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[D-ALA^2]DELTORPHIN II ANALOGS WITH HIGH AFFINITY AND SELECTIVITY FOR DELTA-OPIOID RECEPTOR

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SUMMARY: Nine [D-Ala^2]deltorphin II (DL-II :Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) analogs having various aliphatic amino acids at positions 5 and 6 were synthesized to gain more information about the role of hydrophobic Val^{5,6} residues for the δ-opioid receptor selectivity. Binding assays of analogs replaced by Ala demonstrated the importance of hydrophobic Val^{5,6} residues in DL- II for δ-affinity and selectivity, and especially critical importance of Val^5 residue for higher δ-selectivity. By enhancing the hydrophobicity of residues at positions 5 and 6, we have developed analogs with very high δ-affinity and selectivity over those of DL-II, e. g., [Ile^{5,6}], [norleucine^{5,6}] and [γ-methyl-leucine^{5,6}]DL-II, which will be useful as δ-selective ligands for investigation of the physiological role of opioid receptors.

It is now believed that there are three major opioid receptor types, μ, δ and κ. The developement of highly selective ligands for the three receptor types is of key importance for the elucidation of physiological role of the opioid receptors.

Deltorphin (DL) [1], also named dermenkephalin [2] or dermorphin gene-associated peptide [3], a new natural δ-opioid peptide, is a heptapeptide of a sequence, Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2. Recently, two other δ-selective opioid peptides, [D-Ala^2]deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2, DL-I) and II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2, DL-II) were isolated from the skin extracts of Phyllomedusa bicolor [4]. Dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), a μ-selective opioid peptide [5,6], and the three deltorphins (DLs)

Abbreviations: DAGO = [D-Ala^2,MePhe^4, Gly-ol^5]enkephalin, DADLE = [D-Ala^2, D-Leu^5]enkephalin, DIPCDI = diisopropylcarbodiimide, HOBt = 1-hydroxybenztriazole, t-Boc = tert-butoxycarbonyl.

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share common generallized N-terminal tripeptide sequences, Tyr^1-D-Xaa^2-Phe^3 (where D-Xaa is either D-Ala or D-Met), which comprise the "message domain" [7,8] of these peptides. And it is considered that the critical structural differences between these two classes of peptides lie in the C-terminal tetrapeptide region, the "address domain" [7,8], adjacent to the N-terminal message domain. Recent investigations on the structure-activity relationships of DLs are providing much information [9—14]. During these investigations, it was revealed that the C-terminal hydrophobic residues, Leu^5-Met^6 in DL or $Val^{5,6}$ in DL-I and II, are of importance to the address domain for the receptor selectivity [13,14].

In the present investigation, in order to expand our understanding of the role of hydrophobic residues at positions 5 and 6, we synthesized nine analogs of DL-II, in which $Val^{5,6}$ residues were replaced by other amino acids with alkyl side chains as shown in Fig. 1, i.e., Ala (**1-3**), Nva (norvaline, **4**), Nle (norleucine, **5**), Ile (**6**), Leu (**7**), Tle (tert-leucine, **8**) and Mle (γ-methyl-leucine, **9**). These analogs were tested for their receptor binding properties by displacement experiments with [^{3}H]DAGO for μ- affinity and [^{3}H]DADLE for δ-affinity using rat brain membrane preparations.

MATERIALS AND METHODS

Peptide Synthesis. Nva, Nle, Tle and Mle were purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo. All peptides were prepared by the usual solid phase method with DIPCDI-mediated t-Boc strategy as previously reported [15] with the exception of analog **8**. For the syntheis of analog **8**, Fmoc-Tle with DIPCDI/HOBt method had to be used for the incorporation of Tle^5 residue to overcome the exceptional difficulty of a DIPCDI-mediated coupling reaction of Boc-Tle with the corresponding peptide resin due to highly steric hindrance of the Tle residue. Such a synthetic difficulty was not observed with the other analogs. Peptides were cleaved from the resin and simultaneously deprotected by treatment with an HF/anisole mixture and purified by medium-pressured HPLC. Homogeneity of the peptides was assessed by analytical HPLC,TLC, amino acid analysis and fast atom bombardment mass spectrometry. Details of the synthesis of these analogs will be reported in a separate paper.

Receptor Binding Assay. Crude synaptosomal fractions were prepared from rat decerebrated whole brain as previously described [16]. Binding assays were performed by incubating an aliquot of the membrane fraction (600μg of protein) in each assay containing 500μg of BSA, 50μg of bacitracin, 5μg of bestatin and 2 nM of [^{3}H]DAGO or [^{3}H]DADLE in a final volume of 500μl (50 mM Tris-HCl buffer at pH 7.4). A specific μ-ligand, [N^{α}-Me-Phe^3, D-Pro^4]morphiceptin (2.6 μM) was included in the δ-binding assay to suppress binding to μ-receptor site [17]. After 60 min at 25 ℃, the reaction mixture was filtered

over Whatman GF/B filter soaked with 0.1 % polyethyleneimine (μ- assay) or 0.5 % BSA (δ-assay). The filter was washed twice with cold Tris-HCl buffer (pH 7.4) and counted after overnight extraction with liquid scintillation fluid (3 ml each). All assays were performed in duplicate. Specific binding was determined from the difference between total binding and that in the presence of excess (1 μM) unlabelled ligand. Inhibition constants (Ki) were calculated according to the equation of Cheng and Prusoff [18]. Kd values of the [^{3}H]DAGO and [^{3}H]DADLE used are 0.52 and 1.38, respectively.

RESULTS AND DISCUSSION

Table I shows the results of the receptor binding assay. Simultaneous substitution of Val^{5,6} by Ala resulted in an analog (**1**) with greately decreased δ-affinity and significantly unaltered μ-affinity. Single substitution of Val^5 or Val^6 by Ala yielded analog **2** or **3** with a slightly decreased, but still high δ-affinity. However, the μ-affinity of **2** definitely increased resulting in a lower δ-selectively than that of **3** which still retained a high δ-selectivity being nearly comparable to that of DL-II. These results strongly suggest that the Val^5 residue is more important than the Val^6 residue in DL-II for δ-selectivity although both Val residues are required for high δ-

Table I. Opioid receptor assay of synthetic analogs

Peptides	[^{3}H]DAGO		[^{3}H]DADLE		Ki(μ)/Ki(δ)
	Ki(μ), nM±S.E.	R. P.[a]	Ki(δ), nM±S.E.	R. P.[b]	
DAGO	0.42 ± 0.08	100	——	——	——
DADLE	——	——	0.58 ± 0.08	100	——
DL-II	418 ± 56	0.10	0.54 ± 0.11	107	774
1 [Ala^{5,6}]	568 ± 186	0.07	21.0 ± 1.9	2.8	27
2 [Ala^5]	200 ± 20	0.21	1.60 ± 0.34	36.4	125
3 [Ala^6]	582 ± 95	0.07	1.07 ± 0.06	54.2	542
4 [Nva^{5,6}]	217 ± 27	0.20	0.74 ± 0.22	78.4	293
5 [Nle^{5,6}]	275 ± 18	0.15	0.085 ± 0.021	688	3256
6 [Ile^{5,6}]	251 ± 20	0.17	0.067 ± 0.021	871	3760
7 [Leu^{5,6}]	250 ± 32	0.17	0.227 ± 0.040	257	1105
8 [Tle^{5,6}]	352 ± 59	0.12	6.20 ± 0.80	9.4	57
9 [Mle^{5,6}]	433 ± 85	0.10	0.157 ± 0.042	370	2750

[a] Relative potencies (%) to DAGO. [b] Relative potencies (%) to DADLE.

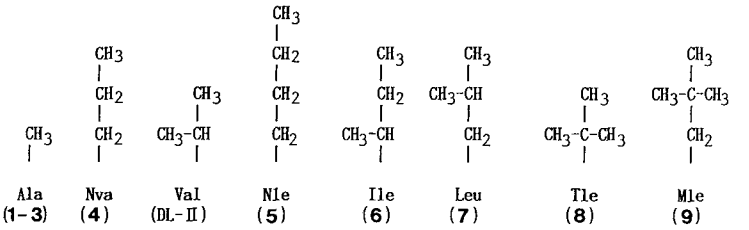

Fig. 1. Aliphatic amino acid side chains at positions 5 and/or 6 of the synthetic analogs.

affinity. To examine further the effects of hydrophobicity on the addressing role in DL-II, $Val^{5,6}$ residues were simultaneously replaced by various aliphatic amino acids with equal to or more hydrophobic side chains than that of Val (**4-9**). This series of analogs contained three- to five-membered linear or branched alkyl side chains (Fig. 1) at positions 5 and 6. Most of these analogs showed more increased δ-affinities and selectivities than the parent peptide without significant changes in μ-affinity with the exception of **4** and **8**. Among these, **6** showed the most potent δ-affinity and selectivity, being 9 and 5 times more potent than those of DL-II, respectively. **4** showed relatively low δ-selectivity in consequence of its somewhat increased μ-affinity as compared with DL-II. When comparing isomeric analogs, differences in both μ- and δ-affinities between linear and branched side chains (DL-II and **4**, **5**-**7**) were rather small. However, it should be noted that **8**, which has tert-butyl side chains at α-carbon of amino acids in the address domain, showed an exceptionally significant decrease in its δ-affinity, resulting in 13 times lower δ-selectivity than DL-II. In contrast, **9** showed 4 times higher δ-affinity and selectivity than DL-II despite the fact that this analog has also tert-butyl groups at β- carbon of amino acids. These observations can best be interpreted by assumption that the $Tle^{5,6}$ residues in **8** disrupt the proper peptide conformation of the molecule due to its highly steric hindrance around the α-carbon atom while the Mle residues in **9** permit the molecule to arrange for the active conformation due to an apparently lower steric hindrance of Mle than that of Tle as can be assumed by peptide bond formation described in the MATERIALS AND METHODS. This interpretation supports a proposal that the C-terminal tripeptide of DL influences the three-dimentional structure of the entire molecule [19]. These lines of evidence suggest that the address domain of DLs requires not only hydrophobicity, which may directly interact with

the receptor through hydrophobic bonding as suggested by Lazarus et al. [13], but also a proper peptide conformation which fits the peptide to form an active conformation at the receptor site for the high δ-affinity.

In summary, our data demonstrated the importance of hydrophobic $Val^{5,6}$ residues in DL-II for δ-affinity and selectivity, especially the critical importance of the Val^{5} residue for high δ-selectivity. By enhancing the hydrophobicity of residues at positions 5 and 6, we have developed analogs (**5**, **6** and **9**) with very high δ-affinity and selectivity over those of DL-II, which will be useful as δ-specific ligands for investigation of the physiological role of opioid receptors.

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