the ED_{50} value was estimated to be 19.3 nM, almost the same as the reported value.[31] This agonist activity of nociceptin appears to be due to the specific interaction with ORL1 receptor in MVD, since nociceptin exhibited absolutely no binding affinity for the opioid receptors.

In contrast to the considerably high agonist activity in the GTPγS binding assay, Ac-RYYRIK-NH_2 was almost completely inactive in MVD. It exhibited only weak agonist activity (an approximately 40% reduction, but only at its highest concentration (10 μM)). Amino-free derivative H-RYYRIK-NH_2 did not at all modify the electrically induced twitches of MVD. Most of the other analogues were also found to be almost completely inactive. By contrast, Pr-RYYRIK-NH_2 and Bz-RYYRIK-NH_2 exhibited considerably high activity (over 90% inhibition of the electrically evoked twitches) all at once at their 10 μM concentration. This abrupt activity was eventually assumed to be due to their binding to the δ opioid receptor (data not shown).

The antagonist ability of acyl-RYYRIK-NH_2 analogues in the MVD assay was assessed by co-administration with nociceptin at specific concentrations. It was found that isoVa-RYYRIK-NH_2 shifts the dose–response curve of nociceptin rightward in a concentration-dependent manner, the curves being parallel to the control (Fig. 4A). This shift demonstrates that isoVa-RYYRIK-NH_2 occupies the binding site of the receptor to which nociceptin competitively binds. The extrapolated pA_2 value from the Schild plot analysis was calculated to be 9.70 (Fig. 4B). Together with the prominent antagonist activity in the GTPγS binding assay, strong antagonist activity in the MVD assay suggests that isoVa-RYYRIK-NH_2 is a highly potent competitive antagonist for nociceptin.

3. Discussion

3.1. Assay system to evaluate the antagonist activity of Ac-RYYRIK-NH_2 analogues

In this study, we attempted to develop a potent peptidic antagonist of ORL1 receptor. For evaluation of antagonist activities, there are two different types of in vitro assays—the GTPγS binding assay and the MVD muscle assay. Since the GTPγS binding assay is carried out for the recombinant receptor preparations, especially by using G protein-fused receptor (hORL1-$G\alpha_o$ in this study), we are able to evaluate the ability of the compound solely for a single type of receptor. By contrast, the muscle preparations usually contain several different types of receptor families. For instance, MVD consists of all three opioid receptor subtypes, particularly the δ-opioid receptor,[31] in addition to the ORL1 receptor. In the present study, in both the GTPγS binding assay and the MVD muscle assay, isoVa-RYYRIK-NH_2 was almost completely inactive and at the same time showed specific antagonist activity, indicating that this compound is a genuine antagonist of the nociceptin/ORL1 ligand–receptor system. This result is in sharp contrast to the activities of the parent compound Ac-RYYRIK-NH_2, which is partially active in both the assays.

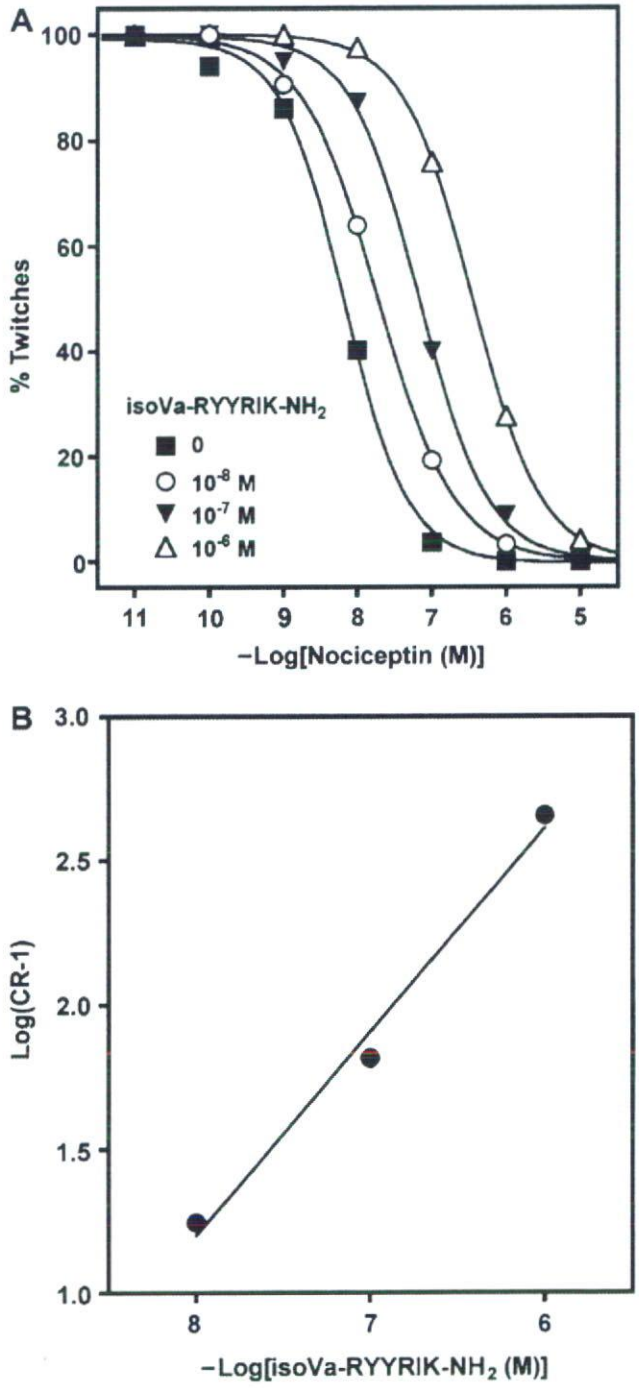

Figure 4. The antagonist activity isoVa-RYYRIK-NH_2 in the MVD muscle assay (A) and the Schild plot analysis of this assay (B). The extrapolated pA_2 value from the plot analysis was calculated to be 9.70 ± 0.32.

Antagonist with no agonist activity is a highly specific molecular tool important for exploration of the inhibition mechanism of receptor activation. We first tried to establish the proper assay system to evaluate the antagonist activity. The results described above indicate that the assays using GTPγS-fused receptor are fundamentally important for evaluating both agonist and

antagonist activities. Since the report indicated that there are some species differences between human and rodent ORL1 receptors,[10] we examined both types. However, in the present study we found no crucial differences in the receptor binding and the GTPγS binding assays using rat and human ORL1 receptors. As long as we use recombinant ORL1 receptors, it appears to be possible to use the receptor molecules of any aminal species.

Eventually we decided to utilize the human ORL1 receptor to evaluate the activities of acyl-RYYRIK-NH_2 series. In order to more effectively assess G-protein activation, we fused ORL1 with the G protein α subunit. Obviously, fusion genes between the GPCR receptor molecule and its coupled G protein α-subunit do not exist in nature. However, such fusion proteins show a much enhanced signaling efficiency in the cells transfected. Indeed, the GTPγS-fused ORL1 receptor exhibited a high stimulatory enhancement in a dose-dependent manner much more effectively than the ORL1 receptor with no GTPγS fusion. The present results are the first data obtained from assays using the human GTPγS-fused ORL1 receptor, the assay using a rat GTPγS-fused ORL1 receptor being reported by Molinari et al.[33] This assay system enabled us to estimate the detailed activation levels against the acyl-RYYRIK-NH_2 series having similar N-terminal acyl groups.

3.2. Structural determinants of N-terminal acyl–alkyl group for antagonist activity

We attempted to optimize the size of the N-terminal acyl group that may fit the binding pocket of the human ORL1 receptor. We first selected a linear acyl–alkyl group series for acyl-RYYRWK-NH_2. As shown in Table 2, the highest receptor-binding activity was attained by For-RYYRIK-NH_2 (IC_{50} = 0.66 nM), but it immediately became apparent that even Ada-RYYRIK-NH_2 shows significantly potent binding ability (3.42 nM) to the ORL1 receptor. Since the N-terminal free analogue H-RYYRIK-NH_2 is intrinsically inactive, these results imply that one of the most important structural elements for the acyl group in acyl-RYYRIK-NH_2 is the binding of the carbonyl group (C=O) to the ORL1 receptor. Since the different sizes of the acyl–alkyl group afford different strengths of antagonist activity, the size of the alkyl group appears to be the determinant of the inability of acyl-RYYRIK-NH_2 to activate the receptor as a basal condition of the antagonism. Our results indicate that there is an optimal size of alkyl group for the antagonism. The analogue having the vareryl group, Va-RYYRIK-NH_2, was found to be the strongest antagonist among a series of C_nH_{2n+1}-CO-RYYRIK-NH_2 with the non-branched alkyl (C_nH_{2n+1}) group.

Although the literal reason is not apparent, N-terminal modification has also been reported for Ac-RYYRWK-NH_2,[34,35] a derivative of Ac-RYYRIK-NH_2. The length of the aliphatic methylene chain was characterized as a determinant of efficacy, which decreases with acetyl through pentanoyl(=vareryl) and then increases up to heptanoyl. However, in the present study, isoVa-RYYRIK-NH_2 was eventually found to be the strongest antagonist. As an antagonist, isoVa-RYYRIK-NH_2 was definitely stronger in the GTPγS binding assay than Va-RYYRIK-NH_2. It is clear that the length of the aliphatic methylene chain is not merely a determinant of the receptor efficacy. The important determinant for the antagonism induction is the molecular size and shape of the acyl–alkyl group.

When the biological activities of Bu(=$CH_3CH_2CH_2CO$)-RYYRIK-NH_2 (E_{max} = 21%), isoVa(=$(CH_3)_2CHCH_2CO$)-RYYRIK-NH_2 (≈0%), and *t*-BuAc(=$(CH_3)_3CCH_2CO$)-RYYRIK-NH_2 (33%) were compared (Table 2), only isoVa-RYYRIK-NH_2 was found to be a pure antagonist. The methyl branching at the Cγ position is a crucial determinant for the antagonism, indicating that the binding site for the isovareryl group with the N-terminal $(CH_3)_2CHCH_2$– captures or arrests the peptide RYYRIK-NH_2 so as to not activate the receptor.

3.3. A possible binding site of isoVa-RYYRIK-NH_2

By the photo-affinity labeling method using [Bpa^2, ^{125}I-Tyr^3]Ac-RYYRWK-NH_2, the binding site of Ac-RYYRWK-NH_2 was suggested to be the portion limited from Gln^{107} in the transmembrane #2 (TM2) to Leu^{113} in the extracellular loop #1 (EL1) of human ORL1 receptor, Gln-Gly-Thr-Asp-Ile-Leu-Leu.[36] This portion is different from the nociceptin-binding site reported by Mouledous et al.[37] They utilized [Bpa^{10}, ^{125}I-Tyr^{14}]nociceptin for labeling and identified the portion of ORL1[296–302] (Thr^{296}-Ala-Val-Ala-Ile-Leu-Arg^{302}, EL3-TM7) as a nociceptin-binding site. There is no overlap between these two binding sites. However, these two ligands, Ac-RYYRI(or W)K-NH_2 and nociceptin, should share the same binding site, at least in part, because they can displace [^3H]nociceptin in the human ORL1 receptor-binding assay.

Displacement of [^3H]nociceptin is feasible only when acyl-RYYRIK-NH_2 or acyl-RYYRWK-NH_2 occupies the same receptor site for [^3H]nociceptin in ORL1. Thus, if the portions ORL1[107–113] and ORL1[296–302] are specific for acyl-RYYRI(or W)K-NH_2 and nociceptin, respectively, the binding site shared by these peptides must exist in region(s) other than these portions in ORL1. Both acyl-RYYRWK-NH_2 and nociceptin are rich in basic amino acids (Arg and Lys). The most likely portion shared by these peptides is to bind such residues rich in basic amino acids.

One of the most important residues in acyl-RYYRI(or W)K-NH_2 and nociceptin is their N-terminus. The N-terminal free amino group is essential for nociceptin to bind to ORL1 for the receptor activation, but the N-terminal acyl group, particularly the isovaleryl group, is crucial to bind to ORL1 for the receptor inactivation. To identify the particular receptor site for binding of the N-terminal region is key to revealing the receptor activation/inactivation mechanisms of ORL1 receptor.

isoVa-RYYRIK-NH_2 is definitely superior to the peptide and nonpeptide antagonists reported for ORL1 receptor to date. Among the peptide antagonists, [Nphe^1]nociceptin(1–13)–NH_2 has been announced as a pure and potent antagonist. However, as a pure antagonist, isoVa-RYYRIK-NH_2 is much more potent (three orders of magnitude more potent) than [Nphe^1]nociceptin(1–13)–NH_2. When compared with the most potent antagonist of J-113397,[30] isoVa-RYYRIK-NH_2 was found to be as active as this nonpeptide J-113397, exhibiting almost the same antagonist activity. The ligand-binding site of ORL1 receptor is thought to be different between the peptide ligand and the nonpeptide ligand. Thus, the usefulness of isoVa-RYYRIK-NH_2 as an antagonist should be emphasized as a specific competitor of nociceptin. This is particularly important to elucidate the molecular mechanism of the nociceptin/ORL1 ligand–receptor system.

4. Conclusion

isoVa-RYYRIK-NH_2 was found to be an efficient nociceptin antagonist with high affinity for the ORL1 receptor. isoVa-RYYRIK-NH_2 is also significant due to its pure antagonist activity. These results were obtained by the two different efforts to eliminate the agonist activity of Ac-RYYRIK-NH_2 and to retain antagonist activity. isoVa-RYYRIK-NH_2 appears to be a valuable molecular tool in structure–activity studies for the nociceptin/ORL1 ligand–receptor system.

5. Experimental

5.1. Peptide syntheses

All peptides used in this study were synthesized (0.15 mmol scale) by the manual solid-phase method using Fmoc-chemistry. Peptides were synthesized using Fmoc-NH-SAL resin, and the coupling reaction was carried out with 2-(1*H*-benzotriazole)-1-1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of 1-hydroxy benzotriazole (HOBt) dissolved in *N*-methylpyrrolidone (NMP) and *N*,*N*-dimethylformamide (DMF). Each coupling reaction was examined for completion by means of the Kaiser ninhydrin test. N-terminal modifications of acylation were carried out at the end of each synthesis cycle by using acyl chloride (R-CO–Cl). A N-terminal free analogue of Ac-RYYRIK-NH_2 was obtained together with the parent peptide without acetylation.

After completion of the synthesis, the peptides were liberated from the resin using a cocktail reagent containing 90% trifluoroacetic acid (TFA), 2.5% water, 5% phenol, 5% thioanisole, and 2.5% ethanedithiol. 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). 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, 5 ml/min; temperature, 25 °C; and UV detection, 230 nm.

The peptide purity was verified by analytical RP-HPLC (4 × 250 mm, Cica-Merck LiChrospher 100 RP-18, 5 μm) using the same elution conditions, except for a flow rate of 0.5 ml/min. The mass spectra of peptides were measured on a mass spectrometer Voyager™ DE-PRO (PerSeptive Biosystems Inc., Framingham, MA, USA) using the matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) method.

5.2. Cell culture and transfection

All receptors were transfected in COS-7 cells with human receptor cDNA. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in the 100 U/ml penicillin, and 100 μg/ml streptomycin. The plasmid DNAs (20 μg) of human ORL1-Gα receptor were transiently transfected into 90% confluent COS-7 cells (0.5×10^5 per cm^2) in a 60 cm^2 culture plate by using TransFectin Lipid Reagent (Bio-Rad Laboratories, Hercules, CA, USA). 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, 1 mM dithiothreitol (DTT), and 11% saccharose (pH 7.4), and homogenized with the Potter–Elvehjem homogenizer (50 strokes). The homogenate was centrifuged for 10 min at 1000*g* (4 °C). The supernatant was centrifuged again for 20 min at 24,000*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, USA). The prepared membrane was frozen at −80 °C until use.

5.3. Receptor-binding assay

The receptor-binding assay with cell membranes was conducted in a 96-well format. The receptor-binding potencies of synthetic peptides were assessed by the radio-ligand receptor-binding assay using COS-7 cell membrane preparations expressing human ORL1-Gα fusion receptors. Each well of the 96-well plate (300 μl) containing 2–3 μg/ml membrane protein, a series of concentrations of synthetic peptide, and 0.05 nM [^{3}H]nociceptin (158 Ci/mmol; Perkin-Elmer Life and Analytical Sciences, Boston, MA) were incubated for 90 min at 25 °C in 50 mM Hepes–Tris buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA). Bacitracin (100 μg/ml) was added as a protease inhibitor. To coat the filter surface, plates were soaked in 0.5% ethyleneimine polymer aqueous solution for 30 min.

After incubation, the mixture was filtered through the glass fiber UniFilter GF/B plate using the FilterMate Harvester (Packard Instrument, Meriden, CT, USA).

Twenty microliters of MicroScinti40 (Packard) was added to each well. The plates were sealed with TopSeal (Packard) and read on the TopCount (Packard) for 3 min per well. The computer program ALLFIT[38] 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.

5.4. [^{35}S]GTPγS binding assay

The in vitro biological activity of synthetic peptides was assessed by the [^{35}S]GTPγS binding assay. Receptor-mediated G-protein activation was measured as described previously.[28] The membranes (5–10 μg) were suspended in 50 mM Hepes–Tris buffer (pH 7.4) containing 100 mM NaCl, 10 mM $MgCl_2$, 200 μM EGTA, and 200 μM DTT. Each well (100 μl) was incubated for 1 h at 25 °C with peptides of appropriate concentrations in the presence of 3 μM GDP and 200 pM of [^{35}S]GTPγS (1000 Ci/mmol, GE Healthcare Biosciences, Buckinghamshire, UK). Nonspecific binding was determined in the presence of 10 μM GTPγS.

After incubation, the reaction mixture was filtered through the glass fiber UniFilter GF/B plate and washed in a similar manner as described for the radio-ligand receptor-binding assay. The activity was estimated by calculating the EC_{50} value, which exhibits the concentration inducing a 50% activity of its own maximal stimulation. The antagonist activity was measured by the concentration–response curves of the nociceptin, which were pictured in both the absence and presence of increasing concentrations of the test compound. The pA_2 value was also estimated to reveal antagonist activity according to the method of Arunlakshana and Schild.[39]

5.5. MVD muscle assays

The in vitro biological assay was carried out using mouse vas deferens (MVD) of male ICR mouse (25–35 g) as described by Hughes et al.[40] The tissue was mounted in a 5-ml organ bath (Panlab s.l., Barcelona, Spain) containing aerated (95% O_2/5% CO_2) Krebs–Ringer solution ((concentrations in mM) NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.8, and glucose 10) at 37 °C. An initial tension of 300 mg was applied. The tissue was stimulated between the alloy wire electrodes using pulses of 1-ms duration with a frequency of 0.1 Hz at the maximal voltage. The electrically induced contractions were recorded using a force transducer (Panlab s.l.) and a PowerLab/4sp (ADInstruments Pty, Chastle Hill, Australia) multichannel polygraph. Digital stimulators (Panlab s.l.) were used for the electrical stimulation.

The agonist potency of compounds was determined by depicting a concentration–response curve to calculate the ED_{50} value. The percent inhibition of the stimulation-induced contraction produced by each agonist was plotted against the log agonist concentration. ED_{50} is defined as the concentration of agonist producing 50% of the maximum effect attainable by that agonist. For experiments to measure the antagonism, the test sample was added to the bath 15 min prior to addition of nociceptin as agonist. The concentration–response curves of the agonist were pictured in both the absence and presence of increasing concentrations of the test compounds, and the pA_2 values were then calculated.

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The Output Mechanism of Circadian Pacemaker Neuropeptide PDF in the Regulation of Bimodal Locomotor Distribution

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Animals usually exhibit a bimodal morning and evening activity profile in the circadian cycle. Pigment-dispersing factor PDF is an 18-mer neuropeptide involved in the circadian output of insect locomotor activity. However, the mechanism how does PDF regulate such bimodal activities have not been well understood. We here propose a model output mechanism in relation to the circadian clock protein PERIOD isoforms which exhibit a bimodal expression.

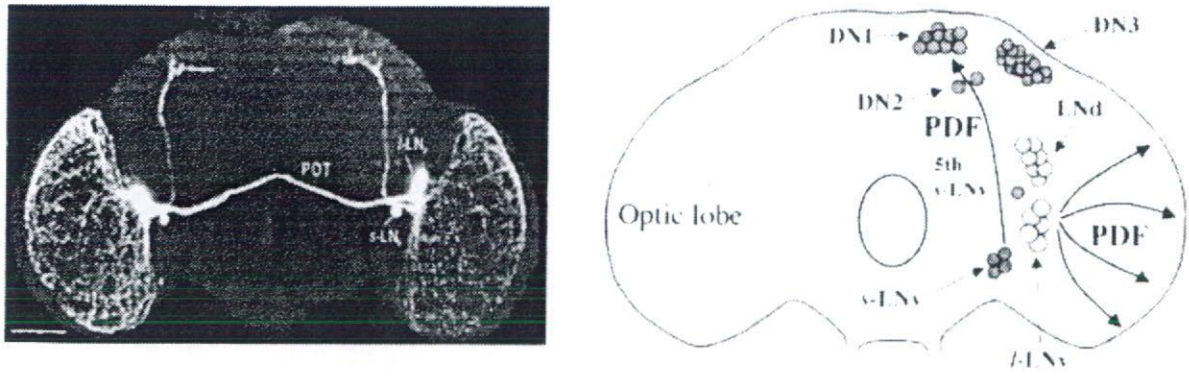
Keywords: circadian rhythm, evening oscillator, morning oscillator, *pigment-dispersing factor* (PDF), *period*

Introduction

Pigment-dispersing factor PDF is an 18-mer peptide involved in the circadian clock as a principal neuromodulator [1-3]. Its importance in transmitting the circadian rhythm of the locomotor activity is well acknowledged. The small (*s*-) and large (*l*-) LNv (ventral lateral neuron) uniquely express PDF that drives a rhythmic behavior especially under the constant darkness (Fig. 1). The protein PERIOD is an essential component in the circadian molecular mechanisms of the fruit fly *Drosophila melanogaster* (Fig. 2A). The circadian clock forms a transcriptional negative-feedback loop containing PERIOD as one of the clock protein components [4].

Animals usually exhibit a bimodal distribution of activities in the circadian cycle. The idea that two circadian oscillators exist individually has been proposed to explain such bimodal activity rhythms. In *Drosophila*, specific neuronal groups, namely the group of four *s*-LNv neurons and the group of LNd and 5th *s*-LNv, has been dedicated to morning and evening behaviors, respectively [5,6]. However, little is known about the molecular mechanisms of the oscillator systems present in these neurons.

We have hypothesized that the alternative splicing variants of PERIOD protein are required independently to drive the two oscillators, since the time-dependent expression and secretion of PDF in the LNv should be regulated by the two independent oscillators, involving individual PERIOD protein. We assumed the



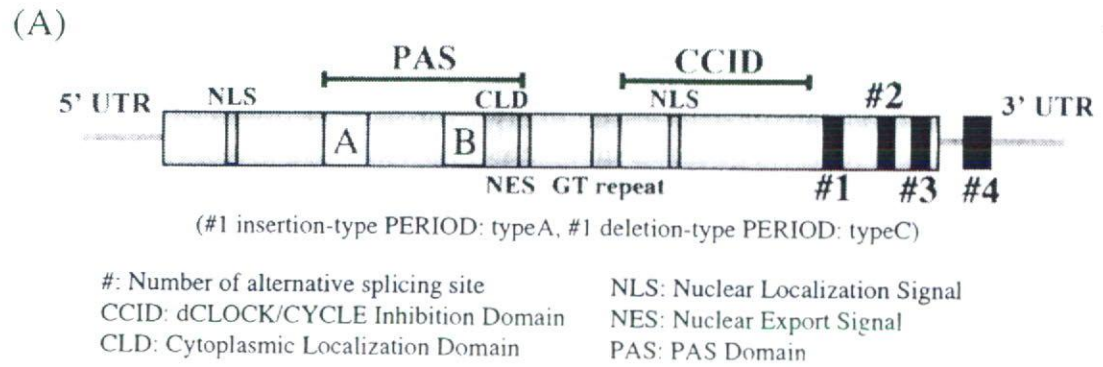
PDF-expressing neurons	Projection
s-LNvs	Dorsal neuron
l-LNvs	Optic lobe

Fig. 1. PDF-expressing neuronal groups in the fly D. melanogaster

variant isoforms to be such key proteins. Then, we attempted to propose a model for the regulation of PDF expression and secretion by PERIOD bimodality. We here describe that *Drosophila* indeed has several different alternative splicing variants of *period* and the resulting PERIOD, both of which are in a bimodal distribution of expression day by day.

Results and Discussion

In the present study, for elucidation of the output mechanism of PDF, we first attempted to reveal the bimodal distributions of *period* mRNA in the fruit fly *D. melanogaster*. The circadian expression profiles of *period* mRNA were measured by



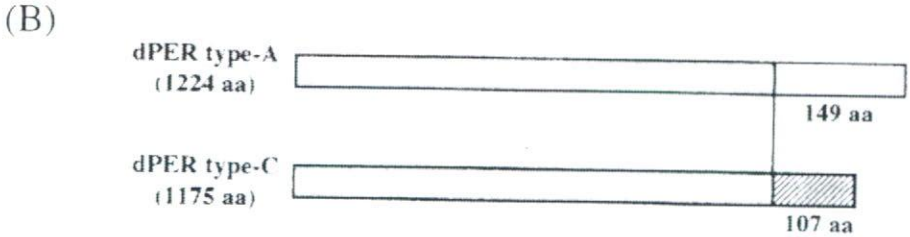


Fig. 2. Schematic structures of D. melanogaster *PERIOD and its isoforms*

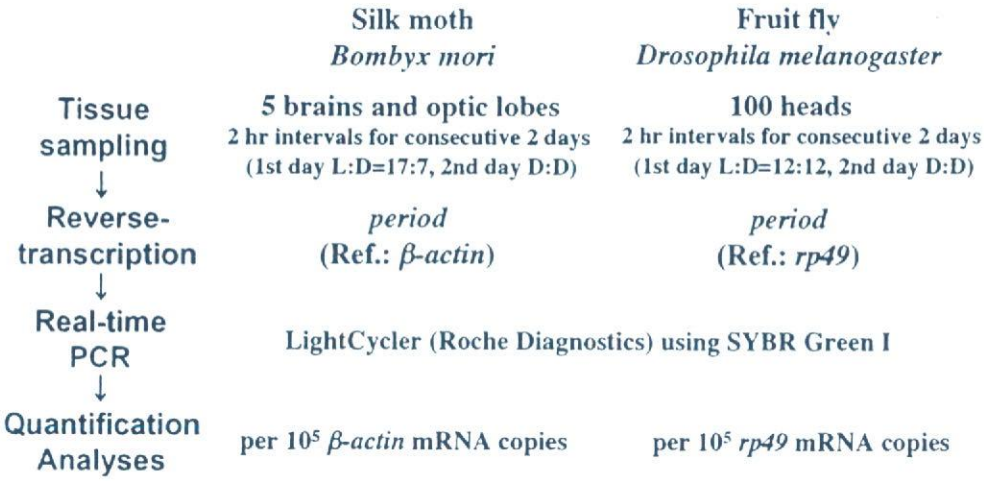


Fig. 3. Experimental procedures for quantitative real-time PCR analysis.

the quantitative real-time PCR. Total RNA samples were extracted from heads 100 in total everytime of the fruit fly every two hours during the first day in L:D=12:12 (a light/dark cycle of 12 h and 12 h) and the second day in DD (constant darkness) (Fig. 3).

We eventually found four alternative splicing sites for *Drosophila period* mRNA. Three successive introns at the C-terminal region were denoted as the site #1, #2, and #3, which involve 70-base, 64-base, and 58-base, respectively. The alternative splicing site #4 has been previously reported as a 89-base site in the 3' UTR. We succeeded to identify all of 16 possible splicing variants produced by all the insertion/deletion combinations at the 4 respective sites. Based on these gene structures, we predicted that there are 5 PERIOD protein isoforms. For real-time PCR, the alternative splicing sites were examined to quantify the insertion-type and deletion-type mRNAs.

We designed PCR primers for quantification of the each insertion- and deletion-type mRNA isoforms. For *Drosophila period* mRNA genes, real-time PCR analyses revealed that the total mRNA exhibits the expression peaks at ZT 13 or CT 13. ZT (zeitgeber time) denotes the time entrained by environmental time cues, whereby lights on is ZT0 and lights off is ZT17. CT (circadian time) denotes the time entrained by no environmental time cues. As to the quantification of each deletion-type isoform at the alternative splicing sites #1, #2, and #3, the expression profiles were found to be similar to that of the total mRNA described above. On the other hand, in the quantifications of insertion-type isoforms, a unique sub-peak was found at the morning. The evening peak appeared to have rather larger sub-peaks. However, it should be noted that these sub-peaks rapidly disappeared to place the condition from LD to DD. The analysis of biosynthesis of mRNA genes clearly indicated that the alternative splicing events at the sites #1, #2, and #3 are light-sensitive. This should be followed by PERIOD protein biosynthesis.

To elucidate whether or not the morning peak is made by PERIOD protein isoforms, we carried out the western blotting analyses. We focused on the insertion and deletion of the alternative splicing site #1. The deletion-type PERIOD protein was denoted as type-A, while the insertion-type was type-C (Fig. 2B). They have different amino acid sequences at the C-terminals. The antibodies used were anti PERIOD N-terminus antibody and newly prepared anti PERIOD type-C mouse monoclonal antibody.

We carried out the western blotting analysis for quantification profile of the type-C PERIOD protein isoforms. We depicted an expression profile for each type of

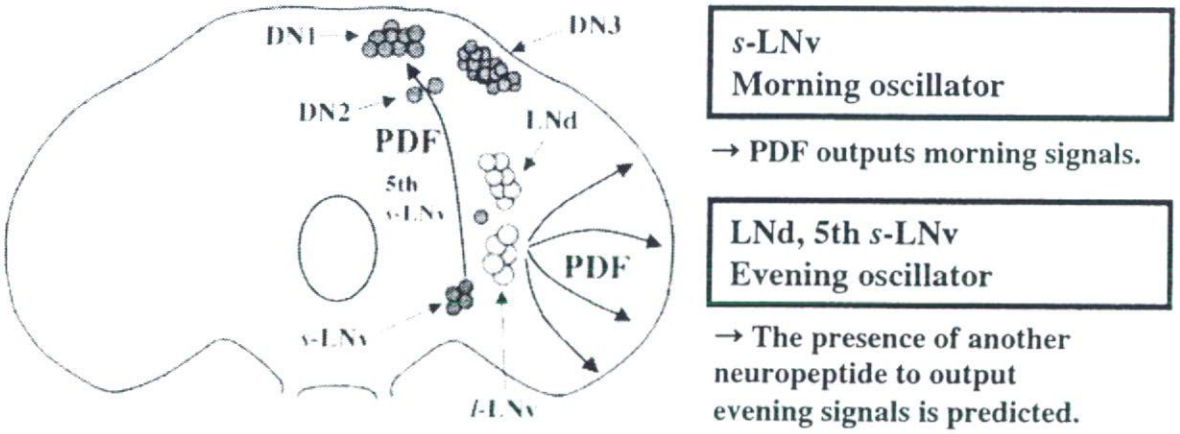


Fig. 4. Schematic figures of D. melanogster *PERIOD*

isoforms. The peak profiles were found to differ under the conditions of either LD or DD. Under the L:D=12:12 condition, there were two protein peaks at ZT 5 and ZT 15. The profile was definitely different from that of PERIOD type-A. Under DD condition the bimodal expression was disappeared. At the first day in DD, the expression profile of PERIOD type-C came to be similar to that of type-A. Interestingly, the results suggested that biosyntheses of type-C mRNA and proteins are light-sensitive, but not thermo-sensitive.

For the *period* mRNA genes in the silk moth *Bombyx mori*, we have also identified three alternative splicing sites containing 15-base, 15-base, and 129-base. The isoform produced by the alternative splicing at the site #3 were found to differ in their production. The expression of insertion-type peaks in the morning, whereas that of deletion-type peaks in the evening. Therefore these *period* isoforms are expected to involved in the morning and evening oscillators.

All the present results suggest that the specific set of PERIOD isoforms should be required for either morning oscillator or evening oscillator. Since PDF knockout flies exhibit only evening activity in the locomotor rhythm, PDF-expressing neurons should drive the morning oscillator. Although we have not revealed which neuronal group expresses PERIOD type-C, it is predicted that PERIOD type-C expresses in either *s*-LNv or LNd and 5th *s*-LNv (Fig. 4). The evening oscillator is said to be present at the LNd and 5th *s*-LNv, and these neurons do not express PDF. The model strongly suggests the presence of another unidentified neuropeptide to output evening activity (Fig. 4).

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Differential Receptor Recognition by Dmt-Containing Enkephalin Dimers Cross-Linked by Phenylenediamines

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Opioid receptors such as δ, μ, and κ subtypes are suggested to exist as a dimer, but its molecular determinants are still unknown. We previously reported that bivalent ligands with two binding cores cross-linked by a spacer exhibited the bivalent interaction mode for each specific receptor. In particular, the inactive enkephalin fragment Tyr-D-Ala-Gly was found to activate the μ receptor by its dimerization. In the present study, we attempted to prepare the structurally constrained analogs of DTRE2 in order to attain a molecular tool for evaluating the organization of receptor dimerization.

Keywords: enkephalin, enkephalin dimers, G protein-coupled receptors (GPCRs), opioid receptors

Introduction

G protein-coupled receptors (GPCRs) comprise the largest family of transmembrane receptors. Opioid receptors (ORs) such as δ, μ, and κ are member of GPCR and all of these receptor subtypes are suggested to exist as dimers. Many lines of evidence have revealed that opioid receptors are present as either homodimer or heterodimer in rat brain. A bivalent ligand with two binding cores cross-linked by a spacer would be capable to interact with two receptor molecules simultaneously [1]. This would lead the increase in affinity, selectivity, and probably biological activity.

We previously reported that DTRE2, (H-Tyr-D-Ala-Gly-NH-CH_2-)$_2$, a dimer of inactive N-terminal tripeptide of enkephalin (TRE) cross-linked by ethylenediamine, bound to the μ receptor considerably strongly [2]. In this DTRE2 dimer, the structural element essential for receptor binding is definitely the N-terminal Tyr

ortho-, meta-, para-DTREϕ *ortho-, meta-, para-Dmt*-DTREϕ

Fig. 1. Chemical structure of DTREϕ and Dmt-DTREϕ

residues.

Results and Discussion

We first dimerized TRE monomer with *ortho*-, *meta*-, and *para*-phenylenediamines, and then replaced Tyr with 2',6'-dimethyl-L-Tyr (Dmt) [3]. These Dmt-DTREϕ analogs were synthesized by three-step coupling reaction; *i.e.*, coupling, elongation, and then deblocking. Here, 'ϕ' designates the benzene ring. Dmt-DTREϕ dimers synthesized were purified by silica gel column chromatography (Silica Gel 60, 1.6 x 60 cm) eluted with $CHCl_3$:MeOH (19:1 v/v), and protected peptides were verified by the elementary analysis. The final products of Dmt-DTREϕ dimers were purified by preparative RP-HPLC with a preparative column (25 x 250 mm, Cica-Merck LiChrospher RP-18(e), 5 μm). The purity of peptides was verified by analytical (RP)-HPLC (4 x 250 mm, Cica-Merck LiChrospher 100RP-18, 5 μm). The mass spectra were measured on a mass spectrometry Voyager^{TM} DE-PRO (PerSeptive Biosystems Inc., Framingham, MA) with the method of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) to identify the mass speak of final compounds.

The opioid receptors are known to couple with $G_{\alpha o}$ protein, which is heterogeneous αβγ trimer with a GDP molecule bound to the α-subunit noncovalently. In the present study, we utilized not only rat δ, μ and κ ORs, but also $G_{\alpha o}$-fused ORs in order to assess the receptor activation more effectively.

All receptors were transiently expressed on COS-7 cells, and the membrane preparations were used for saturation binding assay, competitive binding assay, and [^{35}S]GTPγS functional assay. The expression efficiency of δ, μ, κ-OR and δ, μ, κ-OR-G_{α}o were evaluated by the saturation binding assay using [^{3}H]deltorphine II for δ receptor, [^{3}H]DAGO for μ receptor, and [^{3}H]U69593 for κ receptor. The dissociation constant K_d of each receptor was calculated from the Scatchard analysis. It was found that both fused and non-fused receptors interact with the ligands equally well.

In the receptor binding assay, newly synthesized ortho- and meta-Dmt-DTREϕ dimers exhibited drastically increased binding affinity for μ receptor subtype (Table 1). In particular, *ortho*-Dmt-DTREϕ became extremely active (almost 80 times more potent than *ortho*-DTREϕ) for the μ opioid receptor. This is quite likely, since the parent *ortho*-DTREϕ was considerably potent for the μ receptor (Table 1), despite that other DTRE dimers (*meta*-DTREϕ and *para*-Dmt-DTREϕ) were almost completely inactive. Indeed, *para*-Dmt-DTREϕ exhibited no affinity for all the receptor subtypes in spite of Dmt-substitution.

However, surprisingly, *meta*-Dmt-DTREϕ increased remarkably the binding affinity for the μ receptors. It's IC_{50} value was almost compatible with that of *ortho*-Dmt-DTREϕ. The reason of this very high binding activity attained by *meta*-Dmt-DTREϕ is quite unexpected, since its Tyr-containing analogue

Table 1. Binding potency of Dmt DTRE dimers with ORs.

	Binding Potency (IC_{50}: nM)		
	μ: [^{3}H]DAMGO	δ: [^{3}H]Deltrophin II	κ: [^{3}H]U-69593
ortho-Dmt-DTREϕ	1.05 ± 0.98	18.6 ± 8.36	6.05 ± 3.06
ortho-DTREϕ	78.4 ± 0.60	1430 ± 55	>10,000
meta-Dmt-DTREϕ	1.59 ± 0.89	37.6 ± 36.4	6.06 ± 1.19
meta-DTREϕ	>10,000	>10,000	N. B. *
para-Dmt-DTREϕ	>10,000	N. B. *	N. B. *
para-DTREϕ	N. B. *	N. B. *	N. B. *
Deltorophin II	2.26 ± 0.98	-	-
DAMGO	-	5.36 ± 1.88	-
U-69593	-	-	5.80 ± 3.05

*N.B.: Not Bound

meta-DTREϕ was inactive. The activation by the Tyr→Dmt substitution is so dramatic, since the structural change is very subtle in their chemical structures. Apparently, the incorporation of Dmt brought about the conformation change, in which Dmt constrained the steric structure of dimer only to the bioactive conformation.

Dmt-DTREϕ dimers were also examined in the [^{35}S]GTPγS binding assay to assess their receptor activation ability. For the $G\alpha_o$-fused μ receptors, *para*-Dmt-DTREϕ was almost completely inactive as expected from the receptor binding activity. On the other hand, *ortho*-Dmt-DTREϕ and *meta*-Dmt-DTREϕ were both very active in this functional assay. *ortho*-Dmt-DTREϕ exhibited very high activity (approximately 140% E_{max} of μ-ligand DAGO) for the μ receptor. When we

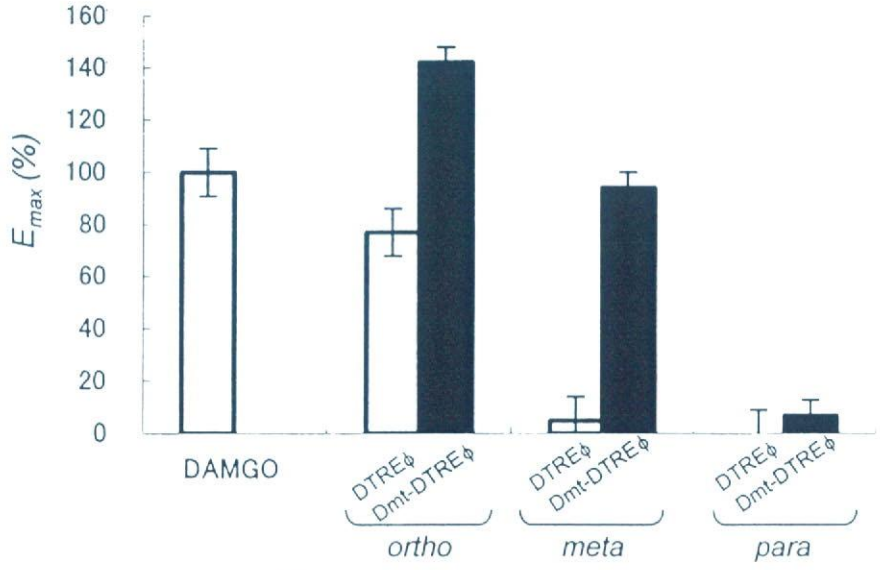

Fig. 2. Stimulation of [^{35}S] GTPγS binding to μ-OR-$G_{\alpha o}$ by Dmt-DTRE dimers.

compared its activity with that of DTREϕ dimer, about 60% activity enhancement was attained by the Tyr→Dmt substitution. It is now important to examine the question whether or not this activity enhancement was brought about by the bivalent interaction.

meta-Dmt-DTREϕ was found to be biologically active, implying that this analogue is a distinct agonist. Although Tyr → Dmt substitution for *meta*-DTREϕ cause this dramatic activity conversion, namely, from the inactivity to the high activity almost equivalent to DAGO, the exact reason is not clear. At this moment, it is important to clarify whether or not *ortho*-Dmt-DTREϕ and *meta*-Dmt-DTREϕ do interact with the μ-receptor in the same way. To answer these question, detailed structure-activities studies of these dimeric peptides are in progress in our laboratory.

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Structural Requirement of Housefly FMRFamide Peptides in Its Receptor Activation

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FMRFamide peptides contain the C-terminal structure of Phe-Met-Arg-Phe-NH_2. We have recently achieved the cDNA cloning of FMRFamide and its receptor in the housefly Musca domestica. *In the present study, we performed the structure-activity studies by using the truncated analogues of* Musca *FMRFamides and site-directly mutated* Musca *FMRMamide recepor. It was shown that the C-terminal hexapeptide sequence of DNFMRF-NH_2 is crucial for receptor activation.*

Keywords: FMRFamide, G protein-coupled receptor, insect, reporter gene assay

Introduction

Peptides containing C-terminal FMRF-NH_2 and its related structures are members of a large family of structurally related peptides found in both invertebrate and vertebrate species. In insects, these peptides are called FMRFamide-related peptides (FaRPs) and three major families have been reported to date, including FMRFamides, sulfakinins, and myosuppressins [1]. We have recently elucidated the whole structure of FaRPs in the housefly *Musca domestica* by the cDNA cloning analysis, and found 13 kinds of 17 FaRPs [2]. It is remarkable that only one peptide is common to both *Drosophila* FMRFamides and *Musca* FMRFamides, although both species belong to the same taxonomic groups of Diptera.

We succeeded recently in cDNA cloning of the *Musca* FMRFamide receptor (designated as *Musca* FR) by reverse transcribe-PCR using degenerated primers designed from the *Drosophila* FR cDNA sequence [3]. This is the second fastest finding of insect FRs. *Musca* FR is a member of G protein-coupled receptor (GPCR), and is very similar to *Drosophila* FR. The amino acid sequence identity and similarity of these two FRs are 64% and 83%, respectively. These FRs are predicted as a class-A GPCR by the computer-assisted Internet program GRIFFIN (http://griffin.cbrc.jp/). It should be noted that no disulfide bond is present between the first extracellular loop (EL1) and EL2, unlike most of other GPCRs.

We have established a novel reporter gene assay system for FRs and FMRFamide peptides by using a cAMP response element (CRE)-luciferase construct. *Musca* FR was found to be activated by all of 13 kinds of *Musca* FMRFamides, but the

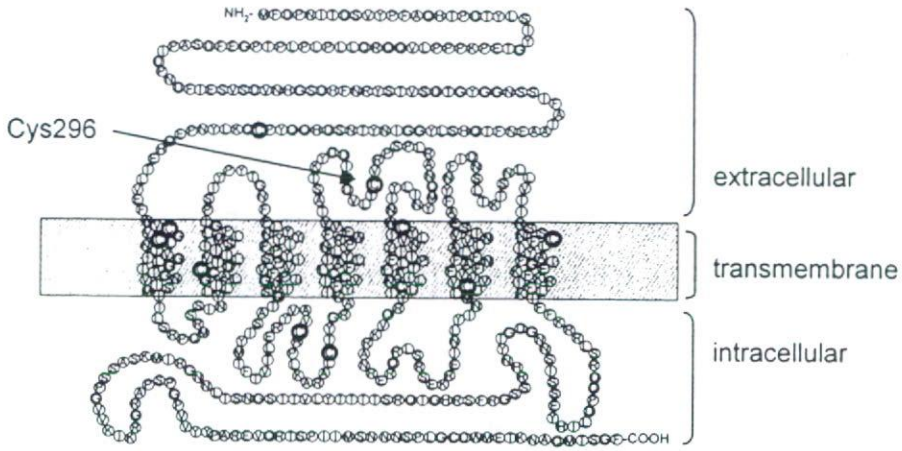

Fig. 1. The structural topology prediction of the Musca *FMRFamide receptor.*

levels of luciferase activities of FaRPs found to vary and PDNFMRF-NH_2 showed the highest activity. In the present study, we performed the structure-activity studies to elucidate the structural elements important for receptor activation. To this end, we prepared the truncated analogues of PDNFMRF-NH_2. The site-directly mutated *Musca* FR with Cys→Ala substitution in EL2 was also prepared to examine whether or not the mutant FR attains a full activation.

Results and Discussion

As to PDNFMRF-NH_2, one of *Musca* FMRFamide peptides, we synthesized the truncated analogues DNFMRF-NH_2 and NFMRF-NH_2. For direct measurement of the specific activity by the reporter gene assay, HEK293 cells expressing *Musca* FR was used together with a CRE-luciferase construct. N-terminal Pro(=P)-lacking DNFMRF-NH_2 was found to elicit almost the same activity as the parent PDNFMRF-NH_2. However, NFMRF-NH_2 lacking N-terminal Pro-Asp(=PD) dipeptide showed significantly weakened activity (about 5-fold less active). These results clearly indicated that the minimum bioactive structure of PDNFMRF-NH_2 is DNFMRF-NH_2, but not NFMRF-NH_2, and thus the Asp residue adjacent to Pro and Asn is definitely important for this activation. It should be noted that PDNFMRF-NH_2 is the sole peptide that is encoded in both *Musca* and *Drosophila* FMRFamides genes. PDNFMRF-NH_2 may be the most sophisticated peptide to activate fly FR *in vivo*, and the other fly species may have a FMRFamide peptide of this amino acid sequence.

The disulfide bond between EL1 and EL2 has been said to be important to retain a bioactive activation conformation of GPCR. However, in *Musca* FR, there appears no such a disulfide SS bond. This is because Cys296 in EL2 is a sole Cys residue, and there is no Cys in EL1 (Fig. 1). In the present study, we prepared the Cys296→Ala mutant FR. As a result, the mutant receptor was found to retain the full activity. The present results implied some other interactions between EL1 and EL2 may compensate for the disulfide bonding to keep up the receptor activation conformation.

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Optimization of the N-Terminal Group of Ac-RYYRIK-NH_2 as ORL1 Receptor Antagonist

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Ac-RYYRIK-NH_2 is firstly reported as an antagonist that inhibits the nociceptin activities mediated through ORL1 receptor. However, Ac-RYYRIK-NH_2 has also acts as a partial agonist. We previously reported that the N-terminal moiety of this peptide is crucially important for specific receptor interaction, and found that isovareloyl-RYYRIK-NH_2 exhibits high affinity and strong antagonist activity. In the present study, we intended to determine the structural elements required for this antagonist by optimizing the acyl group for antagonist activity.

Keywords: antagonist, nociceptin, ORL1 (opioid receptor like 1), partial agonist

Introduction

Nociceptin, a 17-mer neuropeptide with the sequence FGGFTGARKSARKL ANQ, is an endogenous ligand of the ORL1 (opioid receptor-like 1) receptor. This receptor belongs to the G protein-coupled receptor (GPCR) superfamily, and couples specifically with G_i or G_o protein. 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]. In general, for better understanding of such different functions of biologically active peptides, it is imperative to obtain a highly selective and specific receptor antagonist. Antagonist is an important and indispensable molecular tool for investigation of the inhibition mechanism of receptor activation. Because of the intrinsic hyperalgesic activity of nociceptin, its antagonists are expected to be highly effective analgesics.

Acetyl hexapeptide amide Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Ac-RYYRIK-NH_2) has been reported as an effective nociceptin antagonist [2]. Since Ac-RYYRIK-NH_2 displaces [^{3}H]nociceptin in a dose-dependent manner, these two peptides Ac-RYYRIK-NH_2 and nociceptin should share and thus compete for the binding site in ORL1 receptor. However, Ac-RYYRIK-NH_2 *per se* was found to exhibit partial agonist activity in the [^{35}S]GTPγS binding assay [3].

In our previous study, based on the fact that the analogue lacking the acetyl group, H-RYYRIK-NH_2, showed drastically reduced binding potency, we noted the importance of the N-terminal acetyl group, CH_3CO-, as a structural element essential for binding to ORL1 receptor. Then, we found that isovareloyl-RYYRIK-NH_2 with

substituted N-terminal isovareloyl group, $(CH_3)_2CH_2CO$-, exhibited strong antagonist activity as compared to Ac-RYYRIK-NH_2.

In the present study, focusing on the N-terminal isovareloyl group of the strongest antagonist, we attempted to design-synthesize even stronger antagonists. We selected seven different acyl groups with slightly changed structure (Fig. 1) to evaluate the influences to their binding potency and activation efficacy. We here describe the structure-activity relationships of acyl-RYYRIK-NH_2 peptides for the best selection of ORL1 nociceptin antagonism.

Results and Discussion

All acyl-RYYRIK-NH_2 analogues were prepared by the SPS method using Fmoc-amino acids. N-Terminal acylation was carried out at the end of each synthesis cycle by using the corresponding carboxylic acid (R-COOH). Peptides were liberated from the resin and protecting groups by Reagent K, and purified by gel filtration followed by reversed-phase HPLC. The measurement of MALDI-TOF mass spectroscopy guaranteed the theoretical value of the molecular weight of each peptide synthesized (Table 1).

For the receptor binding assay and [^{35}S]GTPγS binding assay, we used the membrane preparations from the COS-7 cells expressing human ORL1 fused with the α subunit of G_o protein ($G_{\alpha o}$). This fusion receptor ORL1-$G_{\alpha o}$ was constructed to evaluate the peptides effectively in the [^{35}S]GTP$_\gamma$S binding assay. We could verify the receptor population sufficiently enough to carry out a series of binding assays; *i.e.*, the saturation binding assay, the competitive binding assay, and the GTPγS binding assay.

Referring the chemical structure of the isovareloyl group, we designed three different types of N-terminal acyl groups for RYYRIK-NH_2. Firstly, to evaluate the methyl group at the γ position as a trigger for receptor binding and activation, C_β was converted to much more electrically negative atoms N, O, and S. Secondly, focusing on the chemical bond between C_α and C_β, the angelicyl group with the C=C double bond and the tetrolicyl group with the triple C≡C bond were introduced. Thirdly, we placed the amino acid valine, which contains the carbon backbone structure of the isovareryl group. L-Val and D-Val were used to evaluate the difference induced by their spatial variety.

In the radio-ligand receptor binding assay, methylthio-RYYRIK-NH_2 was found to exhibit the strongest activity with the IC_{50} value of 0.74 nM (Table 2).

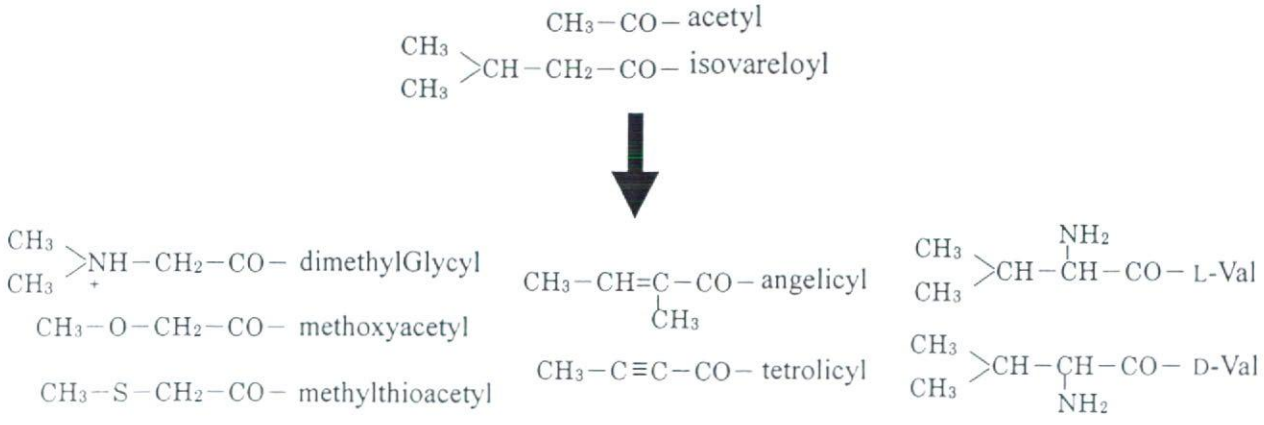

Fig. 1. Chemical structure of N-terminal acyl group for RYYRIK-NH_2.

Table 1. Molecular weight determination of acyl-RYYRIK-NH_2 peptides by the MALDI-TOF mass spectroscopy.

acyl-RYYRIK-NH_2 (acyl groups)	MALDI-TOF-MS	
	Found (m/z)	Theoretical ($m+H^+$)
acetyl	940.39	940.55
isovareloyl	982.03	982.20
dimethylGlycyl	983.07	983.62
methoxyacetyl	969.07	970.94
methylthioacetyl	986.07	986.82
angelicyl	979.07	980.79
tetrolicyl	963.07	964.97
L-Val	996.07	997.96
D-Val	996.07	997.04

This analogue is as strong as the parent peptide Ac-RYYRIK-NH_2 (Table 2). The peptide having the methoxy acetyl group also showed a considerably high binding potency, but the *N,N*-dimethyl glycyl analogue showed almost 100 times reduced binding potency as compared to the isovareloyl analogue. A very high receptor binding affinity observed for methylthioacetyl-RYYRIK-NH_2 made us enthusiastic about its biological activity, anticipating a pure antagonism. However, unfortunately, this expectation was immediately downed, since the analogue was found to activate the ORL1-$G_{\alpha o}$ receptor up to approximately 50% in the GTPγS binding assay. The result implied that this analogue is only a partial agonist. The methoxy acetyl analogue also exhibited a considerable receptor activation activity in the GTPγS binding assay.

The analogue tetrolicyl-RYYRIK-NH_2 was as active as methylthioacetyl-RYYRIK-NH_2 (Table 2), and angelicyl-RYYRIK-NH_2 was also exhibited considerably high binding activity. However, these analogues containing unsaturated triple and double bonds in the N-terminal acyl group activated the receptor much more (about 10-fold) strongly as compared to isovareloyl-RYYRIK-NH_2. Apparently, placing the triple and double bonds is disadvantageous to diminish the biological activity, presumably enhancing the hydrophobic interaction to elicit an agonist activity.

L-Val-RYYRIK-NH_2 and D-Val-RYYRIK-NH_2 led considerably reduction of the binding potency to the ORL1-$G_{\alpha o}$ receptor. Although these analogues possess the amino group at the C_α atom of the isovareloyl group, this substitution is apparently disadvantageous to gain a binding potency enough to bind to the receptor. These two peptides also acted as partial agonist. Since L-Val-RYYRIK-NH_2 and D-Val-RYYRIK-NH_2 exhibited almost the same receptor binding potency (Table 2), the configurational difference in arrangement of the alkyl group between L-Val and D-Val did not affect the receptor binding. This means that the isovareloyl group attached to RYYRIK-NH_2 binds to the receptor site with no any stereo-specificity to discriminate the ligand's spatial arrangements.

Table 2. Binding potency of nociceptin, Ac-RYYRIK-NH_2, isovareloyl- RYYRIK-NH_2 and its analogues for the human ORL1 receptor fused with G_α protein.

Peptides acyl-RYYRIK-NH_2 (acyl groups)	ORL1 receptor binding potency IC_{50} (nM)		
nociceptin	0.60	±	0.08
acetyl	0.79	±	0.18
isovareloyl	7.42	±	0.87
dimethylGlycyl	73. 6	±	0.54
methoxyacetyl	2.31	±	0.35
methylthioacetyl	0.74	±	0.21
angelicyl	5.82	±	1.89
tetrolicyl	2.09	±	0.20
L-Val	76.3	±	7.10
D-Val	78.5	±	9.50

In summary, we designed and synthesized seven novel N-terminal acyl-substituted analogues of isovareloyl-RYYRIK-NH_2 to obtain a stronger antagonist and at the same time to clarify the structural elements essential for the receptor binding potency and receptor activation efficacy. The results afforded rather complicated structural information, suggesting the presence very subtle conformational difference between the agonism and antagonism.

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The Molecular Mechanism of ORL1 Nociceptin Receptor in Activation: Residual Essentials in the Sixth Transmembrane Domain

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Nociceptin is an endogenous ligand of ORL1 receptor, a member of G protein-coupled receptors. In order to elucidate the molecular mechanism of ORL1 activated by nociceptin, we achieved the complete Ala-substitution series for the transmembrane No. 6 (TM6) in ORL1. This systematic mutagenesis enabled us to identify several amino acid residues crucial for receptor activation, but not for nociceptin binding.

Keywords: site-directed mutagenesis, ORL1 receptor, nociceptin, receptor activation

Introduction

ORL1 (opioid receptor-like 1) receptor is a seven transmembrane G protein-coupled receptor (GPCR) and couples to $G_{i/o}$ protein. Its endogenous ligand is a heptadecapeptide named nociceptin, which produces hyperalgesia and various physiological functions. One of its essential binding sites for nociceptin is an acidic amino acid cluster present in the extracellular loop 2 (EL2) [1]. We discovered the amino acid residues present in the transmembrane domain No. 5 (TM5) as structural elements essential for receptor activation. Those include a cluster of aromatic amino acids on the same ridge of TM5 α-helix [2], and are not involved in the ligand-binding.

Several studies by others have suggested that TM5 is important to hold GPCR in the active conformation by interacting tightly with the adjacent TM6 α-helix [3, 4]. Thus, in this study, we attempted to explore the amino acid residues in TM6 that interact with the TM5 aromatic amino acid(s) essential for driving such an active receptor conformation. We prepared a series of mutant ORL1 receptors, in which 30 amino acids in TM6 (254-285 in rat ORL1 receptor) were all mutated to Ala one by one.

We previously succeeded in the preparation of the ORL1 mutant receptors that were conjugated with $G_{\alpha o}$ protein at the C-terminus. These mutants were proved to be excellent in the performance of the GTPγS binding assay. Thus, we also prepared