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Differential receptor binding characteristics of consecutive phenylalanines in μ-opioid specific peptide ligand endomorphin-2

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Abstract—Endogenous opioid peptides consist of a conserved amino acid residue of Phe^3 and Phe^4, although their binding modes for opioid receptors have not been elucidated in detail. Endomorphin-2, which is highly selective and specific for the μ opioid receptor, possesses two Phe residues at the consecutive positions 3 and 4. In order to clarify the role of Phe^3 and Phe^4 in binding to the μ receptor, we synthesized a series of analogs in which Phe^3 and Phe^4 were replaced by various amino acids. It was found that the aromaticity of the Phe-β-phenyl groups of Phe^3 and Phe^4 is a principal determinant of how strongly it binds to the receptor, although better molecular hydrophobicity reinforces the activity. The receptor binding subsites of Phe^3 and Phe^4 of endomorphin-2 were found to exhibit different structural requirements. The results suggest that [Trp^3]endomorphin-2 (native endomorphin-1) and endomorphin-2 bind to different receptor subclasses.

1. Introduction

The presence of a bioactive conformation in neuropeptides is prerequisite to specific receptor interactions, and these interactions are stabilized by distinct structural elements binding to the receptor. Phenylalanine (Phe), an aromatic amino acid, often plays a crucial role in neuropeptides in their receptor binding and activation.[1] Elucidation of the interaction mode of such Phe residues appears to be one of the definite goals to clarifying the molecular mechanism of receptor activation. The interaction of Phe is usually characterized by the term 'hydrophobic', the intrinsic meaning of which is rather obscure. We have postulated a means of differentiating such a hydrophobic interaction of Phe, replacing the residue with a series of amino acids having side-chain varieties.[2] Furthermore, the presence of the edge-to-face CH/π interaction was demonstrated between the Phe-phenyl group of thrombin receptor-tethered ligand peptide and the receptor aromatic group,[3–5] and the face-to-face π/π stacking interaction was proven by X-ray crystallographic analysis and ^1H NMR analysis between the Phe-phenyl group of inhibitor peptide and the His-imidazole group of the enzyme chymotrypsin.[6,7]

Opioid peptides induce analgesia by interacting with opioid receptors such as the endogenous μ, δ, and κ subtypes. It is generally accepted that the N-terminal portion of opioid peptides such as enkephalins, endorphins, and dynorphins is the message sequence for binding to opioid receptors, and that Tyr at position 1 and Phe at positions 3 or 4 are essential for receptor

Abbreviations: Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Cha, cyclohexylalanine; DMF, *N*,*N*-dimethylformamide; End-1, endomorphin-1; End-2, endomorphin-2; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MBHA, *p*-methylbenzhydrylamine; Nle, norleucine; NMP, *N*-methylpyrrolidone; (F_5)Phe, pentafluorophenylalanine; RP-HPLC, reversed-phase high performance liquid chromatography; RT, retention time; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Keywords: Endomorphins; Opioid peptide; Phenylalanine; Structure–activity relationships.

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binding and activation.[8–11] However, despite such a residual importance, their role in the receptor interaction has not necessarily been elucidated in detail.

In our previous studies of a δ-specific opioid peptide named deltorphin II, Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 isolated from African frog skin,[12] the replacement of Phe^3-phenyl group by the cyclohexyl ($-C_6H_{12}$) or *n*-propyl ($-CH_2CH_2CH_3$) was found to retain the receptor binding ability of the parent peptide.[2] In contrast, the same replacement of Phe^4 of δ-specific DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr-OH, a synthetic analog of endogenous opioid peptide enkephalin)[13] did not sustain the binding affinity, reducing by approximately 90% the activity of DSLET.[14] These results indicated that the receptor interaction modes of Phe^3 in deltorphin II and Phe^4 in DSLET residues are different from each other.

Endomorphin-1 (End-1: Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (End-2: Tyr-Pro-Phe-Phe-NH_2) are endogenous ligands specific for the μ receptor. Although they were isolated from bovine brain,[15] the genetic sources have not yet been clarified. Among natural opioid peptides, the sequences of endomorphins are unique, having aromatic residues at the consecutive positions 3 and 4. It is thus intriguing to elucidate the mode of interaction between these aromatic amino acids and the receptor. In the present study, in order to clarify the role of Phe^3 and Phe^4 of endomorphin-2 in receptor binding, we designed and synthesized a series of analogs in which these Phe residues were replaced by various amino acids (Fig. 1). When the activity profiles of each series of analogs were compared, the same substitutions were found to have different effects, with much stronger activity for

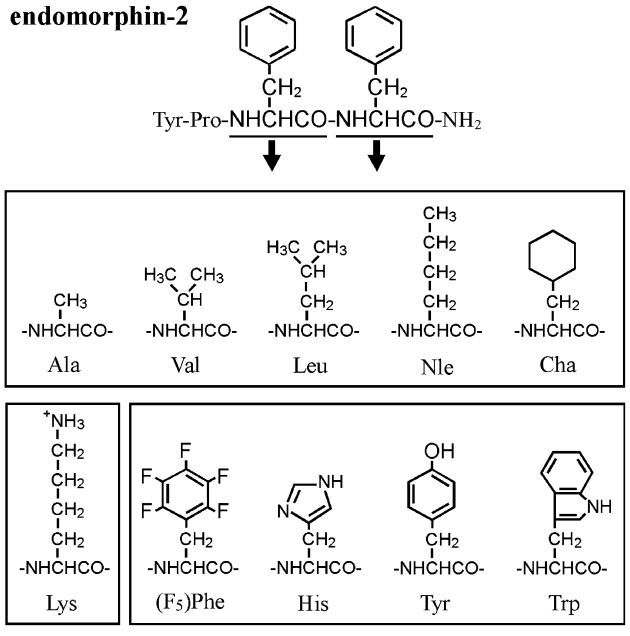

Figure 1. The structures of endomorphin-2 and the side-chains of the amino acid residues at positions 3 and/or 4. Each box classifies a series of amino acids with the side-chains such as aliphatic, basic, and aromatic groups.

Phe^3 than for Phe^4, suggesting their differential receptor binding characteristics.

2. Results

2.1. Peptide synthesis

Endomorphin-2 and its analogs were prepared by the manual solid phase peptide synthesis method. Starting from the MBHA resin, all the coupling reactions were carried out by the HBTU/HOBt method.[16] Completion of the reaction was monitored by the Kaiser test.[17] In the syntheses of Phe^3-substituted analogs, various Boc-amino acids were introduced at the first coupling step starting from Phe-preloaded MBHA resin, while this was done directly for the MBHA resin in the syntheses of Phe^4-substituted analogs. Including native endomorphin-2, all 21 tetrapeptides were synthesized in an average yield of approximately 35%.

Analogs containing cyclohexylalanine (Cha) or pentafluorophenylalanine ((F_5)Phe) were obtained in somewhat lower yields (about 25%). Although the exact reason is not clear, their relatively high hydrophobicity might bring about this lower recovery of the compounds. Table 1 shows the analytical data of all the synthesized tetrapeptides. The mass numbers measured were coincident with the values calculated. The purity of peptides was also verified by analytical HPLC, in which all the peptides emerged as a single peak. Amino acid analyses revealed a good coincidence of the number of amino acid constituents.

The retention time in RP-HPLC usually exhibits the characteristic structural nature of the peptide hydrophobicity. When the same substitution took place for the Phe residues at both positions 3 and 4, the values of the retention time of Phe^3-substituted analogs were slightly larger than those of Phe^4-analogs (Fig. 2). This difference was particularly prominent for Phe → Ala substitutions; that is, RT = 20.74 min for [Ala^3]End-2, and RT = 12.86 min for [Ala^4]End-2. These findings might indicate that the removal of Phe^3-phenyl exposes the peptide in the structure to the solvent more than expected.

2.2. Receptor binding affinities of Phe^3-substituted endomorphin-2 analogs

The binding affinity of peptides was evaluated by measuring the ability to displace the radio-labeled ligand specific for either μ or δ opioid receptor. Radio-labeled ligands utilized were tritium-labeled enkephalin analog [^{3}H]DAGO for μ receptor and tritium-labeled frog-skin peptide [^{3}H]deltorphin II for δ receptor. From the analysis of the obtained dose–response curves, the IC_{50} values of the synthetic peptides were estimated, and Table 2 summarizes these values together with the selectivity expressed by the ratio of $IC_{50}(\delta)$ versus $IC_{50}(\mu)$.

The importance of Phe-phenyl in endomorphin-2 can be explored by assessing the activity profiles of the set of

Table 1. Physical constants of synthetic endomorphin-2 and its Phe[3]- and Phe[4]-substituting analogs

Peptides[a]	Phe[3]-substituents		Phe[4]-substituents	
	HPLC[b] (t_R)	MS[c] (Found/calcd)	HPLC[b] (t_R)	MS[c] (Found/calcd)
Phe (End-2)	30.30	571.63/571.93	30.30	571.63/571.93
Ala	20.74	495.80/495.58	12.86	495.86/495.58
Val	22.56	523.80/523.64	20.98	523.80/523.64
Leu	28.12	538.28/537.66	26.88	537.84/537.66
Nle	29.67	538.27/537.66	28.37	537.89/537.66
Cha	37.92	577.52/577.73	36.42	577.54/577.73
(F_5)Phe	37.35	661.62/661.64	37.24	661.63/661.64
His	12.71	561.55/561.65	9.27	561.66/561.65
Tyr	23.61	587.89/587.68	20.60	587.88/587.68
Trp	33.04	610.80/610.72	32.16	610.91/610.72
Lys	11.19	553.01/552.68	9.04	552.83/552.68

[a] Peptides are designated by the amino acid residues at position 3 or 4 of endomorphin-2.
[b] Retention time (t_R) of RP-HPLC was measured on an analytical column (Cica-Merck, LiChrospher 100 RP-18 (5μ): 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 $(M+H)^+$ by MALDI-TOF-MS.

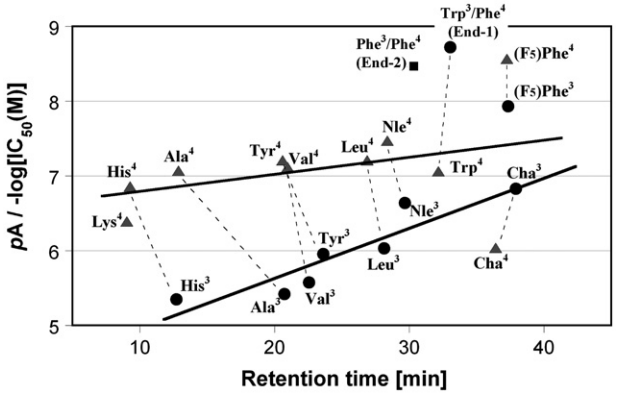

Figure 2. The relationship between the binding affinity and the hydrophobicity of endomorphin-2 and its analogs. The pA values of endomorphin-2 analogs with the substituents at positions 3 (closed circle) and 4 (closed triangle) are shown together with that of Phe[3]/Phe[4] (closed square) of native endomorphin-2. Two solid lines were estimated by the least-square method using values of coordinates of analogs with the substituents at positions 3 (His[3], Ala[3], Val[3], Tyr[3], Leu[3], Nle[3], and Cha[3]) and 4 (Lys[4], His[4], Ala[4], Val[4], Tyr[4], Leu[4], Nle[4], and Trp[4]), respectively. The pA value of Lys[3]-analog is smaller than 3, and thus it was eliminated from this plotting.

Table 2. Opioid receptor binding affinity and selectivity of endomorphin-2 and its Phe[3]-substituting analogs

Peptides[a] (Phe[3]-substituents)	IC_{50} (nM)		Selectivity[b]
	μ receptor	δ receptor	
Phe (End-2)	3.46 ± 1.21	10,300 ± 2770	2980
Ala	3810 ± 159	Unbound[c]	(μ)[d]
Val	2650 ± 339	>1 mM	(μ)
Leu	926 ± 28.3	>1 mM	(μ)
Nle	230 ± 17.7	15,200 ± 4950	66.1
Cha	148 ± 16.3	3150 ± 156	21.3
(F_5)Phe	11.7 ± 0.503	11,700 ± 1010	1000
His	4490 ± 70.7	>1 mM	(μ)
Tyr	1100 ± 113	5720 ± 326	5.20
Trp	1.89 ± 0.731	3580 ± 211	1890
Lys	>1 mM	Unbound[c]	Inactive
DAGO[e]	1.13 ± 0.263	727 ± 109	643

[a] Peptides are designated by the amino acid residues at position 3 of endomorphin-2 (Tyr-Pro-/Xaa/-Phe-NH_2).
[b] Receptor selectivity was estimated by calculating the ratio of the IC_{50} values for μ receptor versus those for δ receptor.
[c] 'Unbound' exhibits that tracer ligands ([^{3}H]DAGO and [^{3}H]deltorphin II) are not replaced with 10 mM of the synthetic peptides.
[d] (μ) means 'extremely or considerably weak selectivity for μ receptor'.
[e] DAGO is a μ-specific opioid peptide with the sequence of Tyr-D-Ala-Gly-(*N*-Me)Phe-Gly-ol.

analogs in which the Phe residue is replaced by various amino acids. We used amino acids with a variety of substituents at the side-chain β-position (Fig. 1). When Phe was replaced by Ala at position 3, eliminating the β-phenyl group, the resulting [Ala[3]]End-2 exhibited a drastically reduced binding affinity (IC_{50} = 3810 nM) as compared with endomorphin-2 (IC_{50} = 3.46 nM). The presence and absence of Phe[3]-phenyl cause the activity difference of three-order magnitude, indicating the critical importance of the Phe[3]-phenyl group in binding to the μ receptor.

Cha possesses the saturated substituent (cyclohexyl group) of the phenyl group. Cha is nearly isosteric with Phe, but lacks the aromaticity and the quadrupole moment associated with an aromatic ring. The Phe → Cha replacement at position 3 resulted in a sharp drop (43-fold) in the receptor binding affinity (Cha[3]; IC_{50} = 148 nM) as compared with endomorphin-2, indicating that the presence of π-electrons and/or the rigid planarity of Phe[3]-phenyl are key structural characteristics for interaction with the receptor. Thus, a series of endomorphin-2 analogs containing aromatic amino acids such as His, Tyr, Trp, and (F_5)Phe were further examined.

Histidine (His) possesses a five-membered aromatic ring (imidazole group), and Tyr has a phenol ring. Both are rather hydrophilic as compared with Phe. On the other hand, Trp is more hydrophobic than Phe, and its β-indole group is distinctly larger than Phe-phenyl. (F_5)Phe is nearly isosteric with Phe, while it has an enhanced hydrophobicity and the inverse quadrupole moment.[18] All the aromatic amino acids were introduced at

position 3 of endomorphin-2 instead of Phe. Neither His nor Tyr substitutions resulted in retainment of the parent binding affinity of endomorphin-2. [His^3]End-2 and [Tyr^3]End-2 were extremely weak to bind to the μ receptor. [His^3]End-2 exhibited only 0.08% activity of native endomorphin-2, and [Tyr^3]End-2 showed 0.3% activity. It is obvious that His and Tyr cannot compensate Phe^3 for binding to the receptor.

In contrast, [Trp^3]End-2 was found to be extremely potent as compared to these analogs. It should be noted that this analog is more potent than native endomorphin-2 (about 180%). [Trp^3]End-2 is native endomorphin-1, and Trp at position 3 appears to preferentially interact with the μ receptor than Phe. Replacement with another aromatic amino acid (F_5)Phe resulted in a drop in activity, showing an approximately threefold weaker affinity than endomorphin-2.

Non-aromatic alkyl amino acids were also incorporated at position 3. These included Val, Leu, and Nle, with the isopropyl, isobutyl, and butyl groups, respectively, at the β-position in the side-chain. It was previously found that replacement of the Phe^3-phenyl group in deltorphin II by alkyl groups fully retains the receptor binding.[2] However, any substitutions with the same amino acid at position 3 of endomorphin-2 were found to reduce drastically the binding affinity; that is, 0.13% with Val^3, 0.37% with Leu^3, and 1.50% with Nle^3 substitutions. Although the binding activities of these analogs are quite weak, there is a clear tendency for the binding affinities of these analogs to be dependent upon the size of the alkyl groups of the substituents and the molecular hydrophobicity of peptides.

2.3. Receptor binding affinities of Phe^4-substituted endomorphin-2 analogs

Table 3 summarizes the results of the binding affinity of endomorphin-2 analogs with Phe^4-substituents for the μ and δ opioid receptors. The elimination of Phe-phenyl at position 4 also resulted in a drastic drop in activity. [Ala^4]End-2 showed a 25-fold drop in binding affinity (IC_{50} = 85.4 nM). Clearly, the Phe^4-phenyl group is essential for binding to the μ receptor. [Cha^4]End-2 also exhibited a drastically reduced (about 270-fold) binding affinity (IC_{50} = 917 nM), indicating the importance of the aromaticity of Phe^4-phenyl.

These results led to an assay to examine the analogs containing a series of aromatic amino acids at position 4. The Phe → His and Phe → Tyr replacements resulted in activity decreases (40-fold and 18-fold, respectively). Surprisingly, Phe → Trp replacement brought about a drastic activity drop (ca. 25-fold). [Trp^4]End-2 showed very weak (ca. 4% of endomorphin-2) binding to the μ receptor, but this was not the case at position 3, as mentioned above. Eventually, [Trp^3]End-2 is much more potent (ca. 1900-fold) than [Trp^4]End-2. It is clear that Trp cannot compensate for the loss of Phe^4 for binding to the receptor. In contrast, another aromatic amino acid (F_5)Phe at position 4 did not change the activity of Phe-containing native endomorphin-2.

Table 3. Opioid receptor binding affinity and selectivity of endomorphin-2 and its Phe^4-substituting analogs

Peptides[a] (Phe^4-substituents)	IC_{50} (nM)		Selectivity[b]
	μ receptor	δ receptor	
Phe (End-2)	3.46 ± 1.21	10,300 ± 2770	2980
Ala	85.4 ± 13.5	Unbound[c]	(μ)[d]
Val	76.5 ± 3.39	>1 mM	(μ)
Leu	62.0 ± 9.66	>1 mM	(μ)
Nle	33.9 ± 5.73	>1 mM	(μ)
Cha	917 ± 181	11,400 ± 4070	21.3
(F_5)Phe	2.74 ± 0.511	8040 ± 1610	1000
His	138 ± 70.7	>1 mM	(μ)
Tyr	62.2 ± 13.9	54,500 ± 3200	5.20
Trp	87.1 ± 10.0	>1 mM	(μ)
Lys	408 ± 95.5	Unbound[c]	(μ)
DAGO[e]	1.13 ± 0.263	727 ± 109	643

[a] Peptides are designated by the amino acid residues at position 4 of endomorphin-2 (Tyr-Pro-Phe-/Xaa/-NH_2).
[b] Receptor selectivity was estimated by calculating the ratio of the IC_{50} values for μ receptor versus those for δ receptor.
[c] 'Unbound' exhibits that tracer ligands ([^{3}H]DAGO and [^{3}H]deltorphin II) are not replaced with 10 mM of the synthetic peptides.
[d] (μ) means 'extremely or considerably weak selectivity for μ receptor'.
[e] DAGO is a μ-specific opioid peptide with the sequence of Tyr-D-Ala-Gly-(*N*-Me)Phe-Gly-ol.

Non-aromatic alkyl amino acids were also incorporated at position 4. Neither Val-, Leu-, nor Nle-substitution resulted in retainment of the binding affinity of endomorphin-2. These substitutions reduced the affinity of endomorphin-2 to 4.5% with Val^4, 5.6% with Leu^4, and 10% with Nle^4.

3. Discussion

Endomorphin-2 has a characteristic structure in which the aromatic amino acid Tyr is connected to the Phe-Phe aromatic dipeptide. The connecting unit is Pro, which usually creates a bent conformation and is important in the determination and stabilization of the structures of endomorphins.[19] The interrelationship between Tyr and Phe-Phe might be crucial to recognizing the receptor, and the binding site would independently be constructed specifically for these aromatic amino acids.[15,20–22] The importance of aromatic–aromatic interactions such as Tyr^{1}Phe^3, Tyr^{1}Phe^4, and Phe^{3}Phe^4 in the association between the receptor and ligand recognition has recently been reported.[19] In order to elucidate the interaction mode of C-terminal consecutive Phe residues, we evaluated the receptor activity of a series of Phe^3 or Phe^4-substituted analogs. It is particularly important to assess the structural importance of each Phe residue to gain insight into the characteristic receptor activity. The present results clearly indicate that the β-phenyl groups of Phe^3 and Phe^4 are critical to binding to the μ receptor, and the aromaticity of Phe is a key structural characteristic essential for this binding ability.

To show the importance of the aromaticity, not the hydrophobicity, we analyzed the interrelationship between the receptor binding affinities and the HPLC retention time of endomorphin-2 analogs (Fig. 2).

The retention time in RP-HPLC is usually characteristic in revealing the molecular hydrophobicity of peptides. It is clear that there is a rough linearity among a series of analogs with substitutions at positions 3 and 4, suggesting that the HPLC retention time correlates to the molecular hydrophobicity. When comparing the activity profiles of each series of endomorphin-2 analogs with Phe^3- and Phe^4-substituents (Fig. 2), it is obvious that the substitutions much more strongly affect the Phe^3 residue than the Phe^4 residue. The line showing activity–hydrophobicity interrelationships for Phe^3-substituents demonstrates a much lower activity region than for Phe^4-substituents, indicating that the receptor activities of analogs with Phe^3-substituents are much weaker than those with Phe^4-substituents.

It should be noted that the linearities between this molecular hydrophobicity and receptor binding activity are not complete. [Phe^3, Phe^4]End-2 (endomorphin-2 itself), [Trp^3, Phe^4]End-2 (endomorphin-1 itself), [$(F_5)Phe^3$]End-2, and [$(F_5)Phe^4$]End-2 do not show such linearity, exhibiting much more enhanced activity than analogs. These results suggest that the receptor binding sites for both Phe^3 (or Trp^3) and Phe^4 (or (F_5)Phe) are constructed just for these aromatic amino acids, not for hydrophobic amino acids.

The substitutions of Phe^4 with Trp and Cha showed unexpectedly diminished receptor affinity (Table 3). Although the affinity of [Cha^3]End-2 lay on the Phe^3-correlation line, as shown in Fig. 2, [Cha^4]End-2 was extremely weaker than expected from the Phe^4-correlation line. With the Cha^4-side-chain it might be hard to retain the interaction with certain receptor sites in the receptor. For Trp-substitutions, [Trp^3]End-2 was found to increase the affinity as compared to parent endomorphin-2. This trend is not surprising because [Trp^3]End-2 is a natural peptide ligand for the μ receptor, namely, endomorphin-1. At position 3 of endomorphins, Trp appears to be much more favorable than Phe. However, the fact that the same substitution at position 4 drastically decreased the binding affinity is somehow surprising, since the Trp^4 residue may conserve both the aromaticity and high hydrophobicity. This result is consistent with the case of $Phe^4 \rightarrow$ Trp replacement in endomorphin-1.[15] Tyr-Pro-Trp-Trp-NH_2 was extremely weak, approximately 100-fold weaker than endomorphin-1. The inverse preference of the μ-receptor for Trp and Phe in endomorphins would be brought about by their differences in size, aromaticity, and perhaps hydrophobicity.

It has not previously been clear whether endomorphin-1 and endomorphin-2 play different roles under physiological conditions, and whether they interact with different receptor subclasses. For such discrimination, the present results definitely help to differentiate the structural characteristics of the receptors. The Trp-indole ring is larger than the Phe-phenyl ring, and the receptor-binding site specific for Phe^3 in endomorphin-2 would be just the actual size of the Phe side-chain, but not the Trp side-chain. On the other hand, the receptor-binding site specific for Trp^3 in endomorphin-1 should be the size of the Trp side-chain in order to accept the Trp-indole group. Indeed, it has been proposed that endomorphin-1 and endomorphin-2 may act through two distinct μ_1- and μ_2-opioid receptor subclasses, respectively.[21,23,24] We used rat brain membrane preparations for receptor binding assay in the present study. If such subclasses were present, both of them would be contained together. Thus, a combined result of receptor affinities would be obtained for each analog. Since the two series of analogs with the substituents at positions 3 and 4, namely, (Xaa^3-Phe^4) and (Phe^3-Xaa^4), were aligned on different lines, their structural requirements appear to be distinct from each other. It should be noted that both native endomorphins have the Phe residue at position 4. The present results strongly suggest that endomorphins distinguish putative μ-receptor binding subsites by amino acids Phe and Trp at position 3. In other words, there would be two distinct μ opioid receptor subclasses in rat brain.

4. Experimental

4.1. Materials

The Boc derivatives of fluorine-containing phenylalanines were prepared as described,[25–27] and all other Boc-amino acids were purchased. *p*-Methylbenzhydrylamine (MBHA) resin, *N*-methylpyrrolidone (NMP), dichloromethane, diisopropylethylamine, trifluoroacetic acid (TFA), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 1-hydroxybenzo-triazole (HOBt) were also purchased. *N*,*N*-dimethylformamide (DMF) and acetonitrile were purchased from Kanto Chemical (Tokyo). Anhydrous hydrogen fluoride (HF), *p*-cresol, Tris–HCl, bacitracin, and bovine serum albumin (BSA) were purchased from Hashimoto Kasei Co. (Osaka), Kishida Chemical Co. (Osaka), Nacalai Tesque Co. (Kyoto), Sigma (St. Louis, MO, USA), and Wako Pure Chemical Co. (Osaka), respectively. All other chemicals were of the best grade available.

4.2. Peptide synthesis

All peptides were synthesized by the method of manual solid phase synthesis. Amino acids were protected at their amino group with the Boc group, and the side-chain protecting groups were 2,6-dichlorobenzyl for Tyr, 2-chlorobenzyloxycarbonyl for Lys, benzyloxymethyl for His, and formyl for Trp. To obtain C-terminal peptide amides, MBHA resin was utilized. The peptide amides synthesized were endomorphin-2 (YP/Phe/Phe) and its analogs: that is, YP/Ala/F, YP/Val/F, YP/Leu/F, YP/Nle/F, YP/Cha/F, YP/Lys/F, YP/(F_5)Phe/F, YP/His/F, YP/Tyr/F, YP/Trp/F (corresponding to endomorphin-1), YPF/Ala, YPF/Val, YPF/Leu, YPF/Nle, YPF/Cha, YPF/Lys, YPF/(F_5)Phe, YPF/His, YPF/Tyr, and YPF/Trp. Coupling reactions were carried out by using HBTU/HOBt in a mixture of NMP and DMF (1:2, v/v) for 30 min.[16] Each coupling reaction was checked by means of a ninhydrin test for completion.[17]

Peptides were liberated from the resin by treatment with anhydrous liquid HF containing 10% *p*-cresol at 0 °C for 1 h. The products were purified first by gel filtration on a column (2.0 × 100 cm) of Sephadex G-15 (Pharmacia, Uppsala, Sweden) eluted with 30% AcOH and then by preparative reversed-phase high performance liquid chromatography (RP-HPLC) (Cica-Merck, LiChrospher RP-18 (e) (5μ): 25 × 250 mm). The elution conditions employed for RP-HPLC were as follows: solvent system, 0.1% aqueous TFA (A solution) and acetonitrile containing 20% A solution (B solution); flow rate, 3 ml/min; temperature, 25 °C; and UV detection, 230 nm. Elution was carried out with 5% B solution for the first 5 min and then with a linear concentration gradient of B solution, 20–60% for 40 min.

The purity was verified by analytical RP-HPLC (LiChrospher RP-18 (5μ): 4 × 250 mm), using the same conditions except for a flow rate of 0.7 ml/min. Amino acid analyses of peptides were carried out by RP-HPLC of phenylthiocarbamoyl derivatives of amino acids using a PICO-TAG™ system (Waters, Milford, MA) 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, Framingham, MA) with the method of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy.

4.3. Receptor binding assays

Radio-ligand receptor binding assays were carried out essentially as described previously.[28] Membranes were prepared from rat brains purchased (Rockland, Gilbertsville, PA). Peptides were evaluated using [^{3}H]DAGO (55.3 Ci/mmol, DuPont/NEN Research Products, Wilmington, DE) for μ receptors and [^{3}H]deltorphin II (49.5 Ci/mmol, Amersham, Buckinghamshire, UK) for δ receptors. Each tube containing the membrane preparations, synthetic peptides, and 0.25 nM respective tritium-labeled ligand was incubated at room temperature for 60 min in Tris–HCl buffer (pH 7.55) containing 0.1% BSA. Bacitracin (100 μg/ml) was added as an enzyme inhibitor. After incubation, solutions were filtered by glass fiber filters (GF/B; Whatman, Clifton, NJ) and washed twice with 10 mM Tris–HCl buffer (pH 7.55, 4 ml). Filters were placed in scintillation vials containing a 4-ml scintillation cocktail (Scintisol EX-H; Dojindo, Kumamoto) for scintillation counting. Dose–response curves were analyzed by the computer program ALLFIT.[29]

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