S]GTPγS assay (IC_{50} = 380 nM) and the guinea pig ileum assay (K_e = 244 nM). Discrepancies between GPI and binding assay have often been ascribed to differential κ receptor subtypes prevailing in central vs peripheral neurons. Since the [^{35}S]GTPγS assay uses the same membrane preparations as the binding assay, differential κ subtypes can be ruled out as an explanation in this case, and the observed behavior rather seems to reflect an intrinsic property of the ligand.

Introduction

The effects of opioids are mediated by at least three distinct receptors: μ, the primary receptor of morphine; δ, targeted by enkephalin; and κ, the primary target of dynorphin.[2] There is considerable interest in selective ligands for the κ opioid receptor. While κ agonists show some promise as nonaddictive analgesics,[3] κ antagonists might be useful in the treatment of addiction.[4]

The heptadecapeptide dynorphin A (Dyn A(1−17): H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH) is a potent peptide opioid[5−7] which exhibits high binding affinity for the κ receptor.[8] It is poorly selective because it also binds well to μ and δ opioid receptors. Truncation of the last six amino acids does not cause a significant loss in activity or selectivity.[9] Dyn A(1−11)-NH$_2$ is therefore widely used as a lead compound for structure−activity studies.[10,11] Goldstein et al. suggested that the active portion of dynorphin consists of a "message sequence" (residues 1−4), which is necessary but not sufficient for potent and selective κ binding, and a potency enhancing "address sequence" (residues 5−13).[9] The most potent and selective peptidic κ ligands known to date, N^1-alkylated dynorphin analogues[12,13] and [D-Ala^3]Dyn A(1−11)-NH$_2$,[14,15] contain modifications in the message sequence, demonstrating a great potential for modifications in this region. Following an approach devised by Marshall,[16] we hypothesized that the effect of the D-Ala substitution in the 3-position may be conformational, possibly inducing a turn about residues 2 and 3. To test this hypothesis, we prepared a series of analogues containing Pro and D-Pro at these positions. Herein, we report the results of this study, which led to a highly κ-selective analogue.

Results and Discussion

Opioid Receptor Binding Affinities. The peptides were evaluated for their binding affinities at κ, μ, and δ opioid receptors by measuring the inhibition of binding of [^{3}H]diprenorphine to cloned human opioid receptors expressed in CHO cell membranes (Table 1). As this table shows, proline substitution at position 2 or 3 reduces affinity to all opioid receptors, particularly to μ and δ receptors, for which micromolar affinities are obtained throughout the series. Although its κ affinity is reduced 36-fold relative to that of the parent compound, the analogue [Pro^3]Dyn A(1−11)-NH$_2$ retains a high κ affinity (K_i = 2.7 nM) and is one of the most κ-selective peptide ligands reported to date. Using a different type of reference ligand ([^{3}H]U69,593), a comparably high κ affinity (K_i = 7.5 nM) was found for this compound.

In the case of the other analogues, κ affinity is weaker and there is no gain in selectivity. Interestingly, the D-Pro^3 analogue is the weakest ligand in this series ($K_i(\kappa)$ = 160 nM). In proline, the dihedral angle φ is constrained to −65 ± 15°, causing a bent conformation of the peptide backbone.[17] This is obviously much better tolerated than the +65 ± 15° which would be expected for the corresponding D-Pro analogue. Apart from this known constraint, our NMR studies of Dyn A(1−11)-NH$_2$ and its proline analogues did not detect signs of a well-defined secondary structure.[18]

While the configuration at position 3 has a profound influence on κ affinity, the κ selectivities and affinities of the Pro^2 and D-Pro^2 analogues are very similar to each other. This differs from the results obtained for other residues, where D-amino acids in position 2 led to dual

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Table 1. Opioid Receptor Binding Affinities and Selectivities of Dynorphin Analogues in CHO Cell Membranes

	K_i (nM)[a]			K_i ratio
compd	κ	μ	δ	$\kappa/\mu/\delta$
Dyn A(1−11)-NH_2	0.074 ± 0.012 (n = 15)	7.6 ± 1.5 (n = 11)	2.9 ± 0.44 (n = 10)	1/103/39
[Pro³] analogue[b]	2.7 ± 0.33 (n = 9)	5700 ± 860 (n = 7)	8800 ± 2100 (n = 5)	1/2110/3260
[D-Pro³] analogue	160 ± 28 (n = 7)	8300 ± 1500 (n = 8)	7100 ± 200 (n = 3)	1/52/44
[Pro²] analogue	94 ± 18 (n = 5)	3200 ± 390 (n = 5)	6800 ± 350 (n = 3)	1/34/72
[D-Pro²] analogue	65 ± 8.7 (n = 7)	1700 ± 160 (n = 4)	3900 ± 300 (n = 4)	1/26/60
[D-Ala³] analogue	0.15 ± 0.01 (n = 7)	67 ± 6.0 (n = 5)	407 ± 24 (n = 6)	1/450/2710
nor-BNI	0.36 ± 0.024 (n = 5)	56 ± 4.6 (n = 8)	19 ± 3.7 (n = 7)	1/155/53

[a] K_i values are given ±SEM with number of determinations in parentheses. Reference ligand: [³H]diprenorphine. [b] With reference ligand [³H]U69,593: $K_i(\kappa)$ = 7.5 ± 2.3 nM (n = 4).

Table 2. Opioid Agonist or Antagonist Properties of Dynorphin Analogues in the [³⁵S]GTPγS Binding Assay[a]

			IC_{50} (nM)	
compd	EC_{50} (nM)	max stim[b] (%)	U50,488[c]	[D-Ala³]Dyn A(1−11)-NH_2[d]
U50,488	14.4 ± 2.2 (n = 37)	100		
nor-BNI			4.6 ± 0.59 (n = 15)	4.7 ± 0.80 (n = 7)
Dyn A(1−11)-NH_2	0.74 ± 0.16 (n = 6)	73 ± 9 (n = 3)		
[D-Ala³] analogue	6.1 ± 1.5 (n = 7)	87 ± 3 (n = 3)		
[Pro³] analogue		16 ± 9 (n = 3)	380 ± 54 (n = 16)	1400 ± 170 (n = 13)
[D-Pro³] analogue		20 ± 12 (n = 4)	2200 ± 1300 (n = 4)	
[Pro²] analogue		11 ± 9 (n = 4)	760 ± 220 (n = 5)	
[D-Pro²] analogue		30 ± 25 (n = 3)	940 ± 470 (n = 5)	

[a] Values are given ±SEM, with numbers of determinations in parentheses. [b] Maximum agonist response at c = 10 μM, relative to that obtained with standard agonist U50,488. (The stimulation achieved by U50,488 is set as 100%.) [c] Antagonist activity determined against 50 nM U50,488. [d] Antagonist activity determined against 25 nM [D-Ala³]Dyn A(1−11)-NH_2.

high affinity for κ and μ receptors.[19] Interestingly, these proline analogues have a very low affinity for the μ receptor, though their message sequence is structurally related to endomorphins I and II (Tyr-Pro-X-Phe-NH_2, X = Phe or Trp), highly potent and selective μ ligands.[20]

For comparison with our functional assays, we also determined the binding profile of the known compounds [D-Ala³]Dyn A(1−11)-NH_2, developed by Hruby et al.,[15] and nor-binaltorphimine (nor-BNI), developed by Portoghese et al.[21] Though our assay conditions differ considerably from those in ref 15, our results for [D-Ala³]-Dyn A(1−11)-NH_2 are in good agreement with the published data.

Functional Assays. 1. Receptor-Mediated [³⁵S]-GTPγS Binding Assays. Initial functional characterization of our analogues was performed by determining κ receptor-mediated [³⁵S]GTPγS binding[22] in the same membrane preparations that were used to measure [³H]-diprenorphine binding to the κ receptor. This assay detects the interaction of receptor with G-protein and the subsequent exchange of GTP for GDP bound to the G-protein. Stimulation of [³⁵S]GTPγS binding in the presence of a series of concentrations of agonist is used to measure potency and efficacy of the agonist.

The peptides Dyn A (1−11)-NH_2 and [D-Ala³]Dyn A (1−11)-NH_2 stimulated [³⁵S]GTPγS binding to membranes obtained from CHO cells expressing the human κ opioid receptor to a comparable extent (73% and 87%, respectively) as the well-characterized κ agonist U50,-488. (The stimulation achieved by U50,488 is set as 100% here.) The EC_{50} values were 14.4 nM for U50,-488, 0.74 nM for Dyn A (1−11)-NH_2, and 6.1 nM for [D-Ala³]Dyn A (1−11)-NH_2 (Table 2). The analogues of Dyn A (1−11)-NH_2 with proline in position 2 or 3 stimulated [³⁵S]GTPγS binding only marginally, implying that they are not agonists at the κ receptor. Since binding data indicated that these compounds had relatively high affinity for the κ receptor, we evaluated the proline-containing analogues for antagonist activity.

The proline analogues were assayed in the presence of 50 nM U50,488, a concentration that stimulates [³⁵S]-GTPγS binding by 80% of the maximal stimulation (EC_{80}). In this assay, the standard κ receptor antagonist nor-BNI inhibited the κ receptor mediated stimulation of [³⁵S]GTPγS binding to basal levels with an IC_{50} value of 4.6 nM. The proline analogues were found to be less potent antagonists in this assay. The [D-Pro²], [Pro²], [D-Pro³], and [Pro³] analogues inhibited U50,488-stimulated [³⁵S]GTPγS binding with IC_{50} values of 940, 760, 2200, and 380 nM, respectively. This rank order of potency is nearly the same as that obtained in the binding assay. Again, the 2-substituted analogues are very similar to each other, while the Pro³ and D-Pro³ analogues are the strongest and weakest member of the series, respectively. For most of the antagonists tested, including nor-BNI, the IC_{50} values obtained in this assay are by a factor of 8−14 larger than the corresponding K_i values obtained in the binding assay. The notable exception is the Pro³ analogue. Its IC_{50} is 141 times larger, indicating surprisingly weak antagonist potency in view of its relatively high κ affinity.

Studies with chimeric receptors and point mutations have shown that U50,488 and dynorphin depend on different binding epitopes. Particularly, U50,488 does not seem to bind to an extracellular loop of the κ receptor which is essential for dynorphin binding.[23] Assuming that different binding epitopes might account for the weak antagonism observed, we tested the Pro³ analogue against the structurally similar agonist [D-Ala³]Dyn A(1−11)-NH_2 in the GTPγS assay. Interestingly, using this reference ligand even weaker antagonism (IC_{50} = 1400 nM) was observed. Furthermore, the K_i of 7.5 nM obtained in the receptor binding assay against [³H]U69,-593 demonstrates that the Pro³ analogue can displace arylacetamide ligands effectively. Thus, different binding epitopes do not seem to account for our observations. The standard κ antagonist nor-BNI is equally potent

Table 3. Opioid Agonist or Antagonist Properties of Dynorphin Analogues in the GPI Assay[a]

		K_e (nM)	
compd	IC_{50} (nM)	U50,488[b]	[D-Ala^3]Dyn A-(1−11)-NH_2[c]
Dyn A(1−11)-NH_2	0.376 ± 0.071		
[D-Ala^3]Dyn A-(1−11)-NH_2	2.38 ± 0.44		
nor-BNI[d]		0.357 ± 0.056	0.572 ± 0.036
[Pro^3]Dyn A-(1−11)-NH_2[e]		244 ± 51	494 ± 74

[a] Mean of 3 determinations ± SEM. [b] Antagonist activity, determined against U50,488. [c] Antagonist activity, determined against [D-Ala^3]Dyn A(1−11)-NH_2. [d] $c = 1$ nM. [e] $c = 1$ μM.

against both U50,488 and [D-Ala^3]Dyn A(1−11)-NH_2 in the GTPγS assay.

Recent studies of μ antagonists led to similar observations.[24] Large differences in the ratios of the K_i's found in binding assay and [^{35}S]GTPγS assay (K_i(binding)/K_i([^{35}S]GTPγS)) were observed for several known antagonists (e.g. naloxone: 277, diprenorphine: 0.2). The authors suggest that each of the antagonists achieves 50% inhibition in the [^{35}S]GTPγS assay at a different level of receptor occupancy.

2. Biological Activity in the Guinea Pig Ileum (GPI) Assay. The GPI bioassay[25] was performed as reported in detail elsewhere.[26] The analogue [Pro^3]Dyn A(1−11)-NH_2 did not inhibit the electrically evoked contractions in the GPI assay at concentrations up to 10 μM. It was subsequently tested for antagonist properties. The K_e values were determined from the IC_{50} values of the reference agonists ([D-Ala^3]Dyn A(1−11)-NH_2 or U50,488) in the presence and absence of a fixed concentration of antagonist (nor-BNI or [Pro^3]Dyn A(1−11)-NH_2).[27] The results are given in Table 3.

In this assay, K_e values of 0.357 nM against U50,488 and 0.572 nM against [D-Ala^3]Dyn A(1−11)-NH_2 are obtained for the standard κ antagonist nor-BNI. Consistent with the results obtained in the GTPγS assay, [Pro^3]Dyn A(1−11)-NH_2 is a weak antagonist in the GPI assay. Again, the K_e values of 244 nM against U50,488 and 494 nM against [D-Ala^3]Dyn A(1−11)-NH_2 show a somewhat lower antagonist activity against the peptide agonist.

Low activity in the GPI assay despite strong binding has been observed for a number of dynorphin analogues (e.g. see refs 10−12, 15, 19). The analogue *N*-benzyl[D-Pro^{10}]Dyn A(1−11) displays subnanomolar affinity in κ-receptor binding but an IC_{50} of 990 nM in the guinea pig ileum assay.[12a,b] This compound was later found to be a partial κ agonist.[12c] Kawasaki et al.[11] report that analogues of Dyn A (1−11)-NH_2 with substitutions at a variety of positions have IC_{50} values in the GPI assay that are 20−63000-fold higher than their IC_{50} values determined in the receptor binding assay using guinea pig brain membranes. Similar observations have also been made for cyclic dynorphin analogues.[10] Although the analogue [D-Ile^3]Dyn A(1−11)-NH_2 is closely related to the D-Ala^3 analogue and has a relatively high κ affinity (K_i = 55 nM), it does not show significant agonist properties in the GPI assay (5.7% at 60 μM).[15] These results suggest that small residues in position 3 are required to achieve both high affinity *and* high agonist potency. As compared to the corresponding L-amino acids, lipophilic D-amino acids can lead to reduced κ affinity and markedly reduced potency in the GPI assay.[15] This is consistent with the results for our Pro analogues.

In the past, discrepancies between binding assay and GPI assay have been ascribed to putative differences between central and peripheral κ receptors or to specific requirements for the opioid signal transduction mechanism.[11] However, our data for both receptor binding and the [^{35}S]GTPγS functional assay were obtained using the same cloned receptor in the same membrane preparations. Thus, different subtypes cannot be the explanation of our results.

The cloned receptor expressed in our system was originally obtained from a peripheral source (human placenta).[28] Its protein sequence differs from the κ receptor cloned from human brain[29] only in a Glu^2 residue in place of an Asp^2 residue. A conservative change in this location would not be expected to have any effect on ligand binding. Furthermore, the antagonist potency of [Pro^3]Dyn A(1−11)-NH_2 in the [^{35}S]-GTPγS binding assay agrees well with its potency in the guinea pig ileum assay, in contrast to the results obtained in the receptor binding assay. These discrepancies seem to reflect an intrinsic property of [Pro^3]Dyn A(1−11)-NH_2 rather than an effect that can be traced to an assay property such as a particular receptor subtype or reference ligand. Possibly, the incorporation of proline in position 3 causes adverse steric interactions with the receptor, which compromise functional response much more strongly than receptor binding, i.e. by prevention of a conformational change which the receptor−ligand complex has to undergo in the course of signal transduction.

Conclusion

Systematic substitution at positions 2 and 3 of Dyn A(1−11)-NH_2 with Pro and D-Pro led to the discovery of [Pro^3]Dyn A(1−11)-NH_2 as one of the most selective peptide ligands known for the κ opioid receptor. In this analogue, incorporation of proline reduced binding affinity to μ and δ opioid receptors to micromolar values, while κ binding was reduced much less severely. With $K_i(\kappa) = 2.7$ nM, the Pro^3 analogue retained a higher κ affinity than the other members of this series, particularly the D-Pro^3 analogue. This observation demonstrates the importance of stereochemical factors in this region for κ opioid receptor binding.

The GTPγS assay data demonstrate that all the new analogues are κ antagonists. However, the observed antagonist potency of the Pro^3 analogue is surprisingly low as compared to its binding affinity. For this analogue, weak antagonism was also observed in the GPI assay. Since the same membrane preparations were used for both radioligand binding and GTPγS assays, it is suggested that the observed behavior reflects an intrinsic property of this ligand and that differential κ subtypes are not involved in this case. A better understanding of these effects would be helpful for the development of potent new drugs. The analogues presented here are useful leads for the discovery of new κ-selective ligands. Studies with analogous structures are underway in our laboratory and will be reported soon.

Experimental Section

General Procedures for Peptide Synthesis and HPLC. The peptides were synthesized on solid support using the Fmoc method. Syntheses were carried out on a 0.1-mmol scale using an Applied Biosystems 433a synthesizer (FastMoc synthesis). Side chains were protected using *tert*-butyl (^{t}Bu) for Tyr, (2,2,5,7,8-pentamethylchroman-6-yl)sulfonyl (Pmc) for Arg, and Boc for Lys. All coupling reactions were carried out using HOBt/HBTU with 1 h of coupling time and subsequent capping with acetic anhydride. Protected amino acids were purchased from Novabiochem (San Diego). Fmoc-PAL-PEG-PS (PerSeptive Biosystems, Hamburg, Germany) was used as the solid support. Cleavage and deprotection were carried out using reagent B (88% TFA, 5% H_2O, 5% phenol, 2% triisopropylsilane) as described by Barany et al.,[30] using 90 min of cleavage time and ether extraction for workup. The aqueous solution of crude material so obtained was concentrated and lyophilized to give a yellow-white powder. The peptides were purified and analyzed by RP-HPLC using Vydac Protein Peptide C_{18} columns. Column dimensions were 4.5 × 250 mm (90 Å silica, 5 μm) for analytical and 22 × 250 mm (90 Å silica, 10 μm) for preparative HPLC. A binary system of water and acetonitrile, both containing 0.1% TFA was used throughout. Analytical HPLC was carried out on a Waters Millenium PDA system using a linear gradient of 10−50% acetonitrile over 30 min at 1 mL/min flow rate. The results are given in the Supporting Information. Preparative HPLC was carried out at 8 mL/min flow rate using the same gradient. The material so obtained was further purified by isocratic elution using 23% acetonitrile in the aforementioned system. UV detection at 220 nm was used throughout.

Other Analytical Methods. Fast atom bombardment (FAB) positive ion mass spectra were obtained on a VG ZAB-VSE double focusing high-resolution mass spectrometer using 3-nitrobenzyl alcohol/NaI as a matrix. The results are given in the Supporting Information.

NMR experiments were carried out on a Bruker AMX 500 spectrometer at the Department of Chemistry and Biochemistry, UC San Diego. The structure of the peptides was confirmed and all residues assigned using DQF-COSY, ROESY and TOCSY experiments. Tables with detailed results are available as Supporting Information.

Hydrolyses of peptides were performed under argon in conical reaction vials (Wheaton) containing 6 M HCl for 60 min at 160 °C. The resulting hydrolysates were analyzed using a modular HPLC system composed of a Spectra-Physics model SP8875 autosampler, a Spectra-Physics model SP8800 ternary pump, a Spectra-Physics model SP8792 column heater, a Thermo Separation Products model UV2000 absorbance detector, and a Spectra-Physics ChromJet printer−plotter−integrator. This system was equipped for postcolumn ninydrin derivatization by Pickering Laboratories, and the chromatographic separation of the amino acids was accomplished using a Pickering Laboratories sodium cation-exchange column (3 mm × 250 mm) and a sodium citrate buffer system. Amino acids were quantified as their ninhydrin derivatives, and norleucine was used as an internal standard. Absorbance detection was performed at the wavelengths of 470 and 570 nm. The results are given in the Supporting Information.

Radioligand Binding Assay. 1. Preparation of Cell Membranes Expressing Opioid Receptors. This method is a modification of the method of Raynor et al.[31] CHO cells stably expressing cloned human μ, δ and κ receptors were harvested by scraping from the culture flasks, centrifuging at 1000*g* for 10 min, resuspending in assay buffer (50 mM tris(hydroxymethyl)aminomethane HCl, pH 7.8, 1.0 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA-free acid), 5.0 mM $MgCl_2$, 10 mg/L leupeptin, 10 mg/L pepstatin A, 200 mg/L bacitracin, 0.5 mg/L aprotinin) and centrifuged again. The resulting pellet was resuspended in assay buffer and homogenized with a Polytron homogenizer (Brinkmann, Westbury, NY) for 30 s at a setting of 1. The homogenate was centrifuged at 48000*g* for 10 min at 4 °C and the pellet resuspended at 1 mg protein/mL of assay buffer and stored at −80 °C until use.

2. Radioligand Binding to μ, δ, and κ Opioid Receptors. After dilution in assay buffer and homogenization as before, membrane proteins (50−100 μg) in 250 μL of assay buffer were added to mixtures containing test compound and radioligand (1.0 nM, 40 000−45 000 dpm) in 250 μL of assay buffer in 96-well deep-well polystyrene titer plates (Beckman) and incubated at room temperature for 60 min ([^{3}H]diprenorphine). The incubation time for [^{3}H]U69,593 was 90 min. Reactions were terminated by vacuum filtration with a Brandel MPXR-96T Harvester through GF/B filters that had been pretreated with a solution of 0.5% polyethylenimine and 0.1% bovine serum albumin for at least 1 h. The filter-bottom plates were washed 4 times with 1.0 mL each of ice-cold 50 mM Tris-HCl, pH 7.8, 30 μL of Microscint-20 (Packard Instrument Co., Meriden, CT) was added to each filter, and radioactivity on the filters was determined by scintillation spectrometry in a Packard TopCount.

[^{3}H]Diprenorphine was purchased from Amersham Life Science, Inc. (Arlington Heights, IL) and had a specific activity of 39−45 Ci/mmol. K_D values for diprenorphine binding are 0.33 nM for κ and μ receptors and 0.26 nM for δ receptors. Preliminary experiments were performed to show that no specific binding was lost during the wash of the filters, that binding achieved equilibrium within the incubation time and remained at equilibrium for at least an additional 60 min, and that binding was linear with regard to protein concentration. Nonspecific binding, determined in the presence of 10 μM unlabeled naloxone, was less than 10% of total binding.

The data from competition experiments were fit by nonlinear regression analysis with the program Prism[32] using the four-parameter equation for one-site competition and subsequently calculating K_i from EC_{50} by the Cheng−Prusoff equation.

Assays using the radioligand [^{3}H]U69,593 (specific activity 54 Ci/mmol) were carried out in the same way. Concentration of this radioligand in the assay was 1.0 nM; K_D for κ receptors: 1.2 nM; incubation time: 90 min.

3. Receptor-Mediated [^{35}S]GTPγS Binding. Assays contained 80−100 pM [^{35}S]GTPγS, 30 μM GDP, 100 mM NaCl, 0.2 mM EGTA, 5.0 mM $MgCl_2$, 1.1 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 200 μg/mL bacitracin, 0.5 μg/mL aprotinin, 50−150 μg of CHO cell membrane protein prepared as described above, and test compounds as appropriate in a final total volume of 0.5 mL of 50 mM Tris HCl, pH 7.8. After incubation at 30 °C for 1 h, the samples were filtered through GF/B filters that had been presoaked in 1.0% bovine serum albumin and the filters rinsed 4 times with 1 mL of ice-cold 50 mM Tris HCl, pH 7.8. The filters were placed in 7-mL scintillation vials, 3.5 mL of ReadySafe (Beckman, Fullerton, CA) was added, and radioactivity on the filters was determined by scintillation spectrometry.

Agonist potency and efficacy was assessed by measuring stimulation of [^{35}S]GTPγS binding by a series of concentrations of agonist. The concentration to give half-maximal stimulation (EC_{50}) was determined by nonlinear regression using the program Prism.[32] To obtain an IC_{50} value, antagonist was titrated in the presence of sufficient agonist to give 80% of its maximal stimulation and the data were analyzed by nonlinear regression fit using the program Prism.[32]

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Supporting Information Available: Tables containing NMR chemical shifts, coupling constants, and temperature coefficients for Dyn A(1−11)-NH_2 and the new analogues; description and summary of NMR experiments; and tables containing analytical data (HPLC, MS, amino acid analyses).

This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC−IUB Commission on Nomenclature (*Biochem. J.* **1984**, *219*, 345−373). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations: Boc, *tert*-butoxycarbonyl; CHO, Chinese hamster ovary; Dyn A, dynorphin A; Fmoc, 9-fluorenylmethyloxycarbonyl; GPI, guinea pig ileum; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxy-1*H*-benzotriazole; HPLC, high-performance liquid chromatography; nor-BNI, nor-binaltorphimine; PAL, peptide amide linker; PEG, poly(ethylene glycol); PS, polystyrene; RP, reverse-phase; SEM, standard error of the mean; ^{t}Bu, *tert*-butyl; TFA, trifluoroacetic acid.

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