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Spare interactions of highly potent [Arg^{14},Lys^{15}]nociceptin for cooperative induction of ORL1 receptor activation

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ABSTRACT

[Arg^{14},Lys^{15}]Nociceptin is a very potent for ORL1 receptor, showing a few times stronger binding activity and much more enhanced biological activity than endogenous nociceptin. This synergistic outcome has been suggested to be due to the interaction with the receptor aromatic and/or acidic amino acid residues crucial to receptor activation. In order to identify such receptor residues in the second ORL1 extracellular loop, we prepared a series of recombinant mutant receptors. The mutant receptor Gln205Ala was found to be as active as wild-type ORL1 for both nociceptin and [Arg^{14},Lys^{15}]nociceptin. In contrast, Asp206Ala and Tyr207Ala exhibited considerably reduced activity for [Arg^{14},Lys^{15}]nociceptin, exhibiting no synergistic activity enhancement. These results suggest that Asp206 and Tyr207 are directly involved in the interaction with nociceptin-[Arg^{14},Lys^{15}]. Trp208Ala was found to bind strongly both nociceptin and [Arg^{14},Lys^{15}]nociceptin, although it elicited no biological activity. All these results indicate that the consecutive amino acid residues Asp206, Tyr207, and Trp208 are critical to the activation of the ORL1 receptor, but not to nociceptin-binding.

1. Introduction

The ORL1 (opioid receptor-like receptor 1) receptor is a G protein-coupled receptor (GPCR) and belongs to the opioid receptor family.[1–3] Its endogenous ligand is heptadecapeptide nociceptin, F^{1}GGFT^{5}GARKS^{10}ARKLA^{15}NQ, which produces hyperalgesia or inhibition of opioid-induced analgesis.[4,5] Mutational analysis of ORL1 has elucidated a number of the ligand-binding sites for nociceptin.[6–8] However, the specific receptor activation mechanism followed by ligand binding has not yet been clarified.

We have previously found that [Arg^{14},Lys^{15}]nociceptin is highly potent, especially in receptor activation.[9] Moreover, we have demonstrated that such a stimulus effect inevitably occurs in response to simultaneous incorporation of two consecutive basic amino acids at positions 14–15.[10] [Arg^{14},Arg^{15}]-, [Lys^{14},Lys^{15}]-, and [Lys^{14},Lys^{15}] nociceptins have been found to be very potent, similar to [Arg^{14},Lys^{15}]nociceptin, and to exhibit similar activity enhancement in receptor activation. Results similar to these for the so-called superagonists were also obtained with [Trp^{14},Arg^{15}]- and [Trp^{14},Lys^{15}]nociceptins.[11]

As compared to native nociceptin, the analogs displayed 3–5-fold higher binding activity in the competitive receptor-binding assay and 10–12-fold greater biological activity in a functional GTPγS binding assay. Thus, their biological activity is several times stronger than that expected based on their receptor-binding activity. The results indicated that the simultaneous replacements of Leu^{14} with Arg, Lys, or Trp and of Ala^{15} with Arg or Lys enhance the interaction of nociceptin with the receptor in a synergistic fashion. Only the parallel presence of these amino acids at the two neighbouring positions, 14 and 15, yielded an optimal effect. This result implies that the complementary receptor-binding sites of these incorporated nociceptin residues are crucially important for receptor activation, and suggests that these residues must be spatially adjacent to each other in the ORL1 receptor.

The identification of such receptor residues would greatly facilitate the clarification of receptor mechanisms to trigger intrinsic signal transduction machinery. This kind of structure–activity study for the ORL1 receptor is feasible simply by using superagonists like [Arg^{14},Lys^{15}]nociceptin, and thus in the present study we attempted to explore the binding sites of Arg-Lys$^{14–15}$ present in highly potent [Arg^{14},Lys^{15}]nociceptin.

Abbreviations: EL2, The second extracellular loop; GPCR, G protein-coupled receptor; ORL1, Opioid receptor-like receptor 1; TM5, The fifth transmembrane domain.

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Nociceptin originally contains two Arg-Lys (RK) dibasic pairs at positions 8–9 and 12–13. This basic region has been demonstrated to interact with the acidic amino acid cluster in the second extracellular loop (EL2: 188-AQVEDEEIECLVEIPAPQDYW-208) of ORL1 receptor (Fig. 1).[12–14] Arg-Lys$^{14–15}$ is adjacent to this basic region in [Arg^{14},Lys^{15}]nociceptin, leading us to speculate that its binding site in ORL1 is in the same EL2. Indeed, in the energy-minimized model complexes between human ORL1 receptor and nociceptin, the ORL1 receptor structural element for Leu^{14} and Ala^{15} of nociceptin was identified as EL2.[12] Since acidic and aromatic amino acid residues have been suggested as receptor residues complementary to Arg-Lys$^{14–15}$, we expected 205-QDYW-208 to be the binding site of Arg-Lys$^{14–15}$. We changed these Gln205, Asp206, Tyr207, and Trp208 residues to Ala by the site-directed mutagenesis method to ensure their roles in receptor binding and activation.

2. Results

2.1. Expression efficiency of ORL1 and its mutant receptors

Among the amino acid residues present in the surfactant between EL2 and the fifth transmembrane domain (TM5) of ORL1, -Pro^{204}-Gln-Asp-Tyr-Trp-Gly^{209}-, four consecutive residues at positions 205–208 were mutated to Ala, respectively, by the PCR method. The resulting mutant receptors include Gln205Ala, Asp206Ala, Tyr207Ala, and Trp208Ala. Here, the mutant receptors are designated using the following format: original amino acid###mutated amino acid (e.g., Gln205Ala). The sequence of the PCR-amplified region of each mutant was determined to be entirely the same as that in the original sequence, except for the mutated nucleotides.

ORL1 and its mutant receptors were transiently expressed in COS-7 cells. A saturation binding assay followed by the Scatchard plot analysis was performed to determine the dissociation constant K_d (nM) and the receptor density B_{max} (fmol/mg protein) of [^{3}H]nociceptin. For all the receptors, [^{3}H]nociceptin exhibited an appropriate specific-binding curve, and the Scatchard plot showed a linear straight line, indicating the presence of a homogenous population of nociceptin binding sites (data not shown). For wild-type ORL1, the B_{max} value of [^{3}H]nociceptin was 723 fmol/mg protein (Table 1). The level of receptor expression was practically maintained for Gln205Ala and Asp206Ala mutant receptors (B_{max} = 742, and 878 fmol/mg protein, respectively), indicating that these Gln-Ala and AspAla substitutions are free from damage in the stable receptor structure. In contrast, the Tyr207Ala mutant showed a somewhat lower expression level (B_{max} = 413 fmol/mg protein), suggesting that the TyrAla substitution at position 207 perturbs the ORL1 receptor structure, thus disturbing its placement in the cell membrane. Furthermore, the Trp208Ala mutant showed quite low expression levels (B_{max} = 98 fmol/mg protein), indicating that the TrpAla substitution at position 208 significantly destabilizes the ORL1 structure for insertion into the cell membrane.

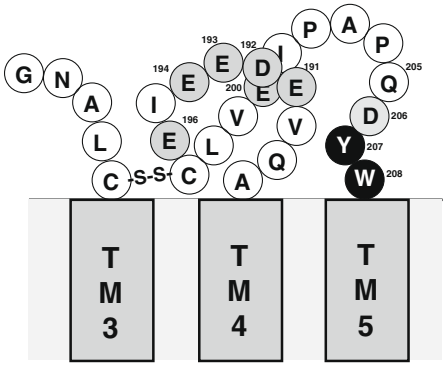

Figure 1. The structure of the ORL1 nociceptin receptor extracellular portion showing the complete amino acid sequence of the second extracellular loop (EL2). EL2 is cross-linked with EL1 by the disulphide bond between Cys197 and Cys120. EL2 bridges TM4 and TM5 at the extracellular side. The acidic amino acid cluster, Glu191-Glu196 and Glu200, is reported to be a binding site for the basic amino acid cluster of nociceptin, Arg^8-Lys-Ser-Ala-Arg-Lys^{13}. In the present study, the amino acid residues at positions of 205–208 of ORL1 were mutated to Ala by the site-directed mutagenesis technique.

Table 1
The kinetic parameters of [^{3}H]nociceptin for the wild-type ORL1 receptor and its mutant receptors expressed in COS-7 cells

ORL1 receptors	K_d (nM)	B_{max} (fmol/mg)
Wild type	0.092 ± 0.004	723 ± 10
Gln205Ala	0.088 ± 0.004	742 ± 15
Asp206Ala	0.67 ± 0.05	878 ± 10
Tyr207Ala	0.71 ± 0.07	413 ± 10
Trp208Ala	0.49 ± 0.03	98 ± 5

Data were first taken from at least three experiments, and then the mean K_d and B_{max} values were calculated to show with SEM among the experiments.

2.2. Receptor-binding characteristics of nociceptin and [Arg^{14}, Lys^{15}]nociceptin

The K_d value reveals the binding ability of [^{3}H]nociceptin for receptors expressed firmly and steadily in the COS-7 cell membranes. As shown in Table 1, Gln205Ala (K_d = 0.088 nM) was found to bind [^{3}H]nociceptin as well as the wild-type ORL1 did (0.092 nM). Although [^{3}H]nociceptin also exhibited very high receptor-binding affinity for Asp206Ala (0.67 nM) and Tyr207Ala (0.71 nM), their binding affinities were several times weaker than those for wild-type ORL1 and Gln205Ala (Table 1). It should be noted that even Trp208Ala bound [^{3}H]nociceptin very strongly (0.49 nM). These results clearly indicate that Tyr207Ala and Trp208Ala sustain their specific conformations to bind nociceptin, although difficulties arise with regard to inserting themselves into the cell membrane.

Similar results were obtained in the competitive binding assay for non-labelled nociceptin to inhibit the binding of [^{3}H]nociceptin. The half-maximal concentrations (IC_{50}) of nociceptin were 0.72 nM for wild-type ORL1 and 0.65 nM for Gln205Ala (Table 2). For other mutant receptors, nociceptin was fairly potent, showing IC_{50} values of 0.80–1.02 nM (Table 2).

[Arg^{14},Lys^{15}]nociceptin was very potent for wild-type ORL1 (IC_{50} = 0.21 nM) (Table 2). It was also found to be very potent for all the mutant receptors (0.22–0.54 nM). When the binding activities of nociceptin and [Arg^{14},Lys^{15}]nociceptin were compared for each receptor, [Arg^{14},Lys^{15}]nociceptin was a few times (1.7–3.6-fold) more potent than nociceptin. However, when the functional activities of [Arg^{14},Lys^{15}]nociceptin were compared among receptors, this nociceptin analog was found to be approximately two times less potent in the mutant receptors Asp206Ala and Tyr207-Ala than in the receptors such as the wild-type ORL1, Gln205Ala, and Trp208Ala (Table 2). These decrements in binding activity are definitely due to the AspAla and TyrAla substitutions, respectively, and thus the Asp206 and Tyr207 residues are likely to interact directly with [Arg^{14},Lys^{15}]nociceptin.

2.3. Biological activities of nociceptin and [Arg^{14},Lys^{15}]nociceptin

When nociceptin was evaluated for its functional biological activity in the [^{35}S]GTPγS binding assay, diverse results were obtained. First, Gln205Ala was found to be as potent as wild-type ORL1 receptor (Table 3). On the other hand, Asp206Ala and Tyr207Ala were less potent (52–62%) than native ORL1 Gln205Ala.

Table 2
The receptor-binding affinity of nociceptin and [Arg^{14},Lys^{15}]nociceptin for the wild-type ORL1 receptor and its mutant receptors expressed in COS-7 cells

ORL1 receptors	Nociceptin		[Arg^{14},Lys^{15}]nociceptin	
	IC_{50} (nM)	Relative potency	IC_{50} (nM)	Relative potency
Wild type	0.72 ± 0.22	100	0.21 ± 0.02	100
Gln205Ala	0.65 ± 0.08	111	0.22 ± 0.04	95
Asp206Ala	1.02 ± 0.05	71	0.54 ± 0.01	39
Tyr207Ala	0.91 ± 0.05	79	0.46 ± 0.07	46
Trp208Ala	0.80 ± 0.09	90	0.22 ± 0.06	95

Relative potency was calculated by dividing the IC_{50} of either nociceptin or [Arg^{14},Lys^{15}]nociceptin in the wild-type ORL1 receptor with those in each mutant receptors. Data were first taken from 4 to 6 experiments, and then the mean IC_{50} values were calculated to show with SEM among the experiments.

Table 3
The GTPS binding activity of nociceptin and [Arg^{14},Lys^{15}]nociceptin for the wild-type ORL1 receptor and its mutant receptors expressed in COS-7 cells

ORL1 receptors	Nociceptin		[Arg^{14},Lys^{15}]nociceptin	
	EC_{50} (nM)	Relative potency	EC_{50} (nM)	Relative potency
Wild type	13 ± 1.4	100	1.1 ± 0.14	100
Gln205Ala	13 ± 1.9	100	1.2 ± 0.14	92
Asp206Ala	21 ± 1.6	62	5.6 ± 0.23	20
Tyr207Ala	25 ± 1.2	52	5.1 ± 0.82	22
Trp208Ala	Inactive	0	Inactive	0

Relative potency was calculated by dividing the EC_{50} of either nociceptin or [Arg^{14},Lys^{15}]nociceptin in the wild-type ORL1 receptor with those in each mutant receptors. Data were first taken from 5 to 8 experiments, and then the mean EC_{50} values were calculated to show with SEM among the experiments.

Surprisingly, Trp208Ala was completely inactive against nociceptin, eliciting absolutely no activation from the basal activity.

[Arg^{14},Lys^{15}]nociceptin also exhibited varied results, just like those for nociception, for mutant receptors (Table 3). It was almost equipotent for both wild-type ORL1 and Gln205Ala, but exhibited significantly reduced activities (approximately 20%) for Asp206Ala and Tyr207Ala. This distinct activity decrement implies that Asp206 and Tyr207 are the site to which Arg-Lys$^{14–15}$ of [Arg^{14},Lys^{15}]nociceptin binds. For Trp208Ala, [Arg^{14},Lys^{15}]nociceptin was also inactive. It showed no activation in this [^{35}S]GTPγS binding assay. It seems that Trp208 is critical for receptor activation, but not for ligand binding.

2.4. Synergistic activity enhancement by [Arg^{14},Lys^{15}]nociceptin

[Arg^{14},Lys^{15}]nociceptin is a kind of superagonist exhibiting much more potentiated functional biological activity than expected based on results regarding its receptor-binding affinity. This was also clearly revealed for the wild-type ORL1 receptor in the present study. As shown in Figure 2, [Arg^{14},Lys^{15}]nociceptin was 3.4 times more potent than nociceptin in the competitive binding assay for the wild-type ORL1 receptor, while it was 11.8 times more potent than nociceptin in the functional [^{35}S]GTPγS assay. Distinct activity enhancement is clearly visible as an intense projecting bar chart in Figure 2. A similar result was obtained for the mutant Gln205Ala receptor, indicating that the Gln205 residue was not involved in the binding of [Arg^{14},Lys^{15}]nociceptin to ORL1.

For the mutant receptor Asp206Ala, however, such an activity enhancement was not observed. Although [Arg^{14},Lys^{15}]nociceptin was more potent than nociceptin in both the competitive binding assay (1.3-fold) and the functional [^{35}S]GTPγS binding assay (2.3-fold), there was no marked enhancement, as shown in the bar chart (Fig. 2). Evidently, the AspAla substitution reduced the potentiation power of [Arg^{14},Lys^{15}]nociceptin, indicating that Asp-β-COOH at the position of ORL1 receptor 207 is crucial for such potentiation by [Arg^{14},Lys^{15}]nociceptin. A similar result was obtained when the mutant receptor Tyr207Ala was assayed for nociceptin and

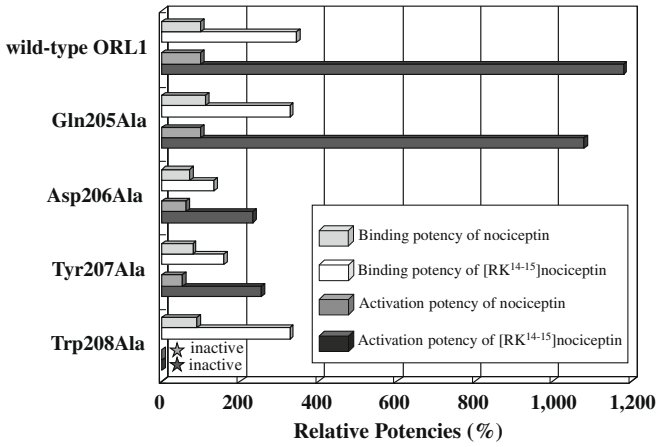

Figure 2. Relative potencies of native nociceptin and superagonist [Arg^{14},Lys^{15}]nociceptin for the wild-type ORL1 receptor and its mutant receptors. The activities are expressed as the % potency against the activity of native nociceptin for the wild-type ORL1 receptor. The white bar shows the binding potency of [Arg^{14},Lys^{15}]nociceptin in the competitive binding assay using [^{3}H]nociceptin as a tracer, and the black bar shows the functional biological potency of [Arg^{14},Lys^{15}]nociceptin in the [^{3}H]GTPγS binding assay. On the other hand, the light grey bar shows the binding potency of native nociceptin in the competitive binding assay using [^{3}H]nociceptin, and the dark grey bar shows the functional biological potency of nociceptin in the [^{3}H]GTPγS assay. [Arg^{14},Lys^{15}]nociceptin exhibits greatly enhanced binding potency in the wild-type ORL1 receptor and Gln205Ala mutant receptor, but not in Asp206Ala and Tyr207Ala mutant receptors. [Arg^{14},Lys^{15}]nociceptin and nociceptin are fully active for Trp208Ala mutant receptor in the receptor-binding assay, but completely inactive in the [^{3}H]GTPγS assay.

[Arg^{14},Lys^{15}]nociceptin, indicating the importance of Tyr-β-phenol in activity enhancement. All these results clearly indicate that Asp206 and Tyr207 are directly involved in a dominant interaction with the Arg^{14} and Lys^{15} residues of [Arg^{14},Lys^{15}]nociceptin to induce an activity synergistic enhancement.

3. Discussion

Agonists are described by two independent parameters: affinity and efficacy. The efficacy of an agonist is defined as its relative potency to activate a receptor when an agonist molecule binds to the receptor.[15,16] Since a superagonist is an agonist that possesses a greatly increased efficacy and an ability to activate much more strongly the receptor than the native ligand, there have been many attempts to discover superagonists as potent therapeutic candidates. Thus far, several superagonists have been announced for some GPCRs such as MSH receptor,[17] somatostatin receptor,[18,19] urotensin II receptor,[20] and GnRH receptor.[21] Superagonists should possess certain structural elements to elicit strongly enhanced receptor activity.[21] Although receptors also must possess complementary structural elements, there has been no attempt to clarify such elements in the target receptor. Elucidation of a

complementary receptor structure would greatly help with the design of a more potent agonist.

[Arg^{14},Lys^{15}]nociceptin and related peptides have been found to function as superagonists for ORL1 receptor.[9–11] The efficacy of natural nociceptin is known to be not particularly high for ORL1 receptor; that is, in spite of its high binding affinity, the activity in the GTPγS binding assay is somewhat weak. A relatively large deviation between the receptor-binding affinity and the biological activity is characteristic of the activity profile of nociceptin. By contrast, [Arg^{14},Lys^{15}]nociceptin exhibited much more increased GTPγS binding activity, being roughly equipotent in both binding and biological assays.[9] Clearly, Arg-Lys placed at position 14–15 is a cause of this superagonist activity. We have previously tried to determine why [Arg^{14},Lys^{15}]nociceptin enhances the biological activity. We assumed two possibilities; that is, a reinforcement of the ligand-receptor interaction, and an increased resistance for endogenous proteases. We evaluated these possibilities by the receptor-binding assay and the trypsin enzyme digestion assay.[10] It was found that the synergistic enhancement in biological activity occurs only when basic amino acids are simultaneously incorporated at consecutive positions 14–15, regardless of the sequence. Protease resistance did not explain the synergistically enhanced functional activity of [Arg^{14},Lys^{15}]nociceptin.

In the present study, we demonstrated that the consecutive Arg^{14} and Lys^{15} residues of superagonist [Arg^{14},Lys^{15}]nociceptin interact with Asp206 and Tyr207 in the second extracellular loop of ORL1 receptor. It is strongly expected that Arg-Lys in nociceptin and Asp-Tyr in ORL1 are caught up with each other in the electrostatic and cation/π interactions. This structural communication between the dipeptide units of the ligand nociceptin and the ORL1 receptor has been found to be substantiated by replacement of the original Leu-Ala with dipeptides such as Arg-Lys, Arg-Arg, Lys-Arg, Lys-Lys, Trp-Arg, or Trp-Lys. No activity enhancement was attained by single amino acid replacement of Arg, Lys, or Trp. It appears that ORL1 Asp-Tyr residues at positions 206–207 are engaged in machinery to activate the receptor and to further facilitate such activation. The capture of this dipeptide unit requires a dipeptide Arg-Lys at position 14–15 of nociceptin.

In the energy-minimized model complexes between human ORL1 receptor and nociceptin, Topham et al.[12] have listed ORL1 receptor residues within 5 Å of each amino acid residue of nociceptin. The ORL1 receptor structural element for Leu^{14} and Ala^{15} of nociceptin was identified to be in EL2. The ORL1 receptor residues suggested for Leu^{14} were Glu194, Pro202, and Thr203, and that for Ala^{15} was Pro202 (the numbering of receptor residues here followed that of rat ORL1). Asp206 and Tyr207 are adjacent to Pro^{202}-Thr-Pro-Gln^{205} in the ORL1 EL2 loop structure. Since Arg and Lys possess considerably larger side chains as compared to Leu and Ala, it is quite likely that their guanidino and butylamino groups reach up to the Asp206 and Tyr207 receptor residues.

Nociceptin exhibited somewhat reduced activity for Asp206Ala and Tyr207Ala ORL1 mutant receptors (Tables 2 and 3). In the ORL1 energy-minimized model, Asp206 and Tyr207 were judged to be in close proximity (within 5 Å) to Arg^{8} and Lys^{9} of nociceptin. Since the AspAla and TyrAla substitutions must influence the activity of nociceptin, it is evident that Arg-Lys$^{8–9}$ is interacting with ORL1 Asp-Tyr$^{206–207}$. In the present study, it was also found that these Asp206 and Tyr207 receptor residues are involved in the interaction with Arg-Lys placed at position 14–15 of nociceptin. Thus, multiple interactions of Asp206 and Tyr207 with two Arg-Lys units at positions 8–9 and 14–15 must be a cause of synergistic activity enhancement observed for [Arg^{14},Lys^{15}]nociceptin. These interactions might bring about a conformation alteration allowing receptor activation to occur much more easily.

Trp208 is a unique residue in the ORL1 receptor residue. It was found that Trp208 is critical for receptor activation, but not for ligand binding. Both nociceptin and [Arg^{14},Lys^{15}]nociceptin could bind fully to the Trp208Ala ORL1 receptor, but this mutant receptor was completely inactive for these peptides. Together with the fact that Trp208 was not found within 5 Å of nociceptin in the energy-minimized model, these results indicate that Trp208 is not involved in the nociceptin binding site. From the crystal structure analysis studies of membrane proteins, it has been suggested that some Trp residues are distributed at the ends of the transmembrane helices.[22] It has been suggested that the main role of such Trp residues is to act as an 'anchor' at the interface region of the membrane.[23] Since ORL1 Trp208 is indeed present at the interface between EL2 and TM5, the Trp-indole group must play a similar role in stabilizing the receptor activation conformation. To confirm that Trp plays such a role, further structural examinations by site-directed mutagenesis are necessary, and such structure–activity studies are in progress in our laboratory.

4. Conclusion

By means of the site-directed mutagenesis experiments, it was found that the consecutive amino acid residues Asp206, Tyr207, and Trp208 in the ORL1 receptor are critical in receptor activation, but not in nociceptin-binding. Arg-Lys$^{14–15}$ residues of superagonist [Arg^{14},Lys^{15}]nociceptin directly interact with Asp206 and Tyr207 in the ORL1 receptor second extracellular loop EL2 and enhance receptor activity. These interactions are not just extra or additional, but are actually critically important for cooperative induction of receptor activation. These findings substantiated the electrostatic and cation/π interactions between the Arg-Lys residues in nociceptin and the Asp-Tyr residues in ORL1 receptor.

5. Materials and methods

5.1. Preparation of ORL1 mutant receptors

Mutated cDNA fragments were synthesized as products of the polymerase chain reaction (PCR) according to the PCR-mediated mutagenesis technique[24] using rat ORL1 receptor as a template by *Pfu* Turbo™ DNA polymerase (Stratagene, La Jolla, CA). The sequence of the mutagenic primer, for example, for Tyr207Ala, is 5'-CT CAG GAC GCT TGG GGA CCT GTA TTC-3′, and at the underlined bases, the codon TAT for Tyr was replaced with GCT for Ala. In order to eliminate *Apa*I and *Pst*I sites, ORL1/pBluescript II SK^{+} was digested at *Xba*I/*Kpn*I sites. The construct was blunted by T4 DNA polymerase and ligated with T4 DNA ligase. Mutated fragments were digested with the reaction enzymes *Apa*I and *Pst*I, and agarose gel-purified fragments (363 bp) were inserted into the wild-type construct rat ORL1/pBluescript II SK^{+} at *Apa*I/*Pst*I sites. The construct was digested with *Afl*II and *Bst*PI, and resulting fragments (1941 bp) were ligated to the wild-type construct ORL1/pcDNA3^{+}. Each subcloned mutant construct was cloned in *Escherichia coli* bacterial strain XL1 Blue (Stratagene). The resulting single clone was verified by restriction enzymatic digestion. Mutations were confirmed by sequencing the targeted regions of the receptor cDNA using Thermo Sequenase™ (Amersham Biosciences, Piscataway, NJ, USA).

5.2. Receptor expression and membrane preparation

The cDNA clones of recombinant ORL1 receptors were inserted into mammalian expression vector pcDNA3 by a method similar to that for wild-type ORL1. COS-7 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 atmosphere of 90% humidity at 37 °C. Plasmid DNA was transiently transfected into confluent COS-7

(ca. $5 \times 10^4/cm^2$) cells using the DEAE-dextran method. After 48 h, cells were harvested and homogenized to prepare membrane fractionation. The concentrations of membrane protein were estimated by the Lowry method. The prepared membrane was frozen at −70 °C before use.

5.3. Saturation binding assay

The saturation-binding assay was carried out for all the newly prepared mutant receptors. A series of reaction mixtures containing 5 μg/ml membrane protein were incubated with increasing concentrations of [^{3}H]nociceptin (158 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK) (0.05–2.0 nM) for 90 min at 25 °C in 50 mM HEPES-Tris buffer (pH 7.4) containing 0.1% BSA. Bacitracin (100 μg/ml) was added as an enzyme inhibitor. After incubation, each incubation mixture (500 μl) was filtered through glass fibre filters (GF/B; Whatman Inc., Clifton, NJ) that were presteeped with 0.5% ethylene imine polymer for 1 h, and rinsed twice with 50 mM Tris–HCl buffer (pH 7.4). Non-specific binding was determined in the presence of 10 μM nociceptin.

5.4. Competitive receptor-binding assay

The receptor-binding potencies of the synthetic peptides were assessed by competitive binding assay. Briefly, membranes (5 μg/ml), serial concentrations of synthetic peptide, and 0.05 nM [^{3}H]nociceptin were incubated for 90 min at 25 °C in the same buffer (2 ml in each tube). The computer program ALLFIT[25] was used to draw dose–response curves for the analysis. The binding potency of each peptide was estimated as the IC_{50} value, the peptide concentration at which the half-maximal inhibition is achieved.

5.5. In vitro [^{35}S]GTPγS-binding assays

The in vitro biological activities of the synthetic peptides were appraised by [^{35}S]GTPγS-binding assay. The membranes (5 μg) were suspended in 50 mM HEPES-Tris buffer (pH 7.4) containing 100 mM NaCl, 10 mM $MgCl_2$, 200 mM EGTA, and 200 μM dithiothreitol. Bacitracin (100 μg/ml) was added as an enzyme inhibitor. Each tube (100 μl) was incubated for 60 min at 25 °C with the appropriate concentration of peptides in the presence of 3 μM GDP and 100 pM of [^{35}S]GTPγS (1000 Ci/mmol; Amersham Pharmacia Biotech). Non-specific binding was determined in the presence of 10 μM GTPγS. After incubation, each mixture was filtered through glass fibre filters (GF/B; Whatman) and rinsed in the same manner as described for the radio-ligand receptor-binding assay. The functional activity was estimated as EC_{50}, that is, the peptide concentration generating 50% of the maximal observed stimulation of GTPγS binding.

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