Expedited Articles

[2′,6′-Dimethyltyrosine]Dynorphin A(1−11)-NH_2 Analogues Lacking an N-Terminal Amino Group: Potent and Selective κ Opioid Antagonists

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Recent studies showed that dermorphin and enkephalin analogues containing two methyl groups at the 2′,6′-positions of the Tyr[1] aromatic ring and lacking an N-terminal amino group were moderately potent δ and μ opioid antagonists. These results indicate that a positively charged N-terminal amino group may be essential for signal transduction but not for receptor binding and suggested that its deletion in agonist opioid peptides containing an N-terminal 2′,6′-dimethyltyrosine (Dmt) residue may represent a general way to convert them into antagonists. In an attempt to develop dynorphin A (Dyn A)-derived κ opioid antagonists, we prepared analogues of [Dmt[1]]Dyn A(1−11)-NH_2 (**1**), in which the N-terminal amino group was either omitted or replaced with a methyl group. This was achieved by replacement of Tyr[1] with 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp) or (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid [(2*S*)-Mdp]. Compounds were tested in the guinea pig ileum and mouse vas deferens bioassays and in rat and guinea pig brain membrane receptor binding assays. All analogues turned out to be potent κ antagonists against Dyn A(1−13) and the non-peptide agonist U50,488 and showed only weak μ and δ antagonist activity. The most potent and most selective κ antagonist of the series was [(2*S*)-Mdp[1]]Dyn A(1−11)-NH_2 (**5**, dynantin), which showed subnanomolar κ antagonist potency against Dyn A(1−13) and very high κ selectivity both in terms of its K_e values determined against κ, μ, and δ agonists and in terms of its ratios of κ, μ, and δ receptor binding affinity constants. Dynantin is the first potent and selective Dyn A-derived κ antagonist known and may complement the non-peptide κ antagonists norbinaltorphimine and GNTI as a pharmacological tool in opioid research.

Introduction[1]

Dynorphin A (Dyn A)[2] is the putative endogenous ligand for κ opioid receptors. However, it is relatively nonselective since it also binds to μ and δ opioid receptors with quite high affinity. Systematic truncation of Dyn A at the C-terminus revealed that the shorter 13-peptide Dyn A(1−13) and 11-peptide Dyn A(1−11) and their corresponding analogues with a C-terminal carboxamide function have an in vitro activity profile similar to that of the parent 17-peptide.[3−5] Therefore, these shorter Dyn A peptides are often used as parent structures for structure−activity studies of Dyn A. Numerous structure−activity studies have been performed during the past two decades in an effort to develop Dyn A analogues that would have improved κ receptor selectivity. These efforts led to the identification of Dyn A analogues that retained potent κ agonist activity and showed much improved κ receptor selectivity[6,7] (for a recent review, see ref 8).

On the other hand, the development of Dyn A analogues with κ antagonist properties has met with only limited success so far. Several Dyn A-derived κ opioid antagonists have been described,[5,6,9−15] but none of them showed both high κ antagonist potency and high κ receptor selectivity. Very recently, the Pro[3]-analogue of Dyn A(1−11)-NH_2 was reported to have high κ receptor binding affinity and κ selectivity.[16] Surprisingly, however, this compound showed only modest κ antagonist activity in functional assays. Thus, it is obvious that a Dyn A analogue with high κ antagonist activity and κ receptor selectivity remains yet to be developed. Among various non-peptide κ antagonists reported to date, norbinaltorphimine (norBNI)[17] and GNTI[18] display high κ antagonist activity and κ selectivity. A κ-selective Dyn A-derived antagonist would complement norBNI and GNTI as a pharmacological tool to study the role of Dyn A in various physiological and pharmacological processes that are mediated by κ receptors.

Des-amino analogues of enkephalins that contain an N-terminal tyrosine residue have been reported to have no affinity for opioid receptors[19] and no opioid agonist or antagonist activity in the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays.[20] In an effort to reexamine the role of the positively charged N-terminal amino group of opioid peptides in the interaction with

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Table 1. Agonist or Partial Agonist Potencies of Dyn A(1−11)-NH_2 Analogues in the GPI and MVD Assays

no.	compd	GPI IC_{50} (nM)[a]	MVD IC_{50} (nM)[a]	MVD/GPI IC_{50} ratio
1	[Dmt[1]]Dyn A(1−11)-NH_2	0.502 ± 0.129	0.377 ± 0.032	0.751
2	[Hpp[1]]Dyn A(1−11)-NH_2	1740 ± 490 (IC_{30})[b]	722 ± 46	0.415
3	Dyn A(1−11)-NH_2	0.456 ± 0.051	12.9 ± 1.0	34.3

[a] Mean of three to six determinations ± SEM. [b] Partial agonist (maximal inhibition of contractions = 60%).

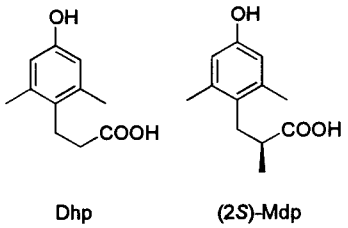

Figure 1. Structural formulas of Dhp and (2*S*)-Mdp.

their receptors, we recently prepared analogues containing an N-terminal 2′,6′-dimethyltyrosine (Dmt) residue in place of Tyr[1], in which the N-terminal amino group was either deleted[21] or replaced with a methyl group.[22] Opioid peptides containing an N-terminal Dmt residue were chosen as parent structures in these studies because the replacement of the Tyr[1] residue in the cyclic enkephalin analogue H-Tyr-c[D-Pen-Gly-Phe-D-Pen]OH (DPDPE) with Dmt had been shown to increase μ and δ receptor binding affinity by at least an order of magnitude,[23] presumably due to an additional binding interaction of the two methyl groups in the 2′,6′-positions. A des-amino analogue of the cyclic β-casomorphin peptide H-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly-], a mixed μ agonist/δ antagonist, was prepared by replacement of Dmt with 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp, Figure 1).[21] The resulting compound turned out to be a μ antagonist/δ antagonist with μ and δ receptor binding affinities in the 100 nM range. Replacement of the N-terminal amino group of the enkephalin analogue H-Dmt-D-Ala-Gly-Phe-Leu-NH_2, a potent μ and δ agonist, with a methyl group was achieved by substitution of Dmt with (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid [(2*S*)-Mdp].[22] The resulting compound turned out to be a quite potent δ opioid antagonist (K_e = 28.1 nM in the MVD assay) and a somewhat less potent μ antagonist. These results indicate that a positively charged N-terminal amino group is not a *conditio sine qua non* for the binding of opioid peptides to δ and μ receptors but may be required for signal transduction. Furthermore, these findings suggest that replacement of the N-terminal tyrosine residue of opioid peptides with Dhp or (2*S*)-Mdp may represent a general way to convert opioid peptide agonists into antagonists. In the present paper we explore the possibility that Dhp[1]- or (2*S*)-Mdp[1]-analogues of Dyn A might have κ opioid antagonist properties. One of the prepared compounds, [(2*S*)-Mdp[1]]-Dyn A(1−11)-NH_2 (**5**), turned out to be the by far most potent, selective Dyn A-derived κ antagonist reported to date.

Chemistry

Dhp was synthesized according to a published scheme.[21] A stereospecific synthesis of (2*S*)-Mdp using Evans' 4-benzyl-2-oxazolidinone chiral auxiliary in the key step for the formation of the stereogenic center was performed as described elsewhere.[22,24] Peptides were synthesized by the solid-phase method using *tert*-butyloxycarbonyl (Boc)-protected amino acids and 1,3-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) as coupling agents. Peptides were cleaved from the resin and completely deprotected by HF/anisole treatment in the usual manner. The final products were purified by preparative reversed-phase HPLC.

Biology

For the determination of their in vitro opioid activities, compounds were tested in bioassays based on inhibition of electrically evoked contractions of the GPI and MVD. The GPI contains μ and κ opioid receptors and, therefore, the GPI assay permits the determination of both μ and κ receptor-mediated agonist or antagonist effects. In the MVD assay, opioid effects are primarily mediated by δ receptors; however, μ and κ receptors also exist in this tissue. κ antagonist activities (K_e values) of compounds were determined in the GPI assay against both Dyn A(1−13) and U50,488 as κ-selective agonists. For the determination of μ antagonist potencies of compounds, their K_e values against the specific μ agonist TAPP (H-Tyr-D-Ala-Phe-Phe-NH_2)[25] were also measured in the GPI assay. K_e values of compounds with δ antagonist properties were determined in the MVD assay against the selective δ agonist DPDPE. Binding affinities of compounds for μ and δ opioid receptors were determined by displacing [^{3}H]DAMGO and [^{3}H]DSLET, respectively, from rat brain membrane binding sites, and κ opioid receptor binding affinities were measured by displacement of [^{3}H]U69,593 from guinea pig brain membrane binding sites.

Results and Discussion

[Dmt[1]]Dyn A(1−11)-NH_2 (**1**) was found to have about the same agonist potency as Dyn A(1−11)-NH_2 (**3**) in the GPI assay (Table 1). This result is in agreement with the receptor binding data (Table 3) which indicate that, in comparison with the parent peptide, the Dmt[1]-analogue has 3.7-fold lower κ affinity but slightly higher μ affinity. On the other hand, [Dmt[1]]Dyn A(1−11)-NH_2 displayed 34-fold higher agonist potency than Dyn A(1−11)-NH_2 in the MVD assay, in good agreement with its 11-fold higher δ receptor binding affinity as compared to the parent peptide. Thus, as in the case of relatively short μ- and δ-selective peptide ligands,[23,26] substitution of the N-terminal Tyr[1] residue with Dmt produced a significant increase in binding affinity and agonist potency at the δ receptor, but not at the κ-receptor.

An analogue of Dyn A(1−11)-NH_2 lacking the N-terminal amino group was prepared by substituting 3-(4-hydroxyphenyl)propanoic acid (Hpp) for Tyr[1]. The resulting compound, [Hpp[1]]Dyn A(1−11)-NH_2 (**2**), turned out to be a weak partial agonist in the GPI assay, capable of producing a maximal inhibition of the electrically evoked contractions of only 60% (Table 1). At a concentration where compound **2** produced its maximal

Table 2. Antagonist Potencies (K_e Values) of Dyn A(1−11)-NH_2 Analogues Determined in the GPI and MVD Assays[a]

		K_e (nM)[b]			K_e (nM)[c]	K_e ratio	
no.	antagonist	Dyn A(1−13)	U50,488	TAPP	DPDPE	$\kappa/\mu/\delta$[d]	$\kappa/\mu/\delta$[e]
4	Dhp[1]]Dyn A(1−11)-NH_2	17.4 ± 2.7	2.96 ± 0.44	445 ± 102	5310 ± 940	1/26/305	1/150/1790
5	[(2*S*)-Mdp[1]]Dyn A(1−11)-NH_2	0.632 ± 0.136	3.92 ± 0.65	925 ± 94	3220 ± 520	1/1460/5090	1/236/820
6	[Dhp[1],D-Ala[3]]Dyn A(1−11)-NH_2	11.6 ± 1.4	8.61 ± 1.06	582 ± 155	PA (32%)[f]	1/50/-	1/68/-
7	[(2*S*)-Mdp[1],D-Ala[3]]Dyn A(1−11)-NH_2	3.31 ± 0.24	4.44 ± 0.14	1970 ± 180	4220 ± 390	1/595/1270	1/444/950
	NorBNI	0.368 ± 0.053	0.213 ± 0.032	26.1 ± 3.1	9.36 ± 1.46	1/71/25	1/123/44

[a] Values represent means of three to six determinations ± SEM. [b] Determined in the GPI assay. [c] Determined in the MVD assay. [d] Determined with Dyn A(1−13) as κ agonist. [e] Determined with U50,488 as κ agonist. [f] Maximal inhibition of contractions = 32%.

Table 3. Binding Affinities of Dyn A(1−11)-NH_2 Analogues at μ, δ, and κ Opioid Receptors

		K_i (nM)[a]			K_i ratio
no.	compd	κ	μ	δ	$\kappa/\mu/\delta$
1	[Dmt[1]]Dyn A(1−11)-NH_2	0.322 ± 0.019	0.435 ± 0.057	1.18 ± 0.07	1/1/4
2	[Hpp[1]]Dyn A(1−11)-NH_2	30.7 ± 5.8	286 ± 31	1500 ± 160	1/9/49
3	Dyn A(1−11)-NH_2	0.0869 ± 0.0112	0.653 ± 0.029	13.5 ± 1.7	1/8/155
4	[Dhp[1]]Dyn A(1−11)-NH_2	3.49 ± 0.12	27.9 ± 3.3	122 ± 24	1/8/35
5	[(2*S*)-Mdp[1]]Dyn A(1−11)-NH_2	0.823 ± 0.162	213 ± 50	163 ± 15	1/259/198
6	[Dhp[1],D-Ala[3]]Dyn A(1−11)-NH_2	3.84 ± 0.90	73.0 ± 1.1	419 ± 57	1/19/109
7	[(2*S*)-Mdp[1],D-Ala[3]]Dyn A(1−11)-NH_2	2.47 ± 0.38	167 ± 17	364 ± 101	1/68/147
	NorBNI	0.811 ± 0.018	28.4 ± 3.9	17.7 ± 0.5	1/35/22

[a] Means of three to four determinations ± SEM.

effect (4×10^{-6} M), it inhibited the contractions merely to the extent of 27% in the presence of the κ antagonist nor-BNI (50 nM). No agonist effect was observed with this compound at the same concentration in the presence of 100 nM naloxone. These observations indicate that [Hpp[1]]Dyn A(1−11)-NH_2 is a weak partial agonist both at the μ and at the κ receptor. It was found to be a weak full agonist in the MVD assay. In the receptor binding assays, this compound showed modest affinity for κ receptors and weak affinity for μ and δ receptors (Table 3).

Deletion of the N-terminal amino group of [Dmt[1]]Dyn A(1−11)-NH_2 resulted in a compound, [Dhp[1]]Dyn A(1−11)-NH_2 (**4**), which in the GPI assay showed quite high κ antagonist potency (K_e = 17.4 nM) against Dyn A(1−13) and even higher κ antagonist potency (K_e = 2.94 nM) against the non-peptide κ agonist U50,488 (Table 2). Its antagonist potency at the μ receptor is much lower (K_e = 445 nM) and it is a very weak δ antagonist (K_e = 5310 nM). The calculated K_e selectivity ratios ($\kappa/\mu/\delta$) indicate that the Dhp[1]-analogue is a very selective κ antagonist, particularly when the calculation of the ratios was based on the K_e^{κ} value obtained against U50,488. In the opioid receptor binding assays, this compound displayed high affinity for κ receptors, lower affinity for μ receptors, and weak affinity for δ receptors (Table 3). Thus, the rank order of the receptor binding affinities is the same as that of the K_e values determined in the functional assays, but the receptor binding selectivity ratios are somewhat smaller than the K_e ratios. Comparison of the opioid activity profile of **4** with that of **2** indicates that the two methyl groups in the 2′,6′-positions of Dhp in [Dhp[1]]Dyn A(1−11)-NH_2 strengthen its binding to all three opioid receptors as compared to [Hpp[1]]Dyn A(1−11)-NH_2. Furthermore, these two methyl groups play an important role in the peptide's binding to and stabilization of inactive receptor conformations, resulting in the antagonism observed at all three receptors.

The analogue [(2*S*)-Mdp[1]]Dyn A(1−11)-NH_2 (**5**) turned out to be a highly potent κ antagonist against Dyn A(1−13) with a K_e value of 0.632 nM and a slightly less potent κ antagonist against U50,488 (K_e = 3.92 nM) (Table 2). It showed very poor μ and δ antagonist activity (in the micromolar range), and the calculated K_e ratios demonstrate its very high selectivity as a κ antagonist. Furthermore, this compound did not produce any agonist effect in the GPI assay at concentrations up to 10 μM, indicating that it is a pure antagonist. In agreement with its high κ antagonist activity, compound **5** displayed subnanomolar κ receptor affinity (K_i^{κ} = 0.823 nM) in the κ receptor binding assay (Table 3). Its binding affinities for μ and δ receptors are lower by at least 2 orders of magnitude and, consequently, the κ vs μ and κ vs δ binding selectivity ratios of this compound are high. Interestingly, it displays higher κ receptor binding selectivity than the Dyn A(1−11)-NH_2 parent peptide. [(2*S*)-Mdp[1]]Dyn A(1−11)-NH_2 (dynantin) (**5**) represents the first highly potent and selective dynorphin A-derived κ opioid antagonist known.

In comparison with the non-peptide κ antagonist norBNI, dynantin has similar κ antagonist potency and κ receptor binding affinity in the subnanomolar range (Tables 2 and 3); however, under our assay conditions it shows markedly higher κ vs μ and κ vs δ selectivity ratios than the non-peptide κ antagonist. In particular, dynantin shows higher κ selectivity in antagonizing Dyn A(1−13) as compared to norBNI.

It is interesting to note that compounds **4** and **5** have similar κ antagonist potency against U50,488, whereas against Dyn A(1−13) **5** is a 28-fold more potent κ antagonist than **4**. This discrepancy is difficult to explain but may be related to the fact that U50,488 and Dyn A(1−13) interact with different κ receptor binding epitopes.[27,28]

Since [D-Ala[3]]Dyn A(1−11)-NH_2 has been reported to display higher κ selectivity than Dyn A(1−11)-NH_2,[7] we also prepared the Dhp[1]- and (2*S*)-Mdp[1]-analogues of the D-Ala[3]-peptide. [Dhp[1],D-Ala[3]]Dyn A(1−11)-NH_2 (**6**) showed κ and μ antagonist potencies similar to those of [Dhp[1]]Dyn A(1−11)-NH_2 (**4**); however, it behaved as a weak partial agonist in the MVD assay (Table 2). In the receptor binding assays, this compound showed about the same κ receptor affinity as compound **4** but slightly higher κ receptor selectivity. [(2*S*)-Mdp[1],D-Ala[3]]Dyn

A(1−11)-NH_2 (**7**) displayed about 5 times lower κ antagonist potency against Dyn A(1−13) and 3 times lower κ receptor binding affinity than analogue **5**. This compound was a weak antagonist at both μ and δ receptors with K_e values similar to those of **5**. Both its selectivity ratios based on the K_e values and its receptor binding selectivity ratios are slightly lower than those of dynantin (**5**). Thus, the relatively higher κ receptor selectivity of the [D-Ala^3]Dyn A(1−11)-NH_2 agonist, as compared to Dyn A(1−11)-NH_2, did not translate into further improved κ receptor selectivity of the [D-Ala^3]-Dyn A(1−11)-NH_2-derived antagonists. As in the case of the Gly^3-analogues (**4** and **5**), the (2*S*)-Mdp^1-analogue (**7**) showed higher κ antagonist potency than the Dhp^1-analogue (**6**) against Dyn A(1−13) but only slightly higher antagonist potency against U50,488.

Conclusions

The results of this study indicate that all Dhp^1- and (2*S*)-Mdp^1-analogues of Dyn A(1−11)-NH_2 and of [D-Ala^3]-Dyn A(1−11)-NH_2 are potent and selective κ opioid antagonists, very weak μ antagonists, and very weak δ antagonists or, in one case, a weak partial δ agonist with low efficacy. The lack of a positively charged N-terminal amino group is primarily responsible for the antagonist behavior of these compounds, in agreement with the results of previous studies indicating that the N-terminal amino group of opioid peptide ligands plays a crucial role in signal transduction at μ and δ receptors.[21,22] However, the two methyl groups in the 2′,6′-positions of the Dhp^1- or (2*S*)-Mdp^1 aromatic ring also play an important role in conferring κ antagonist properties to these Dyn A (1−11)-NH_2 analogues by strengthening binding to an inactive conformation of the κ receptor.

The compounds described here are the first reported examples of Dyn A-derived κ antagonists with both high antagonist potency and high κ receptor selectivity. The most potent and most selective κ antagonist of the series is [(2*S*)-Mdp^1]Dyn A (1−11)-NH_2 (**5**, dynantin), which has subnanomolar κ receptor binding affinity and subnanomolar κ antagonist activity against Dyn A(1−13). Dynantin is a highly selective κ antagonist, as indicated by both the ratios of the K_e values determined against κ, μ, and δ agonists and the ratios of the κ, μ, and δ receptor binding affinity constants. In comparison with norBNI, dynantin is an about equipotent κ antagonist against Dyn A-(1−13) and slightly less potent against the non-peptide κ agonist U50,488. Under the assay conditions used in this study, dynantin was found to be a somewhat more selective κ antagonist than nor-BNI, particularly against Dyn A(1−13) as agonist. Its κ vs μ and κ vs δ receptor selectivities appear to be similar to those recently reported for the indolomorphinan κ antagonist GNTI which is more selective than norBNI.[18] It is expected that dynantin may complement norBNI and GNTI as a pharmacological tool in opioid research.

Experimental Section

General Methods. A Varian ProStar liquid chromatograph was used for the purification and the purity control of the peptides. Preparative reversed-phase HPLC was performed on a Vydac 218-TP column (22 × 250 mm) with linear gradients of 20−50% or 20−75% MeOH in 0.1% TFA over 40 min at a flow rate of 7 mL/min, absorption being measured at 220 and 280 nm. Analytical reversed-phase HPLC was carried out on a Vydac 218TP column (10 × 250 mm) using the following linear gradients: (1) 20−75% MeOH in 0.1% TFA over 40 min at a flow rate of 1.5 mL/min and (2) 10−60% acetonitrile in 0.1% TFA over 30 min at a flow rate of 1.5 mL/min. The determined capacity factors *K*′ are listed in the Supporting Information. Molecular weights of compounds were determined by FAB mass spectrometry on an MS-50 HMTCTA mass spectrometer interfaced to a DS-90 data system (Dr. M. Evans, Department of Chemistry, University of Montreal). $[M + H]^+$ values are indicated in the Supporting Information. For amino acid analyses, peptides (0.3 mg) were hydrolyzed in 6 N HCl (0.5 mL) for 24 h at 110 °C in deaerated tubes. Hydrolysates were analyzed on a Thermo Separation (TSP) P4000 HPLC system using an IB-Sil column (C18, 4.6 × 250 mm; Phenomenex). The results of the amino acid analyses are given in the Supporting Information.

Amino Acids and Derivatives. Boc amino acids were purchased from Bachem Bioscience. Boc-L-Dmt-OH was obtained from RSP Amino Acid Analogues, Inc. Dhp(OBoc) and (2*S*)-Mdp(OBoc) were synthesized as described elsewhere.[21,22,24]

Peptide Synthesis. Peptide synthesis was performed by the manual solid-phase technique using a *p*-methylbenzhydrylamine resin (1% cross-linked, 100−200 mesh, 0.54 mequiv/g of titratable amine) obtained from Bachem Bioscience. Peptides were assembled using Boc-protected amino acids and DIC and HOBt as coupling agents. The hydroxyl functions of Dhp, (2*S*)-Mdp, and Boc-Dmt-OH were also Boc-protected. Other side chain protection was as follows: tosyl (Arg) and 2-chlorobenzyloxycarbonyl (Lys). The following steps were performed in each cycle: (1) addition of Boc amino acid in CH_2Cl_2 (2.5 equiv); (2) addition of HOBt (2.5 equiv); (3) addition of DIC (2.5 equiv) and mixing for 2−3 h; (4) washing with CH_2Cl_2 (3 × 1 min); (5) washing with EtOH (1 min); (6) monitoring completion of the reaction with the ninhydrin test; (7) Boc deprotection with 50% (v/v) TFA in CH_2Cl_2 (30 min); (8) washing with CH_2Cl_2 (5 × 1 min); (9) neutralization with 10% (v/v) DIEA in CH_2Cl_2 (2 × 5 min); and (10) washing with CH_2Cl_2 (5 × 1 min). After final deprotection with 50% (v/v) TFA in CH_2Cl_2 (30 min), the resin was washed with CH_2Cl_2 (3 × 1 min) and EtOH (3 × 1 min) and was dried in a desiccator. Peptides were cleaved from the resin and completely deprotected by treatment with HF for 60 min at 0 °C (20 mL of HF plus 1 mL of anisole/g of resin). After evaporation of the HF, the resin was extracted three times with Et_2O and, subsequently, three times with glacial AcOH. The crude peptide was obtained in solid form through lyophilization of the acetic acid extract. Crude peptides were purified by preparative HPLC. Each peptide was at least 98% pure as assessed by analytical HPLC. The structures of the final products were confirmed by FAB-MS and by amino acid analysis of the hydrolysates.

In Vitro Bioassays and Receptor Binding Assays. The GPI[29] and MVD[30] bioassays were carried out as reported in detail elsewhere.[31,32] A dose−response curve was determined with [Leu^5]enkephalin as standard for each ileum and vas preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.[33] K_e values for antagonists were determined from the ratio of IC_{50} values obtained with an agonist in the presence and absence of a fixed antagonist concentration.[34] κ antagonist K_e values of compounds were determined in the GPI assay against the κ agonist Dyn A(1−13) using antagonist concentrations ranging from 3 to 50 nM and against the κ agonist U50,488 using antagonist concentrations ranging from 1 to 40 nM. μ antagonist K_e values of compounds were also measured in the GPI assay using antagonist concentrations ranging from 100 nM to 5 μM against the μ agonist TAPP. δ antagonist K_e values of compounds were determined in the MVD assay against the δ agonist DPDPE using antagonist concentrations ranging from 30 nM to 10 μM. The concentrations of each antagonist used against the four different agonists are indicated in the Supporting Information.

Opioid receptor binding studies were performed as described in detail elsewhere.[31] Binding affinities for μ and δ receptors

were determined by displacing, respectively, [^{3}H]DAMGO (Multiple Peptide Systems, San Diego, CA) and [^{3}H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor affinities were measured by displacement of [^{3}H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0 °C with [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,-593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC_{50} values were determined from log dose-displacement curves, and K_i values were calculated from the obtained IC_{50} values by means of the equation of Cheng and Prusoff,[35] using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593, respectively.

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Supporting Information Available: Tables of characterization and amino acid analyses data of Dyn A(1−11)-NH_2 analogues as well as the concentrations used of antagonists **4**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC−IUB Joint Commission on Biochemical Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345−373. The other abbreviations are as follows: Boc, *tert*-butoxycarbonyl; DAMGO, H-Tyr-D-Ala-Gly-N$^{\alpha}$MePhe-Gly-ol; Dhp, 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid; DIC, 1,3-diisopropylcarbodiimide; Dmt, 2′,6′-dimethyltyrosine; DPDPE, H-Tyr-c[D-Pen-Gly-Phe-D-Pen]OH; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; Dyn A, dynorphin A; dynantin, [(2*S*)-Mdp^1]Dyn A(1−11)-NH_2; FAB-MS, fast atom bombardment mass spectrometry; GPI, guinea pig ileum; Hpp, 4-hydroxyphenylpropanoic acid; HOBt, 1-hydroxybenzotriazole; (2*S*)-Mdp, (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid; MVD, mouse vas deferens; 2-Nal, 2-naphthylalanine; norBNI, norbinaltorphimine; Pen, penicillamine; TAPP, H-Tyr-D-Ala-Phe-Phe-NH_2; TFA, trifluoroacetic acid; U50,488, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide; U69,593, (5α,7α,8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

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