Sensitivity of Opioid Receptor-like Receptor ORL1 for Chemical Modification on Nociceptin, a Naturally Occurring Nociceptive Peptide*

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Nociceptin or orphanin FQ is a novel neuropeptide that activates an opioid-like G protein-coupled receptor ORL1. This heptadecapeptide FGGFTGARKSARKLANQ resembles κ-opioid peptide dynorphin A but exhibits an opposite effect to make animals hyperreactive to nociceptive stimulations (Meunier, J.-C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.-L., Guillemot, J.-C., Ferrara, P., Monsarrat, B., Mazarguil, H., Vassart, G., Parmentier, M., and Costentin, J. (1995) Nature 377, 532–535; Reinscheid, R. K., Nothacker, H.-P., Bourson, A., Ardati, A., Henningsen, R. A., Bunzow, J. R., Grandy, D. K., Langen, H., Monsma, F. J., Jr., and Civelli, O. (1995) Science 270, 792–794). In the present study, it was found that guinea pig brain contains receptors to which nociceptin binds much more strongly than to ORL1 receptors expressed in human 293 cells. Although the Tyr1 → Phe substitution for dynorphin A eliminates almost completely an ability to bind to opioid receptors, the Phe1 → Tyr substitution in nociceptin was found to retain almost fully both receptor binding affinity and in vivo hyperalgesic activity in tail-flick assay. Nociceptin was extremely weak to bind to opioid receptors, while Tyr1-nociceptin exhibited 10–40 times increased affinity, especially for μ receptors, due to its N-terminal sequential identity to opioid peptides. Shortened analogs of dynorphin A are known to retain receptor binding ability and analgesic activity, whereas the removal of C-terminal hexa- or decapeptide from nociceptin totally abolished the affinity for the ORL1 receptor. These results indicated that the mode of interaction between nociceptin and ORL1 receptor is quite different from that between dynorphin and opioid receptor and that the C-terminal portion of nociceptin is crucial for receptor recognition.

The recent finding of a naturally occurring nociceptive peptide named nociceptin (1) or orphanin FQ (2) has highlighted a possibility to design a novel analgesic. Nociceptin was isolated as a ligand for ORL1 receptor whose primary structure is most closely related to those of opioid receptors (3–5). It produced hyperalgesia in rat and mouse in both hot-plate and tail-flick assays and was found to inhibit CAMP accumulation in the Chinese hamster ovary cell-expressing ORL1 receptor.

Nociceptin resembles dynorphin A, a κ-receptor selective opioid peptide (6, 7) (see Fig. 1). Both nociceptin and dynorphin A are 17-amino acid peptides and contain 4–5 basic amino acids at the C-terminal portions. It is a question of great interest whether structural similarities between nociceptin and dynorphin A not only in peptide sequences but also in their receptor structures are reflected in the structure-activity relationships of nociceptin. The N-terminal sequences of nociceptin and dynorphin A are very similar, having the same sequence of Gly-Gly-Phe at positions 2–4. Gly3 and Phe4 are essential for activities of opioid peptides. As to Gly at position 2, structural requirements appear to be diverse among opioid peptides and receptors. Chavkin and Goldstein (8) reported that Gly2 having no side chain is important to the potency of dynorphins for κ receptors. This is in contrast to the fact that small side chain substituents for Gly2 of enkephalin analogs lead to a favorable steric interaction at δ and μ receptors (9). The major difference between nociceptin and opioid peptides exists, however, in the N-terminal amino acid: Phe for nociceptin and Tyr for dynorphin A and other opioid peptides. It has been well known that the replacement of Tyr in opioid peptides by Phe, which means the removal of the para-hydroxyl group from β-phenyl, results in inactivity (10).

The importance of basic amino acid residues (Lys13, Lys11, and Arg7) to the potency of dynorphin A was demonstrated by successive removal of C-terminal amino acids (8). In particular, Arg7 was found to be an essential structural element of dynorphin A for specific recognition of κ opioid receptors. When this Arg7 was retained, the removal of the C-terminal octapeptide containing two of the other four basic amino acids sustained a full activity of dynorphin A (11–13). Dynorphin A has a basic amino acid pair of Arg-Arg at position 6–7. If dynorphin A was proteolytically excised here, the resulting peptide fragment is no longer dynorphin, producing a leucine-enkephalin molecule. On the other hand, nociceptin contains a couple of such basic amino acid pairs, namely two Arg-Lys sequences at positions 8–9 and 12–13 (see Fig. 1). It is interesting to know whether or not these pairs split nociceptin into inactive peptides.

In the present study, in order to elucidate fundamental structural essentials of nociceptin, we have synthesized Tyr1-nociceptin, nociceptin-(1–7), and nociceptin-(1–11), all by the automated peptide synthesizer ABI 470A with the Fmoc (9-fluorenylmethoxycarbonyl) synthetic strategy. Peptides were first purified by the Sephadex G-15 (Pharmacia, Uppsala, Sweden) column (2.0 × 138 cm) eluted with 30% AcOH after removal of resin and deprotection by reagent K. Further purification was carried out by...

MATERIALS AND METHODS

Peptide Syntheses—All peptides, nociceptin, Tyr1-nociceptin, Tyr14-nociceptin, nociceptin-(1–7), and nociceptin-(1–11), were synthesized by the automated peptide synthesizer ABI 470A with the Fmoc (N-(9-fluorenyl)methoxycarbonyl) synthetic strategy. Peptides were first purified by the Sephadex G-15 (Pharmacia, Uppsala, Sweden) column (2.0 × 138 cm) eluted with 30% AcOH after removal of resin and deprotection by reagent K. Further purification was carried out by...
DNA (5 μg) with 10% fetal calf serum. To generate stable transfectants, plasmid was sequenced, and a cDNA clone without unwanted mutations was selected.

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Receptor binding activities of nociceptin and its analogs for human 293 cells expressing ORL1 receptors and for guinea pig brain membranes are shown in Table I. The data are means ± S.E. determined from the assays at least in triplicate.

| Peptides           | IC50  |
|--------------------|-------|
|                    | 293 Cells | Guinea pig brain |
| Nociceptin         | 0.9 ± 0.3 | 0.016 ± 0.006   |
| Tyr1-nociceptin    | 3.0 ± 0.9 | 0.060 ± 0.006   |
| Tyr1'-nociceptin   | 1.1 ± 0.3 | 0.055 ± 0.005   |
| Nociceptin-(1-11)  | >1000    | 200 ± 100       |
| Nociceptin-(1-7)   | >1000    | >1000           |

The abbreviations used are: HPLC, high pressure liquid chromatography; i.c.v., intracerebroventricularly.

Reversed-phase HPLC was performed with a prepacked column (2.5 × 25 cm, C18-Merk LiChrospher RP-18e, 5 μm) with a linear gradient of 0.1% trifluoroacetic acid and 80% acetonitrile. The fractions containing pure peptide were pooled and lyophilized. The purity was verified by analytical reversed-phase HPLC (0.4 × 25 cm, LiChrospher 100 Rp-18e, 5 μm) and amino acid analysis.

Expression Plasmid—The cDNA clone of human ORL1 receptor was obtained by reverse transcription and polymerase chain reaction. The first strand cDNA was synthesized with 5 μg of total human brain RNA (Clontech, Palo Alto, CA) using the First strand synthesis kit (Pharmacia) according to the manufacturer's instruction. The oligonucleotide primers corresponding to the 5′-end (5′-CGGAATTCGCCTCTTCCCCGCCGCCG-3′) and the opposite strand of the 3′-end of the coding region (5′-GGTCTTAGATCATGCGGGCFCGC-3′) were synthesized based on the published sequence (3). Polymerase chain reaction was carried out with the first strand cDNA reaction mixture and these primers for 25 cycles. The amplified DNA fragment was isolated by electrophoresis on a 1.2% agarose gel, digested with EcoRI and XhoI, and cloned into the EcoRI/XhoI site of pcDNA3 (Invitrogen, San Diego, CA). Several cDNA clones were sequenced, and a cDNA clone without unwanted mutations was selected.

Cell Culture and Transfection—Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. To generate stable transfectants, plasmid DNA (5 μg) was transfected into 293 cells using the Lipofectamine method (Life Technologies, Inc.) in a 3-cm culture dish. After 24 h, cells were seeded into 10-cm dishes, and geneticin (G418) was added as an enzyme inhibitor. Dose-response curves were constructed utilizing 7–8 animals in triplicate. Statistical significance was determined by one-way analysis of variance test.

Receptor Binding Assays—Radioligand receptor binding assays were carried out essentially as described previously (14). Used radioligands were as follows: [125I-Tyr1]nociceptin (74 TBq/mmol, Amersham, Buckinghamshire, United Kingdom) for ORL1 receptor; [3H]-[H-D-Ava2, MePhe4, Gly-ol5]enkephalin ([3H]HIDAGO) (1.80 TBq/mmol, DuPont NEN) for μ opioid receptor; [3H]-[D-Ser2, Leu5]enkephalin-Thr6 ([3H]DSLET) (1.51 TBq/mmol, DuPont NEN) for δ opioid receptor; and [3H]U69593 (1.70 TBq/mmol, DuPont NEN) for κ opioid receptor. For assays using [125I-Tyr1]nociceptin, human 293 cells expressing ORL1 receptor or guinea pig brain were utilized, whereas rat and guinea pig brains were utilized for assays with opioid ligands. The membranes of these brains were obtained by reverse transcription and polymerase chain reaction. The first strand cDNA was synthesized with 5 μg of total human brain RNA (Clontech, Palo Alto, CA) using the First strand synthesis kit (Pharmacia) according to the manufacturer's instruction. The oligonucleotide primers corresponding to the 5′-end (5′-CGGAATTCGCCTCTTCCCCGCCGCCG-3′) and the opposite strand of the 3′-end of the coding region (5′-GGTCTTAGATCATGCGGGCFCGC-3′) were synthesized based on the published sequence (3). Polymerase chain reaction was carried out with the first strand cDNA reaction mixture and these primers for 25 cycles. The amplified DNA fragment was isolated by electrophoresis on a 1.2% agarose gel, digested with EcoRI and XhoI, and cloned into the EcoRI/XhoI site of pcDNA3 (Invitrogen, San Diego, CA). Several cDNA clones were sequenced, and a cDNA clone without unwanted mutations was selected.

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agonists of almost equivalent potency in the cAMP inhibition assay, and [125I-Tyr14]nociceptin was demonstrated to bind to ORL1 receptors in a saturable manner with high affinity (2). This was confirmed in the present study when [125I-Tyr14]nociceptin was assayed for intact human 293 cells expressing ORL1 receptors. The dissociation constant (Kd) was 0.52 ± 0.05 nM, and the maximal binding capacity (Bmax) was 5.4 × 10⁶ sites/cell.

In the radioligand binding assay using [125I-Tyr14]nociceptin and ORL1-expressing 293 cells, nociceptin and Tyr14-nociceptin exhibited very high binding affinity and were almost equipotent (Table I). Interestingly, Tyr1-nociceptin showed considerably high affinity for ORL1 receptors (IC50 = 3.0 nM) and was only slightly less potent than nociceptin. This indicates that the Phe1 → Tyr substitution, namely the replacement of the para-hydrogen atom by the hydroxyl group on the Phe1-phenyl group, does not affect much the receptor binding characteristics of nociceptin. This is quite different from the fact that the Tyr1 → Phe replacement eliminates completely the ability of opioid peptides to bind to opioid receptors. ORL1 receptor is clearly insensitive to the Phe1 → Tyr substitution at the first position of nociceptin.

In sharp contrast, nociceptin-(1–11) was found to be inactive, lacking completely an ability to bind to ORL1 receptor (Table I). Nociceptin-(1–11) has a sequence cleaved at the amino side of a basic amino acid pair Arg-Lys at positions 12–13 (Fig. 1). It is clear that a structural element essential for receptor binding exists in the C-terminal sequence of Arg-Lys-Leu-Ala-Asn-Gln. Nociceptin-(1–7), which has a sequence cleaved at the amino side of another Arg-Lys at positions 8–9, was also completely inactive. Although dynorphin A is also a 17-amino acid peptide, its shortened analogs lacking C-terminal tetra- to nonapeptides have been reported to be almost as potent as parent dynorphin A (11–13). All these data indicate a distinct divergence in structural frames between nociceptin and dynorphin A in interacting with their specific receptors.

**Receptor Binding Activity of Nociceptin and Its Analogs in Guinea Pig Brain.**—When the binding assays were carried out using guinea pig brain membrane preparations, [125I-Tyr14]nociceptin bound in a dose-dependent manner and showed a very high binding affinity. Kd was 0.014 ± 0.002 nM, and Bmax was 2.8 fmol/mg protein. Using this [125I-Tyr14]nociceptin, nociceptin and Tyr1-nociceptin were assayed in guinea pig brain. They exhibited a strong inhibition of the binding of [125I-Tyr14]nociceptin in a dose-dependent manner, and the IC50 values were 0.0016 and 0.0055 nM, respectively (Table I). Clearly, guinea pig brain contains the receptors to which nociceptin and its analogs can bind. Tyr1-nociceptin was here again as potent as nociceptin, while shortened analogs were extremely weak or almost inactive (Table I).

It should be noted that all these nociceptins are much more potent (about 20–50-fold) in guinea pig brain than in human 293 cells. Meunier et al. (1) have reported that the sequence of nociceptin appears to be conserved across mammalian species, showing that central amino acids of the peptide ARKSAR were identical in the human, rat, and mouse species. The present results may imply that there is a slight difference in sequence between rodent and human nociceptins. Alternatively, the receptors expressed in guinea pig brain might be different from ORL1. It is also possible that receptors of the intact cells has a ligand sensitivity different from that of receptors in membranes prepared from guinea pig brain.

**In Vivo Hyperalgesic Effect of Nociceptin and Tyr1-nociceptin.—**Nociceptin and Tyr1-nociceptin were evaluated for the in vivo activity in mice after intracerebroventricular administration. Nociceptin elicited hyperalgesia with 1 ng to 1 µg i.c.v. per mouse. At a dose of 1 ng, the reaction time was reduced by about 50%, and reaction time was further reduced with as doses increased (Fig. 2A). At the highest dose (1 µg), nociceptin induced a decrease in activity. When Tyr1-nociceptin was tested, a similar activity profile was obtained (Fig. 2B). Tyr1-nociceptin also made mice hyperreactive to nociceptive stimulation. This is certainly the reflection of its high binding affinity for ORL1 receptor.

Zhang and Yu (16) have recently reported that dynorphin A activates the potassium channel by stimulating the ORL1 receptor expressed in Xenopus oocytes. This appears to be quite rational for explaining a high activity of Tyr1-nociceptin. The sequence identity between dynorphin A and Tyr1-nociceptin is more than 40%, sharing the N-terminal tetrapeptide YGGF.

Shortened analog nociceptin-(1–11) was completely inactive. Zhang and Yu (16) reported that dynorphin A-(1–13) lacking C-terminal tetrapeptide elicits a similar efficacy for ORL1 receptor as dynorphin A, while dynorphin A-(1–11) (Fig. 1) exhibits substantially reduced receptor efficacy. This is highly relevant to the present result of inactivity of nociceptin-(1–11). These data suggest that both nociceptin and dynorphin A interact with ORL1 receptor in a similar manner.

**Binding Affinity of Nociceptin and Tyr1-nociceptin for Opioid Receptors.**—Nociceptin and Tyr1-nociceptin were assayed for opioid receptors in guinea pig brain, using specific radioligands each for δ, μ, and κ subtypes. Nociceptin bound to δ and μ receptors extremely weakly (IC50 = 0.8–7.4 µM) (Table II). Among the subtypes, μ receptors were most favorable to accept nociceptin. Nociceptin-(1–11) was completely inactive for all subtypes of opioid receptors.

When Tyr1-nociceptin was assayed, it exhibited considerably increased (8–37-fold) affinity to all these receptors. In particular, it bound to μ receptors in guinea pig and rat brains considerably strongly (IC50 = 21.7 nM). As mentioned above, Tyr1-nociceptin possesses exactly the same N-terminal sequence of opioid peptides, namely Tyr-Gly-Gly-Phe, and became much more similar to dynorphin A, a κ receptor-selective opioid ligand (Fig. 1). In spite of such sequence similarity, Tyr1-nociceptin bound to μ receptors about 10 times more preferentially than to κ receptors. These results emphasize the difference in binding abilities of nociceptin and dynorphin A against nociceptive and opioid receptors. Apparently, the C-
terminal sequence of nociceptin is crucial for recognition of ORL1 receptor.

The present study indicates that nociceptin is distinct from opioid peptides, and at least the N-terminal 12-amino acid sequence is required for full activation of ORL1 receptors.

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