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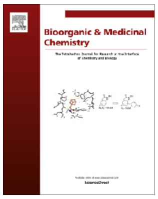

N-methylthioacetylation of RYYRIK-NH_2 with enhanced specific binding affinity and high antagonist activity for nociceptin ORL1 receptor

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ABSTRACT

Antagonists of the neuropeptide nociceptin are expected to be potential analgesic and antineuropathic drugs acting on ORL1 GPCR receptors. The peptide library-based antagonist Ac-RYYRIK-NH_2 inhibits the nociceptin activity mediated through ORL1, but preserves a considerably high level of agonist activity. We previously reported that the N-terminal acyl group is important for interaction with specific receptors, and developed isovarelyl-RYYRIK-NH_2, which exhibits strong antagonist activity with negligible agonist activity. In the present study, in order to obtain a more potent antagonist, we further modified the isovarelyl group by replacing its Cβ atom with an oxygen, nitrogen, or sulfur atom to give the methyl group improved interaction ability. The methyl group bound to such heteroatoms was expected to enhance the hydrophobic interaction between the peptide and the ORL1 receptor. The RYYRIK-NH_2 peptide with a methylthioacetyl group, CH_3SCH_2CO, revealed a higher receptor-binding affinity with strong antagonist activity, and the results suggested the presence of a receptor aromatic group as a complementary residue of this CH_3S group.

1. Introduction

Nociceptin,[1] also known as orphanin FQ,[2] is a 17-mer neuropeptide with the sequence of FGGFTGARKSARKLANQ. Nociceptin is an endogenous ligand of the opioid receptor-like 1 (ORL1) receptor, and nociceptin/ORL1 regulates a wide range of biological functions.[3,4] Due to these functional variations, ORL1 agonists, antagonists, and even partial agonists are anticipated to be effective therapeutics.[5–7] Antagonists are also an indispensable molecular tool for investigation of the mechanism of receptor activation and inactivation. To better understand the molecular mechanisms of nociceptin, it is imperative to obtain a highly selective and specific antagonist. A number of non-peptidic small molecules have been identified as nociceptin/ORL1 antagonists. These include J-113397,[8] JTC801,[9–13] SB-612111,[14–16] and Trap-101.[17,18]

A number of peptidic antagonists have also been reported. These include [$Nphe^1$]-nociceptin-(1-13)-NH_2,[19,20] [$Phe^1\psi(CH_2$-NH)Gly^2]nociceptin(1-13)-NH_2,[19,21–23] and [$NPhe^1$,Arg^{14},Lys^{15}]nociceptin.[24] Another type of peptidic antagonists are compounds selected from the peptide libraries. Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Ac-RYYRIK-NH_2) has been reported as an effective nociceptin/ORL1 antagonist.[25] However, this Ac-RYYRIK-NH_2 was immediately found to exhibit partial agonist activity—for example, in a [^{35}S]GTPγS-binding assay[26,27]—and we also confirmed its considerably high agonist activity.[28] Focusing on Ac-RYYRIK-NH_2, we previously attempted to search for a pure antagonist that retained no agonist activity, and identified isoVa-RYYRIK-NH_2 as such an analog.[28,29] In this peptide, the acetyl group of Ac-RYYRIK-NH_2 was replaced with an isovarelyl (isoVa) group.

Our previous study indicated that an N-terminal free analog of Ac-RYYRIK-NH_2, namely, H-RYYRIK-NH_2, showed drastically reduced binding affinity (approximately 280-fold weaker than Ac-RYYRIK-NH_2).[28] This revealed the importance of the N-terminal acetyl group, CH_3CO–, as a structural element essential for binding to the ORL1 receptor. In a subsequent study on the modification of

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Abbreviations: DTT, dithiothreitol; Fmoc, 9-fluorenylmethoxycarbonyl; GPCR, G protein-coupled receptor; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MVD, mouse vas deferens; Nphe, *N*-benzylglycine; RP-HPLC, reversed-phase high performance liquid chromatography; RT, retention time; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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this acetyl-methyl group, we found that isoVa-RYYRIK-NH_2 exhibits a strong antagonist activity.[29] Since its receptor binding affinity for ORL1 was not very high, we intended to design an analog having an affinity superior to that of isoVa-RYYRIK-NH_2. In the present study, in order to obtain such a high affinity acyl group, the Cβ atom of the isovarelyl groups, isoVa or $(CH_3)_2CHCH_2CO$–, was replaced with a nitrogen, oxygen, or sulfur atom.

2. Results and discussion

2.1. Peptide syntheses

Hexapeptides were synthesized by the manual solid-phase method using Fmoc-amino acids (Fmoc-AA-OH) by the HBTU–HOBt method. At the final step of peptide synthesis, the carboxylic acid was also coupled by HBTU–HOBt to afford the corresponding acyl group. HPLC-purified peptides were obtained in an average yield of approximately 12% from the resin. Among the peptides, $(CH_3)_2NCH_2CO$-RYYRIK-NH_2 was obtained with the best yield (17%), while CH_3SCH_2CO-RYYRIK-NH_2 was obtained with the lowest yield (8%) (Table 1). The other two synthesized peptides were $(CH_3)_3N^+CH_2CO$-RYYRIK-NH_2 and CH_3OCH_2CO-RYYRIK-NH_2, and these were obtained with modest yields, that is, 10% and 11%, respectively (Table 1). The peptides were all water-soluble and assayed without any trouble.

2.2. Receptor-binding activities of acyl-RYYRIK-NH_2 peptides

We prepared human ORL1 fused with the Gαo subunit for efficient measurements of agonist and antagonist activities in the receptor responses, and the resulting ORL1-Gαo was used for both the receptor-binding assay and the functional in vitro GTPγS biological assay.[30–32] When assayed in an ordinary ligand-saturation experiment using COS-7 cells, [^{3}H]nociceptin exhibited a highly specific binding with the dissociation constant K_d of 0.37 nM, which was almost the same value (0.41 nM) as reported previously for the solo rat ORL1 receptor without fusion of the G protein.[28] Also, almost the same result (K_d = 0.40 nM) was obtained for the human ORL1 receptor with no fused G protein. These results imply that both the G protein-fused and non-fused receptors interact with [^{3}H]nociceptin equally well. Ac-RYYRIK-NH_2 exhibited a very high affinity (IC_{50} = 0.79 nM) (Table 2) in the ligand-receptor competitive binding assay, and non-labeled nociceptin showed an almost equivalent affinity in the same assay (0.60 nM).

Four different synthesized analogs of Ac-RYYRIK-NH_2 were evaluated for their binding ability in the competitive receptor-binding assay using [^{3}H]nociceptin. CH_3SCH_2CO-RYYRIK-NH_2 exhibited the highest affinity (IC_{50} = 1.81 nM) among the analogs. Indeed, its activity was almost four times more potent than that of the parent isoVa-RYYRIK-NH_2 (Table 2). CH_3OCH_2CO-RYYRIK-NH_2, which showed the second highest affinity (4.08 nM), was also more potent than isoVa-RYYRIK-NH_2. In contrast to these highly potent analogs, $(CH_3)_2NCH_2CO$-RYYRIK-NH_2 and $(CH_3)_3N^+CH_2CO$-RYYRIK-NH_2 showed drastically decreased binding affinity (188 and 325 nM, respectively) (Table 2).

Table 1

Synthesis of a series of acyl-RYYRIK-NH_2 derivatives: the structure, synthetic yield, and molecular weight determination

Structure of acyl of acyl-RYYRIK-NH_2	Name of acyl	Yield (%)	MALDI-TOF MS	
			Found (*m*/*z*)	Calcd (m+H$^+$)
$(CH_3)_2NCH_2CO$	*N*,*N*-Dimethylglycyl	17	983.07	983.62
$(CH_3)_3N^+CH_2CO$	*N*,*N*,*N*-Trimethylglycyl	10	996.88	997.62
CH_3OCH_2CO	Methoxyacetyl	11	970.07	970.94
CH_3SCH_2CO	Methylthioacetyl	8	986.07	986.82

Table 2

Binding potency and biological activity of nociceptin and analogues of acyl-RYYRIK-NH_2 for the human ORL1 receptor fused with Gαo protein

Peptides acyl-RYYRIK-NH_2 (acyl groups)	ORL1 receptor binding potency IC_{50} (nM)	[^{35}S]GTPγS binding activity	
		EC_{50} (nM)	E_{max} (%)
Nociceptin	0.60 ± 0.08	3.91 ± 0.34	100
CH_3CO [Ac]	0.79 ± 0.18	12.9 ± 2.8	58
$CH_3CH_2CH_2CO$ [Bu]	1.86 ± 0.60	32.4 ± 12.1	21
$(CH_3)_2CHCH_2CO$ [isoVa]	7.42 ± 0.87	Inactive	≈0
$(CH_3)_2NCH_2CO$	188 ± 12	Inactive	≈0
$(CH_3)_3N^+CH_2CO$	325 ± 6.0	Inactive	≈0
CH_3OCH_2CO	3.71 ± 0.26	18.4 ± 2.6	54
CH_3SCH_2CO	1.81 ± 0.12	Inactive	≈0

For the receptor-binding assay, [^{3}H]nociceptin was used as a tracer. Data are the means ± SEM of at least three experiments (*n* = 3–5).

2.3. Biological activities of acyl-RYYRIK-NH_2 peptides

The in vitro functional activity was evaluated by measuring the fold-stimulation of [^{35}S]GTPγS binding for ORL1. In this [^{35}S]GTPγS-binding assay, nociceptin exhibited approximately 10-fold stimulation with an EC_{50} value of 3.91 nM. Ac-RYYRIK-NH_2 retained roughly 60% stimulation of the maximum response of nociceptin, and thus clearly performed as a partial agonist (EC_{50} = 12.9 nM) as reported previously[28] (Table 2). When newly synthesized peptides were assayed, very distinct results were noted. CH_3OCH_2CO-RYYRIK-NH_2 was moderately active (EC_{50} = 18.4 nM), and roughly equipotent to Ac-RYYRIK-NH_2 (Table 2). Both exhibited almost the same maximum response (54–58% stimulation of the maximum response by nociceptin). Since CH_3OCH_2CO-RYYRIK-NH_2 was about four times weaker than Ac-RYYRIK-NH_2 in the competitive receptor-binding assay (Table 2), the efficacy in receptor activation of CH_3OCH_2CO-RYYRIK-NH_2 was much higher than that of Ac-RYYRIK-NH_2. This was almost certainly due to the stimulatory effect of the CH_3O group in the receptor interaction.

In contrast to CH_3OCH_2CO-RYYRIK-NH_2, CH_3SCH_2CO-RYYRIK-NH_2 was almost completely inactive at concentrations of 0.01–10 μM (Table 2). This inactivity was reproduced repeatedly in this [^{35}S]GTPγS-binding assay, as was the inactivity of isoVa-RYYRIK-NH_2. The inactivity was also shown by $(CH_3)_2NCH_2CO$-RYYRIK-NH_2 and $(CH_3)_3N^+CH_2CO$-RYYRIK-NH_2. However, the reasons for the inactivity appear to be differ completely among these peptides. CH_3SCH_2CO-RYYRIK-NH_2 and isoVa-RYYRIK-NH_2 exhibited very high ability to bind to ORL1 receptors, whereas both $(CH_3)_2NCH_2CO$-RYYRIK-NH_2 and $(CH_3)_3N^+CH_2CO$-RYYRIK-NH_2 appeared to lack the fundamental ability to bind to these receptors. These analogs having N-methylated acyl groups exhibited dramatically diminished binding affinity (Table 2).

2.4. Antagonist activity in the [^{35}S]GTPγS-binding assay

Because of the high receptor-binding affinity and inactivity in the [^{35}S]GTPγS-binding assay, CH_3SCH_2CO-RYYRIK-NH_2 appeared to be an effective antagonist against nociceptin/ORL1. Thus, CH_3SCH_2CO-RYYRIK-NH_2, together with the reference compound isoVa-RYYRIK-NH_2, was first tested using the same cell membrane preparations as for nociceptin. These examinations were carried out using three different concentrations of 10^{-8}, $10^{-7.5}$, and 10^{-7} M for each peptide. Nociceptin showed a superlative