The Role of Deltorphin II Phenylalanine Residue in Binding to the δ Opioid Receptor

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Deltorphin II is a peptide ligand specific for the δ opioid receptor. In order to elucidate the role of Phe^3 in binding to the δ opioid receptor, we synthesized a series of analogs in which Phe^3 was replaced by various amino acids such as Ala, cyclohexylalanine (Cha), fluorophenylalanines, and other alkyl-side chain amino acids (Val, Leu, and norleucine (Nle)). It was found that [Cha^3]deltorphin II and [Nle^3]deltorphin II retain almost a full receptor binding affinity. The results indicated that the Phe^3-phenyl group of deltorphin II can be substituted by the alkyl groups such as cyclohexyl and propyl in the interaction with the δ receptor.

An aromatic amino acid phenylalanine (Phe) in ligand peptides often plays a crucial role in the receptor binding and activation.[1,2] The importance of Phe-phenyl in binding to the receptor would be explored by activity profiles of the set of analogs in which the Phe residue is replaced by alanine (Ala) and cyclohexylalanine (Cha).[3] Ala lacks the phenyl of Phe, whereas Cha possesses the saturated analog (cyclohexyl group) of the phenyl group. Cha is nearly isosteric with Phe, but lacks the quadrupole moment associated with an aromatic ring.

Opioid peptides such as enkephalins, endorphins, and endomorphins consist of the Phe residue at position 3 or 4 in the N-terminal message signal portions responsible for binding to opioid receptors. Although these Phe residues are admitted to be essential for receptor interaction, their roles in the receptor interaction have not been elucidated in detail in many cases. In our attempts to clarify the role of Phe in naturally-occurring opioid peptides, we found that the analog of deltorphin II with Cha/Phe^3 substitution retains almost the full receptor binding affinity. Since such an activity expression by non-aromatic substituent for Phe is rather peculiar, we proceeded to carry out the structure-activity study on the deltorphin II peptide.

Deltorphin II (Tyr–D-Ala–Phe–Glu–Val–Val–Gly–NH_2) is a peptide specific for the δ opioid receptors; it was isolated from frog skin.[4] Although the study to examine the importance of Phe^3 has been carried out for deltorphins, no definite role of Phe^3 in the receptor interaction has been described.[5] Schullery et al. also synthesized the Cha-containing analog, but its activity was considerably weaker than that observed by us for [Cha^3]deltorphin II. This discrepancy was thought to be due to the fact that they used [Gly^4]deltorphin as parent compound, in which the Glu^4-residue is substituted by Gly. Thus, we selected a deltorphin peptide in natural form, namely, deltorphin II, the triturated analog of which is available for the binding assay for the δ receptor. In the present study, in order to address the role of this Phe^3-phenyl, we have designed and synthesized a series of analogs in which Phe^3 was replaced by various amino acids such as Ala, Cha, Val, Leu, or norleucine (Nle) (Fig. 1). Since we did know already that [Nle^3]deltorphin II is also potent and since these results suggested the importance of hydrocarbon alkyl groups in the receptor interactions, we synthesized further a series of fluorophenylalanines ((F_n)Phe) to evaluate possible roles of the benzene-hydrogens of Phe-phenyl in deltorphin II. We here describe the structure-activity relationships of these peptides and show that the aromatic Phe^3-phenyl group of deltorphin II is replaceable with the alkyl groups such as cyclohexyl and propyl.

Experimental

Peptide Synthesis. All peptides were synthesized by the method of manual solid phase synthesis. All amino acids were protected at their amino group with Boc group; the side chain-protecting groups were 2,6-dichlorobenzyl for Tyr and cyclohexyl for Glu. Boc–(F_5)Phe–OH and Boc–(3,4,5-F_3)Phe–OH were purchased from Watanabe Chemical Ind. (Hiroshima). To obtain C-terminal peptide amides, Boc–Gly–*p*-methylbenzhydrylamine (MBHA) resin was utilized. Peptide amides synthesized are Ya/Phe/EVVG, Ya/Ala/EVVG, Ya/Cha/EVVG, Ya/Val/EVVG, Ya/Leu/EVVG, Ya/Nle/EVVG, Ya/(F_5)Phe/EVVG, Ya/(3,4,5-F_3)-Phe/EVVG, Ya/(2-F)Phe/EVVG, Ya/(4-F)Phe/EVVG, and Ya/(3-F)Phe/EVVG (shown in one-letter amino acid codes, in which "a" indicates D-Ala). Coupling reactions were carried out by using HBTU–HOBt[6] in a mixture of *N*–methylpyrrolidone and *N*,*N*–dimethylformamide (1 : 2, v/v) for 30 min.

Peptides were liberated from the resin by treatment with anhydrous HF containing 10% *p*-cresol at 0 °C for 1 h, and purified by Sephadex G-15 (2.0×100 cm) eluted with 30% AcOH. Peptides

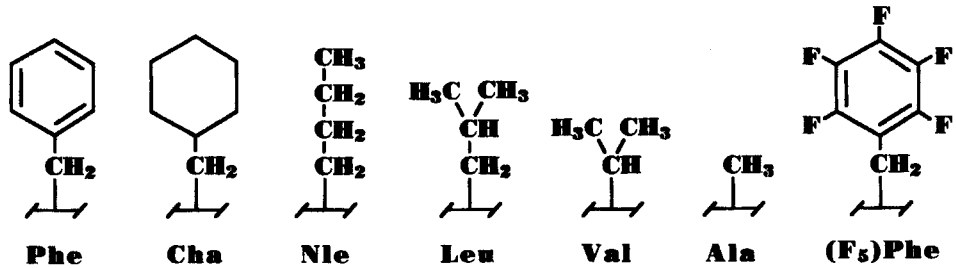

Fig. 1. The side-chain chemical structure of amino acids incorporated into deltorphin II at the position 3.

were further purified by a preparative reversed-phase HPLC (Cica-Merck, LiChrospher RP-18 (e) (5 μ): 25×250 mm). The elution conditions employed were as follows: solvent system, 0.1% aqueous trifluoroacetic acid (A solution) and acetonitrile containing 20% A solution (B solution); flow rate, 3 ml min^{-1}; temperature, 25 °C; and UV detection, 230 nm. Elution was done with a 5% B solution for the first 5 min and then with a linear concentration gradient of B solution (5—55%) for 50 min.

The purity was verified by analytical reversed-phase HPLC (LiChrospher 100 RP-18 (e) (5 μ): 4×250 mm), using the same conditions except for a flow rate of 0.7 ml min^{-1}. Amino acid analyses of peptides were carried out by RP-HPLC of phenylthiocarbamoyl derivatives of amino acids using a Waters PICO-TAG™ system after hydrolysis in a constant-boiling hydrochloric acid at 110 °C for 24 h. Mass spectra of peptides were measured on a mass spectrometer Voyager™ DE-PRO (PerSeptive Biosystems Inc., Framingham, MA) with the method of matrix assisted laser desorption ionization time-of-flight (MALDI-TOF).

Receptor Binding Assays. Radio-ligand receptor binding assays involving membrane preparations were carried out essentially as described.[7] Membranes were prepared from the rat brain purchased (Rockland, Gilbertsville, PA, USA). Peptides were evaluated using [^{3}H]deltorphin II (49.5 Ci $mmol^{-1}$, Amersham, Buckinghamshire, UK) for δ receptors and [^{3}H]DAGO (55.3 Ci $mmol^{-1}$, DuPont/NEN Research Products, Wilmington, DE, USA) for μ receptors (1 Ci = 3.7×10^{10} Bq). Briefly, incubations were carried out at room temperature for 60 min in Tris-HCl buffer (pH 7.55) containing 0.1% bovine serum albumin. Bacitracin (100 μg ml^{-1}) was added as an enzyme inhibitor. After incubation, each incubation mixture was filtered through glass fiber filters (Whatman GF/B) and rinsed twice with 10 mM Tris-HCl buffer pH 7.55 (4 ml) (1 M = 1 mol dm^{-3}). Dose-response curves were analyzed by the computer program ALLFIT.[8]

Results and Discussion

Peptide Synthesis. All eleven heptapeptides including native deltorphin II were synthesized by the solid-phase methodology using Boc–amino acids. Peptides were obtained in an average yield of about 34%. Table 1 shows the analytical data of all heptapeptides synthesized. The mass numbers measured were coincident with the values calculated. The purity of peptides was verified by analytical HPLC, in which all the peptides emerged with a single peak. When the retention times in reversed-phase (RP) HPLC were compared, it was found that the retention time of deltorphin II (45.74 min) is almost in the average (ca. 45 min) of those (about 33—51 min) of analogs containing various amino acids at the position 3. Amino acid analyses revealed a good coincidence of the number of amino acid constituents. Collectively, synthetic deltorphin II and its analogs have been identified physicochemically to reveal the appropriate compounds.

Table 1. Physical Constants of Synthetic Deltorphin II and its Phe^3-Substituting Analogs

Peptides[a)]	RP-HPLC	MALDI-TOF-MS[c)]	
	RT[b)]/min	Found	Calcd
Phe (native)	45.74	782.64	782.98
Ala	32.61	706.77	706.88
Cha	51.18	789.05	789.03
Val	38.31	734.90	734.94
Leu	43.00	749.01	748.96
Nle	43.51	748.99	748.96
(2-F)Phe	46.15	800.77	800.98
(3-F)Phe	46.20	800.92	800.98
(4-F)Phe	46.38	800.69	800.98
(3,4,5-F_3)Phe	50.63	836.92	836.98
(F5)Phe	51.18	872.90	872.94

a) Peptides are designated by the amino acid residues at position 3 of deltorphin II Tyr–D-Ala–/Xaa/–Glu–Val–Val–Gly–NH_2. b) Retention time (RT) was measured on an analytical column (Cica-Merck, LiChrospher 100 RP-18 (e) (5 μ): 4×250 mm) with a linear gradient of 0.1% aqueous trifluoroacetic acid (A solution) and acetonitrile containing 20% A solution (B solution). c) Values express the mass number (*m/z*) of $(M+H)^+$.

Binding Affinity for δ Opioid Receptor. Table 2 summarizes the results of the binding affinity of deltorphin II and its analogs for the δ and μ opioid receptors. For δ receptors, [^{3}H]deltorphin II was utilized as a specific tracer for the competitive radio-ligand binding assay. As shown in Table 2, it is clear that [Ala^3]deltorphin II exhibits a drastically diminished binding affinity (IC_{50} = 158 nM) as compared with deltorphin II (2.59 nM). This indicates that the Phe^3-phenyl group is necessary for binding to the δ receptor. In contrast, [Cha^3]deltorphin II was found to retain an affinity (3.55 nM) almost compatible to that of deltorphin II. These results clearly indicate that, for the δ receptor, the Phe^3-phenyl group of deltorphin II can be substituted by an alkyl group of cyclohexyl.

To further substantiate this replaceability of Phe^3-phenyl group, we tested the analogs substituted with a series of amino acids having non-aromatic alkyl side chain. Those include Val, Leu, and Nle with the isopropyl, isobutyl, and butyl groups, respectively. [Val^3]deltorphin II was very weak (282 nM), being almost 100 times less active than parent deltorphin II. [Leu^3]deltorphin II (17.6 nM) increased the affinity more than ten times, but was still less active than

Table 2. Opioid Receptor Binding Affinities of Deltorphin II and its Phe^3-Substituting Analogs

Peptides[a]	δ Receptor	μ Receptor	Receptor
	IC_{50}/nM	IC_{50}/nM	selectivity[b]
Phe (native)	2.59 ± 0.812	829 ± 312	320
Ala	158 ± 53.5	16,600 ± 4,100	110
Cha	3.55 ± 0.429	524 ± 73.0	150
Val	282 ± 40.6	113,000 ± 23,800	400
Leu	17.6 ± 4.01	11,300 ± 2,810	640
Nle	3.11 ± 1.21	3,380 ± 1,660	1,100
(2-F)Phe	13.7 ± 1.98	806 ± 325	60
(3-F)Phe	6.44 ± 1.13	550 ± 163	85
(4-F)Phe	14.1 ± 2.83	1,010 ± 497	72
(3,4,5-F_3)Phe	12.7 ± 2.86	526 ± 153	41
(F_5)Phe	53.8 ± 4.40	14,600 ± 6,050	270

a) Peptides are designated by the amino acid residues at position 3 of deltorphin II Tyr–D-Ala–/Xaa/–Glu–Val–Val–Gly–NH_2. b) Receptor selectivity was obtained by calculating the ratio of the IC_{50} values for δ receptor versus for μ receptor.

deltorphin II. However, [Nle^3]deltorphin II was found to sustain a high δ receptor binding affinity (3.11 nM), which was almost compatible to those of [Cha^3]deltorphin II and native deltorphin II (Table 2).

Thus, in addition to the cyclic alkyl group of cyclohexyl, it was found that Phe^3-phenyl can be substituted also by a non-cyclic alkyl group of propyl. Schullery et al.[5] reported the synthesis of Cha^3- and Nle^3-containing deltorphin analogs, but their activities were found to be relatively weak (32% and 11% of the parent peptide [Gly^4]deltorphin II, respectively). Since they made additional substitution for the Glu residue at position 4 by Gly, it is suspected that this Gly/Glu^4-replacement led the analogs to their reduced activities.

The analogs containing a series of fluorophenylalanines (F_n)Phe were also examined, but all of them exhibited considerably reduced binding activities (Table 2). Mono- and trifluorophenylalanine-containing analogs exhibited 20—40% activity of deltorphin II, while pentafluorophenylalanine-containing analog showed a considerable reduction (only 5% activity). All these results reveals the insignificant role of benzene-hydrogens of Phe^3-phenyl in the receptor interaction of deltorphin II.

Binding Affinity for μ Opioid Receptor and δ/μ Receptor Selectivity. For μ opioid receptor, the tracer utilized was [^{3}H]DAGO, a highly μ-specific enkephalin analog. Deltorphin II is very weak to bind to the μ opioid receptor with the IC_{50} value of 829 nM (Table 2). This makes the peptide highly selective for the δ receptor, and the selectivity ratio of deltorphin II was calculated to be 320. All other deltorphin II peptides synthesized were found to be also δ-selective (40—1,100-fold). Among the analogs, an extremely high δ-selectivity of [Nle^3]deltorphin II (1,100-fold) should be noted, since [Nle^3]deltorphin II is strong to bind to the δ receptor. Its high δ-selectivity is apparently due to the decrease in binding affinity for the μ receptor (Table 2). In contrast, [Cha^3]deltorphin II rather increased the affinity for μ receptor, and thus it reduced the δ-selectivity to half.

Fluorophenylalanine-containing deltorphin II analogs were found to reduce the binding affinity for the δ receptor to 20—40% of parent deltorphin II, as mentioned above. However, they sustained the affinity for the μ receptor as well as deltorphin II (Table 2). These made the receptor selectivity of the analogs to be relatively small (40—85-fold), although they are still δ-selective. The exception is [(F_5)-Phe^3]deltorphin II, which reduced the affinity for both δ and μ receptors to show a selectivity (270-fold) similar to that of deltorphin II.

Different Structural Roles of Phe Residues in Biologically Active Peptides. The present results clearly show that, when deltorphin II interact with the δ receptor, the Phe–phenyl group of deltorphin II is replaceable with the alkyl groups such as cyclohexyl and propyl. [Cha^3]deltorphin II and [Nle^3]deltorphin II were almost fully active with sustained receptor binding affinity, showing 73% and 83% activities of native deltorphin II, respectively. This kind of activity retainment by the Cha or Nle/Phe-replacement is extraordinary. One of rare examples has been reported for the Cha-containing analog of a neuropeptide substance P. The Cha/Phe-replacement at position 8 of substance P was found to retain a 100% full activity.[9]

Among opioid peptides, however, the Cha/Phe-replacements usually result in a remarkable reduction in receptor activities. δ-Specific enkephalin analog DSLET, H–Tyr–D-Ser–Gly–Phe–Leu–Thr–OH, exhibited only 8% and 1% activities by Cha/Phe^4- and Nle/Phe^4-replacements, respectively.[10] Also, μ-specific endogenous opioid peptide endomorphin 2, H–Tyr–Pro–Phe–Phe–NH_2, showed drastically reduced activities by Cha or Nle/Phe-replacement at both position 3 and 4 (unpublished data). Similar results were reported, for example, for neurokinin A[11] and thrombin receptor-tethered ligand peptide S/Phe/LLRNP.[12] In all these biologically active peptides, the Phe residues appear to be in an interaction in which the aromaticity of Phe plays a crucial role.

Since the Phe residue is replaceable with Cha and Nle in deltorphin II, the aromaticity of Phe–phenyl appears to be insignificant for receptor interaction. Another important structural element is a ring conformation, since the cyclohexyl ring of Cha adopts a chair conformation in energy minimization.[11] This structure appears to be accommodated in the receptor binding site. Although to present a precise molecular mechanism of such a Phe residue in receptor interaction is difficult without the molecular-based information about the receptor, the Phe residue must be in the receptor site to which both the phenyl and cyclohexyl or propyl groups are allowed to bind. The results suggest that the binding interaction between the Phe^3-phenyl group and its specific δ receptor site is primarily van der Waals in nature. Since the Ala/Phe-replacement in deltorphin II eliminate a substantial binding ability of the peptide, this binding interaction appears to be essential for receptor recognition and presumably for activation.

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3 The abbreviations according to biochemical nomenclature by IUPAC-IUB Joint Commission, *Eur. J. Biochem.*, **138**, 9 (1984), are used throughout. Unless otherwise specified, the amino acids are L-stereoisomers. Additional abbreviations are as follows: Cha, cyclohexylalanine; DAGO, [D-Ala^2,*N*–Me–Phe^4,$Gly\text{–}ol^5$]-enkephalin; DSLET, [D–Ser^2,Leu^5]enkephalyl–Thr^6; (F_n)Phe, fluorophenylalanines; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MBHA, *p*-methylbenzhydrylamine; RP-HPLC, reversed-phase high-performance liquid chromatography.

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