Design and Synthesis of Highly Potent and Selective Cyclic Dynorphin A Analogues

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We have designed and synthesized several cyclic disulfide-containing peptide analogues of dynorphin A (Dyn A) which are conformationally constrained in the putative "address" segment of the opioid ligand. Several of these Dyn A analogues exhibit unexpected selectivities for the κ and μ opioid receptors(s) of the central vs peripheral nervous systems. Thus, incorporation of conformational constraint in the putative "address" segment of Dyn A analogues has resulted in the κ/μ opioid receptor ligands [Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 (1) and [Cys^5,Cys^{11},D-Ala^8]Dyn A_{1-11}-NH_2 (2), which possess high κ and μ opioid receptor affinities centrally (guinea pig brain, GPB), but only weak activity at peripheral κ and μ opioid receptors (guinea pig ileum, GPI). On the other hand, [Cys^8,Cys^{13}]Dyn A_{1-13}-NH_2 and [D-Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2 (5) display high κ potencies *and* selectivities at the peripheral (GPI) but not at the central (GPB) κ opioid receptor. The lack of correlation between the pharmacological profiles observed in smooth muscle and in the brain binding assays suggests the existence of different subtypes of the κ and μ opioid receptors in the brain and peripheral nervous systems.

Since the discovery of the endogenous enkephalins,[1] there has been an enormous amount of research done in the peptidic and nonpeptidic opioid areas.[2-4] Shortly after the characterization of the endogenous enkephalins, attempts were made to determine their preferred conformations. By use of various spectroscopic methods, X-ray crystallography, and/or energy calculations, different conclusions were reached.[2,3,5] Now it is accepted that the enkephalins are highly flexible molecules that can assume an ensemble of energetically preferred conformations.[6] There may be definite advantages for a biological system to utilize hormones or neurotransmitters of high conformational flexibility including (1) the availability of thermodynamically accessible pathways to ligand-receptor interactions via a "zipper model";[7] (2) the ability of a specific hormone or neurotransmitter to assume different conformations which could effect different molecular pharmacological events, e.g., ligand-receptor binding, transduction, and reversal of ligand-receptor binding;[8] and (3) the availability of different conformations for a specific ligand to permit binding to multiple receptor subtypes.[9] In regard to the latter, it has been shown that the opioid receptors are heterogeneous and consist of at least three subtypes, namely μ, δ, κ, and possibly others.[10,11] It is thought that the complexity of the pharmacological responses (analgesia, respiratory depression, physiological dependence and tolerance, gut motility, etc.) to the opioids may be due in part to their nonselective binding to these and perhaps other opioid receptor subtypes.[12] Before the physiological role(s) of opioids can be understood, a prerequisite to the rational design of therapeutic opioid drugs, highly receptor-selective ligands (both agonists and antagonists) for the opioid receptor subtypes must be developed.[4]

Most of the structure-function studies of the κ selective ligands have been based on the putative endogenous ligands dynorphin A (Dyn A), dynorphin B (Dyn B), and α-neoendorphin.

Recently the structure-function relationships for the dynorphin peptides have been reviewed.[13] Some of the more important points will be discussed. Sequential removal of the C-terminal amino acids of Dyn A_{1-13} established that the basic residues Arg-7, Lys-11, and Lys-13 were required for high κ selectivity and/or potency,[14] but deletion of residues 14-17 or even 12-17 did not significantly affect Dyn A potency.[14] Substitution of lipophilic residues and certain D-amino acids at position 8 of Dyn A increase κ receptor selectivity.[15] These latter observations may suggest a reverse turn at this position.[16] Similarly the substitution with D-Pro in position 10 of Dyn

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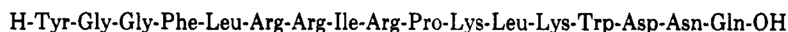
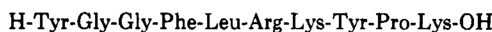

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH

Dyn A

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-OH

Dyn B


H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-OH

α-neoendorphin

A_{1-11} or Dyn A_{1-13} effects greater κ selectivity and is compatible with high κ receptor potency.[15,17,18] Again this may be suggestive of a reverse turn and/or the need for an N-substituted amino acid[16] in position 10 for high κ selectivity and/or potency.

Thus far, the most potent and selective κ opioid receptor agonists have been several analogues of *N*-methyl-*N*-pyrrolidinylcyclohexyl)benzeneacetamide, such as U-50488,[19] of which U-69593 appears to be the most selective for the κ opioid receptor with a κ vs μ selectivity ratio of 484-fold.[20] The most potent and selective peptidic κ opioid receptor agonist reported appears to be [D-Pro^{10}]Cyn A_{1-11}-OH (DPDYN).[17] DPDYN exhibits moderate κ vs μ (62) and κ vs δ selectivities with a binding affinity of 0.032 nM against the κ ligand [^{3}H]bremazocine. In tissue bioassays DPDYN displays a hamster vas deferens (HVD) (δ) vs guinea pig ileum (GPI) (μ and κ) IC_{50} ratio of 280 with an IC_{50} = 3.3 nM in the GPI.[21] When administered intracerebroventricularly (icv), DPDYN did not show any activity against thermal stimulus but, in contrast, produced a dose-related effect against chemical pain.[18]

An indirect approach to obtaining information on the receptor-bound bioactive conformations(s) of opioid ligands has been to utilize constrained or conformationally restricted peptides.[22] The use of modern pharmacological methods (binding assays, in vitro bioassays, etc.) in conjunction with spectroscopic methods (e.g., two-dimensional nuclear magnetic resonance (2-D NMR) for conformational analysis has resulted in the design and syntheses of conformationally constrained opioid peptides that are highly receptor-selective, e.g., the bis-penicillamine-containing enkephalin analogue [D-Pen^2,D-Pen^5]enkephalin,[23] which is a δ opioid agonist, and several somatostatin-related cyclic octapeptides,[24-27] which are μ opioid receptor antagonists. In this study we have explored possible requirements of the bioactive conformation for potency and receptor selectivity at the κ opioid receptors in the brain and in peripheral receptor systems by the synthesis and pharmacological evaluation of two different classes of Dyn A analogues in the putative "address"[28] sequence of the peptide as cyclic disulfides, either as Cys^5,Cys^{11} disulfides or as Cys^8,Cys^{13} disulfides. We have found that several of these analogues display an exceptional selectivity for the central (brain) vs peripheral (ileum) κ receptors.

Results and Discussion

Various spectroscopic methods (FT-IR,[29] ^{1}H NMR,[30] Raman,[31] fluorescence energy transfer,[32] and circular dichroism[33]) support the existence of an extended and/or unordered conformation for Dyn A in an aqueous environment. In this situation where spectroscopy offers sparse information about a preferred secondary structure of a ligand, the method of conformational constraint, in conjunction with bioassays, may provide insight regarding the bioactive conformation of the ligand at its receptor. The first conformationally constrained Dyn A analogue reported was the cyclic disulfide [D-Cys^2,Cys^5]Dyn A_{1-13}.[34] In the GPI and mouse vas deferens (MVD) bioassays [D-Cys^2,Cys^5]Dyn A_{1-13} exhibited high potency in the GPI and possessed a high MVD/GPI IC_{50} ratio. The cyclic analogue displayed a high μ affinity in binding studies using rat brain homogenates.[34] In another study involving selective tolerance in the MVD, [D-Cys^2,Cys^5]Dyn_{1-13} was shown to be selective for the δ receptor with minor κ activity.[35] Recently cyclic lactams of Dyn A, [D-Orn^2,Asp^5]Dyn A_{1-8}, [Orn^5,Asp^8]Dyn A_{1-3}, [Orn^5,Asp^{10}]Dyn A_{1-13}, and [Orn^5,Asp^{13}]Dyn A_{1-13} were prepared.[36] The analogue [D-Orn^2,Asp^5]Dyn A_{1-8} showed high potency in the GPI but displayed a naloxone K_e value of 1.5 nM, which supported interaction with the μ receptor.[10,37] Analogues [Orn^5,Asp^8]-, [Orn^5,Asp^{10}]–, and [Orn^5,Asp^{13}]Dyn A_{1-3} exhibited low potencies in both the GPI and MVD bioassays and also displayed naloxone K_e values consistent with an

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Table I. Opioid Receptor Binding Affinities and Selectivities of Various Cyclic Disulfide-Containing Dynorphin Analogues with Guinea Pig Brain Homogenate

		IC_{50}, nM				
no.	compound	[^{3}H]U-69,593	[^{3}H]PL-17	[^{3}H]DPDPE	μ/κ	δ/κ
	Dyn A_{1-17}-OH	0.231 ± 0.101	5.04 ± 1.10	2.54 ± 0.23	22	11
	Dyn A_{1-11}-NH_2	0.077 ± 0.017	1.08 ± 0.07	6.99 ± 0.901	14	91
	Dyn A_{1-13}-NH_2	0.114 ± 0.036	1.29 ± 0.30	4.07 ± 1.10	11	36
1	[Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2	0.285 ± 0.036	0.270 ± 0.137	1.63 ± 0.53	0.95	5.7
2	[Cys^5,Cys^{11},D-Ala^8]Dyn A_{1-11}-NH_2	0.391 ± 0.059	2.30 ± 0.72	18.6 ± 7.4	5.9	48
3	[Cys^8,Cys^{13}]Dyn A_{1-13}-NH_2	0.074 ± 0.031	0.986 ± 0.115	3.97 ± 0.96	13	54
4	[D-Cys^8,Cys^{13}]Dyn A_{1-13}-NH_2	1.76 ± 0.35	10.3 ± 3.9	104 ± 2	5.8	59
5	[D-Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2	0.110 ± 0.009	0.362 ± 0.127	14.3 ± 1.8	3.3	130

interaction with the μ receptor. In binding studies, analogues [Orn^5,Asp^8]- and [Orn^5,Asp^{13}]Dyn A_{1-13} possessed high μ affinities and moderate μ vs δ selectivities. Binding affinities at κ receptors were not reported. It was concluded that none of the mentioned cyclic lactams of Dyn A possessed κ selectivity.

Initially we chose to apply a conformational constraint in the "address"[28] sequence of Dyn A analogues in the form of cyclic disulfides. Among the analogues prepared, [Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 (**1**) contains a 23-membered cyclic disulfide ring system. Previous structure–activity studies had suggested that the Lys^{11} residue of Dyn A was required for high κ potency and/or κ selectivity.[14] However, structure–function studies of Dyn A analogues[15] suggested the existence of a reversed turn at the 8-position as a feature for κ selectivity, and Chou–Fasman calculations,[38] performed by us, suggested the presence of a reversed turn at the 8-position. This suggested that a disulfide ring system using Cys^5 and Cys^{11} substitutions in Dyn A_{1-11} could result in a preferred conformation with a reversed turn centered about the 8-position and without modification of the important basic residues in the 6-, 7-, and 9-positions. Dyn A analogue **1** was synthesized as the C-terminal carboxamide to impart stability to exopeptidases.[39] A similar rational was used for the design and synthesis of [Cys^5,Cys^{11},D-Ala^8]Dyn A_{1-11}-NH_2 (**2**), except that a D-Ala substitution was incorporated in the 8-position which could stabilize a reversed turn centered at that residue.[16] As previously mentioned, structure–activity relationships also suggested to us that a bioactive conformation of a κ selective ligand might possess a reversed turn centered at position 10. This led to the design and synthesis of the cyclic Dyn A analogues [Cys^8,Cys^{13}]Dyn A_{1-13}-NH_2 (**3**), [D-Cys^8,Cys^{13}]Dyn A_{1-13}-NH_2 (**4**), and [D-Cys^8,D-Cys^{13}]Dyn A_{1-13}-NH_2 (**5**) which contain a 20-membered ring system. D-Cys residues were incorporated to investigate the stereostructural requirements of this ring system for the various opioid receptors and to possibly further stabilize the peptide analogues against biodegradation.

The cyclic disulfide analogues **1–5** were synthesized by use of preformed *N*-hydroxybenzotriazole (HOBt) active esters[40] by the solid-phase method.[41] Peptides were cleaved from the resin and deprotected with liquid HF at −10 to 0 °C. Disulfide cyclizations were performed via oxidation with 0.01 N $K_3Fe(CN)_6$. An HPLC system was used for the final purification of peptides on a semipreparative (10 mg) or a preparative (100 mg) scale. The synthetic peptides eluted as symmetrical peaks (UV detection, 280 or 257, and 220 nm). For the analogues **1** and **5**, a quantitative Ellman test[42] was performed with the final products to confirm that the peptides were not in the sulfhydryl forms.

In competitive binding experiments with guinea pig brain (GPB) homogenates (using [^{3}H]U-69593, [^{3}H]PL-17, and [^{3}H]DPDPE as the κ, μ, and δ selective ligands, respectively) the peptide analogues [Cys^5,Cys^{11}]- (**1**) and [D-Ala^8,Cys^5,Cys^{11}]Dyn A_{1-11}-NH_2 (**2**) displayed very high affinities for the κ (IC_{50} = 0.285 and 0.391 nM, respectively) and μ (IC_{50} = 0.270 and 2.30 nM, respectively) receptors (Table I). Also analogue **1** possessed a high affinity (IC_{50} = 1.63 nM) and **2** a moderate affinity (IC_{50} = 18.6 nM) for the δ receptor. Thus, analogues **1** or **2** showed low κ vs μ selectivities (defined as the ratio of μ/κ IC_{50} values), 0.95 and 5.9, respectively, and low or moderate κ vs δ selectivities, 5.7 and 48, respectively. In the same binding assay, analogues **3**, **4**, and **5** also exhibited high affinities for the κ (IC_{50} = 0.074, 1.76, and 0.110 nM), respectively) and μ (IC_{50} = 0.986, 10.3, 0.362 nM, respectively) opioid receptors (Table I). Thus, analogues **3**, **4** and **5** also showed low κ vs μ, 13, 5.8, and 3.3, and moderate κ vs δ, 54, 59, and 130, selectivities, respectively.

Most strikingly, in the smooth-muscle bioassays (GPI and MVD) (Table II), analogue **1** displayed only weak agonist activity in the GPI with an IC_{50} of 1080 nM. The response of analogue **1** was opioid receptor mediated as evidenced by the antagonism of the concentration–response curve by naloxone (K_e = 66.7 nM). Lack of antagonism of the response to **1** by the highly selective μ antagonist CTAP[27] indicated that the response was mediated via the κ and not the μ opioid receptor. In the MVD bioassay, analogue **1** showed a weak opioid-mediated response (IC_{50} = 421 nM) which was antagonized by the δ antagonist ICI-174,864[43] (K_e = 86.2 nM). Similarly, the high-affinity analogue **2** displayed the pharmacological profile of a very weak μ and/or κ agonist in the GPI bioassay as evidenced by the high IC_{50} (4400 nM) and the antagonism of the response by naloxone and CTAP (K_e = 50 nM). In the MVD bioassay **2** exhibited characteristics of a weak δ agonist as indicated by a high IC_{50} of 1660 nM and strong antagonism of the response by ICI-174,864 (K_e = 21.3 nM). Of the series, the most selective and potent κ agonist in the smooth-muscle bioassays appears to be the

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Table II. Bioassays with the Smooth-Muscle Tissue of the Guinea Pig Ileum and the Mouse Vas Deferens

no.	compd	bioassay	IC_{50}, nM	K_e,[a] nM		
				NLX	CTAP	ICI
	Dyn A_{1-17}–OH	GPI	2.5	62.9	no*	nd
		MVD	22.5	58.8	no*	no
	Dyn A_{1-11}–NH_2	GPI	7.5	r	nr*	nd
		MVD	204	125	no*	625
	Dyn A_{1-3}–NH_2	GPI	1.7	82	2000	nd
		MVD	7.8	100	no	nd
1	[Cys^5,Cys^{11}]Dyn A_{1-11}–NH_2	GPI	1080	66.7	no	nd
		MVD	421	80	2000	86.2
2	[Cys^5,Cys^{11},D-Ala^8]Dyn A_{1-11}–NH_2	GPI	4406	y	50	nd
		MVD	1660	nd	500	21.3
3	[Cys^8,Cys^{13}]Dyn A_{1-13}–NH_2	GPI	1.3	38.9	no	nd
		MVD	20.1	95.2	175	156
4	[D-Cys^8,Cys^{13}]Dyn A_{1-13}–NH_2	GPI	2.27	32.3	256	nd
		MVD	24.5	30.3	370	667
5	[D-Cys^8,D-Cys^{13}]Dyn A_{1-13}–NH_2	GPI	1.75	233	no	nd
		MVD	19.5	18.2	1430	1000

[a] r = reversed a high concentration, antagonist study of entire dose–response curve (drc) not completed. nr = did not reverse at high concentrations. y = shifted drc; no = did not shift drc. * = CTP; no star = CTAP used as μ antagonist. nd = not determined.

cyclic Dyn A_{1-13} analogue **5**. In the GPI bioassay, the low IC_{50} (1.75 nM), the observed antagonism by naloxone (K_e = 233 nM), and the lack of antagonism of the response of CTAP designate analogue **5** as a selective κ agonist. In the MVD bioassay analogue **5** shows a moderate potency (IC_{50} = 19.5 nM) with an opioid receptor mediated response which is antagonized by neither CTAP nor ICI-174,864. Analogue **3**, a diastereoisomer of **5**, also displays high potency (IC_{50} = 1.3 nM) and a κ selectivity in the GPI bioassay since the response was antagonized by naloxone (K_e = 38.9 nM), which can antagonize effects at both κ and μ receptors, but not by CTAP, which has selectivity for μ receptors and hence cannot antagonize at κ receptors. In the MVD bioassay, analogue **3** was shown to be a full agonist with moderate potency (IC_{50} = 20 nM); however, the response appeared to involve a significant interaction with the μ and δ opioid receptors as indicated by antagonism of the response by CTAP (K_e = 175 nM) and ICI-174,864 (K_e = 156 nM). These results together indicate that, in the GPI, **3** was active mainly at κ receptors, while in MVD (which has fewer κ receptors than GPI) it also acted at μ receptors. Analogue **4**, which is a diastereoisomer of both analogues **3** and **5**, did not retain κ selectivity in the GPI bioassay and exhibited characteristics of a μ agonist or μ/κ mixed agonist with high potency (IC_{50} = 2.3 nM). In light of the pharmacological and biochemical profile shown by analogue **2**, experiments were performed to investigate the potential μ and/or κ antagonistic effects of **2** in the GPI bioassay. In this experiment, the concentration–response curve of the κ agonists Dyn A_{1-17} and U-69593 (vs 100, 300, and 1000 nM of **2**), and of the μ agonist PL-17 (vs 100 nM of **2**), were not antagonized by **2** (data not shown).

If the affinities at the κ opioid receptor in the GP brain binding studies are compared to potencies in the GPI bioassay, a measure of the selectivity of the ligand for the central κ vs peripheral κ (or possibly κ and μ) receptor system(s) can be obtained (Table III). Analogue **2** shows an 11 000-fold and **1** a 3800-fold greater affinity in the guinea pig central nervous system vs potency in the peripheral nervous system (Table III). The analogues **3** and **5** possess about 16-fold higher affinities in the central nervous system vs potencies in peripheral nervous system, while the affinity and potency of **4** are about equal in the two systems. These results indicate that the central and peripheral κ (and apparently μ) opioid receptor system(s)

Table III. Central Nervous System (Guinea Pig Brain, GPB) vs Peripheral (Guinea Pig Ileum, GPI) Selectivities at the κ and μ Opioid Receptor(s) for Various Cyclic Dynorphin A Analogues

no.	compound	ratio of IC_{50} GPI/GPB
	Dyn A_{1-17}–OH	11
	Dyn A_{1-11}–NH_2	97
	Dyn A_{1-13}–NH_2	15
1	[Cys^5,Cys^{11}]Dyn A_{1-11}–NH_2	3800
2	[Cys^5,Cys^{11},D-Ala^8]Dyn A_{1-11}–NH_2	11000
3	[Cys^8,Cys^{13}]Dyn A_{1-13}–NH_2	17
4	[D-Cys^8,Cys^{13}]Dyn A_{1-13}–NH_2	1.3
5	[D-Cys^8,D-Cys^{13}]Dyn A_{1-13}–NH_2	16

in the guinea pig may have quite different conformational structure requirements. Other explanations for the large differences of IC_{50} values observed in the central vs peripheral systems for **1** and **2** could be the following: (1) the bioavailabilities of the compounds for the receptor may be much decreased in the bioassays in comparison to the binding assays, and (2) the compounds may be undergoing more biodegradation in the bioassays. These possibilities were addressed in part by performing the GPI bioassay in the presence of a peptidase-inhibitor cocktail similar to the binding studies. There was a small change in potency, but the potency in the bioassay is still vastly lower than in the binding assays.

Thus, the incorporation of conformational constraint in the "address" region of Dyn A has resulted in great differences in affinities and potencies shown by analogues **1** and **2** in the central vs peripheral nervous systems and has given rise to a good κ vs μ selectivity, which is observed for **3** and **5** in only the peripheral (GPI) and not the central nervous system. These results may suggest the existence of distinct κ[44] and μ opioid receptor subtypes for the central and peripheral nervous systems. Recent findings in the δ opioid receptor area have led to a similar proposal in regard to the existence of distinct δ opioid receptor subtypes for the central and peripheral nervous systems.[45,54] The new dynorphin analogues reported here can provide useful new tools for examining the pharmacological and

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physiological roles of central and peripheral κ receptors and potentially important new leads to the further development of potent κ receptor selective ligands. We are currently investigating further conformational constraints of Dyn A analogues and their effect upon selectivity and potency at the κ opioid receptor.

Experimental Section

In Vitro Bioassays. The guinea pig ileum longitudinal muscle/myenteric plexus preparation was used as described previously.[46] The tissues were suspended under a final tension of 1 g in organ baths, bathed with Krebs buffer (118 mM NaCl, 4.7 mM KCl, 4.7 nM KCI, 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), maintained at 37 °C, and aerated with 95% O_2/5% CO_2. Electrical stimuli were 0.4-ms pulses of supramaximal voltage, at a rate 6/min. Isometric contractions were measured via strain gauge force transducers on chart recorders. The mouse vas deferens preparation was also performed as described.[47] The Krebs buffer was made as above, but without magnesium, and the tissues were suspended under a final tension of 500 mg. Pulse duration was 2 ms. Concentration–response testing in all preparations was carried out in a cumulative fashion. The compounds were tested for intrinsic agonist activity in the two in vitro bioassays. Thus, concentrations showing intrinsic activity for each analogue were tested in the presence of naloxone (1000 mM) to define opioid activity. To further define the opioid selectivity of the agonist effect, the δ selective antagonist ICI 174,864[43] (1000 nM) was utilized in the MVD, and the μ selective antagonists CTAP[27] (1000 nM)[48] (1000 nM) were employed in the GPI. K_e values were calculated according to the formula:

$$K_e = [\mathrm{Ant}]/(\mathrm{DR} - 1)$$

where [Ant] is the concentration of the antagonist, and DR is the dose ratio (i.e., IC_{50} after antagonism divided by the IC_{50} before antagonist activity was measured.

Radioligand Binding Assay. Membranes were prepared from whole brains taken from adult male Hartley guinea pigs (300–500 g) obtained from Harlan Sprague–Dawley Inc. (Indianapolis, IN). 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 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 was resuspended in 20 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 (phenylmethyl)sulfonyl fluoride, pH 7.4 (all from Sigma, St. Louis, MO, except captopril that was a gift of Squibb, Princeton, NJ). The radioligands used were [^{3}H][D-Pen^2,D-Pen^5]enkephalin[49] at a concentration of 1.0 nM, [^{3}H]PL-17[50] at a concentration of 2.0 nM, and [^{3}H]U-69,593[20] at a concentration of 0.75 nM (all radioligands from New England Nuclear, Boston, MA). Peptide analogues were dissolved in assay buffer prior to each experiment and added to duplicate assay tubes at 10 concentrations over an 800-fold range. Control (total) binding was measured in the absence of any inhibitor while nonspecific binding was measured in the presence of 10 μM naltrexone (Endo Laboratories, New York, NY). The final volume of the assay samples was 1.0 mL of which 10% consisted of the membrane preparation in 0.1 mL Tris-HCl buffer. Incubations were preformed at 25 °C for 3 h after which the samples were filtered through polyethylenimine (0.1% w/v, Sigma, St. Louis, MO) treated GF/B glass fiber filter strips (Brandel, Gaithersburg, MD). The filtrates were washed three times with 4.0 mL of ice-cold 100 mM KCl before transfer to scintillation vials. The filtrate radioactivity was measured after adding 10 mL of cocktail consisting of 16 g of CrystalFluor (West Chem, San Diego, CA) in 1.0 L of Triton X-100 and 2.0 L of toluene to each vial and allowing the samples to equilibrate over 8 h at 4 °C.

Binding data were analyzed by weighted, nonlinear-regression methods using one and two independent site models as previously described.[51] Statistical comparisons between one- and two-site fits were made with the F ratio test using a p value of 0.05 as the cutoff for significance.[52] Data best fitted by a one-site model was reanalyzed with the logistic equation.[53] Data obtained from independent measurements are presented as the arithmetic mean ± SEM.

Peptide Synthesis and Purification. Peptide syntheses were performed by the solid-phase method[41] utilizing an automated synthesizer (Applied Biosystems Inc. Model 431 A, Applied Protein Technologies Model PSS-80, or Vega Coupler Model 1000). Thin-layer chromatography of synthetic peptides was performed on silica gel plates (0.25 mm, Analtech, Newark, DE) with the following solvent systems: 1-butanol/pyridine/acetic acid/water (15/10/3/8, v/v/v/v), 1-butanol/pyridine/acetic acid/water (6/6/1/5, v/v/v/v), 1-butanol/acetic acid/water (4/1/5, v/v/v), and 2-propanol/concentrated ammonium hydroxide/water (3/10/10, v/v/v). Peptides were detected with ninhydrin reagent. *p*-Methylbenzhydrylamine resin was purchased from Advanced Chem Tech (Louisville, KY) or US Biochemical Corp. (Cleveland, OH). *N*-α-Boc-amino acids (N^α-Boc-*O*-(2,6-dichlorobenzyl)tyrosine, N^α-Boc-N^ϵ-(2,4-dichlorobenzyloxycarbonyl)lysine, N^α-Boc-N^g-tosylarginine) were purchased from Bachem Inc., Torrance, CA, or were synthesized by standard methods. 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 (Model 7300, Beckman Instruments). Mass spectra (fast-atom bombardment, low-resolution full scan, glycerol or dithioerythritol/dithiothreitol matrix) were performed by the Midwest Center for Mass Spectrometry, Lincoln, NE, a National Foundation Regional Instrumentation Facility (Grant No. CHE 8211164), or by the Center for Mass Spectrometry, University of Arizona, Tucson, AZ. HPLC was carried out by use of a ternary pump (Spectra Physics Model 8800) equipped with a UV/vis detector (Spectra Physics 8450) and integrator (Spectra Physics 4270). For analytical HPLC the solvent system used was a binary system, water containing 0.1% TFA (pH 2.0) and acetonitrile as the organic modifier, and solvent programs involved linear gradients as follows: (1) 10% to 30% acetonitrile over 20 min with flow rate of 1.5 mL/min; (2) 0% to 50% acetonitrile over 50 min with flow rate of 1.5 mL/min; (3) 15% to 30% acetonitrile over 15 min with flow rate of 1.5 mL/min. The column used for analytical chromatography had dimensions of 4.5 × 250 mm (Vydac, 10 mM particle size, C-18). HPLC purification on a semipreparative scale (10 mg) was performed with a reverse-phase column (Vydac, 1.0 × 25 cm, C-18, 10 μm particle size) employing the same binary solvent system used for analytical HPLC. Preparative (100 mg), low-pressure (50 psi) chromatographic purification was accomplished with a reversed-phase glass column (Bio-Rex column, 2.5 × 48 cm, Vydac C-18 resin, 20–30 μm particle size). A linear gradient of 0% to 30% acetonitrile over 3 h, involving the mentioned binary solvent system, was routinely used with a flow rate of 2 mL/min. Dyn A_{1-11}-NH_2 and Dyn A_{1-13}-NH_2

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Table IV. Amino Acid Analysis of Synthetic Cyclic Disulfide Dyn A Analogues

no.	compound	amino acids[a]									
		Pro	Gly	Ala	Ile	Leu	Tyr	Phe	Lys	Arg	Cys
1	[Cys^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$	1.1 (1)	2.0 (2)		0.98 (1)		0.91 (1)	1.0 (1)		3.1 (3)	2.2 (2)
2	[Cys^5,Cys^{11},D-Ala^8]Dyn A$_{1-11}$-NH$_2$	1.1 (1)	2.0 (2)	1.1 (1)			1.0 (1)	1.0 (1)		3.1 (3)	2.0 (2)
3	[Cys^8,Cys^{13}]Dyn A$_{1-13}$-NH$_2$	1.0 (1)	2.0 (2)			2.0 (2)	0.91 (1)	0.90 (1)	0.98 (1)	2.9 (3)	2.0 (2)
4	[D-Cys^8,Cys^{13}]Dyn A$_{1-13}$-NH$_2$	1.1 (1)	2.0 (2)			1.9 (2)	1.0 (1)	0.92 (1)	1.0 (1)	2.9 (3)	2.0 (2)
5	[D-Cys^8,D-Cys^{13}]Dyn A$_{1-13}$-NH$_2$	1.0 (1)	2.0 (2)			2.0 (2)	0.96 (1)	0.93 (1)	0.98 (1)	2.8 (3)	2.0 (2)

[a] Theoretical values are in parentheses. Hydrolysis in 4 N methanesulfonic acid [0.2% 3-(2-aminoethyl)indole] at 110 °C for 24 h.

Table V. Analytical Characteristics of Cyclic Dynorphin A Analogues

no.	thin-layer chromatography[a] R_f values				HPLC K' values[b]	FAB-MS[c]
	I	II	III	IV		
1	0.63	0.70	0.17	0.83	4.00[1]	1325 [M + H]$^+$
2	0.58	0.70	0.15	0.83	7.09[2]	1283 [M + H]$^+$
3	0.56	0.72	0.07	0.75	2.89[3]	1565 [M]$^+$ 1566 [M + H]$^+$
4	0.62	0.70	0.10	0.77	2.28[3]	1565 [M]$^+$
5	0.62	0.70	0.12	0.71	8.84[2]	1567 [M + 2H]$^+$

[a] Solvent systems: I, 1-butanol/pyridine/acetic acid/water (15/10/3/8, v/v/v/v); II, 1-butanol/acetic acid/water (4/1/5, v/v/v); III, 2-propanol/concentrated ammonium hydroxide/water (3/10/10, v/v/v); IV, 1-butanol/pyridine/acetic acid/water (6/6/1/5, v/v/v/v). [b] Superscripts 1–3 refer to HPLC solvent systems and solvent programs described in the Experimental Section. [c] Fast-atom bombardment, low-resolution full scan, glycerol or DTE/DTT matrix.

were synthesized and purified by using procedures as described for Dyn A$_{1-11}$-NH$_2$; Dyn A$_{1-17}$ was a gift from Vega Biochemicals.

Protected Dyn A$_{1-11}$-NH$_2$-resin. *p*-Methylbenzhydrylamine resin (1.06 g, 0.5 mequiv) was esterified with N^α-Boc-N^ϵ-(2,4-dichlorobenzyloxycarbonyl)lysine via its *N*-hydroxybenzotriazole (HOBt) active ester.[40] N^α-Boc-amino acids (4 mequiv) were added to the reaction mixture as performed HOBt activated esters. DMF or *N*-methyl-2-pyrrolidinone were used as the solvent, and the coupling reaction times were normally 30–60 min. Diisopropylethylamine was utilized as base and dichloromethane or DMF was used as the solvent for washes. Side-chain protection was as follows: Lys, 2,4-dichlorobenzyloxycarbonyl; Arg, tosyl; Tyr, 2,6-dichlorobenzyl; and Cys, *p*-methylbenzyl. After deprotection of the last N^α-Boc group with 50% trifluoroacetic acid (TFA) in dichloromethane, the peptide-resin was dried in vacuo to yield the protected Dyn A$_{1-11}$-NH$_2$-resins.

Dyn A$_{1-11}$-NH$_2$. The protected peptide-resin was treated with liquid anhydrous hydrofluoric acid (HF) in the presence of anisole (10%, v/v) for 1 h at -10 to 0 °C. After removal of HF in vacuo at 0 °C, the residue was washed three times with ether and extracted with aqueous 6% acetic acid three times, followed by extraction with glacial acetic acid. The acetic acid solution was lyophilized to give a yellow solid (0.80 g) which was dissolved in 30% acetic acid and subjected to gel filtration (Sephadex G-15) with 30% acetic acid as eluant. A cream powder (0.77 g) was obtained after gel filtration. This product was subjected to preparative HPLC under the mentioned conditions to yield a white powder (424 mg, 44%) after lyophilization. The structure assignment was corroborated by the results of the amino acid analysis and mass spectrometry, and the purity of the product was characterized by analytical HPLC and TLC (Tables IV and V).

[Cys^5,Cys^{11}]Dyn A$_{1-11}$-NH$_2$ (1). The crude deprotected peptide was obtained as a white powder (273 mg) by the use of procedures as described by Dyn A$_{1-11}$-NH$_2$. After dissolving the crude product in degassed aqueous 0.1% acetic acid, the solution was diluted to a volume of 2 L with degassed, deionized, distilled water. The pH was adjusted to 8.5 by the addition of 3 N ammonium hydroxide, and 0.01 N $K_3Fe(CN)_6$ (15 mL) was added dropwise to a yellow endpoint. Additional 0.1 N $K_3Fe(CN)_6$ (19 mL) was added and the mixture was stirred at room temperature for 60 min, after which time the solution remained yellow. The pH was adjusted to 4 by the addition of 30% acetic acid, and anion-exchange resin (Amberlite IRA-68, chloride form, 50 mL) was added to the solution. After stirring of the mixture for 60 min, the solution was colorless. The resin was filtered and washed three times with 30% acetic acid, and the solvent was evaporated at 40–45 °C to a volume of about 100 mL. Lyophilization of this solution yielded a pale-green powder which was subjected to preparative HPLC as described to give a white powder (92 mg, 10%). The purity of the final product was verified by TLC and analytical HPLC (Table V), and the structure assignment was corroborated by the amino acid analysis (Table IV) and mass spectral (FAB) (Table V) results. In addition, a quantitative Ellman test[42] was performed, with oxytoceine as standard, to verify the absence of free sulfhydryl groups.

[Cys^5,Cys^{11},D-Ala^8]Dyn A$_{1-11}$-NH$_2$ (2). Compound **2** was synthesized and purified in a manner similar to that employed for compound 1. After purification, the final product was obtained as a white powder (88 mg, 10%). The purity of the final product as verified by TLC and analytical HPLC (Table V), and the structure assignment was corroborated by the amino acid analysis (Table V) and mass spectral (FAB) (Table V) results.

[Cys^8,Cys^{13}]Dyn A$_{1-13}$-NH$_2$ (3). Compound 3 was synthesized and purified in a manner similar to that employed for compound 1. After purification, the final product was obtained as a white powder (64 mg, 6%). The purity of the final product was verified by TLC and analytical HPLC (Table V), and the structure assignment was corroborated by the amino acid analysis (Table IV) and mass spectral (FAB) (Table V) results.

[D-Cys^8,Cys^{13}]Dyn A$_{1-13}$-NH$_2$ (4). Compound **4** was synthesized and purified in a manner similar to that employed for compound 1. After purification, the final product was obtained as a white powder (43 mg, 4%). The purity of the final product was verified by TLC and analytical HPLC (Table V), and the structure assignment as corroborated by the amino acid analysis (Table IV) and mass spectral (FAB) (Table V) results.

[D-Cys^8,D-Cys^{13}]Dyn A$_{1-13}$-NH$_2$ (5). Compound **5** was synthesized and purified in a manner similar to that employed for compound 1. After purification, the final product was obtained as a white powder (43 mg, 4%). The purity of the final product was verified by TLC and analytical HPLC (Table V), and the structure assignment was corroborated by the amino acid analysis (Table IV) and mass spectral (FAB) (Table V) results. In addition a quantitative Ellman test[42] was performed, with oxytoceine as standard, to verify the absence of free sulfhydryl groups.

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Registry No. **1**, 127103-89-3; **2**, 127103-90-6; **3**, 127103-91-7; **4**, 127181-95-7; **5**, 127180-78-3; Dyn A$_{1-11}$-NH$_2$, 79985-48-1; Dyn A, 80448-90-4; Dyn A$_{1-13}$-NH$_2$, 79515-34-7.