Articles

Design, Synthesis, and Biological Properties of Highly Potent Cyclic Dynorphin A Analogues. Analogues Cyclized between Positions 5 and 11[1]

Jean-Philippe Meyer,[†] Nathan Collins,[†] Feng-Di Lung,[†] Peg Davis,[‡] Teresa Zalewska,[‡] Frank Porreca,[‡] Henry I. Yamamura,[‡] and Victor J. Hruby*,[†]

Departments of Chemistry and Pharmacology, University of Arizona, Tucson, Arizona 85721

Received April 20, 1994[⊗]

We have recently reported the synthesis of several cyclic disulfide bridge-containing peptide analogues of dynorphin A (Dyn A), which were conformationally constrained in the putative address segment of the opioid ligand. Several of these analogues, bridged between positions 5 and 11 of Dyn A_{1-11}-NH_2, exhibited unexpected selectivities for the κ and μ receptors of the central over the peripheral nervous systems. In order to further investigate the conformational and topographical requirements for the residues in positions 5 and 11 of these analogues, we have synthesized a systematic series of Dyn A_{1-11}-NH_2 analogues incorporating the sulfydryl containing amino acids L- and D-Cys and L- and D-Pen in positions 5 and 11, thus producing 16 cyclic peptides. In addition, Dyn A_{1-11}-NH_2, [D-Leu^5]Dyn A_{1-11}-NH_2, and [D-Lys^{11}]Dyn A_{1-11}-NH_2 were synthesized as standards. Several of these cyclic analogues, especially c[Cys^5, D-Cys^{11}] Dyn A_{1-11}-NH_2, c[Cys^5, L- or D-Pen^{11}]Dyn A_{1-11}-NH_2, c[Pen^5, L-Pen^{11}]Dyn A_{1-11}-NH_2 and c[Pen^5, L- or D-Cys^{11}]Dyn A_{1-11}-NH_2, retained the same affinity and selectivity (vs the μ and δ receptors) as the parent compound Dyn A_{1-11}-NH_2 in the guinea pig brain (GPB). These same analogues and most others exhibited a much lower activity in the guinea pig ileum (GPI), thus leading to centrally vs peripherally selective peptides, but showed a different structure–activity relationship than found previously. In a wider scope, this series of analogues also provided new insights into which amino acids (and their configurations) may be used in positions 5 and 11 of Dyn A analogues for high potency and good selectivity at κ opioid receptors. The results obtained in the GPB suggest that requirements for binding are not the same for the κ, μ, or δ central receptors.

Introduction

Dynorphin A (Dyn A) is a potent opioid peptide[2] which was first isolated and identified from porcine pituitary.[3] This 17 amino acid peptide interacts preferentially with κ opioid receptors in a variety of tissue preparations and is thus postulated to be an endogenous ligand for these receptors.[4]

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

Though research in the development of selective and potent opioid peptides has been mainly devoted to the μ and δ opioid receptors,[5–9] targeting of the κ receptor may prove to be equally important in mediating analgesia.[10] The pharmacology of κ receptors and their ligands involves a lower abuse potential and a milder form of dependence and withdrawal symptoms in comparison to the prototypic μ opiate morphine. It also has been suggested that selective κ ligands may have therapeutic utility as a new treatment for head injury and stroke.[11] Nevertheless, adverse side effects have been implicated with the κ opioid ligands, e.g., dysphoria, psychotomimesis, and diuresis.[12] It also has been reported that Dyn A can induce a hind limb paralysis and spinal cord injury in the rat that is not opioid receptor mediated.[13] To further examine the role of κ receptors for nociception and adverse side effects, it is necessary to develop stable, highly potent and selective ligands for these receptors and their subtypes.[14,15] The κ receptor has been recently cloned from the mouse brain[16] and is found to belong, as do the μ and δ receptors,[16,17] to the seven helical transmembrane G-protein coupled receptor family, for which a proposed three dimensional model exists.[18] To date, only a few studies regarding the possible interactions between the ligand Dyn A and its receptor have been reported.[19] Structure–function relationships of dynorphin-related peptides have been reviewed extensively,[20] and some of the more relevant points to this study will be discussed. Sequential removal of amino acids from the C terminus has shown that deletion of residues 14–17 or even 12–17 did not significantly affect Dyn A potency.[21] Nevertheless, this study and others also established that the basic residues Arg^6, Arg^7, Lys^{11}, and to a lesser extent Lys^{13} were important for κ selectivity and/or potency,[21–23] although not all of the results could be confirmed.[23,24] In the message sequence (i.e., that of Leu-enkephalin), the two aromatic residues Tyr^1 and Phe^4 are key amino acids for opioid activity.[21] Replacement of Gly^2 by various L-amino acids leads to analogues with weak affinities and potencies in the central and peripheral nervous systems. The results in this series

* Author to whom reprints requests should be addressed at the Department of Chemistry.
† Department of Chemistry.
‡ Department of Pharmacology.
⊗ Abstract published in *Advance ACS Abstracts*, October 1, 1994.

0022-2623/94/1837-3910$04.50/0 © 1994 American Chemical Society

were very different when D-amino acids were used, as the Dyn A analogues were fairly potent in the GPI bioassays and exhibited high μ and κ affinities.[25] Finally, N-monoalkylations of Tyr[1] were reported to lead to analogues of Dyn A that are highly selective for the central κ vs μ and δ receptors ($\kappa/\mu/\delta$ K_i ratio = 1/1070/6080, for [*N*-benzyl-Tyr[1],D-Pro[10]]Dyn A_{1-13}-NH_2).[26] Thus far, only *N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]-dec-8-yl]benzo[*b*]furan-4-acetamide (U-69,593), a non peptide κ agonist, has been shown to possess a similar κ vs μ selectivity (K_i ratio = 520 or 1520, depending on the experimental conditions).[26,27]

Several conformationally constrained Dyn A analogues have been reported. The first, constrained in the putative message sequence of the peptide, was the cyclic disulfide c[Cys[2], Cys[5]]Dyn A_{1-13}-NH_2 analogue.[28] This peptide exhibited high potency in the GPI bioassay and a high MVD/GPI IC_{50} ratio was reported, indicating a strong interaction with the μ and/or κ receptors. The same analogue exhibited a high μ affinity in the rat brain. More recently, several cyclic lactam peptides, including c[D-Orn[2], Asp[5]]Dyn A_{1-8}-NH_2, c[Orn[5], Asp[8]]-Dyn A_{1-13}-NH_2, c[Orn[5], Asp[10]]Dyn A_{1-13}-NH_2 and c[Orn[5], Asp[13]]Dyn A_{1-13}-NH_2 have been prepared.[29] The Dyn A_{1-13}-NH_2 analogues showed only weak activities in both the MVD and GPI bioassays. They also displayed naloxone K_e values consistent with interaction with the μ receptor.[4,30] In binding studies, the analogues c[Orn[5], Asp[8]]Dyn A_{1-13}-NH_2 and c[Orn[5], Asp[13]]Dyn A_{1-13}-NH_2 had a high μ affinity and moderate μ over δ selectivity. Finally, the analogue c[D-Orn[2], Asp[5]]Dyn A_{1-8}-NH_2 showed high potency in the GPI bioassay, but again a naloxone K_e value of 1.5 nM, indicating that the interaction was primarily with the μ receptor. Surprisingly, none of the above peptides retained the κ over μ or δ selectivity of the parent peptide Dyn A, in the peripheral nervous system. Other cyclic analogues also were prepared, such as the cyclic lactam c[D-Asp[2], Dap[5]]Dyn A_{1-13}-NH_2. As many other analogues constrained in their message segment, this peptide displayed very poor κ vs μ selectivity in the brain.[31]

It previously had been shown that substitution by lipophilic residues and certain D-amino acids at position 8 of Dyn A increased selectivity for the κ receptor.[32,33] These observations may suggest a reverse turn about this position important for interactions with the κ receptors. A D-Pro residue in position 10 also enhances κ selectivity and is compatible with high κ receptor potency.[33-35] This again may be suggestive of a reverse turn about this position. Nevertheless, no spectroscopic proof has been found to support these hypotheses, as different spectroscopic methods (FT-IR,[36] ^{1}H NMR,[37] Raman,[38] and fluorescence energy transfer[39]) support the existence of an extended and/or random coil conformation for Dyn A and its shorter analogues. In order to test the potential importance of a reverse turn in positions 8 or 10, several Dyn A_{1-11}-NH_2 analogues were designed and synthesized previously in our laboratory.[40,41] These analogues were constrained in the address segment of the peptide by a disulfide bridge between positions 5 and 11 or 8 and 13. These ring systems could result in a preferred conformation with a reverse turn centered around the 8 or 10 positions and would not affect the important basic residues Arg[6], Arg[7], and Arg[9]. Previously reported analogues c[D-Cys[8], Cys[13]]Dyn A_{1-13}-NH_2 and c[D-Cys[8], Cys[11]]Dyn A_{1-13}-NH_2 displayed high potencies in the GPI bioassay (IC_{50} = 2.27 and 1.75 nM, respectively). They also showed in the same bioassay a high κ vs μ ratio, as measured by the extent of antagonism of the analogue by the μ-selective antagonist CTAP. In the GPB, these peptides retained a high κ affinity (IC_{50} = 1.76 and 0.11 nM, respectively), but a low κ vs μ selectivity (IC_{50} ratio = 5.8 and 3.3, respectively). Furthermore, analogues of Dyn A_{1-11}-NH_2 cyclized between positions 5 and 11 displayed large differences between κ central binding affinities and peripheral κ bioassay potencies. These compounds, including c[Cys[5], Cys[11]]Dyn A_{1-11}-NH_2, c[Cys[5], D-Ala[8], Cys[11]]Dyn A_{1-11}-NH_2, and c[Pen[5], Cys[11]]-Dyn A_{1-11}-NH_2 exhibited binding affinities and selectivities at the GPB κ receptor comparable to those of Dyn A itself. In the GPI bioassays, the potencies were poor (IC_{50} = 1080, 4406, and >10000 nM, respectively), leading to high selectivity ratios between the central and the peripheral κ receptors.

1: Dyn A_{1-11}-NH_2
2: [D-Lys[11]] Dyn A_{1-11}-NH_2
3: [D-Leu[5]] Dyn A_{1-11}-NH_2
4: c[Cys[5,11]] Dyn A_{1-11}-NH_2
5: c[Cys[5], D-Cys[11]] Dyn A_{1-11}-NH_2
6: c[Cys[5], Pen[11]] Dyn A_{1-11}-NH_2
7: c[Cys[5], D-Pen[11]] Dyn A_{1-11}-NH_2
8: c[Pen[5], Cys[11]] Dyn A_{1-11}-NH_2
9: c[Pen[5], D-Cys[11]] Dyn A_{1-11}-NH_2
10: c[Pen[5,11]] Dyn A_{1-11}-NH_2
11: c[Pen[5], D-Pen[11]] Dyn A_{1-11}-NH_2
12: c[D-Cys[5], Cys[11]] Dyn A_{1-11}-NH_2
13: c[D-Cys[5,11]] Dyn A_{1-11}-NH_2
14: c[D-Cys[5], Pen[11]] Dyn A_{1-11}-NH_2
15: c[D-Cys[5], D-Pen[11]] Dyn A_{1-11}-NH_2
16: c[D-Pen[5], Cys[11]] Dyn A_{1-11}-NH_2
17: c[D-Pen[5], D-Cys[11]] Dyn A_{1-11}-NH_2
18: c[D-Pen[5], Pen[11]] Dyn A_{1-11}-NH_2
19: c[D-Pen[5,11]] Dyn A_{1-11}-NH_2

Figure 1. Structure of the linear and cyclic analogues of Dyn A. Prefix c is indicative of the cyclic structure.

These interesting results prompted us to further investigate the conformational and topographical requirements at positions 5 and 11, necessary for high affinities and selectivities. We synthesized a complete series of Dyn A_{1-11}-NH_2 analogues incorporating the sulfydryl containing amino acids L- and D-Cys and L- and D-Pen in positions 5 and 11, leading after cyclization to 16 constrained peptides. We present here the synthesis and structure–biological activity relationships of these compounds.

Results and Discussion

Synthesis. All 16 cyclic peptides (Figure 1) were synthesized by solid-phase methods, cyclized in solution, and purified by RP-HPLC. They were obtained in

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

analogue	IC_{50} (nM)[a] κ	μ	δ	selectivity μ/κ	δ/κ
1	0.58 ± 0.03	9.9 ± 2.0	25.5 ± 3.4	17.1	44.0
2	0.53 ± 0.01	5.2 ± 1.9	18.6 ± 4.3	9.8	35.1
3	15.3 ± 2.0	116 ± 20	1740 ± 190	7.6	114
4	1.0 ± 0.4	12.2 ± 3.7	13.9 ± 6.0	12.2	13.9
5	0.71 ± 0.10	5.2 ± 0.7	15.9 ± 1.1	7.3	22.4
6	1.0 ± 0.2	17.0 ± 0.5	319 ± 75	17.0	319
7	1.1 ± 0.4	31.0 ± 2.0	242 ± 54	28.2	220
8	2.3 ± 0.3	7.1 ± 1.0	232 ± 16	3.1	100
9	2.0 ± 0.1	5.0 ± 0.7	245 ± 15	2.5	123
10	3.1 ± 0.8	67.6 ± 11.1	717 ± 94	21.8	231
11	15.0 ± 6.0	473 ± 45	1000 ± 320	31.5	66.7
12	104 ± 11	128 ± 13	1030 ± 128	1.2	9.9
13	96.2 ± 9.1	27.4 ± 4.5	2190 ± 235	0.3	22.7
14	42.6 ± 0.6	99.0 ± 2.6	2340 ± 98	2.3	54.9
15	100 ± 12	108 ± 15	1370 ± 370	1.1	13.7
16	87.1 ± 16.9	87.0 ± 17.0	2000 ± 320	1.0	22.9
17	18.0 ± 2.6	51.1 ± 10.2	390 ± 99	2.8	21.6
18	26.1 ± 7.0	224 ± 82	1360 ± 430	8.6	52.1
19	70.5 ± 1.5	563 ± 101	1908 ± 313	8.0	27.1

[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).

sufficient quantities for analysis and biological testing and were found to be single peak by HPLC (at least 98% pure) at 225 and 280 nm, using two independent gradients, and single spots by TLC in four different solvent systems. The correct mass in each case was observed by fast atom bombardment mass spectroscopy. Finally, amino acid analysis results were within experimental error limits. All analytical results are summarized in Tables 4 and 5 in the Experimental Section.

Influence of the Residues in Positions 5 and 11 on the Binding in the GPB at the κ, μ, and δ receptors (Table 1). The three linear peptides, Dyn A_{1-11}-NH_2 (**1**), [D-Lys^{11}]Dyn A_{1-11}-NH_2 (**2**), and [D-Leu^5]-Dyn A_{1-11}-NH_2 (**3**), were synthesized as control analogues to assess the importance of the chirality of residues incorporated in the key positions 5 and 11. It is striking to note that all eight cyclic analogues with an L-amino acid in position 5 are more potent (IC_{50} ranging from 0.71 to 15.3 nM) at the κ receptor than the analogues with a D-residue in the same position (IC_{50} ranging from 18.0 to 104 nM). A similar result is obtained for Dyn A_{1-11}-NH_2 (**1**) and [D-Leu^5]Dyn A_{1-11}-NH_2 (**3**) with IC_{50} values of 0.58 and 15.3 nM, respectively. At the same receptor, analogues with L-Cys^5 (analogues **4**–**7**) showed improved binding affinity when compared to analogues incorporating an L-Pen^5 residue (analogues **8**–**11**). In these cases, the IC_{50} values ranged from 0.71 to 1.1 nM with L-Cys (analogues **4**–**7**), comparable to the value obtained for **1** and from 2.0 to 15.0 nM (analogues **8**–**11**). Surprisingly, the opposite result is observed if the amino acid in position 5 is of the D-configuration. For example, **17** has a higher binding affinity than **13** (18.0 vs 96.2 nM), and the same trend is observed for analogues **12**–**19**. In a series of analogues bearing the same residue in position 5, the influence of the amino acid in position 11 appears to be of less importance. Nevertheless, a L-Pen^{11} residue gives slightly improved results compared with its D-counterpart. To illustrate this, analogues **10** and **11** (IC_{50} of 3.1 and 15.0 nM, respectively) can be taken as examples. Therefore, we conclude that an important consideration for binding at the κ receptor is the presence of an L-residue in position 5. The physical size and/or the constraints of this amino acid do not appear to be an important factor. Moreover, the κ receptor seems to be able to accommodate many kinds of residues in position 11. Finally, it is quite remarkable to notice that cyclization does not greatly affect the binding capacities of the most potent analogues **4**–**10**. The IC_{50} values are 0.58 and 0.53 nM for the standards **1** and **2**, respectively, whereas these values range from 0.71 to 3.1 nM for **4**–**10**, which represents a decrease in potency of at most a factor of 5. In general, these cyclic peptides follow the same trend as the standards **1**, **2**, and **3**.

The results obtained with the cyclic analogues at the μ receptor follow to some extent the same pattern as those observed at the κ receptor. It seems nevertheless harder to isolate and identify the relative importance of the different factors (e.g., Cys vs Pen residues, of L- or D-configuration) on binding at the μ receptor. Overall, as at the κ receptor an L-residue is generally better accepted than a D-residue at position 5. The results also clearly indicate that the residue and its configuration at position 11 strongly influence the binding at the μ receptor. The use of L-Pen instead of L-Cys in position 11 decreases the affinity at the μ receptor. This is illustrated by comparing **8** and **10** (IC_{50} values of 7.1 and 67.6 nM at the μ receptor, respectively). The effect is even more dramatic with D-residues: **9** has an affinity almost 100 times better than **11** (IC_{50} values of 5.0 and 473 nM, respectively). In a similar manner to **1** and **2**, and as for the κ receptor, an analogue incorporating a D-Cys amino acid in position 11 shows higher affinity for the μ receptor than its L-counterpart. However, the opposite is observed for Pen residues.

As for the κ and μ receptors, the results obtained for analogues **1**, **2**, and **3** at the δ receptor demonstrate that using a D-Lys residue in position 11 slightly increases the potency at the δ receptor (IC_{50} value decreasing from 25.5 to 18.6 nM) whereas a D-Leu residue in position 5 significantly decreases it. The main difference is that in this case the potency decreases much more drastically, from 25.5 to 1740 nM (**1** and **3**, respectively, Table 1). Results obtained for the cyclic analogues incorporating a D-amino acid in position 5 (analogues **12** to **19**) show that these peptides also have a low potency (submicromolar for some) at the δ receptor (IC_{50} values ranging from 390 to 2340 nM). In addition by comparing analogues **4** and **5** to **8** and **9**, it is noticeable that the small Cys residue in this position is much preferred to the more bulky Pen residue, as the IC_{50} values drop from 13.9 and 15.9 nM to 232 and 245 nM, respectively. These same observations can be made for analogues **6** and **7** together with **10** and **11**. These results should be put in perspective with those previously obtained in our laboratory for peptides such as c[D-Pen^2, D-Pen^5]-Enk, c[D-Pen^2, L-Pen^5]Enk, c[D-Pen^2, D-Cys^5]Enk and c[D-Pen^2, L-Cys^5]Enk.[42,43] For these latter peptides, as for our cyclic Dyn A analogues, an L-residue in position 5 gives higher binding affinities than a D-residue, and a Cys is better than a Pen residue for binding at the δ receptor (16.2, 10.0, 7.2, and 3.4 nM, respectively). However, the loss in affinity induced, for the enkephalin analogues, by the nature and the chirality of the residue present in position 5 is much less than the one observed for the cyclic dynorphin analogues. These results demonstrate the importance of the chirality of the

residue in position 5, when the message segment is followed by an address segment, as in dynorphins. Apparently this residue aligns these two segments in a spatial conformation that is right for binding. On the other hand, it remains unclear why the incorporation of an L-Pen residue in position 5 of cyclic dynorphin induces such a loss in binding affinity. It appears obvious that for analogues **4** to **10**, bearing an L-residue in position 5, the incorporation of a Cys amino acid of L- or D-configuration in position 11 leads to analogues with much higher affinities than those with a D- or L-Pen amino acid residue, as shown by comparing **4** to **6**, **5** to **7**, and **8** to **10**. On the other hand, no conclusion can be drawn with respect to the necessary configuration of the residue in position 11, as results do not demonstrate a general trend. As a conclusion, requirements for good binding at the δ receptor are highly stringent compared to these for binding at the κ receptor. In fact, only analogues **4** and **5** retain affinities comparable to those of **1** and **2**, at the δ opioid receptor.

Influence on the Selectivity in the GPB (Table 1). Though it has been shown to be the endogenous ligand for the κ receptor, Dyn A and its shorter analogue Dyn A_{1-11}-NH_2 are still rather potent in binding to μ and δ receptors (Table 1). For analogue **1**, the IC_{50} ratios are only 17.1 and 44.0 for μ vs κ and δ vs κ, respectively. Introduction of a D-residue in position 11 (analogue **2**) does not dramatically alter these ratios as it improves binding at all three receptors (IC_{50} ratios = 9.8 and 35.1 for μ vs κ and δ vs κ, respectively). On the other hand, for analogue **3**, the δ/κ IC_{50} ratio increases from 44.0 to 114, as this modification affects the binding at the δ receptor more drastically. For the cyclic peptides made in this study, the μ vs κ selectivities vary only a little for analogues **4**–**11** which contain an L-residue in position 5; the ratios range from 2.5 to 31.5, and results are comparable to the values obtained for **1** and **2**, which are 17.1 and 9.8, respectively. For analogues **12** to **19**, the μ vs κ selectivity ratio is lower and below 10 in general. Several peptides, such as **12**, **15**, **16**, or even **14**, are almost equipotent at the two receptors. It therefore seems that a D-residue in position 5 affects binding to the κ receptor more than to the μ receptor. On the other hand, the results are quite different for the δ vs κ selectivities. Among all cyclic peptides with a L-amino acid in position 5, only **4** and **5** show a selectivity analogous to that of **1** and **2** (IC_{50} ratios of 13.9 and 22.4 vs 44.0 and 35.1, respectively). For analogues **6**–**11**, the selectivity ratio is between 66.7 and 319, again reflecting the fact that the structures for good binding at the δ receptor are much more critical than at the κ receptor. For example, the κ receptor can accommodate well an L-Pen residue in position 5 and any other in position 11 (peptides **8** to **11**), whereas the δ receptor cannot. Finally, for analogues 12–19 (with a D-residue in position 5), the δ over κ selectivity returns to the same range (IC_{50} ratios from 9.9 to 54.9) as **1**. This shows that, for the κ and the δ receptor, the binding of our cyclic analogues is primarily and strongly under the influence of the nature and the chirality of the amino acid in position 5.

Activities in the GPI (Table 2). Dyn A_{1-11}-NH_2 has an IC_{50} value of 1.07 nM in the GPI bioassay. The fact that no significant shift in potency can be observed upon addition of the highly μ-selective ligand CTAP[44] proves that this activity is due only to the peripheral κ receptor rather than to the μ receptor. Incorporation of a D-residue in position 5 (**3**) or 11 (**2**) leads to the same changes in potency in the GPI that were observed in the GPB, i.e., a slight increase for **2** (IC_{50} value of 0.30 nM) and a strong decrease for **3** (IC_{50} value of 94.0 nM). As previously described, cyclization via a disulfide bridge between positions 5 and 11 leads to peptides with much lower activities in the GPI.

Overall, the highest activities were obtained for analogues with an L-residue in position 5 (**4**–**11**, IC_{50} values ranging from 213 to 1660 nM) rather than a D-residue (**12**–**19**, except **17**, IC_{50} values ranging from 1460 to >30 000 nM). In general, analogues incorporating an L-Cys residue in this position did not have higher potencies than those incorporating the spatially larger and more constrained L-Pen. Also no clear conclusion can be reached regarding the requirements for the amino acid and its configuration at position 11, since the results were highly variable. Nevertheless, only analogues **4** and **5** in this series show modest potency at the peripheral κ receptor (IC_{50} values of 220 and 213 nM, respectively).

Selectivity between the GPB and the GPI (Table 3). The highest selectivities, measured as the ratio between the IC_{50} values in the GPI and GPB, are obtained for analogues **4**–**10**, which all possess an L-residue in position 5 (ratios ranging from 219 to 1660). Due to their extremely low activities in the GPI, analogues **12**, **14**, and **19** also can be considered in this list (ratios of 183, 238, and >426, respectively). The requirements for activity at the κ peripheral receptor differ dramatically from those of the κ central receptor. First of all, cyclization of Dyn A analogues between positions 5 and 11 strongly reduces the activity in the GPI, and does not, for analogues **4**–**10**, significantly

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

analogue	IC_{50} (nM) GPI	shift[a]	analogue	IC_{50} (nM) GPI	shift[a]
1	1.07 ± 0.31	ns	**11**	615 ± 145	ns
2	0.30 ± 0.02	ns	**12**	19100 ± 5338	nt
3	94.0 ± 18.1	ns	**13**	6240 ± 1402	nt
4	219 ± 20	ns	**14**	10100 ± 1610	ns
5	213 ± 2	ns	**15**	1460 ± 581	nt
6	1660 ± 106	ns	**16**	3640 ± 769	nt
7	686 ± 194	ns	**17**	92.3 ± 17.6	ns
8	940 ± 156	ns	**18**	3450 ± 659	nt
9	1130 ± 328	ns	**19**	>30000 ± 802	nt
10	716 ± 57	ns			

[a] nt, not tested; ns, no significant shift observed with 1000 nM of 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

analogue	ratio of IC_{50} GPI/GPB	analogue	ratio of IC_{50} GPI/GPB
1	1.8	**11**	41
2	0.6	**12**	183
3	6.1	**13**	64
4	219	**14**	238
5	300	**15**	15
6	1660	**16**	42
7	624	**17**	5.1
8	409	**18**	132
9	565	**19**	>426
10	231		

alter the affinity for the central κ receptor. As described in the introduction, and as previously reported, this could be due to different conformational requirements at these receptors, implying that there are in fact subtypes of the κ receptor. An alternate possibility may be that the compounds with poor activity in the GPI bind well to the receptors in this tissue but simply have low efficacy. In this regard, two of the three analogs of lowest potency (compounds **14** and **19**) were tested as antagonists against U-69,593. We used U-69,593 as this has been our standard κ agonist and we have not detected any evidence of κ heterogeneity in the GPI. At concentrations of up to 1 μM, **14** and **19** did not alter the U-69,593 concentration–effect curve (data not shown). There is therefore no evidence of antagonist action, and so differences in efficacy do not appear to be sufficient to explain the results. Notably the most active cyclic peptides in the GPB have affinities for the κ receptor similar to that of Dyn A_{1-11}-NH_2. This study thus indicates that the peripheral κ receptors are much more sensitive to conformational changes induced by cyclization, but also changes in residues and/or their configurations at positions 5 and 11 than are the central receptors. Effectively, the fact that only peptides **4** and **5** retain modest activity in the GPI bioassay shows that the peripheral κ receptor cannot accommodate larger residues such as Pen in positions 5 and 11, whereas the central ones can. It should be noted that analogue **17** also retains modest activity in the GPI and therefore exhibits somewhat anomalous behavior, compared to closely related analogues, showing greater receptor affinity. These results are qualitatively similar to these reported previously[40,41] but quantitatively different in terms of affinity and selectivity as we do not see as large a selectivity ratio as previously described by our laboratories. Unfortunately direct comparisons with previously prepared samples were not possible, so that the reasons for these discrepencies are unclear.

Conclusions. The binding results in the GPB show that, at the κ receptors, the cyclic peptides presented in this study follow the same structure–activity trends as the three standard peptides **1**, **2**, and **3**. Good binding affinity in the GPB requires an L-residue in position 5 while any other residue can be incorporated in position 11. The first condition is also shared by the μ receptor, but the amino acid in position 11 has to be a Cys rather than a Pen residue. For the κ receptor, D-Cys^{11} analogues show only slightly improved binding affinities compared to ones bearing an L-Cys^{11} residue. Nevertheless, this effect is more important at the μ receptor. Finally, analogues that bind to the δ receptor with affinities comparable to that of Dyn A_{1-11}-NH_2 must incorporate an L-Cys^5 residue and an L- or D-Cys^{11} residue.

The only two cyclic peptides fulfilling all of these conditions at the three opioid receptors are **4** and **5**. They both show binding and selectivity profiles extremely similar to those of the control peptides **1** and **2**. This also indicates that the cyclization, *per se*, does not affect binding at the opioid receptors in the GPB. This could therefore prove that the important conformational constraints imposed on the analogues by the cyclization between positions 5 and 11 lead to a spatial arrangement of the peptides that is compatible with potent binding to κ, μ, and δ central opioid receptors. As the activities of the synthesized analogues are much weaker at the peripheral opioid receptors, it can be postulated that this conformation is not suitable for good interactions with these receptors and that therefore central and peripheral receptors are different in their nature. One of the other consequence of these results is that the positively charged residue lysine in position 11 does not appear to be critical for binding to the κ, μ, and δ receptors.

Although the results clearly indicate that the requirements for binding to the central κ and μ receptors are different, these cyclic analogues are unable to discriminate efficiently between the two receptors and therefore exhibit poor selectivity for κ over μ. On the other hand, since no significant shift can be observed when adding the μ antagonist CTAP in the GPI, our analogues are capable of differentiating between the two peripheral receptors. The same results are obtained with Dyn A_{1-11}-NH_2. Finally, it is quite remarkable that the precise structure of the residue in position 5 has less importance *per se* than its configuration. Effectively, the [L-Leu^5]-, c[L-Cys^5]-, and c[L-Pen^5]Dyn A_{1-11}-NH_2 peptides give almost the same binding affinities especially at the central κ receptor (0.6, 1.0, and 2.3 nM for **1**, **4**, and **8**, respectively), whereas the peptides incorporating their D-counterparts disrupt this binding (15.3, 104, and 87.1 nM for **3**, **12**, and **16**, respectively). These results strongly suggest that the amino acid in position 5 plays a pivotal role in the peptide by aligning the message segment (residues 1–4) with respect to the address segment (residues 6–11 in our analogues). This might indicate the existence of primary and secondary binding sites for the message and address segments at the κ opioid receptors.

Material and Methods

Peptide Synthesis and Purification. Peptide syntheses were performed by the solid-phase method[45,46] utilizing an automated synthesizer (Applied Biosystems Inc. Model 431 A), a Boc/Benzyl strategy, and a *p*-methylbenzhydrylamine (*p*-MBHA) resin (Advanced Chem Tech, Louisville, KY), as previously described for Dynorphin analogues.[41] Side-chain protected Nα-Boc amino acids were purchased from Bachem (Torrance, CA), whereas the others amino acids were synthesized by standard methods in our laboratory. The analytical data for the purified peptides synthesized are given in Tables 4 and 5. HPLC was carried out by use of a binary pump (Perkin Elmer LC 250 model) equipped with an UV/vis detector (Perkin-Elmer LC 90 UV model) and integrator (Perkin Elmer LCI 100 model). 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) 10–90% acetonitrile over 40 min; and (2) 10–50% over 40 min. In both cases the flow rate was 1.5 mL/min. The column used 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 binary solvent system (1) described above, 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 Spectroscopy, University of Arizona, Tucson, AZ. Thin-layer chromatography of synthetic peptides was performed on silica plates (0.25 mm, Analtech, Newark, DE) with the solvent systems given in Table 4. Peptides were detected with the ninhydrin reagent. Hydrolysis of the peptides was performed in 4 N methanesulfonic acid (0.2% 3-(2-aminomethyl)indole) at 110 °C for 24 h, and amino acids were analyzed with an automatic analyzer (Bechman Instruments, Model 7300). The results are reported in Table 5.

Table 4. Analytical Properties of Various Dyn A Analogues

analogue	TLC R_f values[a]				K' HPLC values[b]		FAB-MS[c]
	I	II	III	IV	1	2	
1	0.65	0.20	0.80	0.65	3.4	5.0	1362 [M+]
2	0.60	0.15	0.85	0.60	3.5	5.1	1362 [M+]
3	0.70	0.25	0.80	0.65	3.4	4.9	1362 [M+]
4	0.70	0.25	0.85	0.70	3.3	5.4	1325 [M+]
5	0.65	0.25	0.80	0.55	3.2	5.2	1325 [M+]
6	0.55	0.20	0.95	0.75	3.6	5.1	1353 [M+]
7	0.70	0.25	0.90	0.65	2.8	3.6	1353 [M+]
8	0.50	0.15	0.90	0.65	2.9	3.8	1353 [M+]
9	0.55	0.15	0.85	0.70	3.8	4.3	1353 [M+]
10	0.60	0.20	0.80	0.60	3.4	5.5	1381 [M+]
11	0.60	0.25	0.95	0.65	3.7	5.4	1381 [M+]
12	0.65	0.20	0.90	0.60	3.4	5.0	1325 [M+]
13	0.70	0.15	0.85	0.55	3.3	5.1	1325 [M+]
14	0.65	0.20	0.80	0.65	3.1	4.0	1353 [M+]
15	0.60	0.20	0.95	0.70	3.7	5.4	1353 [M+]
16	0.70	0.15	0.90	0.65	2.7	3.6	1353 [M+]
17	0.70	0.15	0.90	0.70	3.4	5.7	1353 [M+]
18	0.65	0.20	0.85	0.70	3.0	4.6	1381 [M+]
19	0.55	0.25	0.85	0.60	4.2	5.7	1381 [M+]

[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 below in Material and Methods. [c] Fast atom bombardment mass spectroscopy.

General Method for Peptide Synthesis. The N$^\alpha$-Boc amino acids (4 equiv) were sequentially added as their preformed HOBt active esters to the resin (ca. 1.0 g, 0.5 mmol). *N*-Methyl-2-pyrrolidinone (NMP) was used as solvent and the coupling time was 1 h. Trifluoroacetic acid (TFA) was used to remove the protecting Boc groups. Diisopropylethylamine (DIEA) was used as a base, and DCM and NMP were used as solvents for washes. Side-chain protection was as follows: Lys, 2,4-dichlorobenzyloxycarbonyl; Arg, tosyl; Cys and Pen, *p*-methylbenzyl; Tyr, 2,6-dichlorobenzyl. After the removal of the last Boc group, the peptide-resin was dried *in vacuo*. It was then treated with liquid anhydrous hydrofluoric acid (HF) in the presence of cresol and *p*-thiocresol (5% each, w/v) for 50 min at 0 °C. After removal of HF *in vacuo*, the residue was washed three times with diethyl ether and extracted three times with 30% aqueous acetic acid. Then the acetic acid solutions were evaporated to give a yellow residue. For the disulfide bridge formation (preparation of analogues **4–19**), the peptide was taken up in about 20 mL of water and slowly added to a 0.01 M solution of $K_3Fe(CN)_6$ in water. The addition time was about 20 h, and the pH of the solution was kept between 8 and 8.5, by adding concentrated NH_4OH or using a buffer (saturated aqueous solution of ammonium acetate). Once the cyclization was finished, the pH was lowered to 4 by addition of glacial acetic acid. An ion-exchange resin (IRA 60 Amberlite, chloride form) was added to get rid of the excess of $K_3Fe(CN)_6$. After 1 h, the ion-exchange resin was filtered off and the solvent was evaporated. The crude peptide was then purified by semipreparative HPLC under the previously mentioned conditions to yield a white powder after lyophilization. The yields were not optimized. The structure assignment was corroborated by the results of the amino acid analysis and mass spectroscopy, and the purity of the product was characterized by analytical HPLC and TLC. The analogues prepared are given in Figure 1 and all analytical data are summarized in Tables 4 and 5 for the 16 cyclic peptides and the 3 linear peptides prepared as controls.

Binding Assays. Membranes were prepared from whole brains taken from adult male guinea pigs (200–400 g) obtained from SASCO. 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 48000*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 toluenesulfonyl fluoride, pH 7.4 (all from Sigma, St. Louis, MO) except bestatin (Peptides International, Louisville, KY). The radioligands used were [^{3}H]c[D-Pen^2,p-Cl-Phe^4,D-Pen^5]enkephalin[47] (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 concentration of 1.5 nM (all obtained from New England Nuclear, Boston, MA). Peptide analogues were dissolved in the assay buffer prior to each experiment and added to duplicate assay tubes at 10 different concentrations over a 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 (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 were 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 were analyzed by nonlinear least-square regression analysis program named 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 cut-off for significance.[48] Data best fitted by a one site model were re-analyzed using the logistic equation.[49] Data obtained from at least three independent measurements are presented as the arithmetic mean ± SEM.[50] 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 were used as a bioassay.[51] Tissues came from male Hartley guinea pigs weighing 250–500 g and were prepared as described previously.[52] 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. No peptidase inhibitors were used as no reversal of the initial contraction height inhibition, that would be indicative of peptidase activity, could be observed with the passage of time. Drugs were added to the baths in 14–60 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. Maximum inhibition of contraction height is reached within 3 min of dosing, and longer incubation of the drug would not produce a greater response. 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.[53] IC_{50} values represent the mean of two to 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-squares method.[50] As previously stated, the results are not corrected for the actual peptide content.

Acknowledgment. This work was supported by grants from the National Institutes of Drug Abuse DA 04248 and DA 06284.

Table 5. Amino Acid Analysis of Various Dyn A Analogues

analogue	amino acids[a]									
	Tyr	Gly	Phe	Leu	Arg	Ile	Pro	Lys	Pen	Cys
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)		
2	1.0 (1)	2.1 (2)	0.9 (1)	1.0 (1)	3.1 (3)	0.9 (1)	1.0 (1)	0.9 (1)		
3	1.1 (1)	2.1 (2)	0.9 (1)	1.0 (1)	2.9 (3)	0.9 (1)	1.1 (1)	0.9 (1)		
4	0.9 (1)	1.9 (2)	1.0 (1)		3.0 (3)	1.0 (1)	0.9 (1)			1.8 (2)
5	0.9 (1)	2.0 (2)	1.0 (1)		3.1 (3)	1.0 (1)	0.9 (1)			1.9 (2)
6	1.0 (1)	2.0 (2)	1.1 (1)		2.9 (3)	1.1 (1)	1.0 (1)		1.0 (1)	1.1 (1)
7	1.0 (1)	2.1 (2)	1.0 (1)		3.0 (3)	1.0 (1)	1.0 (1)		0.9 (1)	0.9 (1)
8	1.1 (1)	2.1 (2)	1.0 (1)		2.8 (3)	0.9 (1)	1.1 (1)		1.1 (1)	0.9 (1)
9	1.0 (1)	1.9 (2)	1.0 (1)		2.9 (3)	0.9 (1)	1.0 (1)		0.9 (1)	1.0 (1)
10	0.9 (1)	2.0 (2)	1.1 (1)		3.0 (3)	0.9 (1)	0.9 (1)		1.9 (2)	
11	0.9 (1)	1.9 (2)	1.0 (1)		3.1 (3)	0.9 (1)	0.9 (1)		1.9 (2)	
12	0.9 (1)	2.0 (2)	0.9 (1)		2.9 (3)	0.9 (1)	0.9 (1)			2.0 (2)
13	1.0 (1)	2.0 (2)	0.9 (1)		3.0 (3)	1.0 (1)	1.0 (1)			1.9 (2)
14	1.0 (1)	2.1 (2)	1.0 (1)		2.8 (3)	1.0 (1)	1.0 (1)		1.0 (1)	1.1 (1)
15	1.1 (1)	2.1 (2)	1.0 (1)		3.1 (3)	1.1 (1)	1.1 (1)		0.9 (1)	1.0 (1)
16	1.0 (1)	1.9 (2)	1.1 (1)		3.1 (3)	1.0 (1)	1.0 (1)		1.0 (1)	1.1 (1)
17	0.9 (1)	1.9 (2)	1.0 (1)		2.8 (3)	0.9 (1)	0.9 (1)		0.9 (1)	1.0 (1)
18	1.0 (1)	2.0 (2)	1.0 (1)		2.9 (3)	1.0 (1)	1.0 (1)		1.8 (2)	
19	0.9 (1)	2.0 (2)	0.9 (1)		3.0 (3)	0.9 (1)	1.1 (1)		1.9 (2)	

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

References

(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 are Dyn A, dynorphin A; Enk, enkephalin; Pen, penicillamine; GPB, guinea pig brain; GPI, guinea pig ileum; MVD, mouse vas deferens; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; CTAP, c[D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2]; DCM, dichloromethane; NMP, *N*-Methyl-2-pyrrolidinone; NMR, nuclear magnetic resonance.

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