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Capturing of the free cysteine residue in the ligand-binding site by affinity labeling of the ORL1 nociceptin receptor

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ABSTRACT

All of the δ, μ, and κ opioid receptors have a free thiol group of the Cys residue in the ligand-binding site, although its functional role is not yet known. In order to examine whether or not a similar Cys is also present in the ORL1 nociceptin receptor, we attempted to identify it by affinity labeling using a specific antagonist peptide. We first treated ORL1-expressing COS-7 cell membrane preparations with the thiol-alkylation reagent *N*-ethylmaleimide (NEM) to perform a binding assay using [^{3}H]nociceptin as a tracer and nociceptin, an ORL1 agonist, or Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2, a nociceptin/ORL1 antagonist, as a competitor. It was suggested that ORL1 has a free Cys in its ligand-binding site, since the NEM treatment reduced the population of ligand-binding sites. This was further confirmed by affinity labeling using Cys(Npys)-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 with the SNpys group that can react with a free thiol group, resulting in the formation of a disulfide bond. This affinity labeling was approximately 23 times more specific than NEM alkylation. The results revealed that the ORL1 nociceptin receptor does contain a free Cys residue in the ligand-binding site.

1. Introduction

The 17-mer neuropeptide nociceptin,[1] also known as orphanin FQ,[2] is an endogenous ligand of the ORL1 (opioid receptor-like 1) receptor. 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 anti-opioid 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] The structure of the ORL1 receptor is very similar to those of the δ, μ, and κ opioid receptors[9]—all of which belong to the G protein-coupled receptor (GPCR) superfamily with seven-transmembrane domains (TM1-7)—and couples specifically with G_i or G_o protein.

Several characteristic amino acid residues are conserved among these receptors, and such residues are highly likely to be essential with regard to their unique structural construction, ligand binding, and perhaps activation. In particular, the cysteine (Cys) residues within GPCR have been identified as important for inducing and maintaining the three-dimensional receptor conformation. In the ORL1 receptor, there are 14 Cys residues; that is, 7 in the transmembrane domains (TM), 2 in the extracellular loops (ECLs), 1 in the intracellular loop (ICL), and 4 in the C-terminal tail.[10] In general, the Cys residues located in the first and second ECLs, which are preserved among almost all the GPCRs, have been suggested to participate in disulfide bonding,[11–15] although their functional role in construction of a bioactive conformation remains unclear. In ORL1, Cys123 and Cys200 likely form such a disulfide bond. One of the four Cys residues in the C-terminal tail, Cys334, is a parmitoylation site.[16] These three Cys residues are conserved also among opioid δ, μ, and κ receptors.[17] Although the roles of other Cys residues not have been solved yet, these are highly likely to be involved in the construction of a robust receptor conformation.

We have demonstrated that free thiol group(s) exists at or near the ligand-binding sites of opioid receptors. Using SNpys-containing Leu-enkephalin and dynorphin A analogs, the μ, δ receptors in rat brain and the κ receptor in guinea pig brain were found to be affinity labeled discriminatively.[18,19] The in vitro affinity labeling of a single class of opioid receptors was also achieved for the receptors expressed in the COS-7 cells.[20] In these affinity-labeling experiments,

Abbreviations: Ac-RYYRIK-NH_2, the one-letter amino acid code denotes Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; EC_{50}, the concentration for the half maximal effective concentration; ECL, extracellular loops; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HBTU, 2-(1*H*-benzotriazole)-1-1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; IC_{50}, the concentration for the half maximal inhibition; ICL, intracellular loop; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NEM, *N*-ethylmaleimide; NMP, *N*-methylpyrrolidone; Npys, 3-nitro-2-pyridinesulfenyl; ORL1, opioid receptor-like 1; SAL resin, super acid-labile resin; TFA, trifluoroacetic acid; TM, transmembrane domain.

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the Cys(Npys)-containing peptide first binds to the ligand-binding site of receptors, and then if there is a receptor thiol group near the bound peptide, the SNpys group in the peptide reacts with this free thiol group, resulting in the formation of a disulfide bond. The 3-nitro-2-pyridinesulfenyl group (Npys group) was originally developed as an amino-protecting group for peptide synthesis.[21] The SNpys group, in which the Npys is bound to a thiol group, becomes a highly activated electrophilic reagent, and reacts only with a free thiol group via the thiol–disulfide exchange reaction. The reactivity of the SNpys group is due to the asymmetrical mixed disulfide bond, in which the 3-nitro-2-pyridine moiety never binds to the thiol group, leaving the remaining moiety to bind to the thiol group to form a novel disulfide bond (Fig. 1). These characteristics allow specific affinity labeling of the receptor with a ligand attached to the SNpys group.[22–25]

In the present study, we intended to examine whether or not the ORL1 receptor contains a free thiol group, namely, a free Cys residue, at the ligand-binding site. To this end, we selected an effective nociceptin antagonist Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Ac-RYYRIK-NH_2) as a parent peptide for introduction of Cys(Npys). This acetyl hexapeptide amide antagonist compound was selected from the peptide libraries.[26] We replaced the N-terminal acetyl group with Cys(Npys), and found that the resulting Cys(Npys)-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 did indeed affinity label the ORL1 receptor expressed in COS-7 cells. We here describe the synthesis of this Cys(Npys)-containing peptide and the results of the affinity-labeling experiments.

2. Results and discussion

2.1. Synthesis of the Cys(Npys)-containing peptide

For the synthesis of Cys(Npys)-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Cys(Npys)-RYYRIK-NH_2), we applied the manual solid phase procedure using Fmoc-chemistry. Using the super acid-labile resin (SAL resin), namely, Fmoc-NH-SAL resin, the coupling reaction was carried out with 2-(1*H*-benzotriazole)-1-1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of 1-hydroxybenzotriazole (HOBt). By means of this HOBU--HOBt method, the synthesis of Fmoc-Arg(Pbf)-Tyr(tBu)-Tyr(tBu)-Arg(Pbf)-Ile-Lys(Boc)-NH-SAL resin was first completed. After deprotection of the Fmoc group, Boc-Cys(Npys)-OH was coupled by the carbodiimide method, but with no HOBt. It has been reported that the conjugate formed by attachment of an Npys group to a thiol group is unstable against HOBt.[27,28] The final objective peptide was eventually obtained in a high yield (22%) with high quality.

2.2. Activities of the Cys(Npys)-containing peptide on ORL1 receptors

Synthesized Cys(Npys)-RYYRIK-NH_2 was first evaluated for its ability to bind to ORL1 receptors. The peptide was assayed for ORL1 receptors expressed in COS-7 cells by using [^{3}H]nociceptin as a tracer. In the assay in which nociceptin itself is highly potent (IC_{50} = 0.52 ± 0.03 nM), this peptide was not particularly active, exhibiting an IC_{50} value of 133.0 ± 13.6 nM, or approximately 250 times less than that of nociceptin, and approximately 160 times less that even that of the parent Ac-RYYRIK-NH_2.

Since it was found that the peptide was still significantly potent in the binding assay, we further evaluated its receptor activation activity in a [^{35}S]GTPγS-binding assay. The results showed that the Cys(Npys)-attached peptide was almost completely inactive. Even at a concentration of 10 μM, Cys(Npys)-RYYRIK-NH_2 exhibited only 5% of the activity of nociceptin, as shown in Fig. 2A. This was in stark contrast to the considerably high agonist activity (about 60% of the activity of nociceptin) of the parent Ac-RYYRIK-NH_2 (Fig. 2A). On the other hand, 10 μM Cys(Npys)-RYYRIK-NH_2 clearly reduced the activity of nociceptin when the two were incubated together (Fig. 2B and C). As a result, Cys(Npys)-RYYRIK-NH_2 was judged to be a weak antagonist of nociceptin on the ORL1 receptor.

2.3. Non-specific labeling of the ORL1 antagonist-binding site by *N*-ethylmaleimide

If there is a free thiol group of Cys in the ligand-binding site of ORL1, *N*-ethylmaleimide (NEM), a Michael acceptor, would react with this free thiol group, resulting in the formation of a thioether C–S bond.[29–33] Such a reaction would be virtually irreversible, and as for the ORL1 receptor, the resulting alkylation modification of

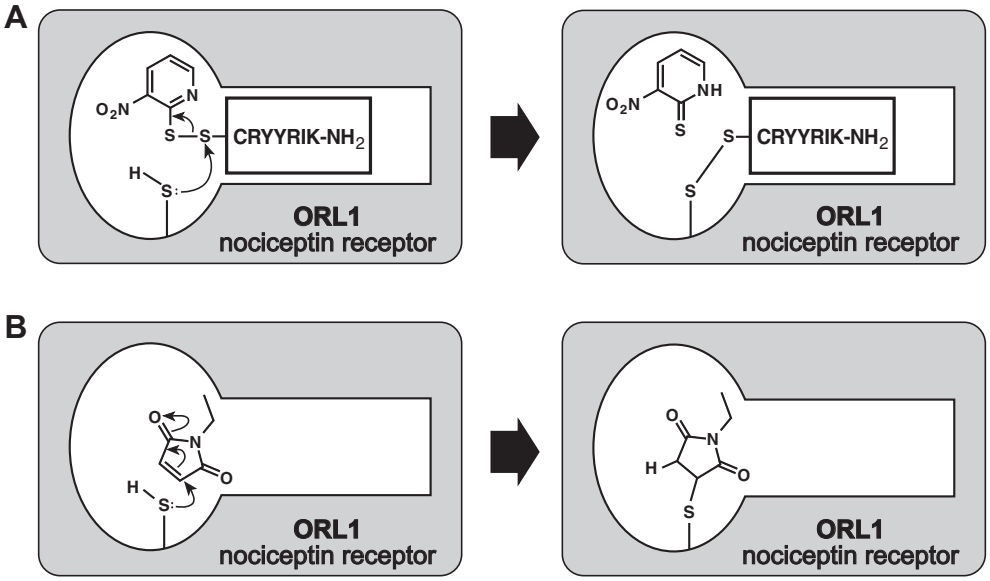

Figure 1. Reaction modes of two different types of thiol reagents against a free thiol group in the ORL1 nociceptin receptor. The thiol reagents used were (A) an affinity-ligand Cys(Npys)-RYYRIK-NH_2, which is an SNpys-containing derivative of the ORL1 nociceptin receptor antagonist Ac-RYYRIK-NH_2, and (B) a non-specific thiol-alkylation reagent *N*-ethylmaleimide (NEM).

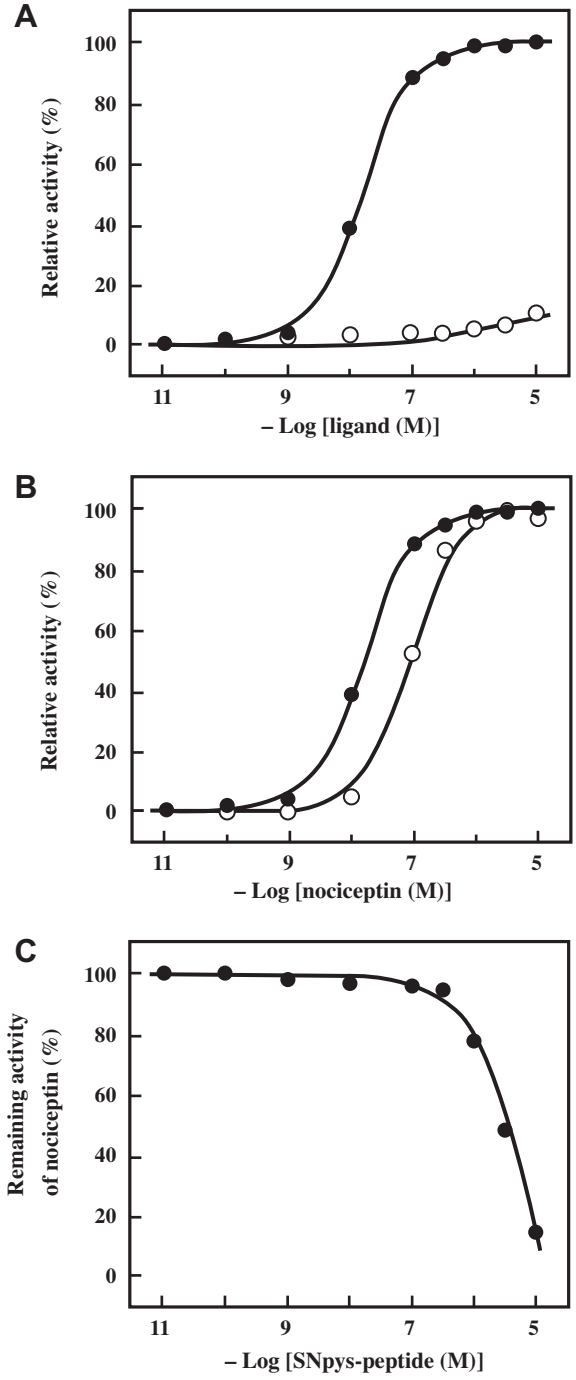

Figure 2. (A) Dose–response curves of nociceptin (●) and Cys(Npys)-RYYRIK-NH_2 (○) in the [^{35}S]GTPγS-binding assay using the Gα-fused ORL1 receptor. (B) The antagonist activity of 10 μM Cys(Npys)-RYYRIK-NH_2 against nociceptin in the [^{35}S]GTPγS-binding assay using the Gα-fused ORL1 receptor. The curves show activities without (●) and with (○) 10 μM Cys(Npys)-RYYRIK-NH_2. (C) The antagonist activity of Cys(Npys)-RYYRIK-NH_2 against 10 nM nociceptin in the [^{35}S]GTPγS-binding assay using the Gα-fused ORL1 receptor. The vertical axis shows a remaining % activity of 10 nM nociceptin, and the horizontal axis shows the concentration of antagonist SNpys-containing peptide, Cys(Npys)-RYYRIK-NH_2 (the concentration is denoted as SNpys-peptide in the square brackets). All the assays were repeated at least three times and each data point is the average of these experiments (SE <8%).

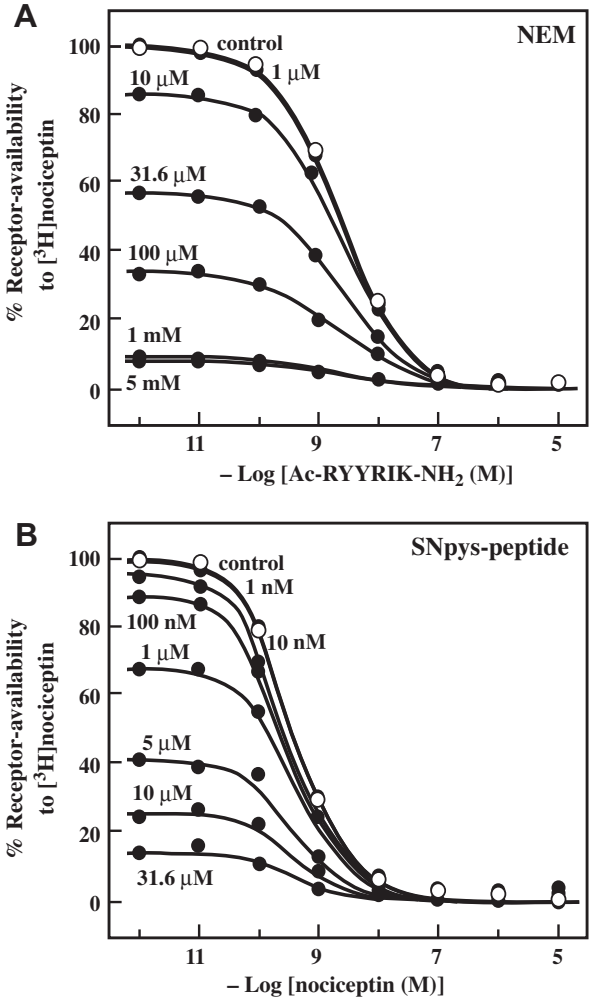

Figure 3. Loss of binding sites for the ORL1 nociceptin receptor expressed in the COS-7 cells in response to preincubation with Cys(Npys)-RYYRIK-NH_2 (A) and *N*-etylmaleimide (NEM) (B). The receptor binding assays were performed by using [^{3}H]nociceptin as a tracer and nociceptin as a competitor in (A), but Ac-RYYRIK-NH_2 was used as a competitor in (B) to ensure the presence of a free thiol in the binding site of this peptide. The vertical axis shows a remaining % receptor concentration (*B*: bound)/total receptor concentration (B_0). The assays were repeated at least three times and each data point is the average of these experiments (SE <17%).

the Cys residue would reduce the number of receptors available for the ligand added afterwards. Thus, after pre-incubation of membranes with NEM, the receptor-binding assays would reveal the loss of ORL1 receptors, and consequently decreases in the amounts of the receptors remaining. In the present study, in order to examine whether a free Cys-β-CH_2SH group was present in the ligand-binding site of ORL1 receptors, we first carried out a heterogeneous competitive binding assay by using radio-labeled [^{3}H]nociceptin as a tracer for Ac-RYYRIK-NH_2 as a competitor. This assay was expected to specifically reveal whether or not the binding site of Ac-RYYRIK-NH_2 in ORL1 consists of a Cys residue.

COS-7 cell membranes were first incubated with varying concentrations of NEM for 90 min at 25 °C. Then, the membranes were washed twice by centrifugation of suspension with ice-cold buffer. In order to determine the number of ORL1 receptors remaining unlabeled, these washed membrane preparations were assayed for Ac-RYYRIK-NH_2 using [^{3}H]nociceptin. As shown in Figure 3A, when the membranes were preliminarily incubated with 100 μM NEM, approximately 35% of the ORL1 receptors were available for the assay by [^{3}H]nociceptin, indicating that the remaining 65% of ORL1 receptors were occupied by NEM. As the concentrations of NEM increased, the receptors available for Ac-RYYRIK-NH_2 decreased sharply. This apparent loss of ORL1 receptors in cell membranes was clearly due to the irreversible chemical modification by NEM. The receptor-binding affinity of Ac-RYYRIK-NH_2, which was calculated as the IC_{50}, did not change at all despite the decrease in

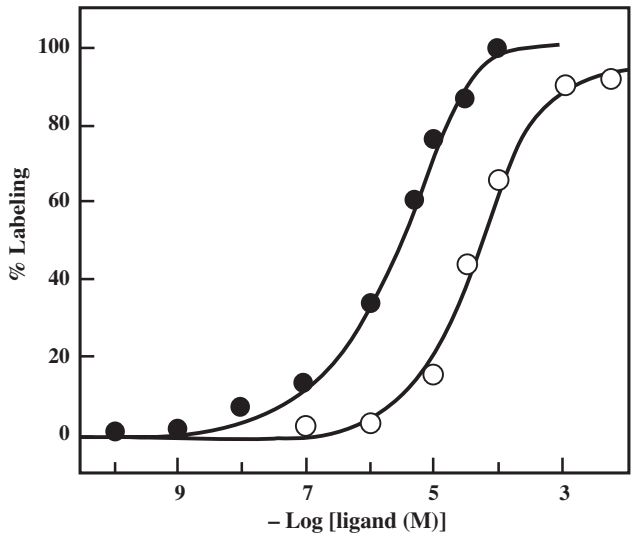

Figure 4. Effectiveness of affinity labeling of the ORL1 nociceptin receptor with Cys(Npys)-RYYRIK-NH_2 (●) and alkylation with *N*-etylmaleimide (NEM) (○). The labeling percentage by either Cys(Npys)-RYYRIK-NH_2 or NEM was calculated by subtracting the percentage of ORL1 receptors available for homologous [^{3}H]nociceptin/nociceptin competitive binding assay from its full binding (100%) without preincubation. The concentration of [^{3}H]nociceptin used was 0.05 nM in these experiments. The assays were repeated at least three times and each % labeling value is the average of these experiments (SE <17%).

receptor population. Also, nociceptin showed unchanged binding characteristics for the same receptor preparation in the homologous competitive binding assay using [^{3}H]nociceptin as a tracer and nociceptin as a competitor (data not shown). All these results indicated that the remaining receptor sites were all sites for the wild-type ORL1.

2.4. Specific affinity-labeling of ORL1 by the Cys(Npys)-containing peptide

The results obtained from the NEM alkylation test indicated that there is at least one free Cys residue in the binding site of Ac-RYYRIK-NH_2 in ORL1. We next attempted to capture this free thiol by using Cys(Npys)-RYYRIK-NH_2. The labeling experiment was carried out by using this Cys(Npys)-containing peptide instead of NEM exactly as described above, except for the choice of the competitor for [^{3}H]nociceptin. We did perform a homologous competitive receptor-binding assay using nociceptin against [^{3}H]nociceptin to examine the affinity labeling of the nociceptin-binding site in ORL1. The results obtained using Cys(Npys)-RYYRIK-NH_2 were similar to those obtained using NEM, as shown in Figure 3B. The only difference was in the effective concentration of Cys(Npys)-RYYRIK-NH_2 required for a specific labeling. That is, for about 60% labeling of ORL1 receptors, namely, for about 40% receptor-availability to [^{3}H]nociceptin, 5 μM Cys(Npys)-RYYRIK-NH_2 was required. This is in clear contrast to the requirement of 100 μM NEM for 65% labeling (see above).

When an SNpys-containing peptide ligand is incubated with membranes expressing a single class of ORL1 receptors, it binds to the ligand-binding site of receptor molecules upon first opportunity. If a receptor thiol group is located near the SNpys group, this SNpys group would react with the free thiol group via a thiol–disulfide exchange reaction, resulting in formation of a disulfide bond (Fig. 1). Because the 3-nitro-2-pyridine moiety of the SNpys group never binds to the thiol group, this reaction provides specific cross-linking of the receptor with a ligand attached to the SNpys group. In this case, RYYRIK-NH_2 is cross-linked to the ORL1 receptor. Since the concentrations of Cys(Npys)-RYYRIK-NH_2 increased, the receptors available for nociceptin decreased sharply, as seen for the NEM treatment. This apparent loss of ORL1 receptors in cell membranes is definitely due to the irreversible cross-linking of RYYRIK-NH_2.

The efficiency of Cys(Npys)-RYYRIK-NH_2 in affinity labeling can be evaluated based on the effective concentration (EC_{50}) sufficient to label the half-maximal amount of total ORL1 receptors. The EC_{50} values are obtained from sigmoidal curves that represent the number of labeled receptors (% labeling), plotted against the concentration of Cys(Npys)-RYYRIK-NH_2 in pre-incubation. The calculated EC_{50} value was approximately 1.9 μM (Fig. 4). By contrast, the calculated EC_{50} value for NEM was 43.7 μM (Fig. 4). These results clearly indicate that Cys(Npys)-RYYRIK-NH_2 is about 23 times more effective or specific for labeling the ORL1 receptor than NEM. Cys(Npys)-RYYRIK-NH_2 is not sufficiently strong to cross-link the ORL1 receptor, and thus we are now trying to prepare a series of peptides containing Cys(Npys) in the middle of the amino acid sequence of Ac-RYYRIK-NH_2 in order to obtain a more specific affinity ligand and identify further Cys residues in the ORL1 receptor.

3. Conclusion

The present results clearly demonstrated that the ORL1 receptor possesses a free Cys residue in the ligand-binding site, the β-SH group of which can be cross-linked covalently by both Cys(Npys)-RYYRIK-NH_2 and NEM. NEM-labeling is non-specific, and all the free Cys residues present at the molecular surface of ORL1 would be chemically cross-linked by NEM via the thioether linkage. However, because the receptor-binding assays were carried out by using [^{3}H]nociceptin, we could detect and identify only the Cys residue in the nociceptin-binding site by NEM-labeling. In contrast, the labeling by Cys(Npys)-RYYRIK-NH_2 is definitely specific for this free Cys residue in the nociceptin-binding site, making a disulfide S–S linkage. For better affinity labeling with reinforced labeling-efficiency, it will be necessary to obtain an affinity-ligand in which the SNpys group is located closer to the receptor Cys residue. Such affinity-ligand would be essential as a molecular tool for identifying the corresponding receptor Cys residue and its functional role.

4. Experimental

4.1. Peptide syntheses

The Npys-containing peptide used in this study, Cys(Npys)-RYYRIK-NH_2, was synthesized (0.15 mmol scale) by the manual solid phase method using Fmoc-chemistry. Using Fmoc-NH-SAL resin (Merck, Darmstadt, Germany), the coupling reaction was carried out with HBTU (Peptide Institute Inc., Osaka, Japan) in the presence of HOBt (Peptide Institute) dissolved in *N*-methylpyrrolidone (NMP; Nakalai Tesque Inc., Kyoto, Japan) and *N*,*N*-dimethylformamide (DMF; Kanto Chemical Co. Inc., Tokyo, Japan). Each coupling reaction was examined for completion by means of the Kaiser ninhydrin test.[34] N-Terminal Cys(Npys) was introduced by the coupling of Boc-Cys(Npys)-OH (Kokusan Chemical Co., Ltd, Tokyo, Japan) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride (Peptide Institute) in dichloromethane. Ac-RYYRIK-NH_2 was also synthesized as described above by acetylation with acetic anhydride (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at the final step instead of Cys(Npys)-elongation. Nociceptin was prepared by the automated peptide synthesizer ABI 433A (Applied Biosystems Inc., Foster City, CA) with the Fmoc synthetic method.

After completion of the synthesis, the peptides were liberated from the resin using a cocktail reagent containing 95% trifluoroacetic acid (TFA) and 5% water. Crude peptide was purified 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 carried out on a preparative HPLC column (25 × 250 mm; Cica-Merck LiChrospher RP-18 (e), 5 μm; Merck, Darmstadt, Germany). The linear elution conditions employed were as follows: solvent system, 0.1% aqueous TFA-(A solution) and acetonitrile containing 20% A solution-(B solution); flow rate, 4 ml/min; temperature, 25 °C; and UV detection, 230 nm.

The purity of peptide was verified by analytical RP-HPLC (4 × 250 mm; Cica-Merck LiChrospher 100 RP-18, 5 μm; Merck), using the same elution conditions except for a flow rate of 0.65 ml/min. 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.

4.2. Cell culture and membrane preparation

The COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich Co. LLC., St. Louis, MO) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA) in 100 U/ml penicillin and 100 μg/ml streptomycin. The plasmid DNAs (20 μg) of the ORL1-Gα receptor were transiently transfected into confluent COS-7 cells in a 100 mm cell culture dish by using TransFectin Lipid Reagent (Bio-Rad Laboratories, Hercules, CA). After 48 h, cells were harvested and centrifuged for 10 min at 500 × *g* (4 °C). Cells were then resuspended in the buffer containing 5 mM Tris–HCl, 1 mM EGTA, 200 μM dithiothreitol (DTT), and 11% saccharose (pH 7.4), and homogenized with a Polytron® PT-3100 (Kinematica, AG, Lucerne, Switzerland). The homogenate was centrifuged for 10 min at 1000 × *g* (4 °C). The supernatant was centrifuged again for 20 min at 31,500 × *g* (4 °C), and the pellet was washed with the buffer containing 5 mM Tris, 1 mM EGTA, and 200 μM DTT (pH 7.4). The concentration of membrane protein was estimated by the Bradford method using Protein Assay CBB solution (Nakalai Tesque). The prepared membrane was frozen at −80 °C until use.

4.3. Receptor-binding assay

The saturation-binding assay was first carried out to obtain a sound ORL1 receptor preparation for affinity-labeling examination. Individual tubes containing 2–5 μg/ml membrane protein were incubated with increasing concentrations of [^{3}H]nociceptin (82.3 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) (0.05–2.0 nM) for 1.5 h at 25 °C in 50 mM HEPES–Tris buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA; Wako) and 100 μg/ml bacitracin (Wako). Non-specific binding was determined in the presence of 10 μM nociceptin. The data on specific binding of [^{3}H]nociceptin were used to perform a one-site binding hyperbola nonlinear regression analysis using the software package Prism (GraphPad Software Inc., La Jolla, CA), estimating a change in a receptor density B_{max} (fmol/mg protein) and equilibrium dissociation constant K_d (nM). Membrane preparations of K_d = 0.1–0.50 nM with B_{max} = 1000–2000 fmol/mg protein were utilized for the following homologous competitive receptor-binding assay.

Competitive receptor-binding assays were conducted in a 96-well format, using a firm and viable membrane preparation of COS-7 cells expressing rat ORL1-Gα fusion receptors. Each well of a 96-well plate (300 μl) containing 5 μg/ml membrane protein, a series of concentrations (10^{-5}–10^{-11} M in final concentration) of non-labeled nociceptin, and 0.05 nM [^{3}H]nociceptin was incubated for 90 min at 25 °C in 50 mM HEPES–Tris buffer (pH 7.4) containing 0.1% BSA. Bacitracin (100 μg/ml) was also added as a protease inhibitor. After incubation, the mixture was filtered and washed with 50 mM Tris buffer (pH 7.4) through a glass fiber UniFilter GF/B plate, by means of a FilterMate Harvester (PerkinElmer Life Sciences Japan, Tokyo, Japan). To coat the filter surface, plates were soaked in 0.5% ethyleneimine polymer aqueous solution for 30 min before use. 20 μl of MicroScinti40 (PerkinElmer) was added to each well. The plates were sealed with TopSeal (PerkinElmer), and read on the TopCount NXT (PerkinElmer) for 3 min per well. The computer program Prism was used to draw dose-response curves for the analysis. The binding potency of nociceptin was estimated as the IC_{50} value, the peptide concentration at which the half-maximal inhibition is achieved.

4.4. Non-specific labeling of free Cys residues with N-ethylmaleimide

For alkylation of the free thiol group of the Cys residue(s) in the ORL1 receptor, NEM (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) at various concentrations (0, 0.1, 1.0, 10, 31.6, 100, 1,000, and 5,000 μM) was incubated at 25 °C for 90 min with membrane preparations (10–30 μg protein/well) in 50 mM HEPES-Tris buffer (pH 7.4) containing 0.1% BSA, 10 mM $MgSO_4$, and bacitracin (100 μg/ml). The membranes were centrifuged at 100,000 × *g* for 15 min and resuspended using a homogenizer in 50 mM HEPES–Tris buffer (pH 7.4) containing 0.1% BSA and bacitracin (100 μg/ml). The washing process was repeated twice, and then the membranes were employed for the competitive binding assay of Ac-RYYRIK-NH_2 with [^{3}H]nociceptin as described above. The binding assay was also carried out for nociceptin as a competitor with [^{3}H]nociceptin.

4.5. Affinity-labeling assay

For affinity labeling of ORL1 receptors, Cys(Npys)-RYYRIK-NH_2 was incubated at various concentrations (10^{-4}–10^{-9} M) as described above. The centrifuged (100,000 × *g*, 15 min) membranes were resuspended in 50 mM HEPES–Tris buffer (pH 7.4) containing 0.1% BSA and bacitracin (100 μg/ml). For the homologous competitive binding experiment with nociceptin and [^{3}H]nociceptin, washed membranes were treated as described for the ordinary receptor-binding assays.

Acknowledgments

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