Effects of Substitution of Hydrophobic Amino Acids by Tryptophan on Receptor Binding and Biological Activity of Neuropeptide Nociceptin

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Nociceptin is a neuropeptide that binds to and activates the opioid receptor-like ORL1 receptor. In order to explore the structural elements necessary for receptor recognition and activation, we designed and synthesized a series of nociceptin analogues, in which nonpolar amino acid residues such as Gly^6, Ala^7, Ala^{11}, Leu^{14}, and Ala^{15} were substituted respectively by Trp. [Trp^6]- and [Trp^7]nociceptins exhibited rather weak activities (5—15% of nociceptin), and [Trp^{11}]- and [Trp^{15}]-nociceptins also showed reduced activities (40—50%). These results suggested that the space available for these particular residues are relatively restricted. By contrast, the Trp/Leu-substitution at position 14 retained full receptor binding activity, and the resulting [Trp^{14}]nociceptin exhibited an increased biological activity in the functional assay using [^{35}S]GTPγS. This suggested that the receptor residue interacting with nociceptin-Leu^{14} is the aromatic amino acid.

Heptadecapeptide nociceptin[1] or orphanin FQ[2] was isolated from the rat and porcine brains as an endogenous ligand of G protein-coupled receptor ORL1 (opioid receptor-like 1),[3] the structure of which is very similar to that of opioid receptors.[4—6] Nociceptin was originally reported to produce hyperalgesia,[1] but later found to have various physiological effects relating, for example, to locomotion and learning.[7,8] Recent study using mice lacking ORL1 suggested that the nociceptin system plays negative roles both in learning and memory.[8]

The amino acid sequence of nociceptin, FGGFTGARKSARKLANQ, is similar to that of an endogenous κ-selective opioid peptide, dynorphin A (YGGFLRRIRPKLKWDNQ). In addition to similar N-terminal amino acid sequences, these peptides contain several residues of basic amino acids Arg and Lys at their C-terminal regions. However, recent structure-activity studies using chimeric nociceptin/dynorphin A peptides have suggested that crucial region for receptor binding of nociceptin differ from that of dynorphin A.[9,10] One of the most striking structural dissimilarities appears in the N-terminus; i.e., phenylalanine for nociceptin, while tyrosine for dynorphin A and also for all other opioid peptides. We found that [Tyr^1]nociceptin retains essentially full activities,[11] while Phe^1-containing opioid peptides were completely devoid of biological activities.

The role of the amino acid residues of nociceptin in activation of ORL1 has also been examined by replacing each residue with other amino acids. Alanine-scanning is a method in which each residue is substituted by alanine (Ala). This screening method for nociceptin has clarified some important residues, the side chain of which is crucial for receptor activation.[12,13] However, nociceptin originally contains three Ala residues (at positions 7, 11, and 15) in its 17 amino acid constituents. To elucidate the function of these Ala residues, residual substitutions with other amino acids would afford the structural information. Since Ala is a hydrophobic amino acid with the methyl group in the side chain, we replaced it with tryptophan Trp (indolyl-substituted amino acid) to evaluate both the hydrophobicity and the molecular size available for receptor interaction. In the present study, we synthesized [Trp^7]-, [Trp^{11}]-, and [Trp^{15}]nociceptins together with [Trp^6]-, and [Trp^{14}]nociceptins (Fig. 1), in which Gly^6 and Leu^{14} with nonpolar side chains were also substituted by Trp, respectively. We here report the synthesis and the results of peptide synthesis, receptor binding assays, and the biological functional assay for these Trp-containing nociceptin analogues.

Experimentals

Peptide Synthesis. All the peptides, namely, nociceptin and its Trp-replaced analogues, were synthesized (0.1 mmol scale) by the automated peptide synthesizer ABI 430A (Applied Biosystems Inc., Foster City, CA) with the Fmoc synthetic strategy. Peptides were liberated from the resin by Reagent K[14] at room temperature for 3 h. After evaporation, the residue was solidified with diethyl ether. The purification was carried out first by gel filtration on a column (2.0×100 cm) of Sephadex G-15 (Pharmacia, Uppsala, Sweden) eluted with 10% acetic acid. For further purification, reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a preparative column (25×250 mm, Cica-Merck LiChrospher RP-18 (e), 5 μm) with a linear gradient of 0.1% trifluoroacetic acid and 80% acetonitrile and the fractions containing pure peptides were lyophilized to obtain the final peptide sample.

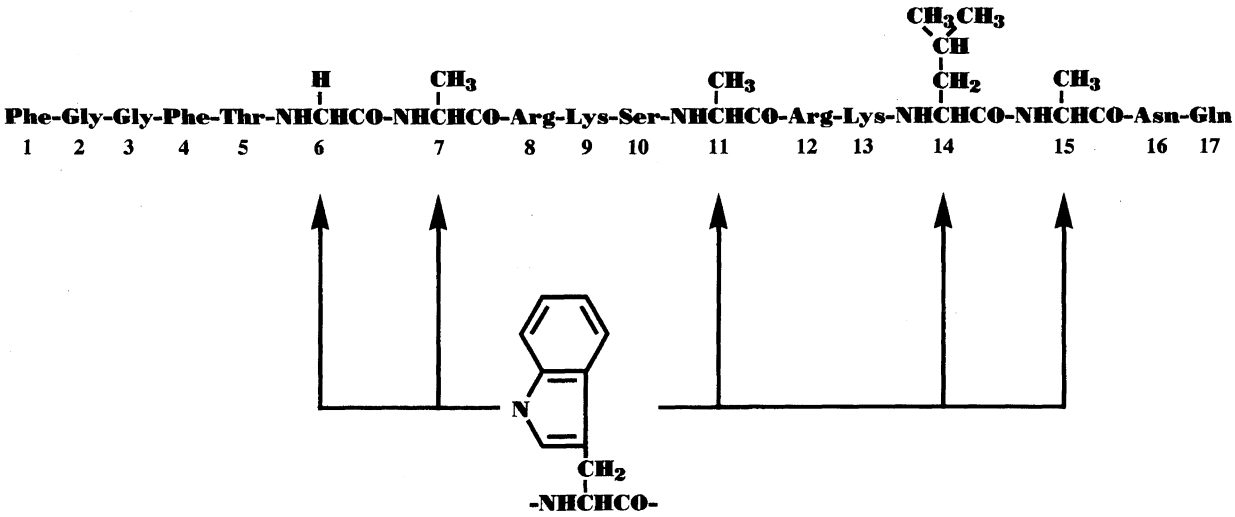

Fig. 1. The structure of nociceptin with amino acid residues that are replaced by Trp. Arrows indicate each residue to be replaced.

The purity was verified by analytical RP-HPLC (4×250 mm, Cica-Merck LiChrospher 100 RP-18, 5 μm). 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). 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. Amino acid sequences of nociceptin and its analogs were determined by Procise™ Model 491 Protein Sequencing System (PE Biosystems, Foster City, CA). The UV spectra were measured on a DU-62 Spectrophotometer (Beckman, Fullerton, CA).

Receptor Binding Assays. Membranes were prepared from the rat brain and human embryonic kidney 293 cells expressing human ORL1, μ, δ, and $\varkappa$ opioid receptors as described previously.[11] Peptides were evaluated using [^{3}H]nociceptin (158 Ci/mmol, Amersham, Buckinghamshire, UK) for ORL1 receptor assays. Binding assays were also carried out for opioid receptor subtypes using [^{3}H]DAGO (55 Ci/mmol, DuPont/NEN Research Products, Wilmington, DE, USA) for human μ receptors, [^{3}H]DPDPE (46 Ci/mmol, DuPont/NEN) for human δ receptors, and [^{3}H]Cl-977 (42 Ci/mmol, Amersham) for human $\varkappa$ receptors. Briefly, for the assay using [^{3}H]nociceptin in the rat brain membrane, 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 (1 M = 1 mol dm^{-3}) pH 7.55 (4 ml). Dose-response curves were analyzed by the computer program ALLFIT.[15]

[^{35}S]GTPγS Binding to Cell Membranes. The [^{35}S]GTPγS binding assay was performed according to the method of SPAG-protein coupled receptor assay provided by Amersham with slight modification. The membranes were suspended in ice-cold 20 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM EDTA. After addition of dithiothreitol (*threo*-1,4-dimercapto-2,3-butanediol; 0.17 mg ml^{-1}) to the solution, membranes were incubated at 25 °C for 30 min with the appropriate concentration of drugs in the presence of 5 μM GDP, 0.4 nM of [^{35}S]GTPγS, and wheat-germ agglutinin-coated SPA beads (1.5 mg) in a 0.2 ml total volume. Basal binding was assessed in the absence of agonist and nonspecific binding was determined with 10 μM GTPγS. The activity was estimated as EC_{50}, the value of which exhibits the concentration inducing 50% of its own maximal stimulation.

Results and Discussion

Peptide Synthesis. All six heptadecapeptides including native nociceptin were synthesized by the solid-phase methodology using Fmoc-amino acids. Nociceptin and its analogues prepared in this study all contain two arginine residues, the side chains of which were protected by the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) group.[16] Tryptophan was introduced as Fmoc-derivative with the indole side chain protected by the butoxycarbonyl group. The treatment of synthetic peptides with Reagent K[14] containing trifluoroacetic acid did not cause any troubles during purification, indicating that the Pmc groups were removed completely.

Peptides were obtained in an average yield of about 35%. They were all easily soluble in water and could be assayed without any trouble. Table 1 shows the analytical data of all six heptadecapeptides synthesized. The mass numbers measured were coincident with the values calculated. The purity of the peptides was verified by analytical HPLC, in which all the peptides emerged with a single peak. When their retention times were compared, it was found that the retention time of nociceptin (16.4 min) is much smaller than those (about 22—28 min) of Trp-replaced nociceptin analogs. This implies that the hydrophobicity of Trp-replaced analogs increased due to the incorporation of Trp. Amino acid analyses revealed a good coincidence of the number of amino acid constituents. This was also confirmed by the amino acid sequencing of the peptides, indicating that the synthetic peptides have correct amino acid sequences. Although Trp was not detected by the amino acid analyses after hydrolysis in constant-boiling hydrochloric acid, the amino acid sequencings clearly indicated the presence of the Trp residue at the right position. Except for nociceptin, all Trp-replaced analogs depicted a UV spectrum typical for the indolyl group at the range of 250—310 nm (ε_{max} = ca. 3,800 M^{-1} cm^{-1}

Table 1. The Analytical Data of Synthetic Peptides from the Mass Spectrometry, Reversed-Phase High Performance Liquid Chromatography, and Amino Acid Analsis

Peptides	MALDI-TOF-MASS[a]		RP-HPLC	Amino acid analysis[c]									
	Found	Calcd	RT[b] (min)	Asn	Gln	Ser	Gly	Arg	Thr	Ala	Leu	Phe	Lys
Nociceptin	1808.7	1810.1	16.4	0.79 (1)	1.23 (1)	1.07 (1)	3.30 (3)	2.29 (2)	1.10 (1)	3.00 (3)	0.98 (1)	2.19 (2)	2.21 (2)
[Trp^{6}]Nociceptin	1938.8	1939.2	28.3	0.76 (1)	1.22 (1)	1.07 (1)	2.19 (2)	2.29 (2)	0.92 (1)	3.00 (3)	0.76 (1)	1.88 (2)	1.71 (2)
[Trp^{7}]Nociceptin	1924.7	1925.2	26.9	0.78 (1)	1.24 (1)	0.94 (1)	3.15 (3)	1.98 (2)	1.02 (1)	2.00 (2)	0.71 (1)	1.75 (2)	1.75 (2)
[Trp^{11}]Nociceptin	1925.4	1925.2	25.2	0.85 (1)	1.09 (1)	1.28 (1)	3.27 (3)	1.90 (2)	1.01 (1)	2.00 (2)	0.75 (1)	2.14 (2)	1.77 (2)
[Trp^{14}]Nociceptin	1882.4	1883.1	21.9	0.74 (1)	0.97 (1)	1.00 (1)	3.07 (3)	1.93 (2)	1.02 (1)	3.00 (3)	-	1.90 (2)	2,01 (2)
[Trp^{15}]Nociceptin	1924.9	1925.2	25.9	0.86 (1)	0.88 (1)	0.88 (1)	2.88 (3)	2.38 (2)	0.98 (1)	2.00 (2)	0.71 (1)	1.72 (2)	1.77 (2)

a) Values express the mass number (m/z) of $(M+H)^+$. b) Retention time (RT) was measured on an analytical column (Cica-Merck LiChrospher 100 RP-18, 4×250 mm) with a linear gradient of 0.1% TFA and 80% acetonitrile. c) Amino acid analysis was performed by PTC method with Pico·Tag^{TM} column, and the values were normalized for alanine as an internal standard.

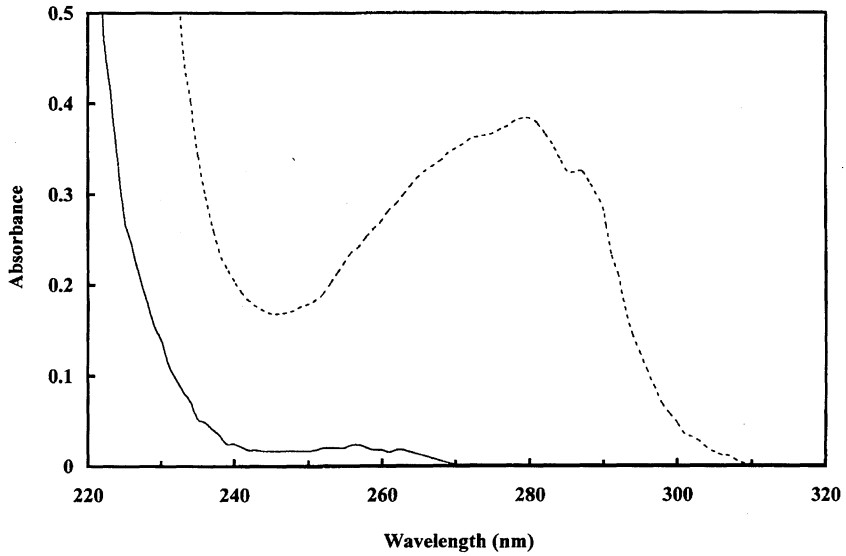

Fig. 2. The UV spectra of nociceptin (solid line) and [Trp^{14}]nociceptin (broken line).

at 280 nm; 1 M = 1 mol dm^{-3}). Figure 2 exhibits the UV spectra of [Trp^{14}]nociceptin and parent nociceptin without the Trp residue. Collectively, synthetic nociceptin and Trp-replaced nociceptin analogs have been identified physicochemically to reveal the right compounds.

Binding Affinity of Nociceptin Peptides for ORL1. Nociceptin exhibited a potent binding affinity to ORL1 receptors expressed in human 293 cells with the IC_{50} value of 0.93 nM, which is the half-maximal concentration for inhibition of the binding of radioactive-labeled ligand [^{3}H]nociceptin (Table 2). Nociceptin also bound strongly to ORL1 receptors in the rat brain membrane preparations (IC_{50} = 1.2 nM). When the Ala residues at positions 11 and 15 were replaced by Trp, respectively, the resulting analogues [Trp^{11}]- and [Trp^{15}]nociceptins exhibited 50—60% decreased affinities in both cells and rat brain as compared to parent nociceptin. [Trp^{7}]nociceptin was found to show much reduced receptor affinity and activity (5—15% of nociceptin). It is clear that the Ala residues in nociceptin are critical constituents for receptor binding. The Ala^{7}, Ala^{11}, and Ala^{15} residues appear to interact with the ORL1 receptor without spare space, suggesting that Trp at these positions causes a steric perturbation in the interaction with receptors.

Trp/Gly^{6}-replacement resulted in a drastic reduction in receptor binding. [Trp^{6}]nociceptin exhibited a considerably diminished receptor affinity (20-fold lower than nociceptin). Dooley et al.[12] have reported that [Ala^{6}]nociceptin also results in reduction in binding affinity (7-fold lower than nociceptin). The importance of Gly^{6} in nociceptin receptor binding and activation was demonstrated recently by using dynorphin A/nociceptin chimeric peptides.[9] When the C-terminal sequence of dynorphin A was substituted by that of nociceptin, a hybridpeptide with the sequence of dynorphin A(1-5)+nociceptin(6-17) attained an intense binding affin-

Table 2. Binding Affinities of Nociceptin and Its Analogues to ORL1 and Opioid Receptors

Peptides	IC_{50} (nM)				
	Rat brain	Human (293 Cells)			
	[^{3}H]Nociceptin	[^{3}H]Nociceptin	[^{3}H]DAMGO	[^{3}H]DPDPE	[^{3}H]Cl-977
Nociceptin	1.2±0.66	0.93±0.50	290± 50	> 10,000	3500± 140
[Trp^6]Nociceptin	18 ±2.5	16 ±3.2	530±150	>9,700	2800±1700
[Trp^7]Nociceptin	15 ±2.1	6.0±2.0	430±110	> 10,000	1800± 590
[Trp^{11}]Nociceptin	2.9±0.64	2.2±0.67	600±110	> 10,000	5500±2700
[Trp^{14}]Nociceptin	1.4±0.46	1.1±0.28	390± 76	> 10,000	3700±1100
[Trp^{15}]Nociceptin	2.4±0.65	2.1±0.64	450± 96	> 10,000	4800± 440

Each value represents mean ±S.D. of three independent experiments.

ity to the ORL1 receptor. A hybridpeptide of dynorphin A(1-6)+nociceptin(7-17) binds to the κ receptor better than ORL1, and thus the Gly residue at position 6 is a determinant of receptor preference of nociceptin for ORL1. Since side-chain-lacking glycine affords a considerable residual conformational freedom in the peptides, Gly^6 in nociceptin may play a role of conformational adjustment in binding to the receptor.

When Leu^{14} was replaced by Ala or Tyr, the binding properties of nociceptin were found to be retained for ORL1 receptor.[12,13] This is also the case for [Trp^{14}]-nociceptin, the receptor binding affinity profile being similar to those of nociceptin (Table 2). These results indicate that any hydrophobic amino acids can compensate for Leu at position 14 of nociceptin.

Biological Activity Shown by GTP$_\gamma$S Binding Affinity. The opioid receptors including ORL1 are known to be coupled with G-proteins. G-proteins are heterogeneous $\alpha\beta\gamma$ trimer with a GDP molecule bound to the α-subunit noncovalently. Stimulation of the G-protein-coupled receptor by a ligand results in a conformation change of the G-protein, that allows cytoplasmic GTP to displace GDP in the α-subunit and further facilitates a dissociation of GTP-bound α-subunit from $\beta\gamma$-subunits. Thus, the GDP/GTP exchange represents a receptor-mediated G protein activation of a receptor ligand. GTP$_\gamma$S is a GTP analogue resistant to the GTPase enzyme activity of G-protein, and dissociates very slowly. The amount of [^{35}S]GTP$_\gamma$S bound to G-protein thus represents the receptor activity of a ligand as demonstrated in many studies of signaling properties in G-protein coupled receptors.[17—20] In the present study, [^{35}S]GTP$_\gamma$S binding assay was carried out to evaluate the functional activation of the ORL1 receptor by nociceptin and its Trp-containing analogues.

It was found that the results from this GTP$_\gamma$S functional assay are consistent with those of binding assays for ORL1 (Tables 2 and 3 and Fig. 3). [Trp^{11}]- and [Trp^{15}]nociceptins were only moderately active (25—35% activity of nociceptin). [Trp^6]- and [Trp^7]nociceptins were very weak, showing 5—10% activity of nociceptin. On the other hand, [Trp^{14}]nociceptin exhibited a slightly different tendency in receptor affinity and activity. [Trp^{14}]nociceptin increased biological activity about 40% as compared to nociceptin, although it was almost as potent as nociceptin in receptor binding assays. This is the first nociceptin analogue possessing a biological activity greater than nociceptin, albeit very slight. It seems that Trp in the position 14 of nociceptin is responsible for causing a different efficacy between the receptor binding affinity and biological activity. The impor-

Table 3. Potency and Efficacy Values for Nociceptin and Its Analogues Using [^{35}S]GTP$_\gamma$S Binding in ORL1 Receptor Membrane Assay

Peptides	EC_{50} (nM)	Relative potency (%)
Nociceptin	17± 2.3	100
[Trp^6]Nociceptin	380±36	5
[Trp^7]Nociceptin	170±56	10
[Trp^{11}]Nociceptin	66±32	26
[Trp^{14}]Nociceptin	13± 1.4	130
[Trp^{15}]Nociceptin	50± 7.9	34

Data were subjected to nonlinear analysis with a computer program Graphpad Prism (Graphpad Software, San Diego, CA). Each value represents mean ±S.D. of three individual experiments.

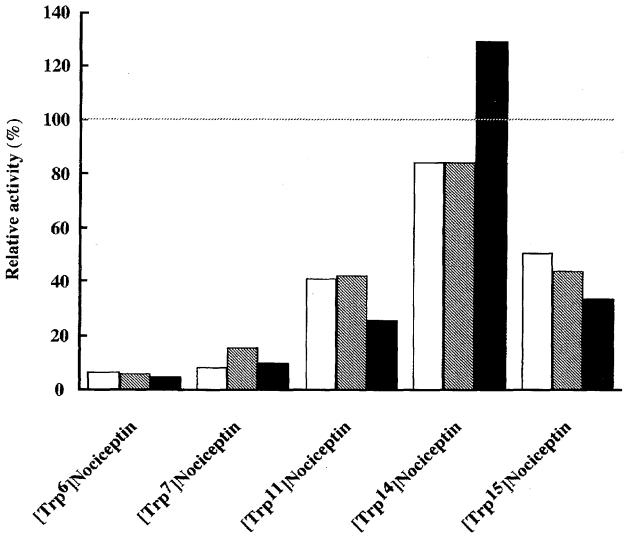

Fig. 3. Relative receptor binding affinity and activity of nociceptin analogues. White columns and slashed columns show relative receptor binding affinities of each nociceptin analogue against rat ORL1 and human ORL1 receptors, respectively. Black columns show relative biological activities of each nociceptin analogue. Each value of any classes of columns is shown as a ratio when nociceptin itself is fixed as 100%.

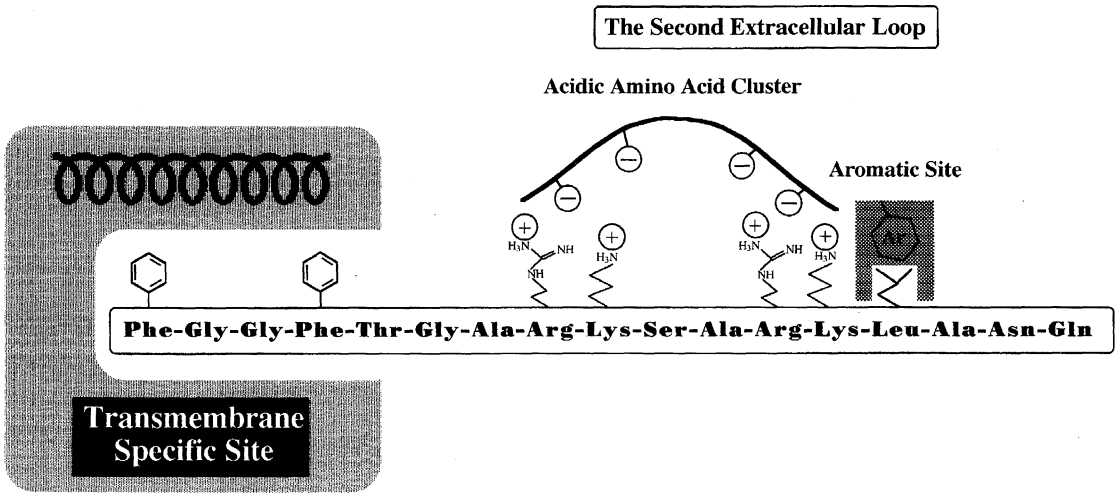

Fig. 4. The putative interaction mode between nociceptin and ORL1 nociceptin receptor. Transmembrane domain is constructed with α-helices and the helical coil in the figure represents the helix structure. The aromatic site in the second extracellular loop would be Tyr, Trp, or Phe, and in the figure the benzene ring represents the site.

tance of the residues at position 14 in receptor activation has been recently emphasized by Arg-substitution (unpublished data).

Binding Affinity of Nociceptin and its Analogues for Opioid Receptors. Nociceptin and Trp-containing analogues were also tested for their binding to μ, δ, and $\varkappa$ opioid receptors. All the peptides were almost completely inactive for δ receptors, and extremely weak for $\varkappa$ receptors with the IC_{50} values of three to four orders of magnitude larger than those in the ORL1 nociceptin receptor assays (Table 2). These results indicate that nociceptin does not bind intrinsically to δ and $\varkappa$ opioid receptors. For the μ opioid receptors, it has been reported that nociceptin binds weakly with the IC_{50} value of about 300 nM. This was confirmed also in the present study, and it was found that all Trp-containing nociceptin analogues bind to μ receptors (390—600 nM) as well as nociceptin. Their weak binding affinities to the μ receptors (30—300-fold weaker than to ORL1), however, definitely differ from their binding spectra against ORL1 receptors. There is no apparent structure-activity relationships of nociceptin and its Trp-containing analogues for the μ receptors.

Structure-Activity Relationships of Nociceptin. Tryptophan is large enough to induce a steric hindrance, while its aromatic and hydrophobic characteristics often impel the peptide to reinforce the interaction with the receptors. The present data obtained by Trp-replacement clearly indicated that nociceptin is a ligand specific to the ORL1 receptor that is distinct from other opioid receptors. The Ala residues in nociceptin appear to possess distinct roles in receptor binding and activation, and thus they are indispensable for the activities. This is also evident for Gly at position 6. In contrast, Leu^{14} in nociceptin compensate for substitutions with other hydrophobic amino acids. Trp/Leu^{14}-replacement, namely the 3-indolyl/isopropyl substitution, found to affect the ligand-receptor interaction.

The N-terminal tetrapeptide Phe–Gly–Gly–Phe of nociceptin appears to bind in a hydrophobic region constructed with transmembrane domains of the ORL1 receptor.[21,22] Meanwhile, a suggestion that nociceptin interacts with the second extracellular loop of the ORL1 receptor has recently been postulated by the study using chimera and mutated ORL1 receptors.[22] Nociceptin-Leu^{14} is adjacent to basic amino acids Arg–Lys at positions 12-13, which are believed to interact with a cluster of acidic amino acids in the second extracellular loop of the ORL1 receptor. It seems that Leu or Trp at position 14 of nociceptin interacts with the aromatic amino acids such as Tyr, Trp, or Phe, present at the C-terminal side of this extracellular loop. In the case, [Trp^{14}]nociceptin may play a important role in receptor activation. Figure 4 shows a putative binding model of nociceptin and ORL1 receptor, depicting the interacting structural elements. To evaluate these assumptions, further structure-activity studies are in progress in our laboratory.

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3 The abbreviations according to biochemical nomenclature by IUPAC-IUB Joint Commission, *Eur. J. Biochem.*, **138**, 9—37 (1984), are used through out. Unless otherwise specified, the amino acids are L-stereoisomers. Additional abbreviations are as follows: Cl-977, *N*-methyl-*N*-[(5*R*)-(5α,7α,8β)-7-(1-pyrrolindinyl)-1-oxaspiro[4.5] dec-8-yl]-4-benzofuranacetamide hydrochloride; DAGO, [D-Ala^2,*N*-Me-Phe^4,$Gly\text{-}ol^5$]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; Fmoc, (9-fluorenyl)methoxycarbonyl; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; ORL1, opioid receptor-like 1; RP-HPLC, reversed-phase high-performance liquid chromatography.

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