Design, Synthesis, and Biological Activities of Cyclic Lactam Peptide Analogues of Dynorphin A(1−11)-NH_2[1]

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We previously have reported four possible binding conformations of dynorphin A (Dyn A) for the central κ opioid receptors, induced by the address sequence, using a molecular mechanics energy minimization approach. The lowest energy conformation was found to exhibit an α-helical conformation in the cyclized address sequence. It was suggested that an α-helical conformation in the cyclized address sequence or a helical conformation induced by the conformational characteristics of the message sequence may be important for binding potency and κ opioid receptor selectivity. Side chain to side chain lactam bridges between the *i* and *i* + 4 positions have been shown to stabilize α-helical conformations. Thus, a series of cyclic lactam analogues of dynorphin A(1−11)-NH_2 have been designed, synthesized and evaluated by the guinea pig brain (GPB) binding assay and guinea pig ileum (GPI) bioassay to evaluate the conformational analysis prediction and, further, to investigate the conformational requirements for high potency and selectivity for κ opioid receptors. Positions 2−6, 3−7, and 5−9 were chosen as the sites for incorporating cyclic conformational constraints. Cyclization between D-Asp[2] and Lys[6] in c[D-Asp[2],Lys[6]]Dyn A(1−11)-NH_2 led to an analogue with pronounced potency and selectivity enhancement for the μ opioid receptor, whereas cyclization between D-Asp[3] and Lys[7] in c[D-Asp[3],Lys[7]]Dyn A(1−11)-NH_2 led to a potent ligand (IC_{50} 4.9 nM) with κ receptor selectivity. The other analogues in the series proved to be less selective. The biological results led to the suggestion that the binding conformation for the κ receptor may have structural requirements that are distinct from those of μ and δ receptors. Interestingly, analogues with a D-Asp at position 2, 3, or 9 were found to be more potent for the κ receptor than analogues with an L-Asp at the same positions. It is suggested that the incorporation of D-Asp into position 2, 3, or 9 of Dyn A(1−11)-NH_2 may have stereochemical and conformational effects on the nearby amino acids which can help discriminate the preference between κ, μ, and δ receptors.

Introduction

The discovery of endogenous opioid peptides in the 1970s[2] has greatly accelerated research in opioid chemistry and biology. Evidence accumulated has led to the suggestion of multiple opioid receptors.[3] At present, it is generally accepted that there are at least three different opioid receptors, namely μ (mu), δ (delta), and κ (kappa).[4] Studies of the physiological and pharmacological roles of these receptors require highly potent and receptor-selective ligands for μ, δ, and κ receptors. Research in the development of potent and selective peptide ligands has led to the development of peptides with high receptor selectivities for the μ and δ opioid receptors.[5] The potential of targeting the κ opioid receptor as an effector for analgesia with the putative endogenous κ opioid ligand dynorphin A (Dyn A)[6] has not been explored to date in great detail. Therefore, it was necessary to design and synthesize highly selective ligands for the κ receptor, especially in relation to the endogenous dynorphins. Dynorphin A is a 17 amino acid peptide that consists of a N-terminal message sequence (Tyr-Gly-Gly-Phe) and an address sequence in the more C-terminal region which imparts selectivity for κ opioid receptors.[7]

Dyn A: H-Tyr-Gly-Gly-Phe-Leu-ArgArg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH

Structure−activity relationships for the dynorphin peptides have been reviewed.[8] It was suggested that the basic residues Arg[7], Lys[11], and Lys[13] were important for high selectivity and/or potency, but the deletion of residues 14−17 or 12−17 of dynorphin A did not significantly affect its selectivity or potency.[7,9,10] Thus, we have used the truncated derivative Dyn A(1−11)-NH_2 as a starting point to design analogues with high potency and selectivity for the κ receptor.

In solution, Dyn A exhibits an extended and/or random coil structure as indicated by several spectroscopic methods (FT-IR,[11,12] NMR,[11,13] CD,[14] Raman,[15] and fluorescence energy transfer[16]). In anisotropic environments such as membranes[17] or phase interfaces,[18] some α-helical or β-sheet structure has been observed for dynorphin and some of its analogues. Conformational analyses examined in our laboratory also have suggested that an α-helical conformation in the cyclized address sequence or a helical structure induced in the message sequence is important for κ opioid receptor binding potency and selectivity.[19] A ^{1}H NMR study of Dyn A(1−17)-NH_2 indicated that residues from Gly[3] to Arg[9] form a helical conformation when bound to lipid micelles.[20] This supports our suggestion that a helical structure in the message sequence of Dyn

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

analogues of Dyn A(1−11)-NH_2	IC_{50} (nM)[a]			selectivity	
	κ	μ	δ	μ/κ	δ/κ
Dyn A(1−11)-NH_2 (**1**)	0.58 ± 0.03	9.9 ± 2.0	26 ± 3	17.1	45.0
c[Asp^2,Lys^6] (**2**)	100 ± 42	2980 ± 614	910 ± 62	29.8	9.1
c[D-Asp^2,Lys^6] (**3**)	110 ± 26	3.4 ± 2.1	12 ± 5	0.03	0.11
c[Asp^3,Lys^7] (**4**)	25 ± 5	740 ± 97	1710 ± 256	30	68
c[D-Asp^3,Lys^7] (**5**)	4.9 ± 0.6	310 ± 40	130 ± 12	64	27
c[Lys5,Asp^9] (**6**)	100 ± 42	120 ± 25	150 ± 7	1.2	1.5
c[Lys^5,D-Asp^9] (**7**)	6.6 ± 2.5	12 ± 4	50 ± 1	1.8	7.6
[Asp^2,Lys^6] (**8**)	220 ± 27	290 ± 70	560 ± 29	1.3	2.5
[D-Asp^2,Lys^6] (**9**)	1.5 ± 0.4	0.08 ± 0.04	1.15 ± 0.02	0.05	0.77
[Asp^3,Lys^7] (**10**)	190 ± 46	1320 ± 421	1800 ± 327	7.0	9.5
[D-Asp^3,Lys^7] (**11**)	47 ± 15	660 ± 202	870 ± 251	14	19
[Lys^5,Asp^9] (**12**)	35 ± 9	400 ± 110	3290 ± 809	12	94
[Lys^5,D-Asp^9] (**13**)	17.5 ± 6.4	555 ± 165	1970 ± 328	32	110

[a] The radioligands used were [^{3}H]U-69,593 (κ receptor), [^{3}H]DAMGO (μ receptor) and [^{3}H]c[D-Pen^2,*p*-Cl-Phe^4,D-Pen^5]enkephalin (δ receptor).

A is biologically important. Side chain to side chain lactam cyclizations between the side chains of aspartic acid and lysine at positions *i* and *i* + 4 have been shown to stabilize α-helical peptides.[21] Very recently we reported several lead compounds with modifications involving the 3 position of Dyn A and suggested that position 3 in the message sequence of Dyn A is a promising site for further modifications.[22] We recently reported in preliminary studies that c[D-Asp^3,Lys^7]Dyn A(1−11)-NH_2 and c[Asp^3,Lys^7]Dyn A(1−11)-NH_2 were the first message-cyclized ligands to retain the κ receptor selectivity.[23] To further analyze these conformational suggestions, to evaluate the importance of global constraints, and to investigate further the conformational requirements for high potency and selectivity for the κ opioid receptor, cyclic [*i*, *i* + 4] lactams of Dyn A analogues have been designed, synthesized, and evaluated for their biological activities. Since Tyr^1 and Phe^4 were reported to be critical for opioid agonist activity and potency, positions 2−6, 3−7, and 5−9 were chosen as the sites for incorporation of a conformational constraint to determine whether the helical conformation is important in the address or message sequence of Dyn A. We report here the results of this comprehensive study.

Results and Discussion

All Dyn A analogues were synthesized as C-terminal carboxamide analogues (to impart stability to exopeptidase[24]) by solid phase methods,[25,26] purified by RP-HPLC, and characterized by FAB mass spectrometry and amino acid analysis. The purity of the synthesized peptides was assessed by TLC (single spot in four different solvent systems, ninhydrin detection) and HPLC (one single peak, UV detection at 280 nm and 225 nm, using two independent gradients) (see the Experimental Section).

Binding Assay

The peptides were evaluated for opioid receptor affinities at κ, μ, and δ 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), [^{3}H]DAMGO ([D-Ala^2,$MePhe^4$,Glyol^5]enkephalin), and [^{3}H]c[D-Pen^2,*p*-Cl-Phe^4,D-Pen^5]enkephalin, respectively, to opioid receptors in plasma membrane homogenates of the guinea pig brain (GPB) (Table 1). The selectivities are defined as the IC_{50} ratios of μ vs κ and δ vs κ receptors. The linear Dyn A(1−11)-NH_2 (**1**) was chosen as the standard for comparison with the synthetic Dyn A analogues.

In the GPB binding assay, it was found that Dyn A analogues with a D-residue at position 2, 3, or 9 (analogue **5**, **7**, **9**, **11**, or **13**) are generally more potent for the κ receptor than the corresponding analogues with an L-residue at the same position (**4**, **6**, **8**, **10**, and **12**). The cyclic c[Asp^2,Lys^6]Dyn A(1−11)-NH_2 analogue (**2**) displayed poor affinities for κ, μ, and δ receptors (IC_{50} are 100, 2980, and 910 nM, respectively), but slightly increased selectivity for κ vs μ receptors (IC_{50} ratio is 29.2). Interestingly, the related cyclic analogue c[D-Asp^2, Lys^6]Dyn A(1−11)-NH_2 (**3**) displayed similar affinity for the κ receptor (IC_{50} value is 110 nM), but greatly improved affinities for μ and δ receptors (IC_{50} values are 3.4 and 12 nM, respectively), leading to a ligand that preferentially interacts with μ and δ receptors. The cyclic analogue c[Asp^3,Lys^7]Dyn A(1−11)-NH_2 (**4**) displayed moderate affinity for the κ receptor (IC_{50} is 25 nM) and greatly decreased affinities for μ and δ receptors (IC_{50} values are 740 and 1710 nM, respectively), leading to increased selectivities for κ vs μ and κ vs δ receptors (IC_{50} ratios are 30 and 69). On the other hand, the corresponding cyclic analogue c[D-Asp^3,Lys^7]-Dyn A(1−11)-NH_2 (**5**) displayed high affinity for the κ receptor (IC_{50} value is 4.9 nM) and also is the most selective for κ vs μ receptors (IC_{50} ratio is 64) of all analogues reported here. To our knowledge, analogues **4** and **5** are the only message-cyclized peptide ligands that retain good κ opioid receptor selectivity. These interesting biological results provide new insights into the requirements for high affinity and selectivity for the κ receptor. These biological results and the preliminary results of NMR studies of analogues **4** and **5** suggest that the conformational changes induced in the message sequence of Dyn A may be stabilized by a D-Asp^3 and demonstrate for the first time that a basic residue in position 7 is not needed for good κ receptor binding and selectivity in dynorphin analogues. On the other hand, the structural effects of L-Asp^2 vs D-Asp^2 and L-Asp^3 vs D-Asp^3 clearly play an important role in discriminating binding preference for κ, μ, and δ receptors. For the 5,9 cyclized analogues we placed a Lys in position 5 which has a side chain more homologous to Leu^5, and because our previous work had shown that a D-amino acid in position 5 was not consistent with high κ receptor potency.[27] The cyclic analogue c[Lys^5,Asp^9]Dyn A(1−

11)-NH_2 (**6**) was found to be not very potent at any of the opioid receptors and was nonselective for the κ receptor (Table 1). Since the L-configuration in position 5 has been reported to be important for κ affinity,[27] the related cyclic analogue **7** with a D-amino acid in position $i + 4$ and L-amino acid in position i was synthesized, although this linkage would be expected to be shorter than an idealized helix would require.[28] Nonetheless, the cyclic analogue c[Lys^5,D-Asp^9]Dyn A(1−11)-NH_2 (**7**) exhibited good affinity (6.6 nM) for the κ receptor, but decreased selectivity for the κ receptor. We suggest that further modifications in the message sequence of cyclic Dyn A analogues may lead to novel peptides with high potency and selectivity. Of all the synthesized analogues, the linear [D-Asp^2,Lys^6]Dyn A(1−11)-NH_2 (**9**) was found to be the most potent for central κ, μ, and δ receptors (IC_{50} values are 1.5, 0.08, and 1.15 nM, respectively). However, this linear analogue preferentially interacted with μ and δ receptors. The high affinities for all three receptors may be due to increased enzymatic stability and/or to stabilization of the helical conformation. Of particular note is the very high affinity of **9** at the μ opioid receptor. It would appear that an acidic amino acid in the 2 position can lead to very strong binding to μ receptors that deserves further examination in other opioid ligands. The structural effects of D-Asp^2 may be responsible for the preference for μ and δ receptors for this analogue. The other message sequence-modified linear analogues, [Asp^2,-Lys^6]Dyn A(1−11)-NH_2 (**8**), [Asp^3,Lys^7]Dyn A(1−11)-NH_2 (**10**), and [D-Asp^3,Lys^7]Dyn A(1−11)-NH_2 (**11**), all displayed decreased affinities for opioid receptors and decreased selectivities for κ vs μ and κ vs δ receptors. It is interesting to note that, with the exception for analogue **9**, the message-cyclized analogues generally display somewhat higher affinity for the κ receptor than the related linear analogues (**2** vs **8**, **4** vs **10**, and **5** vs **11**). This indicates that the conformational change induced in the message sequence may be important for maintaining high affinity for the κ receptor, but not for μ and δ receptors, although cyclic analogue **5** also is more potent than linear analogue **11** at the μ and δ receptor as well. The linear analogue [Lys^5,Asp^9]Dyn A(1−11)-NH_2 (**12**) displayed moderate affinity for the κ receptor (IC_{50} value is 35 nM) and enhanced selectivities for κ vs δ receptors (IC_{50} ratio is 94). Interestingly, the linear analogue [Lys^5,D-Asp^9]Dyn A(1−11)-NH_2 (**13**) exhibited moderate affinity for the κ receptor (IC_{50} value is 17.5 nM) and enhanced selectivities for κ vs μ (IC_{50} ratio is 32) and κ vs δ receptors (IC_{50} ratio is 112). These results indicate that the stereochemical and electrochemical properties produced by the replacement of an Arg^9 with an L-Asp^9 or a D-Asp^9 residue and by the replacement of an Leu^5 with an Lys^5 (**12** and **13**, respectively), though reducing affinity for the κ receptor, generally have an even greater effect on μ and δ receptor bindings. Interestingly, except for **6** at the κ receptor, cyclization of these analogues leads to increased affinities at κ, μ, and δ receptors.

Bioassay

The opioid activities of these peptides also were measured by their ability to inhibit the electrically evoked contraction of the GPI[29] (guinea pig ileum) (Table 2). The selectivity between the central and peripheral κ receptor systems is defined as the IC_{50} ratio of the GPB and GPI (Table 3).

Table 2. Bioassays with the Smooth-Muscle Tissue of the Guinea Pig Ileum

analogues of Dyn A(1−11)-NH_2	IC_{50} (nM)	
	GPI	fold-shift[a]
Dyn A(1−11)-NH_2 (**1**)	1.07 ± 0.31	ns
c[Asp^2,Lys^6] (**2**)	16000 ± 1300	nt
c[D-Asp^2,Lys^6] (**3**)	610 ± 23	17
c[Asp^3,Lys^7] (**4**)	1500 ± 86	nt
c[D-Asp^3,Lys^7] (**5**)	600 ± 51	nt
c[Lys^5,Asp^9] (**6**)	9300 ± 95	nt
c[Lys^5,D-Asp^9] (**7**)	2500 ± 180	nt
[Asp^2,Lys^6] (**8**)	490 ± 74	nt
[D-Asp^2,Lys^6] (**9**)	14.2 ± 1.8	4.5
[Asp^3,Lys^7] (**10**)	13300 ± 182	nt
[D-Asp^3,Lys^7] (**11**)	4900 ± 288	nt
[Lys^5,Asp^9] (**12**)	3170 ± 510	nt
[Lys^5,D-Asp^9] (**13**)	4160 ± 423	nt

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

Table 3. Central (GPB) vs Peripheral (GPI) Nervous Systems Selectivities at the κ Opioid Receptors of Various Dyn A Analogues

analogues of Dyn A(1−11)-NH_2	ratio of IC_{50} GPI/GPB	analogues of Dyn A(1−11)-NH_2	ratio of IC_{50} GPI/GPB
Dyn A(1−11)-NH_2 (**1**)	1.8	[Asp^2,Lys^6] (**8**)	2.2
c[Asp^2,Lys^6] (**2**)	160	[D-Asp^2,Lys^6] (**9**)	9.5[a]
c[D-Asp^2,Lys^6] (**3**)	5.6[a]	[Asp^3,Lys^7] (**10**)	72
c[Asp^3,Lys^7] (**4**)	61	[D-Asp^3,Lys^7] (**11**)	110
c[D-Asp^3,Lys^7] (**5**)	120	[Lys^5,Asp^9] (**12**)	91
c[Lys^5,Asp^9] (**6**)	91	[Lys^5,D-Asp^9] (**13**)	240
c[Lys^5,D-Asp^9] (**7**)	380		

[a] Not really κ receptor selective.

Dyn A(1−11)-NH_2 (**1**) showed an IC_{50} value of 1.07 nM in the GPI bioassay. It has been shown that this activity is due only to a peripheral κ receptor rather than the μ receptor because the highly μ selective antagonist ligand CTAP[30] has no antagonist effect on the activity of **1** in the GPI assay (Table 2). All the analogues synthesized are less potent than Dyn A(1−11)-NH_2 (**1**), but display increased selectivities for the central vs periphery κ receptors (ratios range from 2.2 to 380, measured as the IC_{50} ratios of the GPB and GPI). Except for analogue **13**, analogues with a D-residue at position 2, 3, or 9 are more potent and selective than analogues with an L-residue at the same position. The cyclic analogues c[D-Asp^3,Lys^7]Dyn A(1−11)-NH_2 (**5**) exhibited good selectivity for the central vs peripheral κ receptors (IC_{50} ratio is 122) and was found to be the most potent in the periphery (IC_{50} value is 600 nM) of the cyclic analogues. The cyclic analogue c[Asp^2,Lys^6]-Dyn A(1−11)-NH_2 (**2**) also exhibited good selectivity for the central vs peripheral κ receptors (IC_{50} ratio is 160). These results suggest that the incorporation of conformational constraints into the message sequence may produce or stabilize a conformation which is important for selectivity for central vs peripheral κ receptors. The cyclic analogue c[Lys^5,D-Asp^9]Dyn A_1−11NH_2 (**7**) showed the highest selectivity (IC_{50} value is 380) of all analogues synthesized. Linear analogues [D-Asp^3,Lys^7]Dyn A(1−11)-NH_2 (**11**) and [Lys^5,D-Asp^9]Dyn A(1−11)-NH_2 (**13**) exhibited poor potency for the peripheral κ receptor, but high selectivities for central vs peripheral κ receptors. (IC_{50} ratios are 110 and 240, respectively).

Table 4. Analytical Properties of Various Dyn A Analogues

analogues of Dyn A(1−11)-NH_2	TLC R_f values[a]				K' HPLC[b]		
	I	II	III	IV	1	2	FAB-MS[c]
Dyn A(1−11)-NH_2 (**1**)	0.65	0.20	0.80	0.65	3.4	5.0	1362 $[M]^+$
c[Asp^2,Lys^6] (**2**)	0.66	0.24	0.74	0.77	3.72	3.19	1374 $[M + H]^+$
c[D-Asp^2,Lys^6] (**3**)	0.69	0.29	0.73	0.77	2.91	2.76	1374 $[M + H]^+$
c[Asp^3,Lys^7] (**4**)	0.71	0.37	0.93	0.82	3.51	2.90	1374 $[M + H]^+$
c[D-Asp^3,Lys^7] (**5**)	0.74	0.41	0.92	0.80	2.51	2.32	1374 $[M + H]^+$
[Lys^5,Asp^9] (**6**)	0.61	0.25	0.91	0.73	2.44	3.29	1318 $[M + H]^+$
c[Lys^5,D-Asp^9] (**7**)	0.63	0.28	0.64	0.77	2.39	2.63	1318 $[M + H]^+$
[Asp^2,Lys^6] (**8**)	0.53	0.26	0.79	0.72	2.50	2.25	1392 $[M + H]^+$
[D-Asp^2,Lys^6] (**9**)	0.58	0.22	0.73	0.74	2.53	3.22	1392 $[M + H]^+$
[Asp^3,Lys^7] (**10**)	0.65	0.17	0.68	0.78	2.58	2.64	1392 $[M + H]^+$
[D-Asp^3,Lys^7] (**11**)	0.58	0.39	0.58	0.77	2.60	2.50	1392 $[M + H]^+$
[Lys^5,Asp^9] (**12**)	0.49	0.30	0.71	0.69	1.88	1.64	1336 $[M + H]^+$
[Lys^5,D-Asp^9] (**13**)	0.49	0.16	0.66	0.65	2.59	1.56	1336 $[M + H]^+$

[a] Solvent systems: I, 1-butanol/pyridine/acetic acid/water (15/10/3/8); II, 1-butanol/acetic acid/water (4/1/5); III, 2-propanol/concentrated ammonium hydroxide/water (3/10/10); IV, 1-butanol/pyridine/acetic acid/water (6/6/1/5). [b] 1 and 2 refer to the HPLC gradients as described in the Experimental Section. [c] Fast atom bombardment mass spectroscopy.

Conclusions

The biological results provide some new insights for the future design of conformationally constrained Dyn A analogues. The biological results for the new analogues demonstrate that the configuration, position, and electrochemical properties of the residues that are incorporated into the cyclic lactam analogues of Dyn A have great effects on the potency and selectivity for the κ receptor. It is found that analogues with a D-Asp incorporated into position 2, 3, or 9 of Dyn A(1−11)-NH_2 are more potent at the central κ receptor than analogues with an L-Asp incorporated into the same positions. The cyclic analogues c[Asp^3,Lys^7]- and c[D-Asp^3,Lys^7]Dyn A(1−11)-NH_2 (**4** and **5**) which displayed good affinity and increased selectivity for the κ receptor appear to be lead compounds based on this study. These results demonstrate that the incorporation of conformational constraints involving residues at the 3 and 7 positions of Dyn A into lactam analogues of Dyn A have important effects on the selectivity for κ vs μ and/or κ vs δ receptors. It is suggested that cyclization through these positions to give conformationally constrained analogues will have useful effects for potency and selectivity for κ receptors. To further investigate the effect of ring size of this global conformation on potency and selectivity for the κ receptor, the incorporation of conformational constraints between positions 3 and 6, 3 and 8, or 3 and 9 of Dyn A(1−11)-NH_2 may be a rational approach.

Experimental Section

Peptide Synthesis and Purification. Peptides were synthesized by the solid phase method utilizing a Boc/benzyl strategy and a *p*-methylbenzhydrylamine (*p*-MBHA) resin (Advanced Chem Tech, Louisville, KY), as previously described for dynorphin analogues.[31] Side chain protected N^{α}-Boc amino acids were purchased from Bachem (Torrance, CA), whereas the other amino acids were synthesized by standard methods in our laboratory. The analytical data for the purified peptides are given in Tables 4 and 5.

Thin-layer chromatography of synthetic peptides was performed on silica gel plates (0.25 mm, Analtech, Newark, DE) with the solvent systems given in Table 4. Peptides were detected with ninhydrin reagent. HPLC was carried out by using of a binary pump (Perkin-Elmer LC 250) equipped with an UV/vis detector (Perkin-Elmer LC 90 UV model) and integrator (Perkin-Elmer LCI 100 model). The solvent system used for analytical HPLC was a binary system, water containing 0.1% of TFA (pH 2.0) and acetonitrile as the organic modifier, and solvent programs involved linear gradients as follows: (1) 10−90% acetonitrile over 40 min and (2) 10−50% over 30 min. The flow rate was 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) 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). The results are reported in Table 5.

General Method for Peptide Synthesis. The N^{α}-Boc amino acids (4 equiv) were sequentially coupled to the growing peptide chain using diisopropylcarbodiimide (DIC) and *N*-hydroxybenzotriazole (HOBt) in *N*-methyl-2-pyrrolidone (NMP). The coupling reaction time was 1 h. Side chain protecting groups used were 2,4-dichlorobenzyloxycarbonyl or 9-fluorenylmethyloxycarbonyl (Fmoc) for Lys, tolylsulfonyl (Tos) for Arg, 2,6-dichlorobenzyl (2,6-Cl_2Bzl) for Tyr, and fluorenylmethyl (Fm) for Asp. Trifluoroacetic acid (TFA, 50% in DCM) was used to remove the N^{α}-Boc protecting group, and 20% piperidine was used to remove the Fmoc side chain protecting group from Lys and the OFm group from Asp. Diisopropylethylamine (DIEA) was used as a base, and dichloromethane (DCM) and NMP were used as solvents for washing. The fully protected peptide-resin was dried and separated into two portions for synthesizing the linear and cyclic peptides. The linear peptide-resin was produced by removing the Fmoc side chain protecting group from Lys and the OFm group from Asp and then deprotecting the last N^{α}-Boc group. The cyclic peptides were cyclized on the resin by removing the Fmoc side chain protecting group from Lys and the OFm group from Asp, cyclizing to the lactam ring using 3 equiv of BOP reagent and 6 equiv of DIEA in NMP as coupling reagent, reacting for 7−15 h at room temperature, and then deprotecting the last N^{α}-Boc group. Each peptide-resin was dried *in vacuo*, and the peptide was then cleaved from the resin using liquid anhydrous hydrofluoric acid (HF) in the presence of cresol (10% w/v) for 1 h at 0 °C. After removal of HF *in vacuo*, the residue was washed with anhydrous ether and extracted with 30% aqueous acetic acid. The acetic acid solution was lyophilized to give a white residue. The crude peptide was then purified by semipreparative reverse phase HPLC under the conditions described above to yield a white powder after lyophilization. The average yield for linear and cyclic peptides are 14% and 11%, respectively.

The structures were corroborated by the results of the amino acid analysis and mass spectrometry, and the purity of each product was assessed by analytical HPLC and TLC (Tables 4 and 5).

Table 5. Amino Acid Analysis of Various Dyn A Analogues

analogues of Dyn A(1−11)-NH_2	amino acids[a]								
	Tyr	Gly	Phe	Leu	Arg	Ile	Pro	Lys	Asp
Dyn A(1−11)-NH_2 (**1**)	1.1 (1)	2.0 (2)	1.1 (1)	1.1 (1)	2.8 (3)	1.1 (1)	0.9 (1)	1.0 (1)	
c[Asp^2,Lys^6] (**2**)	0.9 (1)	1.0 (1)	1.1 (1)	0.97 (1)	2.02 (2)	0.8 (1)	0.98 (1)	1.98 (2)	0.95 (1)
c[D-Asp^2,Lys^6] (**3**)	0.95 (1)	0.9 (1)	1.02 (1)	1.01 (1)	2.01 (2)	0.9 (1)	0.98 (1)	1.95 (2)	0.95 (1)
c[Asp^3,Lys^7] (**4**)	0.95 (1)	1.01 (1)	1.01 (1)	0.96 (1)	2.01 (2)	0.90 (1)	0.99 (1)	1.97 (2)	0.95 (1)
c[D-Asp^3,Lys^7] (**5**)	0.95 (1)	0.98 (1)	1.03 (1)	1.01 (1)	2.04 (2)	0.90 (1)	0.99 (1)	2.0 (2)	0.97 (1)
[Lys^5,Asp^9] (**6**)	1.1 (1)	2.0 (2)	1.1 (1)		1.9 (2)	1.0 (1)	0.99 (1)	1.9 (2)	0.9 (1)
c[Lys^5,D-Asp^9] (**7**)	1.2 (1)	2.1 (2)	1.0 (1)		1.95 (2)	1.1 (1)	0.99 (1)	2.0 (2)	0.95 (1)
[Asp^2,Lys^6] (**8**)	0.95 (1)	0.98 (1)	1.01 (1)	1.01 (1)	2.0 (2)	0.90 (1)	0.99 (1)	2.0 (2)	0.9 (1)
[D-Asp^2,Lys^6] (**9**)	0.95 (1)	0.96 (1)	1.03 (1)	1.01 (1)	2.04 (2)	0.95 (1)	0.99 (1)	2.0 (2)	0.97 (1)
[Asp^3,Lys^7] (**10**)	1.1 (1)	0.98 (1)	1.01 (1)	1.01 (1)	1.9 (2)	0.90 (1)	0.99 (1)	1.95 (2)	0.97 (1)
[D-Asp^3,Lys^7] (**11**)	0.95 (1)	0.98 (1)	1.03 (1)	1.01 (1)	2.04 (2)	0.90 (1)	0.99 (1)	2.0 (2)	0.97 (1)
[Lys^5,Asp^9] (**12**)	1.1 (1)	2.0 (2)	1.1 (1)		1.9 (2)	1.1 (1)	1.0 (1)	1.97 (2)	1.1 (1)
[Lys^5,D-Asp^9] (**13**)	1.0 (1)	2.1 (2)	1.0 (1)		2.0 (2)	1.0 (1)	1.0 (1)	2.0 (2)	0.95 (1)

[a] Theoretical values in parentheses. L- or D-amino acid for Asp. Hydrolysis in 4 N methanesulfonic acid (0.2% 3-(2-aminomethyl)indole) at 110 °C for 24 h.

Radioligand Binding Assay

Membranes were prepared from whole brains taken from adult male guinea pigs (200−400 g) obtained from SASCO (Omaha, Nebraska). Following decapitation, the brain was removed, dissected, and homogenized at 0 °C in 20 volumes of 50 mM Tris-HCl (Sigma, St. Louis, MO) buffer adjusted to pH 7.4 using a Teflon−glass homogenizer. The membrane fraction obtained by centrifugation at 48 000*g* for 15 min at 4 °C was resuspended in 20 volumes of fresh Tris-HCl buffer and incubated at 25 °C for 30 min to dissociate any receptor bound endogenous opioid peptides. The incubated homogenate was centrifuged again as described and the final pellet resuspended in 10 volumes of fresh Tris-HCl buffer. Radioligand binding inhibition assay samples were prepared in an assay buffer consisting of 50 mM Tris-HCl, 1.0 mg/mL bovine serum albumin, 30 μM bestatin, 50 μg/mL bacitracin, 10 μM captopril, and 0.1 mM phenylmethanesulfonyl fluoride, pH 7.4 (all from Sigma, St. Louis, MO, except bestatin that was purchased from Peptides International, Louisville, KY). The radioligand used were [^{3}H]c[D-Pen^2,*p*-Cl-Phe^4,D-Pen^5]enkephalin[32] (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). Peptides were dissolved in the assay buffer prior to each experiment and added to duplicate assay tubes at 10 different concentrations over an 800-fold concentration range. Control (total) binding was measured in the absence of any inhibitor, while nonspecific binding was measured in the presence of 10 μM naltrexone (Sigma, St. Louis, MO). The final volume of the assay samples was 1.0 mL, of which 10% consisted of the membrane preparation in 0.1 mL of Tris-HCl buffer. Incubation was performed at 25 °C for 3 h after which the samples were filtered through polyethylenimine (0.5% w/v, Sigma, St. Louis, MO)-treated GF/B glass fiber filter strips (Brandel, Gaithersburg, MD). The filters were washed three times with 4.0 mL of ice-cold 1 M NaCl solution before transfer to scintillation vials. The filtrate radioactivity was measured after adding 7−10 mL of cocktail (EcoLiteTM (+), ICN Biomedicals, Inc) to each vial and allowing the samples to equilibrate over 8 h at 4 °C.

Binding data was analyzed by nonlinear least-square regression analysis using the program Inplot 4.03 (GraphPadTM, 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.[33] Data best fitted by a one-site model was analyzed using the logistic equation.[34] Data obtained from at least three independent measurements in duplicate are presented as the arithmetic mean ± SEM.[35] The results are not corrected for the actual peptide content.

In Vitro GPI Bioassay

Electrically induced smooth muscle contraction of strips of guinea pig ileum longitudinal muscle−myenteric plexus was used as bioassay.[29] Tissues from male Hartley guinea pigs weighing 250−500 g were prepared as described previously.[36] The tissues were tied to gold chain with suture silk, suspended in 20 mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2) Krebs buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.18 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11.48 mM glucose), and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length previously determined to be 1 g tension and again allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum wire electrodes at 0.1 Hz, 0.4-ms pulses of supramaximal voltage. Drugs were added to the baths in 15−60 μL volumes. The agonists remained in contact with the tissue for 3 min before the addition of the next cumulative dose, until maximum inhibition was reached. Percent inhibition was calculated by using the average contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the dose of the agonist. To further define the opioid selectivity of the agonist effect, the μ selective antagonist CTAP was used at the concentration of 1000 nM.[37] IC_{50} values represent the mean of no less than four tissues. IC_{50} estimates, relative potency estimates, and their associated standard errors were determined by fitting the mean data to the Hill equation by a computerized nonlinear least-square method.[35] The results are not corrected for the actual peptide content.

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(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; CTAP, c[D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2]; HOBt, *N*-hydroxybenzotriazole; NMP, *N*-methyl-2-pyrrolidone; Fmoc, 9-fluorenylmethyloxycarbonyl; Tos, tolylsulfonyl; Fm, fluorenylmethyl; DCM, dichloromethane; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine.

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