Effects of the Substitution of Phe^4 in the Opioid Peptide [D-Ala^8]Dynorphin A-(1−11)NH$_2$

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Phenylalanine at position 4 of the peptide dynorphin A (Dyn A) is an important residue for opioid receptor affinity and activity, but there is very little information available on the structure−activity relationships or conformational preference of this residue for interaction with κ-opioid receptors. Based on the hypothesis that the spatial orientation of the aromatic ring at position 4 of Dyn A is important for opioid receptor affinity and selectivity, a series of Dyn A analogues with various Phe derivatives substituted at position 4 were synthesized and evaluated for their opioid receptor affinity and activity. The L- and D-Homophe^4 (homophenylalanine) analogues of [D-Ala^8]Dyn A-(1−11)NH$_2$ were compared to the (*R*)- and (*S*)-Atc^4 (2-aminotetralin-2-carboxylic acid) derivatives (Aldrich et al. *Chirality* **2001**, *13*, 125−129). [L-Homophe^4,D-Ala^8]Dyn A-(1−11)NH$_2$ exhibited higher κ-opioid receptor affinity than the D-Homophe^4 isomer, while [(*R*)-Atc^4,D-Ala^8]Dyn A-(1−11)NH$_2$ exhibited higher κ-opioid receptor affinity than the (*S*)-Atc^4 isomer. Comparing the structure of Atc to those of Phe and Homophe, these results suggest that the Atc isomers are functioning more as constrained Homophe rather than Phe analogues in these Dyn A derivatives. The higher κ-opioid receptor affinity of the (*R*)-Atc^4 analogue suggests that Phe^4 of Dyn A most likely adopts a gauche (−) or trans conformation in the κ-opioid receptor binding site. Comparison of [D-Ala^8]Dyn A-(1−11)NH$_2$ derivatives containing Aic^4 (2-aminoindan-2-carboxylic acid) and Tic^4 (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) with the peptides containing their acyclic counterparts α-MePhe^4 and N-MePhe^4, respectively, suggest that the loss in opioid receptor affinity seen for the Aic^4 and Tic^4 analogues is probably due to an improper orientation of the aromatic ring in these residues. Most of the analogues in this series showed much lower affinity for δ-opioid receptors than the parent peptide, suggesting that κ- and δ-opioid receptors have distinct binding pockets for the residue at position 4 of Dyn A. All of the analogues with high affinity for κ-opioid receptors exhibited full agonist activity in the adenylyl cyclase assay using cloned κ-opioid receptors, indicating that changes in the position or orientation of the phenyl ring in this residue did not alter the ability of the peptides to activate the receptor.

The focus of research on opioids and opioid receptors for several decades has mainly been on the development of strong analgesics free of the abuse potential and side effects of narcotics such as morphine. Compared to μ-opioid agonists, κ-opioid agonists lack respiratory depression, physical dependence, and strong addictive properties.[1] Lately there has been considerable interest in agonists for κ-opioid receptors as peripheral analgesics.[2−7] Further, κ-opioid receptor agonists have been shown to decrease the self-administration of cocaine;[8−10] this could lead to new approaches for the treatment of cocaine addiction for which there is no suitable therapy available. Kappa agonists can also potentially be used as neuroprotective and anticonvulsant agents[11] and for the treatment of HIV-1 and HIV-1 related encephalopathy.[12,13] A better understanding of how κ-opioid receptors function at the molecular level is important in the development of new therapeutic agents for these receptors.

The heptadecapeptide dynorphin A (Dyn A^{14}) is thought to be an endogenous ligand for κ-opioid receptors[15,16] and is involved in a variety of biological functions.[17] The synthesis of κ-opioid receptor selective Dyn A analogues and evaluation of their structure−activity relationships (SAR) will aid in the understanding of receptor−peptide ligand interactions with κ-opioid receptors. Compared to other opioid peptides the SAR studies on Dyn A have been limited.[18] Chavkin and Goldstein in their early studies of Dyn A proposed a "message−address" concept for this peptide (Figure 1).[19] According to this hypothesis the N-terminal sequence Tyr-Gly-Gly-Phe, which is common among most mammalian opioid peptides, acts as the "message" sequence and is responsible for the opioid receptor activity of Dyn A. The C-terminal "address" sequence that is unique to Dyn A is proposed to be the potency-enhancing domain and directs the peptide to κ-opioid receptors.

Stepwise substitution of alanine indicated that Tyr[1]

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Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-NH_2

"Message" "Address"

Figure 1. Dynorphin A-(1−11)NH_2.

and Phe⁴ are key residues in the "message" sequence of Dyn A for opioid receptor affinity and activity.[20] Although the importance of Phe at position 4 of Dyn A is well established, little information is available about the preferred spatial orientation for the aromatic ring of this residue for interaction with κ-opioid receptors. To date the only reported modifications of the aromatic ring of Phe⁴ in Dyn A are substitution by Trp[21,22] and D-Trp,[23] and incorporation of substituents (NO_2, NH_2) on the para position of the aromatic ring.[21,24,25] Yoshino et al. also reported substitution of α-MePhe and D-Phe at position 4 of [D-Leu⁸]Dyn A-(1−8)NH_2,[24] but both analogues, especially the D-Phe⁴ derivative, showed a loss in affinity for all opioid receptors compared to the parent peptide. Replacement of the peptide bond between Gly³-Phe⁴ of Dyn A-(1−11)NH_2 by the reduced amide ψ(CH_2NH) resulted in loss in affinity for all opioid receptors,[26] but the analogue of [D-Leu⁸]Dyn A-(1−8)-NH_2 with this modification retained comparable κ-opioid receptor affinity and showed much higher selectivity for κ- over μ- and δ-opioid receptors than the corresponding parent peptide.[27] Replacement of the peptide bond between Phe⁴-Leu⁵ by the reduced amide ψ(CH_2NH) in both Dyn A-(1−11)NH_2 and [D-Leu⁸]Dyn A-(1−8)NH_2 resulted in analogues which retained affinity for opioid receptors comparable to the parent peptides.[26,27] Although a variety of conformationally constrained Phe analogues have been incorporated in other opioid peptides,[28−33] when we began our research *cyclo*[5,11][Tic⁴,-Cys⁵,Cys¹¹]Dyn A-(1−11)NH_2 was the only reported Dyn A analogue with a conformationally constrained Phe derivative at position 4.[34] This peptide showed large decreases in affinity (180-fold and 392-fold for κ- and μ-opioid receptors, respectively) compared to the parent cyclic peptide.

Since Phe⁴ is a critical residue in Dyn A, the aromatic ring of this residue is expected to make key interactions with opioid receptors. In a computational model of Dyn A-(1−10) bound to the κ-opioid receptor[35] Phe⁴ of Dyn A was postulated to be in a hydrophobic binding pocket consisting of receptor residues near the extracellular−transmembrane interface from transmembrane segments V−VII and from extracellular loop 2, including residues I294 and V201. Therefore the spatial orientation of this group was expected to be important for the κ-opioid receptor affinity of the peptide; the orientation of this side chain might also affect receptor activation and therefore the efficacy of the peptide. On the basis of this hypothesis, we evaluated the effects on opioid receptor affinity after substituting the optically pure constrained Phe analogues (*R*)- and (*S*)-Atc (2-aminotetralin-2-carboxylic acid) (Figure 2) in position 4 of [D-Ala⁸]Dyn A-(1−11)NH_2.[36] Both Dyn A analogues containing Atc at position 4 showed nanomolar affinity for κ- and μ-opioid receptors. Unexpectedly the peptide containing (*R*)-Atc, which corresponds to a D-Phe derivative (Figures 2 and 3b), showed much higher κ-opioid receptor affinity than the (*S*)-Atc⁴ analogue. This reversal of the preferred stereochemistry has been demonstrated for only one other opioid peptide containing Atc, [(*R*)-Atc³,Ile⁵,⁶]deltorphin II[37] (the reversal of the preferred stereochemistry for δ-opioid receptor interaction for this deltorphin II analogue was demonstrated only when [³H][Ile⁵,⁶]deltorphin II was used as the radioligand). In contrast, [D-Phe⁴,D-Ala⁸]Dyn A-(1−11)NH_2 exhibits significantly lower affinity for κ- and μ-opioid receptors than the parent peptide.[36]

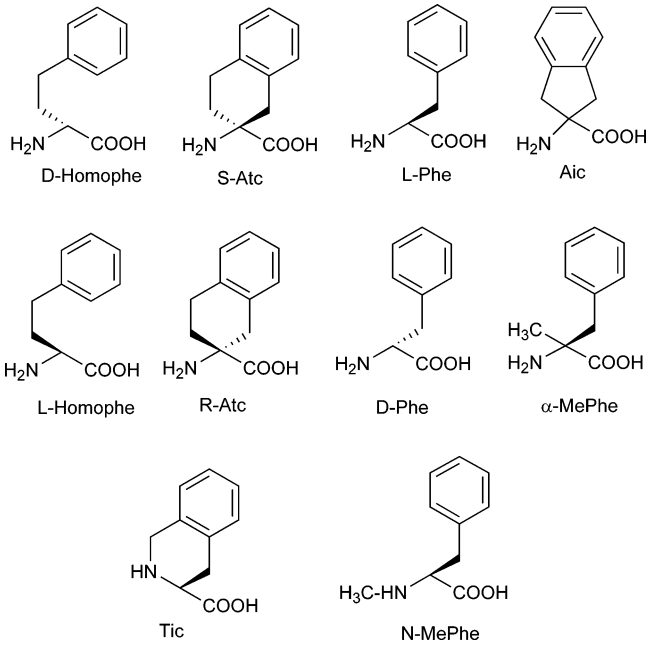

Figure 2. Comparison of phenylalanine derivatives incorporated into position 4 of [D-Ala⁸]Dyn A-(1−11)NH_2.

Atc can be considered as a conformationally constrained analogue of either Phe or homophenylalanine (Homophe), the amino acid with an extra methylene group in the side chain (Figures 2 and 3). As an analogue of Phe, (*S*)-Atc corresponds to L-Phe and (*R*)-Atc corresponds to D-Phe. The reverse is true for Atc as an analogue of Homophe, where (*S*)-Atc is an analogue of D-Homophe and (*R*)-Atc is a constrained derivative of L-Homophe (Figures 2 and 3). To assess how Atc analogues might be binding to κ-opioid receptors, both isomers of Homophe were incorporated in position 4 of [D-Ala⁸]Dyn A-(1−11)NH_2. To further investigate the conformational preferences of κ-opioid receptors for the aromatic residue at position 4 of Dyn A, we also incorporated the symmetric conformationally constrained analogue of Phe, Aic (2-aminoindan-2-carboxylic acid), at this position. This analogue has been incorporated previously in μ- and δ-opioid receptor selective opioid peptides.[38,39] As compared to Atc, Aic is a conformationally constrained analogue of Phe only and lacks a chiral center (Figure 2). Atc and Aic influence both the orientation of the aromatic ring and the backbone conformation of the peptide. Therefore, the acyclic counterpart of Atc/Aic, α-MePhe, was also incorporated in position 4 to assess separately the influence of substitution at C^{α} of Phe⁴ in Dyn A analogues on opioid receptor affinity, selectivity, and efficacy.

Compared to Atc and Aic, in which cyclization is through the C^{α}-carbon, the conformationally constrained Phe derivative Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) involves cyclization through the N^{α}-amino group, resulting in the formation of a tertiary amide and constraint of the Φ dihedral angle when incorporated in the middle of the peptide. The effect of

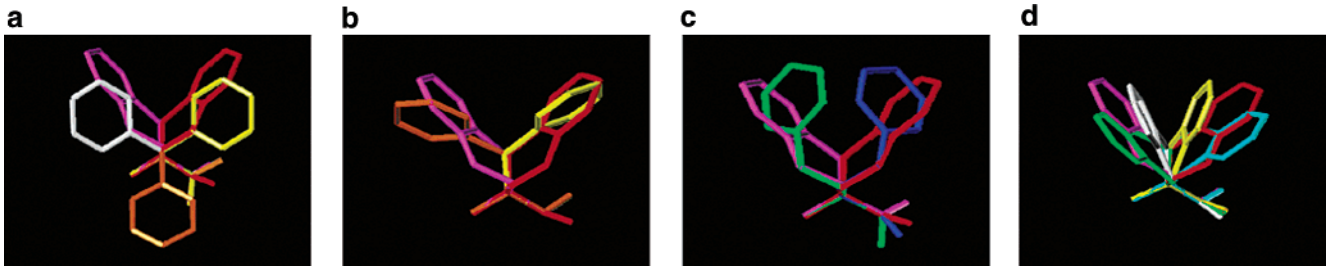

Figure 3. Comparison of the conformations of (*R*)-Atc ($\chi^1 = 71°$ (gauche (+)), $\chi^2 = 20°$ is in magenta and $\chi^1 = 168°$ (trans), $\chi^2 = -20°$ is in red) to (a) L-Phe ($\chi^1 = -57°$ (gauche (−)) in white, $\chi^1 = 57°$ (gauche (+)) in orange, and $\chi^1 = 180°$ (trans) in yellow), (b) D-Phe (the same colors are used as given for L-Phe), (c) L-Homophe (only the two conformations ($\chi^1 = -58°$ (gauche (−)), $\chi^2 = -60°$ in cyan and $\chi^1 = 178°$ (trans), $\chi^2 = 53°$ in blue) that are closest to the Atc conformations are shown), and (d) (*S*)-Atc ($\chi^1 = -71°$ (gauche (−)), $\chi^2 = -20°$ in green and $\chi^1 = -168°$ (trans), $\chi^2 = 20°$ in cyan) and Aic ($\chi^1 = -97°$ (gauche (−)), $\chi^2 = -13°$ in white and $\chi^1 = -134°$ (trans), $\chi^2 = 9°$ in yellow). The conformations of (*R*)-Atc, (*S*)-Atc, and Aic are those reported for the corresponding hydroxylated methylamide derivatives by Mosberg et al.[31] Backbone atoms were used for superposition; N^{α} is on the lower left in each structure.

Table 1. Opioid Receptor Affinities of [D-Ala^8]Dyn A-(1−11)NH_2 Analogues

substitution at position 4 of [D-Ala^8]Dyn A-(1−11) NH_2 analogues	K_i (nM ± SEM)			K_i ratio
	κ	μ	δ	$\kappa/\mu/\delta$
1 Homophe	0.66 ± 0.03	13.7 ± 0.3	190 ± 58	1/21/288
2 D-Homophe	1.73 ± 0.51	47.0 ± 3.0	5570 ± 1620	1/27/3220
3 (*R*)-Atc[a]	0.89 ± 0.14	32.9 ± 4.7	>10 000	1/37/>10 000
4 (*S*)-Atc[a]	9.54 ± 2.77	88.3 ± 5.9	>10 000	1/9.3/>1050
5 Aic	26.5 ± 12.7	364 ± 52	>10 000	1/14/>377
6 α-MePhe	3.20 ± 0.79	3.24 ± 0.87	157 ± 46	1/1/49
7 Tic	4.69 ± 0.75	206 ± 36	3590 ± 580	1/44/765
8 N-MePhe	0.62 ± 0.19	1.03 ± 0.40	56.1 ± 7.1	1/1.7/90
9 D-Phe[a]	8.91 ± 0.07	123 ± 37	>10 000	1/14/>1120
[D-Ala^8]Dyn A (1−11) NH_2[a]	0.11 ± 0.05	4.20 ± 1.9	6.57 ± 0.23	1/38/60

[a] From ref.[36]

Tic substitution was therefore compared to that of Atc and Aic. Since Tic influences both the orientation of the aromatic ring and backbone conformation, the acyclic counterpart of Tic, N-MePhe, was also incorporated in position 4 to assess separately the influence of substitution on the N^{α}-amine on opioid receptor affinity and selectivity of the Dyn A analogues.

Here we report the effects of substitution of these Phe analogues in position 4 of [D-Ala^8]Dyn A-(1−11)NH_2 on opioid receptor affinity, selectivity, and efficacy, and the resulting conclusions concerning the possible conformations of this important residue.

Results and Discussion

Synthesis. All peptides were synthesized by solid-phase peptide synthesis using Fmoc-protected amino acids according to standard procedures.[40] The synthesis was performed on the Tentagel-S AM (5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid) resin using *N*,*N*′-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as the coupling reagents. Following synthesis and purification, the identity and purity of the final compounds was verified using mass spectrometry, analytical HPLC, and capillary electrophoresis (CE). The final purity of all peptides was >98%.

Pharmacological Evaluation. The purified peptides were examined for their affinity for κ-, μ-, and δ-opioid receptors in radioligand binding assays using cloned receptors stably expressed in CHO (Chinese hamster ovary) cells.[41] The affinities for κ-, μ-, and δ-opioid receptors, determined by competitive inhibition of the radioligands [^{3}H]diprenorphine, [^{3}H]DAMGO ([D-Ala^2,N-MePhe^4,glyol]enkephalin), and [^{3}H]DPDPE (*cyclo*[D-Pen^2,D-Pen^5]enkephalin), respectively, are given in Table 1.

Both peptides containing the Homophe isomers exhibited high affinity for κ-opioid receptors. The peptide containing L-Homophe at position 4 showed subnanomolar affinity for κ-opioid receptors and retained selectivity for κ- over μ-opioid receptors similar to that of the parent peptide [D-Ala^8]Dyn A-(1−11)NH_2. [D-Homophe^4,D-Ala^8]Dyn A-(1−11)NH_2 showed 3-fold lower affinity than [Homophe^4,D-Ala^8]Dyn A-(1−11)NH_2 and 16-fold lower affinity than the parent peptide for κ-opioid receptors. However, this loss in affinity for κ-opioid receptors for the peptide with D-Homophe at position 4 was much less than for the analogue with D-Phe at this position (81-fold decrease compared to [D-Ala^8]Dyn A-(1−11)NH_2). This may be due to the ability of D-Homophe (with a longer, more conformationally flexible side chain than D-Phe) to orient the aromatic group in a more favorable position in the receptor binding pocket than D-Phe. The Homophe^4 analogue exhibited similar κ-opioid receptor affinity as the (*R*)-Atc^4 analogue (K_i (κ) = 0.66 and 0.89 nM, respectively). The D-Homophe^4 peptide exhibited lower affinity for all opioid receptors, but still significantly higher (5.5-fold) affinity for κ-opioid receptors than the (*S*)-Atc^4 analogue. The lower affinity of the (*S*)-Atc^4 peptide is most likely due to the restriction of the aromatic ring orientation by this residue, whereas the flexibility of the D-Homophe^4 side chain permits this ring to adopt a more favorable orientation for interaction with κ-opioid receptors. These results suggest that in these Dyn A derivatives the Atc isomers might be behaving as Homophe rather than as Phe analogues

(Figure 3). Further, the orientations adopted by the aromatic ring in the (*R*)-Atc⁴ peptide and its linear counterpart the Homophe⁴ analogue are more favorable for interactions with κ-opioid receptors than orientations adopted by the (*S*)-Atc⁴ derivative and its linear counterpart the D-Homophe⁴ analogue.

Compared to Atc, Aic is a symmetric achiral conformationally constrained analogue of Phe only. Incorporation of Aic at position 4 of [D-Ala⁸]Dyn A-(1−11)NH_2 resulted in much larger decreases in binding affinity for κ- and μ-opioid receptors as compared to the Atc⁴ analogues. These results suggest that the orientation adopted by the aromatic ring of Aic in [Aic⁴,D-Ala⁸]Dyn A-(1−11)NH_2 is not complimentary with κ- and μ-opioid receptor interaction. The slight decrease in the selectivity for κ- over μ-opioid receptors for the Aic⁴ and (*S*)-Atc⁴ analogues may be due to the substitution at the C^{α}, which causes a smaller decrease in μ- than κ-opioid receptor affinity.

Substitution of α-MePhe, an acyclic analogue of Aic and Atc, in position 4 of [D-Ala⁸]Dyn A-(1−11)NH_2 resulted in a decrease in affinity for κ-opioid receptors while maintaining affinity for μ-opioid receptors, resulting in a complete loss of selectivity for κ- over μ-opioid receptors. These results are in agreement with those reported by Yoshino et al., where substitution of α-MePhe at position 4 of [D-Leu⁸]Dyn A-(1−8)NH_2 resulted in a loss in affinity for κ-opioid receptors.[24] The smaller decrease in affinity for κ-opioid receptors for [α-MePhe⁴,D-Ala⁸]Dyn A-(1−11)NH_2 compared to the Aic- and (*S*)-Atc-containing peptides suggested that the large decrease in affinity for κ-opioid receptors seen for the constrained analogues is due to improper orientation of the aromatic ring. On the basis of these results, it appears that among these conformationally constrained analogues of Phe, the orientation adopted by the aromatic ring in the (*R*)-Atc⁴-containing peptide is most favorable for binding to κ- and μ-opioid receptors, while the orientation adopted by the aromatic ring in the Aic-containing peptide is least favorable for binding to these receptors. The differences in μ-opioid receptor affinities were smaller for the four analogues (<7-fold), suggesting that μ-opioid receptor is less sensitive to the orientation of this residue.

Mosberg and co-workers performed a detailed conformational analysis on the hydroxylated derivatives of (*R*)-Atc, (*S*)-Atc and Aic methylamides.[31] They reported that these analogues have two low energy conformers. In one case the χ^1 dihedral angle was close to ±60° (gauche) and in the other χ^1 was close to 180° (trans) (Figure 3). The phenyl rings in the trans conformations of (*R*)- and (*S*)-Atc occupy approximately the same plane, as do the phenyl rings in the gauche conformations of these two isomers (Figure 3d). In contrast the phenyl ring in both conformations of Aic is in a completely different plane (Figure 3d) which may account for the low κ receptor affinity of [Aic⁴,D-Ala⁸]Dyn A-(1−11)NH_2. Comparing the conformations of (*R*)-Atc to L-Phe (Figure 3a) suggests that the gauche (+) conformation (χ^1 approximately 60°) of Phe⁴ is not the bioactive conformation. However, neither the gauche (−) (χ^1 approximately −60°) nor trans conformations could be ruled out based on comparison of the structures of the possible conformations of Atc, Phe, or Homophe.

Compared to Atc and Aic that involve cyclization through the C^{α}-carbon, Tic involves cyclization through the N^{α}-amino group constraining the Φ dihedral angle; when incorporated in the middle of the peptide, Tic also results in a tertiary amide. Tic influences both the orientation of the aromatic ring (χ^1 and χ^2) and the backbone conformation (Φ dihedral angle). Conformational analysis studies on the hydroxylated derivative of Tic methylamide by Mosberg and co-workers[31] indicated that this analogue also has two low energy conformers (gauche (+) and gauche (−)) with χ^1 close to ±30° and χ^2 close to ±40°. Incorporation of Tic in position 4 of [D-Ala⁸]Dyn A-(1−11)NH_2 resulted in a peptide with similar decreases (40−50-fold) in affinity for κ- and μ-opioid receptors compared to the parent peptide. The acyclic analogue of Tic, N-MePhe, was also incorporated at this position to investigate the influence of the backbone conformation on opioid receptor affinity and activity. [N-MePhe⁴,D-Ala⁸]Dyn A-(1−11)NH_2 showed 7.5-fold higher affinity (K_i (κ) = 0.62 nM) than the Tic⁴ analogue, but 5-fold lower affinity than the parent peptide, for κ-opioid receptors. This indicates that the loss in κ-opioid receptor affinity of the Tic⁴ analogue is probably due to a combination of both the spatial orientation of the aromatic group and the effect of Tic on the peptide backbone conformation. [N-MePhe⁴,D-Ala⁸]Dyn A-(1−11)NH_2 exhibited a 3-fold increase in affinity for μ-opioid receptors compared to the parent peptide, resulting in a complete loss of selectivity for κ- over μ-opioid receptors. In contrast, the Tic⁴ analogue showed similar selectivity for κ- over μ-opioid receptors as the parent peptide, indicating that the spatial orientation of the aromatic group in this residue can compensate for the loss in selectivity due to substitution at the α-nitrogen. These results for the α-MePhe⁴ and N-MePhe⁴ analogues suggest that modifications at Phe⁴ of Dyn A that mainly influence the backbone conformation either increase or maintain the affinity for μ-opioid receptors, resulting in decreased selectivity for κ- over μ-opioid receptors.

The peptides containing D-amino acids (D-Phe and D-Homophe) and peptides containing constrained amino acids (Atc, Aic, and Tic) exhibited exceptionally large losses (600−1500-fold) in affinity for δ-opioid receptors, whereas the remaining substitutions (Homophe, N-MePhe or α-MePhe) resulted in only moderate decreases (8−30-fold) in affinity for δ-opioid receptors compared to the parent peptide. These results indicate that modifications in the stereochemistry and/or conformation of the residue at this position can influence the affinity of the peptides for δ-opioid receptors to a great extent. This suggests that there are distinct differences in the binding pocket for the residue at position 4 in κ- and δ-opioid receptors.

The functional consequences of these modifications to the peptides were investigated by measuring their ability to inhibit forskolin-stimulated adenylyl cyclase activity in CHO cells expressing κ-opioid receptors by the procedure described previously.[42] Results from this assay are summarized in Table 2. The EC_{50} values ranged between 3 to >500 nM, with some of the analogues exhibiting weak agonist activity. Except for the three compounds that exhibited low affinity for κ-opioid receptors, the analogues produced similar

Table 2. Inhibition of Forskolin-Stimulated Adenylyl Cyclase Activity by [D-Ala^8]Dyn A-(1−11)NH_2 Analogues in the CHO Cells Expressing κ-Opioid Receptors

substitution at position 4 of [D-Ala^8] Dyn A (1−11) NH_2 analogues	EC_{50} (nM ± SEM)	% max. response[a]
1 Homophe	5.20 ± 1.99	95 ± 5
2 D-Homophe	46.9 ± 7.1	100
3 *R*-Atc	72.4 ± 28.1	96 ± 3
4 *S*-Atc	573 ± 220	74 ± 1
5 Aic	−	39 ± 8
6 α-MePhe	36.5 ± 6.4	100
7 Tic	29.4 ± 10.3	91 ± 5
8 N-MePhe	3.56 ± 0.64	99 ± 1
9 D-Phe	515 ± 330	73 ± 10
[D-Ala^8]Dyn A-(1−11)NH_2	1.24 ± 0.43	100

[a] Maximum percent inhibition of adenylyl cyclase at a concentration of 10 μM relative to 100 nM Dyn A-(1−13)NH_2.

maximum inhibition at the highest concentration (10 μM) as the reference peptide (Dyn A-(1−13)NH_2, 100% inhibition at 100 nM), indicating that these analogues are full agonists. In this assay, the rank order of potency for adenylyl cyclase inhibition (Phe^4 (parent) > N-MePhe^4 ≈ Homophe^4 > Tic^4 ≈ α-MePhe^4 > D-Homophe^4 > (*R*)-Atc^4 > D-Phe^4 ≈ (*S*)-Atc^4 > Aic^4) was similar to that observed for the affinity for κ-opioid receptors (Phe^4 (parent) > N-MePhe^4 ≈ Homophe^4 > (*R*)-Atc^4 > D-Homophe^4 > α-MePhe^4 > Tic^4 > D-Phe^4 ≈ (*S*)-Atc^4 > Aic^4), with the parent peptide and the N-MePhe^4 and Homophe^4 analogues demonstrating the highest potencies in the adenylyl cyclase assay and κ-opioid receptor binding affinities, while the (*S*)-Atc^4 and Aic^4 analogues exhibited the lowest potencies and κ-opioid receptor affinities.

The rank order of potency of pairs of isomers in the adenylyl cyclase assay was similar to that found in the binding assays. Thus, the parent peptide showed much higher potency in the functional assay than the D-Phe^4 analogue, while the (*R*)-Atc^4 analogue showed higher potency than the (*S*)-Atc^4 isomer. This trend was repeated in the Homophe analogues with the Homophe^4 peptide exhibiting both higher affinity and potency at κ-opioid receptors than its D-Homophe^4 isomer. The acyclic counterpart of Atc and Aic, α-MePhe, showed moderately potent full agonist activity. The N-MePhe^4 analogue showed higher potency than the other analogues tested. The adenylyl cyclase assay results indicate that although substitutions at position 4 of [D-Ala^8]-Dyn A-(1−11)NH_2 influence the potency of the peptides they did not appear to affect their efficacy, with all of the analogues exhibiting agonist activity.

Conclusions

We hypothesized that Phe at position 4 of Dyn A makes key interactions with opioid receptors and that therefore the spatial orientation of the aromatic ring of Phe^4 in Dyn A is important for κ-opioid receptor affinity, selectivity, and possibly efficacy. By incorporating various Phe derivatives at position 4 of [D-Ala^8]Dyn A-(1−11)NH_2 we demonstrated that the affinity, selectivity, and potency, but not efficacy, of the peptide was influenced by the spatial orientation of the aromatic group, the stereochemistry of the residue at this position, and the backbone conformation. Substitution of conformationally constrained (*R*)-Atc at position 4 of [D-Ala^8]Dyn A-(1−11)NH_2 resulted in a peptide with much higher affinity and selectivity for κ-opioid receptors than the analogues containing (*S*)-Atc and Aic at this position, suggesting that the spatial orientation of the aromatic ring of (*R*)-Atc^4 analogue is the most compatible with interaction with κ-opioid receptors. The results obtained in this study compared to the conformations of the various conformationally restricted Tyr analogues determined by Mosberg and co-workers[31] suggest that the preferred conformation for the Phe^4 side chain of Dyn A is most likely either gauche (−), with χ^1 of approximately −60°, or trans. The importance of the orientation of the Phe^4 side chain of Dyn A for κ-opioid receptor affinity is consistent with the computational model of Dyn A interacting with κ-opioid receptors proposed by Paterlini et al.[35] In this model the Phe^4 side chain was in the gauche (−) conformation (G. Paterlini, personal communication).

The stereochemistry of the residue at position 4 of [D-Ala^8]Dyn A-(1−11)NH_2 influenced the opioid receptor affinity and selectivity of this peptide. The (*R*)-Atc-, Homophe-, and Phe-containing analogues exhibited higher κ-opioid receptor affinity and selectivity than the (*S*)-Atc-, D-Homophe-, and D-Phe-containing analogues, respectively. These results also suggest that the Atc isomers incorporated at position 4 of Dyn A behave more like Homophe than Phe analogues (Figure 3). Modifications at Phe^4 of Dyn A that mainly influenced the backbone conformation resulted in peptides that either maintained or exhibited increased affinity for μ-opioid receptors, resulting in reduced selectivity for κ- over μ-opioid receptors for these analogues. Although modifications at Phe^4 of [D-Ala^8]Dyn A-(1−11)NH_2 resulted in peptides with only moderate selectivity for κ- over μ-opioid receptors, these modifications had maximal effect on the affinity for δ-opioid receptors, with most of the analogues exhibiting large decreases in affinity for these receptors. Thus while κ- and μ-opioid receptors can tolerate various modifications at position 4, δ-opioid receptors were very sensitive to these modifications. These results suggest that there are distinct differences in the binding pockets of κ- and δ-opioid receptors for this residue, with the δ-opioid receptor binding pocket having stricter structural requirements for the residue at position 4 of Dyn A than either κ- or μ-opioid receptors. Although the modifications at position 4 of [D-Ala^8]Dyn A-(1−11)NH_2 had considerable influence on opioid receptor affinity and selectivity, these modifications had minimal influence on the κ-opioid receptor efficacy of the resulting peptides; all of the peptides exhibited agonist activity with their potencies paralleling their binding affinities for κ-opioid receptors.

Based on these results selected Phe derivatives have been chosen for incorporation into cyclic Dyn A analogues. These conformationally restricted peptides contain both a long range (backbone) as well as a local constraint (constrained amino acid side chain), which will help to better delineate the structure−activity relationships and conformational preferences for this important residue in Dyn A. These cyclic analogues are currently under investigation in our laboratory.

Experimental Section

Materials. All Fmoc-protected amino acids were purchased from Applied Biosystems (Foster City, CA), Anaspec (San Jose, CA), Bachem (King of Prussia, PA), or Novabiochem (La Jolla,

CA). Tentagel-S AM resin was purchased from Peptides International (Lexington, KY). Acetic acid, DIC, diethyl ether, monobasic sodium phosphate, piperidine, and TFA (trifluoroacetic acid) were purchased from Aldrich Chemical Co. (Milwaukee, WI). HOBt was purchased from Novabiochem. HPLC-grade solvents (dichloromethane (DCM), *N*,*N*-dimethylacetamide (DMA), MeCN, and MeOH for synthesis and HPLC analysis were purchased from Burdick and Jackson, Inc. (Muskegon, MI) or EM Sciences (Gibbstown, NJ).

Synthesis. All peptides were synthesized on the Tentagel-S AM resin (0.25 mmol/g) using a Milligen Biosearch 9500 automated peptide synthesizer. The resin was first swollen with 20 mL of DCM/DMA (1:1, 2 × 20 min). A solution of the desired Fmoc-protected amino acid (0.4 M in DMA, 4-fold excess) with 1 equiv of HOBt was mixed with an equal amount of 0.4 M DIC in DCM and reacted with the resin for 2 h; the resin was then washed with DCM/DMA (1:1, 12×). The completion of the coupling reactions was determined by the ninhydrin[43] and/or the chloranil tests.[44] The side chains of Lys, Tyr, and Arg were protected by Boc (*tert*-butyloxycarbonyl), *t*-Bu, and Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) groups, respectively. Following the coupling reaction, the Fmoc group was removed by 20% piperidine in DMA (2 × 10 min), the resin washed with DMA/DCM (1:1, 5 × 2 min), and the next amino acid then coupled to the resin. After completion of the peptide assembly and removal of the Fmoc group from the N-terminal residue, the resin was washed successively with DCM/DMA (1:1), DCM, and finally with methanol to shrink the resin.

Cleavage of the Peptides from the Resin. The protected peptide resins were reacted with 10 mL of Reagent B (88% TFA, 5% phenol, 5% water, and 2% triisopropylsilane)[45] for 2 h. The solutions were then filtered from the resin and washed with TFA (1 mL). The solutions were diluted with 10% acetic acid (30 mL) and extracted with ether (3 × 30 mL), and the ether extracts were then back extracted with acetic acid (2 × 10 mL). The combined aqueous layers were lyophilized to give the crude peptides.

Purification and Analysis of the Peptides. The crude peptides were purified by preparative reversed phase HPLC (Rainin HPXL HPLC system equipped with a Shimadzu SPD-10A detector) on a Vydac C18 column (10 μ, 300 Å, 21 × 250 mm) equipped with a Vydac guard cartridge. For purification a linear gradient of 15−50% aqueous MeCN containing 0.1% TFA over 35 min, at a flow rate of 20 mL/min, was used. The purification was monitored at 214 nm.

The purity of the final peptides was verified by analytical HPLC and CE. For analytical HPLC, a Beckman HPLC System Gold, consisting of a programmable solvent module 126 and a diode array detector module 168, employing a Vydac 218-TP column (5 μm, 300 Å, 4.6 × 250 mm) equipped with a Vydac guard cartridge, was used. A linear gradient of 5−80% solvent B (solvent A, aqueous 0.1% TFA, and solvent B, MeCN containing 0.1% TFA) over 50 min, at a flow rate of 1 mL/min, was used for the analysis. The CE analysis of the peptides was carried out on a Beckman P/ACE 2100 using the following conditions: 50 μm × 57 cm capillary (50 cm to the detector); 50 mM monobasic sodium phosphate buffer, pH 2.5; applied potential, 20 kV; 20 s pressure injection; detection at 214 nm. The final purity of all peptides by both analytical systems was >98%. Molecular weights of the compounds were determined by fast atom bombardment mass spectrometry (FAB-MS) on a Kratos MS50RF mass spectrometer (Environmental Health Sciences Center, Oregon State University, Corvallis, OR).

Molecular Modeling. Molecular modeling was performed with SYBYL 6.9 (Tripos, Inc.). The conformations for the hydroxylated methylamides of (*R*)-Atc, (*S*)-Atc, and Aic were taken from Mosberg et al.[31] For L- and D-Phe, three different side chain conformations (with $\chi^1 = -60°$, 60°, and 180°) of the acetylated methylamides were constructed, minimized using the Tripos force field and Gasteiger−Marsili charges (all other parameters were set at default values), and compared to (*R*)- and (*S*)-Atc. For D- and L-Homophe, nine side chain conformations (with all possible combinations of −60°, 60°, and 180° for χ^1 and χ^2) were constructed, minimized, and compared to (*R*)- and (*S*)-Atc; the $\chi^1 = -58°$, $\chi^2 = -60°$ and $\chi^1 = 178°$, $\chi^2 = 53°$ conformations are shown in Figure 3c. To simplify the structures, the acetyl and methylamides groups are not shown in Figure 3.

Radioligand Binding Assays. Radioligand binding assays were performed as previously described[41] using cloned rat κ- and μ- and mouse δ-opioid receptors stably expressed in CHO cells. [^{3}H]Diprenorphine, [^{3}H]DAMGO, and [^{3}H]DPDPE were used as radioligands in the assays for κ-, μ-, and δ-opioid receptors, respectively. Nonspecific binding was determined in the presence of 10 μM unlabeled Dyn A-(1−13)NH_2, DAMGO, and DPDPE for κ-, μ-, and δ-opioid receptors, respectively. Binding assays were carried out in the presence of peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine) and 3 mM Mg^{2+}. IC_{50} values were determined by nonlinear regression analysis to fit a logistic equation to the competition data using GraphPad Prism software. K_i values were calculated from the IC_{50} values by the Cheng and Prusoff equation,[46] using K_D values of 0.45, 0.49, and 1.76 nM for [^{3}H]diprenorphine, [^{3}H]DAMGO, and [^{3}H]DPDPE, respectively. The results presented are the mean ± SEM from at least three separate assays.

Adenylyl Cyclase Assays. Adenylyl cyclase assays were performed as previously described[42] using cloned rat κ-opioid receptors stably expressed in CHO cells. Adenylyl cyclase assays were carried out in the presence of a phosphodiesterase inhibitor (50 μM RO20−1724) and peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine). The cultures were incubated at 37° for 40 min in the presence of 50 μM forskolin and various concentrations of peptides. [^{14}C]-Cyclic AMP (5000 cpm in 50 μL) was added to each plate to correct for recovery. Concentrations of [^{3}H]- and [^{14}C]cyclic AMP were determined simultaneously using a Beckman LS 6000SC scintillation counter, and counts were corrected for crossover and recovery. The results presented are the mean ± SEM from at least three separate assays (except for the D-Homophe⁴ and N-MePhe⁴ analogues where $n = 2$).

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Supporting Information Available: HPLC, CE, and mass spectrometry data for the [D-Ala⁸]Dyn A-(1−11)NH_2 analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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