Structure−Activity Relationships of Dynorphin A Analogues Modified in the Address Sequence

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The peptide [Pro^3]Dyn A(1−11)-NH_2 **2** exhibits high affinity (K_i = 2.4 nM) and over 2000-fold selectivity for the κ opioid receptor. Stepwise removal of the C-terminal residues from this ligand demonstrated that its positively charged Arg residues, particularly Arg^6 and Arg^7, were crucial for binding to the κ receptor. Analogues shorter than seven amino acids lacked significant affinity for opioid receptors. Comparison with a series of truncated analogues of Dyn A showed that the relative losses in binding potency differed only slightly between the two series. The neutral residues Ile^8 and Pro^{10} could be removed without significant loss in affinity for the κ receptor. Their replacement, in the Pro^3 analogue, with additional Arg residues led to analogues with improved κ affinity (e.g., [Pro^3,Arg^8]Dyn A(1−11)-NH_2 **20**: $K_i(\kappa)$ = 0.44 nM). This type of modification did not compromise the high κ selectivity of the Pro^3 analogues. These findings support the view that a negatively charged domain in the putative second extracellular loop of the κ receptor selectively recognizes residues 6−11 of dynorphin through electrostatic interactions. As with parent compound **2**, analogue **20** and related compounds displayed κ antagonist properties.

Introduction and Background

The analgesic effects of opioids are mediated by at least three distinct receptors, the μ, δ, and κ opioid receptors.[1−3] Unlike μ agonists (e.g., morphine), selective κ agonists mediate analgesia without constipation, respiratory depression, or addictive side effects.[4] This observation caused considerable interest in selective κ agonists. However, other side effects (dysphoria, diuresis, psychotomimesis) have so far limited their therapeutic use.[4] Very few selective κ antagonists are known, and their potential has not been fully explored. One possible application of κ selective antagonists might be the treatment of addiction.[5]

The heptadecapeptide dynorphin A (Dyn A: Tyr-Gly-Gly-Phe-Leu^5-Arg-Arg-Ile-Arg-Pro^{10}-Lys-Leu-Lys-Trp-Asp^{15}-Asn-Gln-OH) binds with subnanomolar affinity to the κ receptor and is presumed to be its endogenous ligand.[6−9] It can be truncated to the 11-peptide amide without significant loss of affinity or selectivity.[10] Since Dyn A is quite active at all three opioid receptors, the synthesis of analogues more selective for the κ receptor is a primary objective in this field. Efforts in this direction have previously led to some very selective analogues, particularly [D-Ala^3]Dyn A(1−11)-NH_2[11,12] and some N^1-alkylated analogues of [D-Pro^{10}]Dyn A(1−11)-OH.[13−16] Recently, our own studies[17] led to the development of [Pro^3]Dyn A(1−11)-NH_2, one of the most κ selective opioid peptides known to date. In this paper, we report the results of a study on modifications of residues 5−11 (often referred to as the "address sequence") on binding affinity and potency.

The term "address sequence" was originally coined by Goldstein et al. because their study of stepwise removal of residues 5−11 of Dyn A indicated that these residues selectively enhanced potency at the κ receptor.[10] Residues 1−4, on the other hand, were called the "message sequence" because they are essential for binding at all opioid receptors. Within the "address sequence", removal of the basic residues Arg^6, Arg^7, and Arg^9 resulted in marked decreases in potency relative to the natural ligand. Cloning and sequencing of the opioid receptors[1−3] showed that there are Asp and Glu residues in the putative second extracellular loop of the κ receptor, leading to the hypothesis that this domain is the binding epitope of the address sequence. This view was supported by experiments with μ/κ receptor chimeras,[18,19] which demonstrated that exchanging EL2 of the μ receptor with that of the κ receptor results in a marked increase in affinity for Dyn A.

On the other hand, a recent study on neutralizing point mutations of the Asp and Glu residues found in the κ-EL2 does not support this view.[20] Though not all seven acidic residues of this loop could be mutated to Asn and Gln at the same time, it was shown that neutralizing up to four acidic residues at a time *does not* result in significant reduction of the affinity of Dyn A(1−13) toward the mutant receptors. The importance of salt bridges for the binding of the address sequence was also questioned by Paterlini et al.[21] The authors presented a docking model in which the primary binding forces for the address sequence arise from hydrophobic interactions between a helical domain in the κ-EL2 and

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the amino acids Phe[4], Leu[5], and Ile[8] in Dyn A. Though this model indicates potential ion pairs between Arg-6, -7, and -9 of Dyn A and oppositely charged Asp and Glu residues in EL2, these interactions were deemed less important.

We previously reported that [Pro[3]]Dyn A(1−11)-NH_2 **2** possesses markedly enhanced κ selectivity compared to Dyn A(1−11)-NH_2 **1**.[17] The κ affinity of **2** is quite high (K_i = 2.4 nM), although there is a 40-fold loss of κ affinity relative to the parent compound Dyn A(1−11)-NH_2 **1**. In this study, we prepared a series of analogues with stepwise removal of the C-terminal residues to find the shortest sequence that retains high affinity and selectivity for the κ receptor and to investigate the relative importance of each of the C-terminal residues for κ binding. The results, as well as older literature pointing at the importance of ion pairing for the binding of the address sequence, prompted us to incorporate additional Arg residues into this domain in order to maximize this type of interaction. In view of the ongoing debate outlined above, it is important that this approach led to new analogues with improved affinity for the κ receptor.

Experimental Procedures

Symbols and Abbreviations. 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 are the following: Dyn A, dynorphin A; GPI, guinea pig ileum.

Peptide Synthesis and Purification. Peptides **1**−**10** and **20**−**23** were synthesized as described previously.[17,22] Analytical data are available in the Supporting Information. Peptides **11**−**17** were obtained from Bachem California, Inc. (Torrance, CA).

Radioligand Binding Assays. Membrane preparations from Chinese hamster ovary (CHO) cells stably expressing human κ, μ, or δ receptors were prepared as described previously.[17] The assay buffer used is composed of 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, and 0.5 mg/L aprotinin. After dilution in assay buffer and homogenization in a Polytron homogenizer (Brinkmann, Westbury, NY) for 30 s at a setting of 1, membrane proteins (10−80 μg) in 250 μL of assay buffer were added to mixtures containing test compound and [^{3}H]-diprenorphine (0.5−1.0 nM, 25000−50000 dpm) in 250 μL of assay buffer in 96-well deep-well polystyrene titer plates (Beckman) and incubated at room temperature for 60 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 filters were washed four times with 1.0 mL each of ice-cold 50 mM Tris-HCl, pH 7.8, and 30 μL of Microscint-20 (Packard Instrument Company, Meriden, CT) was added to each filter. Radioactivity on the filters was determined by scintillation spectrometry in a Packard TopCount.

[^{3}H]Diprenorphine with a specific activity of 50 Ci/mmol was purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA). The K_D values for [^{3}H]diprenorphine binding were 0.33 nM for the κ and μ receptors and 0.26 nM for the δ receptor. Receptor expression levels, determined as B_{max} values from Scatchard analyses, were 4400, 4700, and 2100 fmol/mg of protein for the κ, μ, and δ receptors, respectively. 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. Protein was quantified by the method of Bradford.[23]

The data from competition experiments were fit by nonlinear regression analysis with the program Prism (GraphPad Software Inc., San Diego, CA) using the four-parameter equation for one-site competition, and K_i values were subsequently calculated from EC_{50} values by the Cheng−Prusoff equation.

Receptor-Mediated [^{35}S]GTPγS Binding. Receptor-mediated [^{35}S]GTPγS binding was performed by modifications of the methods of Selley et al.[24] and Traynor and Nahorski.[25] Assays were carried out in 96-well FlashPlates (Perkin-Elmer Life Sciences, Inc, Boston, MA). Membranes prepared from CHO cells expressing the appropriate receptor (50−100 μg of protein) were added to assay mixtures containing agonist with or without antagonists, approximately 100 000 dpm (100 pM) [^{35}S]GTPγS, 3.0 μM GDP, 75 mM NaCl, 15 mM $MgCl_2$, 1.0 mM EGTA, 1.1 mM dithiothreitol, 10 mg/L leupeptin, 10 mg/L pepstatin A, 200 mg/L bacitracin, and 0.5 mg/L aprotinin in 50 mM Tris-HCl buffer, pH 7.8. After incubation at room temperature for 1 h, the plates were sealed and centrifuged at 800*g* in a swinging bucket rotor for 5 min and bound radioactivity was determined with a TopCount microplate scintillation counter (Packard Instrument Co., Meriden, CT).

Agonist potency and efficacy were 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 Prism. The efficacies of these compounds were expressed as the maximal stimulation of [^{35}S]GTPγS binding relative to the maximal stimulation achieved by U50,488.

Antagonist activities were obtained by titration in the presence of a concentration of U50,488 (50 nM) that yielded 80% of its maximal stimulation (EC_{80}), and the data were analyzed by nonlinear regression fit using Prism. Efficacy was expressed as the maximum percent inhibition of the U50,488-stimulated [^{35}S]GTPγS binding, and potency was expressed as the concentration of antagonist that achieved 50% of the maximum inhibition of that antagonist.

Results and Discussion

Radioligand Binding Assays. The analogue [Pro[3]]-Dyn A(1−11)-OH **3** and the corresponding amide [Pro[3]]-Dyn A(1−11)-NH_2 **2** exhibited similar low affinities for the μ and δ receptors (Table 1). However, the affinity for the κ receptor was 41.7 nM for the peptide acid **3** compared with 2.4 nM for the amide **2**. Under physiological conditions, analogue **3** would have a negatively charged C-terminus. This negative charge may interfere with the electrostatic interaction of the positively charged residues Arg[9] and Lys[11] with a putative negatively charged binding epitope on the EL2 of the κ receptor. Removal of Lys[11] reduced affinity for the κ receptor by 4-fold (peptide **4**).

For comparison, we also assayed a series of truncated analogues of Dyn A(1−13)-OH (peptides **11**−**17**). These analogues consistently displayed a κ affinity that was 1−2 orders of magnitude higher and a κ-selectivity that was 1−2 orders of magnitude lower than those of the corresponding Pro[3] amide analogues. With respect to relative loss of κ affinity, both series of peptides responded slightly differently to truncations in the address sequence. In Dyn A analogues, truncating the decapeptide **12** to the hexapeptide **16** reduced κ affinity by a factor of 1280. In [Pro[3]]Dyn A analogues, analogous truncation reduced κ affinity by a factor of 560 (peptides **4** and **8**). Removal of Pro[10] caused a 7-fold decrease in

Table 1. Opioid Receptor Binding Affinities and Selectivities of C-Terminal Truncated Analogues of Dyn A(1−11)-NH_2[a]

	analogue	K_i [nM]			K_i ratio
		κ	μ	δ	$\kappa/\mu/\delta$
1	Dyn A(1−11)-NH_2	0.059 ± 0.015 (n = 19)	5.62 ± 1.57 (n = 11)	3.24 ± 0.83 (n = 11)	1/95/54
2	[Pro^3]Dyn A(1−11)-NH_2	2.40 ± 0.22 (n = 13)	5250 ± 730 (n = 8)	8900 ± 2300 (n = 4)	1/2200/3700
3	[Pro^3]Dyn A(1−11)-OH	41.7 ± 12.7 (n = 3)	15100 ± 5000 (n = 4)	15000 ± 5600 (n = 3)	1/360/360
4	[Pro^3]Dyn A(1−10)-NH_2	10.0 ± 2.3 (n = 7)	6200 ± 860 (n = 5)	13500 ± 1850 (n = 3)	1/620/1300
5	[Pro^3]Dyn A(1−9)-NH_2	10.7 ± 2.7 (n = 7)	6030 ± 840 (n = 5)	13200 ± 300 (n = 3)	1/560/1200
6	[Pro^3]Dyn A(1−8)-NH_2	79.4 ± 33.9 (n = 7)	16200 ± 4200 (n = 5)	16200 ± 1500 (n = 3)	1/200/200
7	[Pro^3]Dyn A(1−7)-NH_2	60.3 ± 18.3 (n = 7)	12600 ± 3200 (n = 5)	15100 ± 3900 (n = 3)	1/210/250
8	[Pro^3]Dyn A(1−6)-NH_2	5600 ± 2700 (n = 4)	19500 ± 900 (n = 3)	12600 ± 2050 (n = 3)	1/3.4/2.3
9	[Pro^3]Dyn A(1−5)-NH_2	>10000 (n = 5)	15500 ± 1400 (n = 3)	18600 ± 4750 (n = 3)	1/<1/<1
10	[Pro^3,Nle^6]Dyn A(1−7)-NH_2	>100000 (n = 4)	>100000 (n = 4)	14100 ± 1300 (n = 3)	
11	Dyn A(1−13)-OH	0.23 ± 0.07 (n = 10)	8.0 ± 1.9 (n = 9)	8.3 ± 2.2 (n = 8)	1/35/36
12	Dyn A(1−10)-OH	0.35 ± 0.07 (n = 11)	11.9 ± 1.6 (n = 6)	24 ± 13 (n = 6)	1/34/69
13	Dyn A(1−9)-OH	2.5 ± 0.99 (n = 12)	23.8 ± 7.2 (n = 10)	9.8 ± 2.0 (n = 8)	1/9.5/3.9
14	Dyn A(1−8)-OH	4.6 ± 2.0 (n = 10)	35.4 ± 11.4 (n = 7)	8.5 ± 1.7 (n = 6)	1/7.7/1.8
15	Dyn A(1−7)-OH	4.96 ± 1.7 (n = 11)	38.0 ± 7.6 (n = 10)	9.7 ± 2.0 (n = 8)	1/7.7/2.0
16	Dyn A(1−6)-OH	404 ± 40 (n = 7)	40.6 ± 9.3 (n = 8)	9.1 ± 2.9 (n = 9)	1/0.10/0.022
17	Dyn A(1−5)-OH (Leu-Enk)	>1000 (n = 5)	55 ± 18 (n = 6)	0.70 ± 0.23 (n = 3)	1/<0.06/<0.0007
18	nor-BNI	0.37 ± 0.02 (n = 19)	79.4 ± 14.7 (n = 12)	19.5 ± 3.6 (n = 10)	1/210/52
19	naloxone	10.7 ± 1.7 (n = 17)	3.63 ± 0.39 (n = 18)	37.0 ± 5.1 (n = 10)	1/0.34/3.5

[a] K_i values were obtained using CHO cell membranes stably expressing κ, μ, and δ receptors with [^{3}H]diprenorphine as reference ligand. Values are given with ±SEM and the number of determinations in parentheses.

Table 2. Opioid Receptor Binding Affinities and Selectivities of Analogues of [Pro^3]Dyn A(1−11)-NH_2 with Additional Arg in Position 8 or 10[a]

	analogue	K_i [nM]			K_i ratio
		κ	μ	δ	$\kappa/\mu/\delta$
20	[Pro^3,Arg^8]Dyn A(1−11)-NH_2	0.44 ± 0.10 (n = 3)	2750 ± 770 (n = 3)	6760 ± 620 (n = 4)	1/6300/15000
21	[Pro^3,Arg^8]Dyn A(1−8)-NH_2	6.61 ± 1.22 (n = 3)	3100 ± 1630 (n = 3)	>100000 (n = 3)	1/470/>15000
22	[Pro^3,Arg^{10}]Dyn A(1−11)-NH_2	1.20 ± 0.14 (n = 3)	1660 ± 76 (n = 3)	3230 ± 1460 (n = 3)	1/1400/2700
23	[Pro^3,Arg^{10}]Dyn A(1−10)-NH_2	3.39 ± 0.71 (n = 3)	2340 ± 430 (n = 3)	6030 ± 700 (n = 4)	1/700/1800

[a] K_i values were obtained using CHO cell membranes stably expressing κ, μ, and δ receptors with [^{3}H]diprenorphine as reference ligand. Values are given with ±SEM and the number of determinations in parentheses.

κ affinity in Dyn A analogues (peptides **12** and **13**), while truncation of Pro^{10} caused no significant loss of κ affinity in [Pro^3]Dyn A analogues (peptides **4** and **5**). Removal of Ile^8 did not reduce κ affinity significantly in either series.

Removal of Arg^9 caused a 7.4-fold decrease in κ affinity in [Pro^3]Dyn A analogues (peptides **5** and **6**), compared to a 1.8-fold decrease in Dyn A analogues (cf. peptides **13** and **14**). Removal of Arg^7 had almost identical effects in the two series of Dyn A analogues, i.e., κ affinity was reduced by a factor of 91 in dynorphin analogues (peptides **15** and **16**), compared to a 93-fold loss of κ affinity observed in [Pro^3]Dyn A analogues (cf. peptides **7** and **8**).

In both series, the decrease in κ affinity observed upon removal of Arg^7 was pronounced and seems to reflect a change in binding mode. We assume that the electrostatic interaction between address sequence and EL2 causes a favorable alignment of ligand and receptor, possibly accompanied by a change of conformation in the transmembrane domain of the κ receptor. This allows the message sequence to dock to the κ receptor, although it alone binds very weakly (cf. peptides **9** and **17**). The observation that [Pro^3,Nle^6]Dyn A(1−7)-NH_2 **10** was devoid of significant opioid receptor affinity demonstrates that *both* Arg^6 and Arg^7 are required to retain sufficient electrostatic interaction in the Pro^3 analogues. In this respect, the Pro^3 analogues differ from the natural sequence, where isosteric replacement of Arg^6 or Arg^7 with Nle led to Dyn A(1−11)-NH_2 analogues with high κ affinity.[26]

With respect to the relative importance of residues for κ affinity, our observations are consistent with earlier studies on the natural sequence using truncation[10,27] and substitution of residues with Ala.[28] For Dyn A(1−6)-OH **16** and Leu-Enk **17**, our results differ from those obtained by Mansour et al.,[27] who found a $K_i(\kappa)$ of 2.88 and 93.6 nM, respectively, for these peptides. In our assay, the $K_i(\kappa)$ values for **16** and **17** were 404 and >1000 nM. The corresponding Pro^3 analogues **8** and **9** showed weak affinities above 5 μM (**8**) and above 10 μM (**9**) at all opioid receptors.

The minor contribution of the neutral residues Ile^8 and Pro^{10} to κ-binding suggested to us that their replacement by Arg should yield analogues with enhanced κ affinity through an increased positive net charge. As a comparison of the results indicated in Table 2 with those with the unsubstituted analogues of similar length shows, this concept led to a 2- to 3-fold increase in κ affinity for replacement of Pro^{10} and to a 5- to 12-fold increase in κ affinity for replacement of Ile^8. The most potent of these analogues, [Pro^3,Arg^8]Dyn A(1−11)-NH_2 **20**, combines subnanomolar κ affinity with several 1000-fold selectivity for the κ receptor. Contrary to an earlier modeling study,[21] these findings further support the view that electrostatic interactions are important for selective recognition of the address sequence. The fact that replacement of Ile^8 by Arg leads to *enhanced* κ affinity makes an involvement of this residue in a critical hydrophobic interaction unlikely.

[^{35}S]GTPγS Assays. We used [^{35}S]GTPγS binding[29,30] under the conditions stated above for functional char-

Table 3. Opioid Antagonist Properties of Selected Analogues in the κ Receptor-Mediated [^{35}S]GTPγS Binding Assay[a]

analogue	IC_{50} [b] [nM]	maximum inhibition[c] [%]
[Pro^3]Dyn A(1−11)-NH_2 **2**	440 ± 60 (n = 24)	60 ± 6
[Pro^3,Arg^8]Dyn A(1−11)-NH_2 **20**	340 ± 60 (n = 6)	70 ± 4
[Pro^3,Arg^8]Dyn A(1−8)-NH_2 **21**	4000 ± 1200 (n = 6)	57 ± 7
[Pro^3,Arg^{10}]Dyn A(1−11)-NH_2 **22**	410 ± 200 (n = 6)	67 ± 8
[Pro^3,Arg^{10}]Dyn A(1−10)-NH_2 **23**	1260 ± 170 (n = 6)	64 ± 4
nor-BNI **18**	4.37 ± 0.50 (n = 20)	100
naloxone **19**	49.8 ± 7.0 (n = 8)	98 ± 1

[a] Values are given with ±SEM, with the numbers of determinations in parentheses. [b] Antagonist activity determined against 50 nM U50,488. [c] Maximum percent inhibition of the stimulation by 50 nM U50,488, achieved by antagonist at 10 μM.

Table 4. Opioid Agonist Properties of Dynorphin Analogues in the [^{35}S]GTPγS Assay[a]

compound	maximum stimulation[b] [%]
Dyn A(1−11)-NH_2 **1**[c]	73 ± 9.0 (n = 3)
[Pro^3]Dyn A(1−11)-NH_2 **2**	16 ± 9.0 (n = 3)
[Pro^3,Arg^8]Dyn A(1−11)-NH_2 **20**	21.5 ± 7.5 (n = 2)
[Pro^3,Arg^8]Dyn A(1−8)-NH_2 **21**	22.0 ± 3.0 (n = 2)
[Pro^3,Arg^{10}]Dyn A(1−11)-NH_2 **22**	19.5 ± 4.5 (n = 2)
[Pro3,Arg^{10}]Dyn A(1−10)-NH_2 **23**	18.0 ± 12.0 (n = 2)
nor-BNI **18**	−13.0 ± 8.0 (n = 3)
naloxone **19**[d]	30.0 ± 3.0 (n = 7)

[a] Values are given with ±SEM, with the numbers of determinations in parentheses. [b] Maximum stimulation of [^{35}S]GTPγS binding at 10 μM, expressed as the percent of the maximum stimulation of [^{35}S]GTPγS binding evoked by 10 μM U50,488. [c] EC_{50} = 0.74 nM.[17] [d] EC_{50} = 6.6 ± 1.2 nM (n = 7).

acterization of our analogues at the κ receptor. This assay detects the agonist-induced interaction of receptor with G protein and the subsequent exchange of GTP for GDP bound to the G protein.

As observed previously with [Pro^3]Dyn A(1−11)-NH_2 **2**, its Arg^8 and Arg^{10} analogues exhibited antagonist activity and their rank order of potencies was consistent with their rank order of potencies in the radioligand binding assay (Table 3). However, their IC_{50} values were 2−3 orders of magnitude higher than that obtained for the standard κ antagonist nor-BNI. It should be noted that the [^{35}S]GTPγS assay uses the same membrane preparations as the radioligand binding assay. Therefore, the apparent discrepancy between high κ receptor affinity in the binding assay and relatively low potency in the functional assay *cannot* be ascribed to different κ receptor subtypes in the two assays. As we demonstrated before,[17] the use of different reference ligands in the two assays also does not account for these results, since [Pro^3]Dyn A(1−11)-NH_2 **2** can displace arylacetamides effectively in the binding assay.

At high concentrations, the peptide [Pro^3]Dyn A(1−11)-NH_2 **2** and its Arg analogues **20**−**23** exhibited partial agonist activity in this assay. The effect was too small to determine a reliable EC_{50} value. The greater maximum stimulation by the analogue Dyn A(1−11)-NH_2 **1** enabled a reliable EC_{50} value (0.74 nM) to be determined. In Table 4, the observed agonist activity is expressed as the percent stimulation of [^{35}S]GTPγS binding at 10 μM relative to the maximum stimulation observed for U50,488. Notably, even the relatively efficacious agonist Dyn A(1−11)-NH_2 **1** achieved only 73% under these conditions and its analogues **2** and **20**−**23** evoked 16−22% of the response observed for U50,488. The incomplete inhibition of [^{35}S]GTPγS binding shown in Table 4 and the relatively high IC_{50} values are quite typical of antagonism by partial agonists. On the other hand, no agonist activity of our ligands was detected in the GPI assay, even at micromolar concentrations (below). The standard κ antagonist nor-BNI did not display any agonist activity in the [^{35}S]GTPγS assay. Instead, it slightly inhibited basal [^{35}S]GTPγS binding, resulting in a negative maximum stimulation (Table 4).

In the interpretation of the partial agonism observed for our dynorphin analogues, consideration should be given to the fact that the [^{35}S]GTPγS assay was performed in a heterologous system overexpressing the κ receptor. The CHO cells used express the κ receptor with a B_{max} value of 4400 ± 460 fmol/mg of membrane protein, compared to 100 to approximately 200 fmol/mg of protein in guinea pig brain.[31,32] Receptor overexpression can reduce the stoichiometric ratio of G protein to receptor, enabling the receptor to bind to proteins with which it does not normally interact.[33] Cellular host effects due to the heterologous expression in CHO cells can also change the pharmacological profile of a receptor.[34] One possible consequence is the observation of apparent efficacy in ligands that do not promote receptor−G-protein interaction in vivo.[34,35] A striking example involves Y-1 cells overexpressing the 5-HT_{1B} receptor, where full agonism has been observed for a number of partial agonists and even for all tested antagonists except one.[35] Similar observations have been reviewed.[36] For a number of partial μ agonists, relative efficacy in the [^{35}S]GTPγS assay was found to be higher in membranes from transfected CHO cells than in membranes from rat thalamus.[37] While nalorphine and nalbuphine acted as pure μ antagonists in thalamic membranes, these drugs were partial μ agonists in CHO membranes.[38] Quite likely, a similar effect accounts for our observations.

The less selective opioid antagonist naloxone also behaved as a partial agonist in our [^{35}S]GTPγS assay (EC_{50} = 6.6 nM, maximum stimulation of 30%, Table 4). Naloxone has been suspected to be a partial agonist, since low doses of this ligand cause weak analgesia in animal models[38] and human subjects.[39] However, a more recent study on cultured neurons and mouse models demonstrates that low doses of naloxone selectively antagonize excitatory effects of opioids.[40] At all concentrations, the mechanism of action would thus be antagonism.

Our observation of partial agonism by naloxone in the [^{35}S]GTPγS assay is in accord with Fukuda et al.,[41] who used an assay based on inhibition of adenylate cyclase by κ receptors expressed in CHO cells at a level of 20 900 fmol/mg of protein. However, no such observation was made by Zhu et al.,[42] who used a [^{35}S]GTPγS assay on membranes from CHO cells expressing the κ receptor at a level of 1292 fmol/mg of protein. The latter system also had few spare receptors, indicating a balanced ratio of G protein to receptors. To conclude, naloxone displays agonist properties in this assay only at high expression levels, a strong indication of an effect related to overexpression of the receptor.

Biological Activity in the Guinea Pig Ileum (GPI) Assay. The GPI bioassay[43] was performed as reported in detail elsewhere.[44] The K_e values were determined from the IC_{50} values of the reference agonist

Table 5. Opioid Agonist or Antagonist Properties of Dynorphin Analogues in the Guinea Pig Ileum (GPI) Assay[a]

compound	agonists IC_{50} [nM]	antagonists K_e [b] [nM]
[D-Ala^3]Dyn A(1−11)-NH_2 **24**	2.38 ± 0.44	
[Pro^3]Dyn A(1−11)-NH_2 **2**[c]		494 ± 74
[Pro^3,Arg^8]Dyn A(1−11)-NH_2 **20**[c]		280 ± 53
nor-BNI **18** [d]		0.572 ± 0.036

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

[D-Ala^3]Dyn A(1−11)-NH_2 **24**[12] in the presence and absence of a fixed concentration of antagonist.[45] The results are given in Table 5.

Only the most potent of our new analogues, [Pro^3,Arg^8]-Dyn A(1−11)-NH_2 **20**, was tested in the GPI assay. Consistent with the results obtained in the [^{35}S]GTPγS binding assay, [Pro^3]Dyn A(1−11)-NH_2 **2** and its Arg^8 analogue **20** were found to be 2−3 orders of magnitude less potent antagonists than nor-BNI. With a K_e of 280 nM, [Pro^3,Arg^8]Dyn A(1−11)-NH_2 **20** was somewhat more potent than the parent compound **2** (K_e = 494 nM). When tested alone, compounds **2** and **20** did not inhibit electrically evoked contractions in the GPI assay at concentrations up to 10 μM and up to 1 μM, respectively. Thus, both compounds behaved as pure antagonists in this assay. Dynorphin analogues with weak potency in the GPI assay despite high κ affinity in the radioligand binding assay have been described in a number of studies,[12,14,26,46,47] and the observed behavior has been tentatively ascribed to different κ receptor subtypes in central versus peripheral neurons. As outlined above, the results obtained in the [^{35}S]GTPγS assay cannot be explained in this way, since it uses the same membrane preparations as the binding assay. The discrepancy between pure antagonism in the GPI assay and partial agonism in the [^{35}S]GTPγS assay is most likely the result of different ratios of receptor to G protein in the two systems.

Conclusions

This study demonstrated that the structure−activity relationships for analogues of [Pro^3]Dyn A(1−11)-NH_2 differ slightly from those obtained for analogues of Dyn A(1−11). Truncated Dyn A analogues gradually become more δ- and μ-selective as the address sequence is removed and their structure approaches that of enkephalin. By comparison, all Pro^3 analogues show micromolar δ and μ affinities, irrespective of their length. Upon removal of Arg^7, κ affinity drops by a factor of >90 in both series. Compared to the unsubstituted analogues, substitution with proline in position 3 improves κ selectivity because it reduces μ and δ affinities by 2−3 orders of magnitude, while κ affinity is reduced by only 1−2 orders of magnitude. As a result, [Pro^3]Dyn A(1−11)-NH_2 **2** combines a κ affinity of K_i = 2.4 nM with several-thousand-fold selectivity for this receptor and is one of the most κ-selective peptide ligands known. In terms of relative loss of binding potency, partial removal of the address sequence has similar effects in Dyn A analogues and [Pro^3]Dyn A analogues. The observation that the neutral residues Ile^8 and Pro^{10} can be removed without significant loss in κ affinity prompted us to replace these residues with Arg and thereby to enhance a presumed electrostatic interaction with the extracellular loop EL2 of the κ receptor. This type of modification enhanced κ affinity by a factor of 2−12. Substitution of Ile^8 with Arg also enhanced κ selectivity and led to the development of [Pro^3,Arg^8]Dyn A(1−11)-NH_2 **20**, a ligand with $K_i(\kappa)$ = 0.44 nM and selectivities κ/μ and κ/δ of 6300 and 15 000, respectively. Compound **20** is the most κ-selective peptide known. Our results support the view that ionic interactions involving the positively charged residues in the address sequence are important for the selective, high-affinity binding of dynorphin analogues to the κ receptor. This observation is significant for modeling the receptor−ligand interaction of dynorphin.

In the [^{35}S]GTPγS assay, parent compound **2** and all of the new compounds tested were κ antagonists. The standard κ antagonist nor-BNI turned out to be 100 times more potent than the best ligands of this series, peptides **2** and **20**. The incorporation of additional Arg improved κ affinity but did not change agonist vs antagonist character. At high concentrations, weak agonist properties were observed in the [^{35}S]GTPγS assay of peptides **2** and **20**−**23**. Our observations with this heterologous overexpression system demonstrate that subtle artifacts have to be taken into account when interpreting results obtained with this type of assay. The GPI assay of compounds **2** and **20** confirmed their character as κ antagonists and did not indicate any partial agonist properties.

Our results have provided new insight into structural factors governing high κ affinity and selectivity as well as agonist vs antagonist activity in dynorphin analogues. The structure−activity relationships found in this study are a firm basis for the development of potent, κ selective peptide ligands and provided valuable clues for molecular modeling of the receptor−ligand interaction of dynorphin.

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Supporting Information Available: HPLC and MS data of the new peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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