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Structural Requirements of Nociceptin Antagonist Ac-RYYRIK-NH_2 for Receptor Binding

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Abstract: Ac-RYYRIK-NH_2 is a peptide isolated from the peptide library as an antagonist that inhibits the biological activities of nociceptin, a hyperalgesic neuropeptide. In order to clarify the structural requirements of this peptide for binding to the nociceptin receptor ORL1, systematic structure–activity studies were carried out. The result of Ala-scanning indicated that the *N*-terminal tripeptide RYY(= Arg-Tyr-Tyr) is crucially important for binding to the ORL1 receptor. Residual truncations from the *N*- or *C*-terminus revealed the special importance of the *N*-terminal Arg residue. The removal of protecting groups indicated that the *N*-terminal acetyl group is essential, but the *C*-terminal amide group is insignificant. These results indicated the conspicuous importance of acetyl-Arg at position 1 of Ac-RYYRIK-NH_2 as a key structure allowing binding to the receptor. To investigate the binding site of this peptide in the ORL1 receptor, we synthesized and assayed a series of analogues of the nociceptin dibasic repeat region, residues 8–13 of RKSARK. None of the derivatives were active. Ac-RYYRIK-NH_2 was inactive for the μ opioid receptor to which nociceptin binds with considerable strength. All the results suggested that the mode of binding between Ac-RYYRIK-NH_2 and the ORL1 receptor is different to that between the ORL1 receptor and nociceptin, and that it may consist of interaction with the receptor site to which nociceptin(1–7) or -(14–17) binds. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: nociceptin; antagonist; Ala-scanning; residual truncation; opioid receptor

INTRODUCTION

Nociceptin [1], also known as orphanin FQ [2], is a neuropeptide consisting of 17 amino acids with the sequence FGGFTGARKSARKLANQ. Nociceptin is an endogenous ligand of the G protein-coupled seven transmembrane ORL1 receptor, the structure of which is highly similar to those of opioid receptors. Nociceptin induces hyperalgesia, and the nociceptin/ORL1 ligand–receptor system is also involved in many other physiological functions, such as analgesia in the spinal cord and antiopioid effects in the brain [1–5]. The actions of nociceptin in the central nervous system also include the inhibition of locomotor activity and impairment of spatial learning [6–8]. To better understand these different types of biological function, it is imperative that we obtain a selective and specific nociceptin receptor antagonist. In addition, such an antagonist would be an effective molecular tool for investigating the structure of the ORL1 receptor. Because of the intrinsic hyperalgesic activity of

Abbreviations: Ac-RYYRIK-NH_2, Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2; DTT, dithiothreitol; ORL1 receptor, opioid receptor-like 1 receptor; RP-HPLC, reversed-phase high performance liquid chromatography.

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nociceptin, its antagonists are expected to be highly effective analgesics.

We have recently reported [Arg-Lys$^{14-15}$]nociceptin as a so-called superagonist that was highly potent in a receptor binding assay and especially in a functional biological assay [9]. The superagonist often elicits the receptor responses such as desensitization and internalization, providing a kind of antagonistic cellular reply [10]. However, it is difficult to utilize such a substance to replace the antagonist, because of its strong intrinsic agonist activity retained at the initial stage.

Several different types of compounds have recently been determined to be antagonists of nociceptin. Non-peptide antagonists include, for example, *N*-(4-amino-2-methylquinolin-6-yl)-2-(4-methylphenoxymethyl) benzamide monohydrochloride (JTC-801) [11,12] and 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (J-113397) [13–15]. These compounds are full antagonists that reverse the activities of nociceptin. As to compounds based on the structure of nociceptin, [Phe$^1\Psi$(CH_2-NH)Gly^2]nociceptin(1–13)-NH_2 [16] and [Nphe^1]nociceptin(1–13)-NH_2 [17] have been reported to function as antagonists in the peripheral nervous system [18–22]. However, other groups have also reported that these peptides act as partial or even full agonists of nociceptin at the central nervous site [23–26].

Another type of compound is the antagonist screened and selected from the peptide library. Acetyl hexapeptide amide Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Ac-RYYRIK-NH_2) has been reported as an effective nociceptin antagonist inhibiting cAMP accumulation and [^{35}S]GTPγS binding activity [27–29]. It should be noted that this peptide has no sequence similarity to nociceptin, except for the presence of three basic amino acids (Figure 1).

Along with recent developments in combinatorial chemistry, various chemical libraries have afforded effective receptor ligands of agonists and antagonists. However, there have been very few studies investigating the reason why these selected ligands show highly potent binding ability to the receptor. In order to explore the molecular mechanism of receptor activation, it is crucial that we answer this question. An intrinsic requisite for receptor antagonists is a specific binding to the target receptor to inhibit the binding of agonists. In the present study, to clarify the structural essentials of the nociceptin antagonist Ac-RYYRIK-NH_2 and particularly those

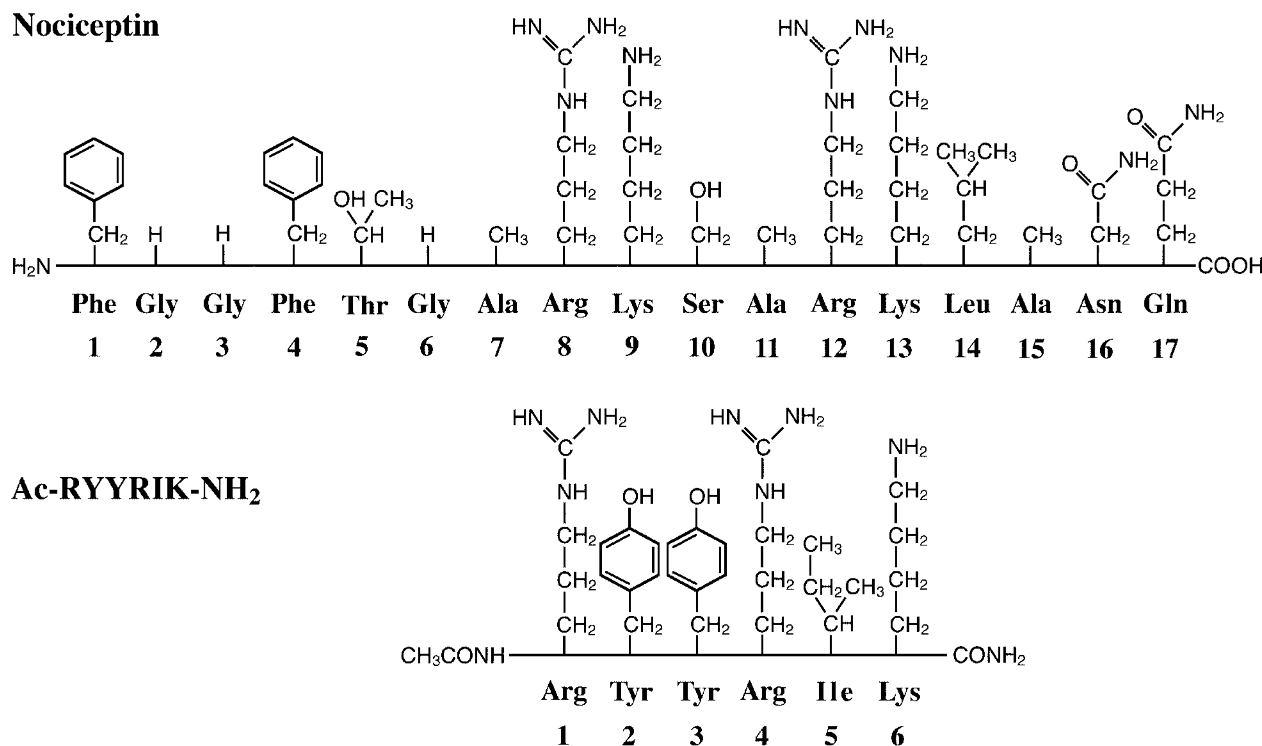

Figure 1 Chemical structure and amino acid sequence of nociceptin and its antagonist Ac-RYYRIK-NH_2. The straight-line indicates the peptide bond main chain.

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involved in the binding to the ORL1 receptor, we carried out structure–activity studies on this antagonist peptide.

MATERIALS AND METHODS

Peptide Syntheses

Ala-substituted derivatives of Ac-RYYRIK-NH_2 were synthesized (0.1 mmol scale) by the manual solid phase method using Boc-amino acids and MBHA-resin. A series of shortened peptides with progressive deletions of amino acid residues from either the *N*-terminus or *C*-terminus was synthesized by the same method. A des-acetyl analogue of Ac-RYYRIK-NH_2 was obtained together with the parent peptide without acetylation, whereas a des-amide analogue was prepared by using ordinary PAM resin. The coupling reaction was carried out with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of 1-hydroxybenzotriazole (HOBt). The peptides synthesized were liberated from the resin by treatment with anhydrous HF containing 10% *p*-cresol. Purification was carried out by gel filtration on a column (2.0 × 100 cm) of Sephadex G-15 (Pharmacia Biotech, 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.

The purity was verified by analytical RP-HPLC (4 × 250 mm, Cica-Merck LiChrospher 100 RP-18, 5 μm). The mass spectra of peptides were measured on a mass spectrometer Voyager™ DE-PRO (PerSeptive Biosystems Inc., Framingham, MA) using the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) method.

Cell Culture and Transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Rat ORL1 plasmid DNA (30 μg) was transiently transfected into confluent COS-7 cells (0.5×10^5 per cm^2) in a 150 cm^2 culture plate according to the DEAE-dextran method as reported previously [30]. After 48 h, the cells were harvested and centrifuged for 10 min at $500 \times g$ (4 °C). Cells were resuspended in the buffer containing 5 mM Tris, 1 mM EGTA, 1 mM dithiothreitol (DTT) and 11% saccharose (pH 7.4), and homogenized with a Potter-Elvehjem homogenizer (50 strokes). The homogenate was centrifuged for 10 min at $1000 \times g$ (4 °C). The supernatant was centrifuged again for 20 min at $24\,000 \times g$ (4 °C), and the pellet was washed with the buffer containing 5 mM Tris, 1 mM EGTA and 1 mM DTT (pH 7.4). The concentration of membrane protein was estimated by the BCA protein assay method using bicinchoninic acid (Pierce, Rockford, IL). The prepared membrane was frozen at −70 °C before use.

Binding Assay for ORL1 Receptor

The receptor binding affinity of synthetic peptides was assessed by a radio-ligand receptor binding assay using COS-7 cell membrane expressing rat ORL1 receptor. Assay tubes each containing 5 μg/ml membrane protein, a series of concentrations of synthetic peptide and 0.05 nM [^{3}H]nociceptin (158 Ci/mmol; Amersham) were incubated for 90 min at 25 °C in 50 mM HEPES–Tris buffer (pH 7.4) containing 0.1% bovine serum albumin. Bacitracin (100 μg/ml) was added as an enzyme inhibitor. After incubation, the mixture was filtered through a glass fibre filter (GF/B; Whatman, Clifton, NJ). To coat the filter surface, filters were soaked in 0.1% ethyleneimine polymer aqueous solution for 30 min and rinsed twice with 50 mM Tris buffer (pH 7.4). Filters were placed in scintillation vials containing 4 ml scintillation cocktail (Scintisol EX-H; Dojindo, Kumamoto, Japan) for scintillation counting. The computer program ALLFIT [31] was used to draw dose-response curves for the analysis. The binding affinity of each peptide was estimated as the IC_{50} value — that is, the drug concentration at which the half-maximal inhibition is achieved.

Receptor Binding Assay for μ Opioid Receptor

Membranes were prepared from rat brains purchased from Rockland (Gilbertsville, PA). Peptides were evaluated using [^{3}H]DAGO (55.3 Ci/mmol; DuPont/NEN Research Products, Wilmington, DE) for μ receptors [32]. Tubes containing the membrane preparations, synthetic peptides and 0.25 nM of the respective radio-ligand were incubated at room temperature for 60 min in 100 mM Tris-HCl buffer (pH 7.55) containing 0.1% BSA. As an enzyme

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inhibitor, bacitracin (100 μg/ml) was used. After incubation for 60 min, solutions were filtered by glass fibre filters (GF/B; Whatman) and washed twice with 100 mM Tris-HCl buffer (pH 7.55, 4 ml). Dose-response curves were analysed by the computer program ALLFIT.

RESULTS AND DISCUSSION

Residue Importance Assessed by Ala-scanning

The importance of each amino acid residue in Ac-RYYRIK-NH_2 was assessed first by the method of so-called Ala-scanning. Six different Ala-substituted analogues were synthesized using the manual solid phase method (Table 1). The receptor binding affinity of these analogues was evaluated in the membrane preparations of COS-7 cells expressing rat nociceptin ORL1 receptors. In this assay system, Ac-RYYRIK-NH_2 was found to bind to the ORL1 receptor with considerable strength (IC_{50} = 1.54 nM) (Table 2). When Ala-substituted analogues were assayed, it was found that all of them exhibited much weaker binding affinity than the parent peptide Ac-RYYRIK-NH_2. Ac-**A**YYRIK-NH_2, Ac-R**A**YRIK-NH_2 and Ac-RY**A**RIK-NH_2 were almost inactive, showing drastically decreased binding affinity (700–5400-fold weaker than that of the parent Ac-RYYRIK-NH_2) (Table 2). Ac-RYY**A**IK-NH_2, Ac-RYYR**A**K-NH_2 and Ac-RYYRI**A**-NH_2 also showed a decreased binding affinity (11–22-fold weaker than the parent). These results clearly indicate that all the amino acid residues in this hexapeptide are required for binding to the ORL1 receptor, although their relative degrees of importance vary.

Ac-RYYRIK-NH_2 possesses two Arg (= R) and two Tyr (= Y). When Arg at position 1 was substituted with Ala, this Arg → Ala replacement decreased the receptor binding affinity of Ac-RYYRIK-NH_2 about 5400 times (Figure 2A). In contrast, the same Arg → Ala substitution at position 4 decreased the affinity about 20 times (Figure 2A). It would thus appear that Arg^1 is much more important than Arg^4 in promoting the receptor binding affinity of Ac-RYYRIK-NH_2. Tyr → Ala substitutions at both positions 2 and 3 resulted in drastic activity reduction (about 1500- and 700-fold, respectively) (Figure 2B). These

Table 1 Synthetic Yields and Analytical Data of Synthetic Nociceptin and Antagonist Peptide Ac-RYYRIK-NH_2 and its Analogues

Peptide	Yield (%)	RT-HPLC RT (min)	MALDI-TOF-MASS	
			Found (m/z)	Calcd ($m+H^+$)
Nociceptin	68	30.05	1809.38	1809.99
Ac-RYYRIK-NH_2	29	32.30	939.91	939.55
Ac-**A**YYRIK-NH_2	25	29.91	855.15	854.49
Ac-R**A**YRIK-NH_2	34	24.20	848.23	847.53
Ac-RY**A**RIK-NH_2	28	23.89	848.25	847.53
Ac-RYY**A**IK-NH_2	36	28.75	855.18	854.49
Ac-RYYR**A**K-NH_2	26	25.65	898.15	897.51
Ac-RYYRI**A**-NH_2	25	35.21	883.02	882.49
Ac-RYYRI-NH_2	39	30.08	812.12	811.36
Ac-RYYR-NH_2	29	23.93	699.09	698.37
Ac-RYY-NH_2	29	23.80	542.91	542.27
Ac-YYRIK-NH_2	29	29.58	784.02	783.45
Ac-YRIK-NH_2	32	24.11	620.81	620.39
Ac-RIK-NH_2	49	13.52	457.90	457.33
Ac-RYYRIK-**OH**	25	29.82	941.10	940.54
H-RYYRIK-NH_2	33	25.96	898.25	897.54
H-RYYRIK-**OH**	23	24.42	899.28	898.53
Ac-RKSARKLA-NH_2	53	24.52	971.31	970.63
Ac-RKSARKRK-NH_2	74	19.16	1070.93	1070.70
Ac-RKYYRKLA-NH_2	59	35.10	1139.36	1138.68

No data are available for H-RKSARK-OH because of the shortage of the sample.

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Table 2 Binding Affinity of Synthetic Peptides to ORL1 Receptor

Peptide	Binding affinity IC_{50} (nM)	Relative affinity (%)
Nociceptin	0.83 ± 0.64	
Ac-RYYRIK-NH_2	1.54 ± 0.54	100
Ac-**A**YYRIK-NH_2	8350 ± 4029	0.018
Ac-R**A**YRIK- NH_2	2280 ± 211	0.067
Ac-RY**A**RIK-NH_2	1080 ± 89	0.14
Ac-RYY**A**IK-NH_2	33.2 ± 6.6	4.6
Ac-RYYR**A**K-NH_2	17.4 ± 0.06	8.9
Ac-RYYRI**A**-NH_2	16.5 ± 5.6	9.3
Ac-RYYRI-NH_2	4.82 ± 0.10	32
Ac-RYYR-NH_2	2420 ± 225	0.063
Ac-RYY-NH_2	N.D.	0
Ac-YYRIK-NH_2	N.D.	0
Ac-YRIK-NH_2	N.D.	0
Ac-RIK-NH_2	N.D.	0
Ac-RYYRIK-**OH**	1.76 ± 1.17	88
H-RYYRIK-NH_2	90.0 ± 30.1	1.7
H-RYYRIK-**OH**	210 ± 95	0.73
H-RKSARK-OH	N.D.	0
Ac-RKSARKLA-NH_2	N.D.	0
Ac-RKSARKRK-NH_2	N.D.	0
Ac-RKYYRKLA-NH_2	N.D.	0

N.D. = not determined because of inactivity.

results indicate that the *N*-terminal tripeptide Arg-Tyr-Tyr is crucial for binding to the ORL1 receptor. This was confirmed also during the selection of the peptide by the positional scanning synthetic combinatorial libraries [27]. Some of amino acid residues of Ac-RYYRIK-NH2 can be replaced by similar types of natural amino acids as evidenced during the peptide selection [27]. The present results from the Ala-scanning substantiate the importance of even these amino acids in their interaction with the ORL1 receptor.

Assessment by *N*-terminal and *C*-terminal Truncations

Additional evidence for the importance of the *N*-terminal residues was provided by the finding that the binding activity was completely abolished in *N*-terminal truncated peptides. We first assayed *N*-terminal shortened peptides of Ac-YYRIK-NH_2 (corresponding to the des-Arg[1] derivative), Ac-YRIK-NH_2 (des-Arg-Tyr$^{1-2}$ derivative) and Ac-RIK-NH_2 (des-Arg-Tyr-Tyr$^{1-3}$ derivative). These analogues showed no binding ability to ORL1 receptor (Table 2). Even at concentrations as high as 1 mM, no apparent displacement of the receptor tracer [^{3}H]nociceptin was observed. The results indicated that the Arg residue at position 1 is crucially important for the peptide to interact with the receptor.

We also examined the effect of deleting the *C*-terminal portion of the amino acid residues on the receptor binding affinity. When *C*-terminal truncated peptides (Ac-RYYRI-NH_2, Ac-RYYR-NH_2 and Ac-RYY-NH_2) were assayed, they decreased the binding affinity progressively. Ac-RYYRI-NH_2, in which the *C*-terminal Lys^6 was deleted, showed considerably weaker (about 32% weaker) binding to ORL1 receptor than did the parent, Ac-RYYRIK-NH_2 (Table 2). However, it should be noted that this *C*-terminal truncated Ac-RYYRI-NH_2 still showed fairly strong binding to ORL1 receptor, with an IC_{50} value of 4.82 nM. Further deletions of residues made the peptides extremely weak: Ac-RYYR-NH_2 (des-Ile-Lys$^{5-6}$ derivative) was about 500 times less potent than the des-Lys^6 derivative of Ac-RYYRI-NH_2, and Ac-RYY-NH_2 (des-Arg-Ile-Lys$^{4-6}$ derivative) was completely inactive. Thus, the pentapeptide Ac-RYYRI-NH_2 appears to reside in a core structure that is essential for receptor binding.

In addition to five peptide bonds, Ac-RYYRIK-NH_2 consists of two additional amide bonds at

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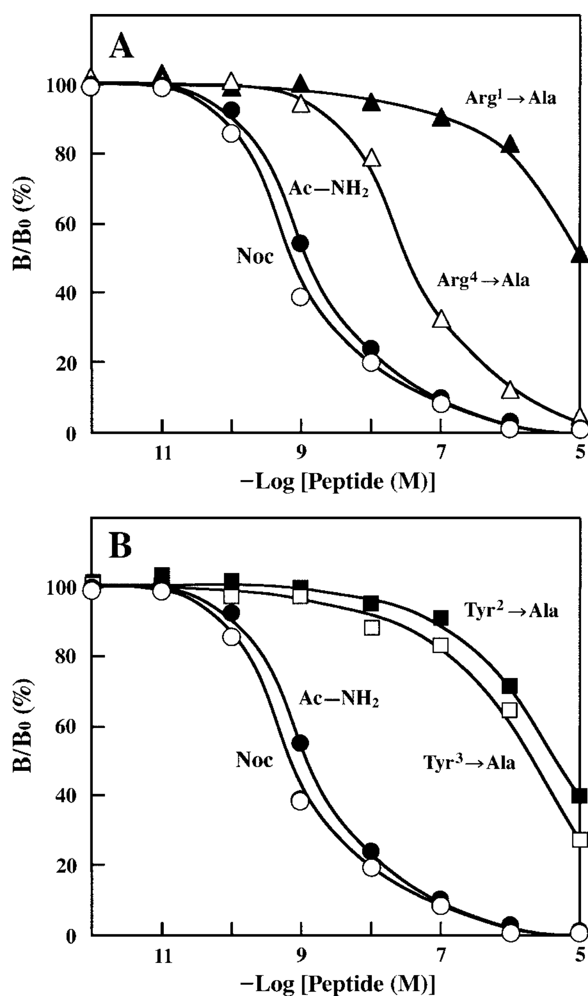

Figure 2 Dose-response curves of Ac-RYYRIK-NH_2 (Ac-NH_2) and Ala-substituted analogues together with nociceptin in the binding assay for the ORL1 nociceptin receptors. (A) Ala-scanning for the Arg residues: Arg→Ala replacements at positions 1 and 4; and (B) Ala-scanning for the Tyr residues: Tyr→Ala replacements at positions 2 and 3.

its *N*- and *C*-termini, namely, those in the *N*-terminal acetyl group and the *C*-terminal carboxyl amide group. We prepared H-RYYRIK-NH_2, Ac-RYYRIK-OH and H-RYYRIK-OH in order to investigate the importance of these amide groups. The receptor binding assay gave very clear results. As shown in Table 2, two analogues lacking the acetyl group, H-RYYRIK-NH_2 and H-RYYRIK-OH, showed drastically reduced binding affinity (about 60-fold and 140-fold weak compared with Ac-RYYRIK-NH_2, respectively). In contrast, Ac-RYYRIK-OH lacking the *C*-terminal amide group was found to be almost equipotent to the parent Ac-RYYRIK-NH_2 (IC_{50} = 1.76 nM). It is clear that the *N*-terminal acetyl group of Ac-RYYRIK-NH_2 is one of the essential structural elements allowing the peptide to bind to ORL1.

Nociceptin is Dissimilar to Ac-RYYRIK-NH_2

A basic region of nociceptin 8–13, namely, Arg-Lys-Ser-Ala-Arg-Lys (= RKSARK) (Figure 1), has been reported to interact with the acidic cluster in the second extracellular loop of the ORL1 receptor [33,34]. Ac-RYYRIK-NH_2 consists of three basic amino acid residues. These led to the hypothesis that Ac-RYYRIK-NH_2 may bind to the same site of ORL1 receptor as do nociceptin Arg-Lys^{8-9} and Arg-Lys^{12-13} to ORL1 receptor [35]. In order to evaluate this hypothesis, we first synthesized the fragment of nociceptin, H-RKSARK-OH, as a reference. However, this *C*,*N*-terminal free peptide was completely inactive (Table 2). We then designed and synthesized a series of analogue peptides that included Ac-RKSARKLA-NH_2 and Ac-RKSARKRK-NH_2. Ac-RKSARK-NH_2 was not prepared, since it was apparent that this hexapeptide was not selected from the peptide library as a ligand with the ability to bind to ORL1 receptor. However, all of these peptides were inactive and unable to bind to ORL1 (Table 2). Ac-RKYYRKLA-NH_2 was also completely inactive. Although it remains unclear whether or not basic amino acid residues (Arg^1, Arg^4 and Lys^6) share the binding site with nociceptin Arg-Lys^{8-9} and Arg-Lys^{12-13}, the results strongly suggested that the interaction mode of the antagonist Ac-RYYRIK-NH_2 is different to that of the nociceptin basic region.

Nociceptin binds to the μ opioid receptor fairly strongly [36]. Although nociceptin is structurally similar to dynorphin A, an endogenous peptide ligand for κ opioid receptor, nociceptin is almost completely inactive in the κ receptor [36]. If the binding ability of nociceptin is unique to this 17-amino acid peptide, it is likely that the μ opioid receptor distinguishes Ac-RYYRIK-NH_2. Thus, we assayed Ac-RYYRIK-NH_2 in the μ opioid receptor. It was found that Ac-RYYRIK-NH_2 is almost completely inactive (Figure 3). This result indicated that the structural characteristics of Ac-RYYRIK-NH_2 are dissimilar to those of nociceptin.

The competition between the ligand and agonist for binding to a particular receptor site is a basal condition of the antagonist. Indeed, Ac-RYYRIK-NH_2 displaces [^{3}H]nociceptin in the receptor binding assay. This means that Ac-RYYRIK-NH_2 and nociceptin share the site, at least in part.

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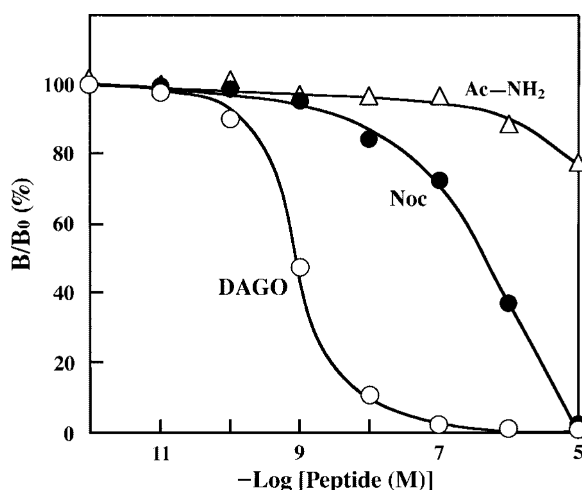

Figure 3 Dose-response curves of Ac-RYYRIK-NH_2 (Ac-NH_2) and nociceptin in the binding assay for the μ opioid receptors. DAGO is an enkephalin analogue, [D-Ala^2,NMePhe^4,Glyol^5]enkephalin, specific for the μ receptors.

The present data strongly suggest that Ac-RYYRIK-NH_2 interacts with the site in ORL1 receptor to which nociceptin(1–7) or -(14–17) binds. It is particularly important to identify the receptor site to which the antagonist Ac-Arg^1 binds. In order to determine the receptor residues complementary to this Arg^1 residue and to explore a rational method for designing an effective antagonist molecule, further structure–activity studies on the ORL1 receptor are in progress in our laboratory.

CONCLUSION

A series of structure–activity evaluations was carried out for the nociceptin antagonist Ac-RYYRIK-NH_2, which was selected from a peptide library. Analysis by Ala-scanning revealed an essential structural requirement of Arg at position 1 for binding to ORL1 receptor. Residual truncation examination revealed that the *N*-terminal pentapeptide was a core structure essential for interaction with ORL1 receptor. The intrinsic importance of the *N*-terminal acetyl group, but not the *C*-terminal amide group, was also clarified by the removal of these protecting groups. All these results indicated that the library-selected peptide Ac-RYYRIK-NH_2 possesses a structural element, acetyl-arginine, that is key for eliciting a molecular contact or interaction with a receptor specific for nociceptin. Since nociceptin consists of a basic region (residues 8–13), several derivatives of this basic fragment were synthesized and assayed for the ORL1 receptor. These derivatives could not bind to the ORL1 receptor, suggesting that the binding mode of Ac-RYYRIK-NH_2 is different to that of native nociceptin. This was also suggested by the results obtained from the binding assay for the μ opioid receptor, in which Ac-RYYRIK-NH_2 was almost completely inactive in spite of fairly high activity by nociceptin. The results indicated that nociceptin and Ac-RYYRIK-NH_2 do not share the binding site in the second extracellular loop of the ORL1 receptor. It is thus very likely that Ac-RYYRIK-NH_2 interacts with the site in the ORL1 receptor to which nociceptin(1–7) or -(14–17) binds.

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