Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 2635–2644
Bioorganic & Medicinal Chemistry

Designed modification of partial agonist of ORL1 nociceptin receptor for conversion into highly potent antagonist

Jinglan Li,[a,†] Kaname Isozaki,[a,†] Kazushi Okada,[a] Ayami Matsushima,[a] Takeru Nose,[a] Tommaso Costa[b] and Yasuyuki Shimohigashi[a,*]

[a]*Laboratory of Structure–Function Biochemistry, Department of Chemistry, Research-Education Centre of Risk Science, Faculty of Sciences, Kyushu University, Fukuoka 812-8581, Japan*
[b]*Laboratorio di Farmacologia, Istituto Superiore di Sanità, Viale Regina Elena 299, Roma, Italy*

Received 6 October 2007; revised 13 November 2007; accepted 15 November 2007
Available online 21 November 2007

Abstract—Nociceptin is an endogenous agonist ligand of the ORL1 (opioid receptor-like 1) receptor, and its antagonist is a potential target of therapeutics for analgesic and antineuropathy drugs. Ac-RYYRIK-NH_2 is a hexapeptide isolated from the peptide library as an antagonist that inhibits the nociceptin activities mediated through ORL1. However, the structural elements required for this antagonist activity are still indeterminate. In the present study, we evaluated the importance of the acetyl-methyl group in receptor binding and activation, examining the peptides acyl-RYYRIK-NH_2, where acyl (R-CO) possesses a series of alkyl groups, R = C_nH_{2n+1} (n = 0–5). The isovaleryl derivative with the C_4H_9 (=$(CH_3)_2CHCH_2$–) group was found to reveal a high receptor-binding affinity and a strong antagonist nature. This peptide achieved a primary goal of eliminating the agonist activity of Ac-RYYRIK-NH_2 and producing pure antagonist activity.

1. Introduction

Nociceptin,[1] also known as orphanin FQ,[2] is a 17-mer neuropeptide with the sequence FGGFTGARKSARKLANQ. Nociceptin is an endogenous ligand of the ORL1 (opioid receptor-like 1) receptor, the structure of which is very similar to those of the δ, μ, and κ opioid receptors.[3] 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 antiopioid effects in the brain.[1,2,4–6] The actions of nociceptin in the central nervous system also include the inhibition of locomotor activity and impairment of spatial learning.[7–9]

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.

Several different types of compounds have recently been identified as antagonists of nociceptin.[10] As full antagonists, a number of nonpeptide compounds have been designed and synthesized,[11] but it has been difficult to identify any general structural elements common to all of these organic compounds. As for compounds based on the structure of nociceptin peptide, [Phe$^1\Psi$(CH_2–NH)Gly^2]nociceptin(1–13)–NH_2[12] and [Nphe^1]nociceptin-(1–13)–NH_2[13] have been reported as antagonists in the peripheral nervous system.[14–18] However, these

Abbreviations: Boc, *tert*-butyloxycarbonyl; Bpa, *p*-benzoyl-L-phenylalanine; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; 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; MBHA, *p*-methylbenzhydrylamine; Nphe, *N*-benzylglycine; RP-HPLC, reversed-phase high performance liquid chromatography; RT, retention time; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Keywords: Acyl group; Antagonist; Nociceptin; Structure–activity relationships.

* Corresponding author. Tel./fax: +81 92 642 2584; e-mail: shimoscc@mbox.nc.kyushu-u.ac.jp
† These authors contributed equally to this study.

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.11.043

peptides appear to act as partial or even full agonists of nociceptin at the central nervous site.[19–22] UFP-101, the compound with the Leu[14] → Arg and Ala[15] → Lys substitutions in [Nphe[1]]nociceptin-(1–13)–NH_2, is a competitive type of nociceptin antagonist.[23] This simultaneous Leu-Ala[14,15] → Arg-Lys substitution was reported originally by us as a structural conversion to turn nociceptin into a super agonist.[24]

In addition to these nociceptin analogues, there is another type of antagonist compound selected from the peptide libraries. For instance, acetyl-hexapeptide amide Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Ac-RYYRIK-NH_2) has been reported as an effective nociceptin antagonist.[25] Since Ac-RYYRIK-NH_2 displaces [^{3}H]nociceptin in a dose-dependent manner, these two peptides 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.[26,27] In addition, it did not exhibit any in vivo activity, presumably due to the rapid degradation.

Our previous study of Ala-scanning for Ac-RYYRIK-NH_2 indicated that the N-terminal tripeptide Arg-Tyr-Tyr is crucially important for binding to the ORL1 receptor.[28] In the present study, based on the fact that the analogue lacking the acetyl group, H-RYYRIK-NH_2, shows drastically reduced binding efficacy (approximately 60-fold weaker than Ac-RYYRIK-NH_2), we noted the importance of the N-terminal acetyl group, CH_3CO–, as a structural element essential for binding to ORL1.[28]

The acetyl group has two different types of structural elements—the methyl (CH_3) group and the carbonyl (CO) group. In this study, focusing on the N-terminal acetyl-methyl group, we synthesized a series of acyl-RYYRIK-NH_2 peptides (acyl = R-CO, where the alkyl group is denoted as R = C_nH_{2n+1}; n = 0–5) (Table 1), and evaluated the structural effectiveness of the acyl–alkyl group (R) for the antagonist activity. We here describe the structure–activity relationships of acyl-RYYRIK-NH_2 peptides for the best selection of ORL1 nociceptin antagonism.

2. Results

2.1. Peptide syntheses

All of the 17 N-terminal modified hexapeptides, including the parent Ac-RYYRIK-NH_2, were synthesized by the manual solid-phase method using Fmoc-amino acids. Peptides in a pure form were obtained in an average yield of approximately 31%. Among the analogues, Ada-RYYRIK-NH_2 was obtained with the best yield of approximately 56%, while *t*-BuAc-RYYRIK-NH_2 was obtained with the worst yield of less than 10% (Table 1). These were all easily soluble in water and could be assayed without any trouble. Table 1 shows the analytical data of all analogues synthesized. The purity of the peptides was verified by analytical HPLC, in which all the peptides emerged with a single peak. The retention time (approximately 34 min) of the N-terminal modified analogues is much larger than that (23.24 min) of H-RYYRIK-NH_2. It is presumed that the increased hydrophobicity of acyl groups brings about an increased retention time on HPLC. The mass numbers measured were coincident with the values calculated (data not shown). Collectively, synthetic Ac-RYYRIK-NH_2 and its analogues have been found to reveal the authentic compounds.

2.2. ORL1 receptor fused with the G protein α_o subunit for better antagonism measurement

For efficient measurements of agonism and antagonism in the receptor responses, GPCR fused with the G pro-

Table 1. Synthetic yield and HPLC analytical data of acetyl-hexapeptide amide Ac-RYYRIK-NH_2, and its analogues

n	Structure of acyl = $C_nH_{2n+1}CO$ of Acyl-RYYRIK-NH_2	Name of acyl	Abbreviations of acyl	Yield (%)	RP-HPLC retention time (min)
0	HCO–	Formyl	For	34	24.89
1	CH_3CO–	Acetyl	Ac	52	26.16
2	CH_3CH_2CO–	Propionyl	Pr	28	28.33
3	$CH_3CH_2CH_2CO$–	Butyryl	Bu	20	29.88
	$(CH_3)_2CHCO$–	Isobutyryl	isoBu	39	29.85
4	$CH_3CH_2CH_2CH_2CO$–	Valeryl	Va	21	32.94
	$(CH_3)_2CHCH_2CO$–	Isovaleryl	isoVa	14	32.35
	$CH_3CH_2CH(CH_3)CO$–	2-Methylbutanoyl	MeBut	41	31.86
	$(CH_3)_3CCO$–	Pivaloyl	Piv	23	33.25
5	$CH_3CH_2CH_2CH_2CH_2CO$–	Hexanoyl	Hex	18	35.57
	$CH_3CH_2CH_2CH(CH_3)CO$–	2-Methylpentanoyl	2-MePen	29	35.15
	$(CH_3)_3CCH_2CO$–	*tert*-Butylacetyl	*t*-BuAc	8	34.73
	$CH_3CH_2C(CH_3)_2CO$–	2,2-Dimethylbutanoyl	2,2-diMeBut	37	34.21
	$(CH_3CH_2)_2CHCO$–	2-Ethylbutanoyl	EtBut	35	34.83
	C_6H_5CO–	Benzoyl	Bz	14	36.52
	$C_{10}H_{15}CO$–	Adamantyl	Ada	56	41.89

Three of the acyl groups are not listed, since those acyl chlorides or acids are not commercially available, and thus the peptides having those acyl groups were not chemically synthesized. These include the 3-methylpentanoyl ($CH_3CH_2CH(CH_3)CH_2CO$–), 4-methylpentanoyl ($(CH_3)_2CHCH_2CH_2CO$–), and 3,3-dimethylbutanoyl ($CH_3C(CH_3)_2CH_2CO$–) groups. Elution conditions for the analytical RP-HPLC to measure the retention time: solvent system, 0.1% aqueous TFA-(A solution) and acetonitrile containing 20% A solution-(B solution) with a gradient elution from 10% to 50% B solution for 40 min; flow rate, 0.5 ml/min; temperature, 25 °C; and UV detection, 230 nm.

tein α subunit has been recognized to afford an excellent assay system.[29] In the present study, we intended to establish such a system for the ORL1 receptor. Using human ORL1 receptor, we succeeded in preparing hORL1 fused with the G protein α_o subunit (hORL1-Gα_o) for both the receptor-binding assay and the functional in vitro biological assay. We first tested this assay system for nociceptin and Ac-RYYRIK-NH_2.

For the receptor-binding assay, the highest expression efficiency of hORL1-Gα_o receptor was pursued by using COS-7 cells for the ordinary ligand-saturation experiment. Under the best conditions using the tritium-labeled ligand [^{3}H]nociceptin, the largest specific binding was obtained by subtracting its nonspecific binding from the total binding. The data were analyzed by Scatchard plot analysis, and the dissociation constant K_d was calculated to be 0.37 nM, being almost the same as the K_d value (0.41 nM) reported previously for solo rat ORL1 receptor with no G protein fused.[28] Also, in this study almost the same result (K_d = 0.40 nM) was obtained for human ORL1 receptor with no G protein fused. The results imply that both G protein fused and nonfused receptors interact with [^{3}H]nociceptin equally well.

In the ligand–receptor competitive binding assay, Ac-RYYRIK-NH_2 exhibited a very high affinity, with an IC_{50} value of 0.79 nM (Table 2). This result indicates that Ac-RYYRIK-NH_2 binds to the ORL1 receptor very strongly, and that its binding ability is almost equivalent to that of nociceptin itself (IC_{50} = 0.60 nM).

The in vitro functional activity was evaluated by measuring the fold-stimulation of [^{35}S]GTPγS binding. The activity was compared with that of nociceptin (EC_{50} = 3.91 nM) (Table 2). The extent of [^{35}S]GTPγS binding in the presence of nociceptin was at least 10 times greater than that in its absence. When the [^{35}S]GTPγS binding of the parent acetyl containing hexapeptide Ac-RYYRIK-NH_2 was measured, it was estimated to reveal approximately 60% stimulation of the maximum response by nociceptin. Obviously, Ac-RYYRIK-NH_2 is a partial agonist, and yet it possesses considerably strong agonist activity. The EC_{50} value of Ac-RYYRIK-NH_2 was estimated to be 12.9 nM, the activity of which is only approximately threefold weaker than nociceptin.

Table 2. Binding potency and biological activity of nociceptin, acetyl-hexapeptide amide Ac-RYYRIK-NH_2, and its analogues for the human ORL1 receptor fused with Gα protein

Peptides acyl-RYYRIK-NH_2 (acyl groups)	ORL1 receptor 1 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
H-	218 ± 78	N.D.	—
For-	0.66 ± 0.09	23.3 ± 4.2	61 ± 2.3
Ac-	0.79 ± 0.18	12.9 ± 2.8	58 ± 3.2
Pr-	1.70 ± 0.66	27.6 ± 6.8	46 ± 3.6
Bu-	1.86 ± 0.60	32.4 ± 12.1	21 ± 3.0
isoBu-	2.81 ± 0.52	44.5 ± 11.7	14 ± 3.8
Va-	5.67 ± 0.26	N.D.	7 ± 2.1
isoVa-	7.42 ± 0.87	N.D.	≈0
MeBut-	21.3 ± 2.3	52.7 ± 16.8	27 ± 1.8
Piv-	14.9 ± 3.5	97.9 ± 20.5	32 ± 5.6
Hex-	21.7 ± 5.3	N.D.	9 ± 2.6
2-MePen-	47.3 ± 8.0	23.8 ± 4.7	17 ± 4.7
t-BuAc-	16.9 ± 4.1	46.5 ± 2.0	33 ± 2.0
2,2-diMeBut-	45.5 ± 7.1	17.7 ± 3.4	17 ± 3.4
EtBut-	92.6 ± 5.4	N.D.	10 ± 1.5
Bz-	14.7 ± 2.6	18.3 ± 4.6	38 ± 5.8
Ada-	3.42 ± 0.49	19.2 ± 5.9	35 ± 4.9

For the receptor-binding assay, [^{3}H]nociceptin was used as a tracer. Data are means ± SEM of at least three experiments (n = 3–8). N.D. (not determined) means that the activity (EC_{50} (nM)) was not calculated due to inactivity.

2.3. Activities of acyl-RYYRIK-NH_2 peptides with non-branched acyl–alkyl groups

In the binding assay using rat ORL1 receptor, the N-terminal free analogue of Ac-RYYRIK-NH_2, namely, H-RYYRIK-NH_2, exhibited a drastically diminished binding potency.[28] A similar result was obtained from the assay using the G protein-fused receptor hORL1-Gα_o. H-RYYRIK-NH_2 showed an IC_{50} value of 218 nM, indicating that it is approximately 280-fold less active than Ac-RYYRIK-NH_2 (Table 2). Since these results clearly indicate that ORL1 receptor possesses a specific binding site for the acetyl group (CH_3CO) of Ac-RYYRIK-NH_2, we attempted to optimize the acyl–alkyl group. We designed and synthesized a series of analogues, in which the acetyl group was substituted with the acyl groups (R-CO) of different alkyl groups (R = C_nH_{2n+1}; n = 0–5): i.e., For (n = 0, R = H), Pr (2, CH_3CH_2), Bu (3, $CH_3CH_2CH_2$), Va (4, $CH_3CH_2CH_2CH_2$), and Hex (5, $CH_3CH_2CH_2CH_2CH_2$) (Table 1).

In the ligand–receptor-binding assay, For-RYYRIK-NH_2 exhibited the highest affinity (IC_{50} = 0.66 nM) (Table 2) among all the analogues, including Ac-RYYRIK-NH_2. Since For-RYYRIK-NH_2 is almost equipotent with nociceptin (IC_{50} = 0.60 nM) in displacing [^{3}H]nociceptin, their total binding energies to attach to ORL1 must be similar to each other. As shown in Figure 1, they exhibited almost the same dose–response curves, revealing a similar binding mode. Other analogues exhibited rather weaker binding affinity as compared with the parent peptide Ac-RYYRIK-NH_2 (Table 2).

Figure 2 shows the activity profiles of nociceptin, Ac-RYYRIK-NH_2, and its analogues in the [^{35}S]GTPγS binding assay. By using the membrane preparations from the cells expressing hORL1-Gα fusion receptor, nociceptin exhibited a strong binding activity, indicating that nociceptin stimulates the G-protein activation in a dose-dependent manner. Ac-RYYRIK-NH_2 exhibited considerably high activity, and its maximum response reached 60% of that by nociceptin. For-RYYRIK-NH_2 elicited almost the same response as Ac-RYYRIK-NH_2. In contrast to their agonist activity, the maximum response of Pr-RYYRIK-NH_2 and Bu-RYYRIK-NH_2 was significantly reduced by 20% and 40%, respectively. Furthermore, it was found that Va-RYYRIK-NH_2 and Hex-RYYRIK-NH_2 were virtually devoid of agonist activity (Fig. 2). Apparently, the receptor efficacy

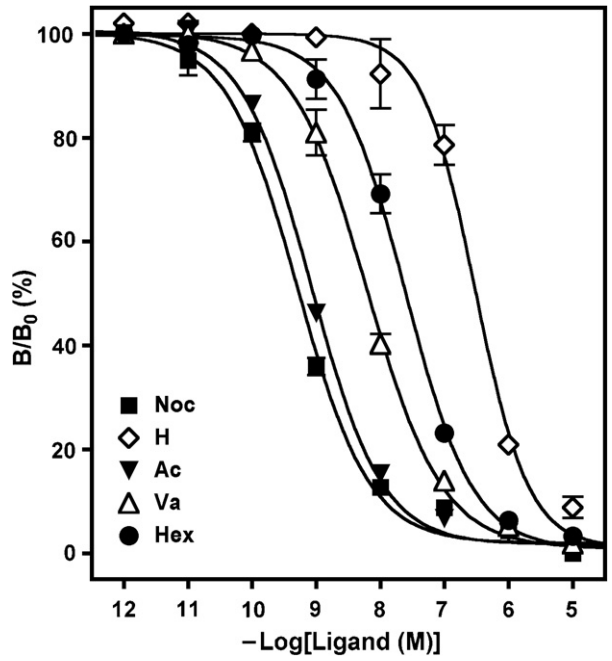

Figure 1. Dose–response curves of nociceptin and acyl-RYYRIK-NH_2 peptides in the binding assay for the hORL1-Gα fusion protein. The receptor tracer is [^{3}H]nociceptin (0.05 nM in the final concentration). The curves are of nociceptin and the parent Ac-RYYRIK-NH_2 and the analogues of acyl-RYYRIK-NH_2 with the N-terminal acyl–alkyl group R = C_nH_{2n+1}: H- (n = 0), Va- (n = 4), and Hex- (n = 5).

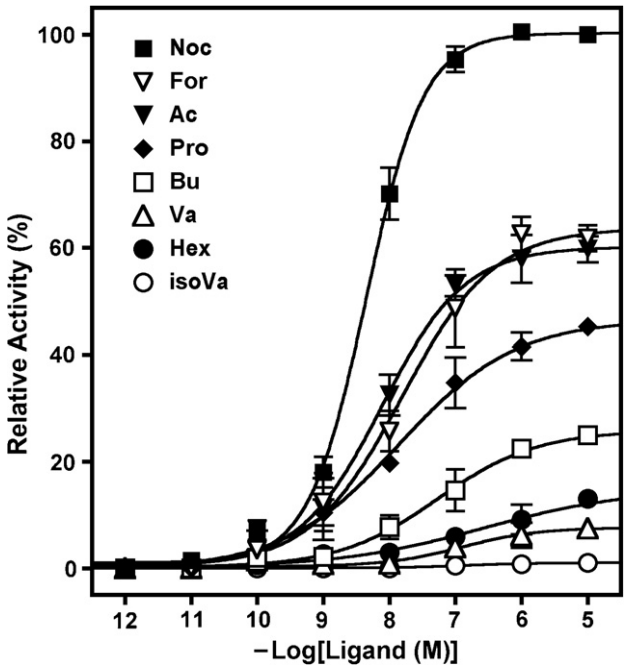

Figure 2. Dose–response curves of nociceptin and acyl-RYYRIK-NH_2 peptides in the [^{35}S]GTPγS binding assay using the hORL1-Gα receptor. Assayed acyl-RYYRIK-NH_2 peptides are with the acyl–alkyl group R = C_nH_{2n+1} (n = 0–5). Data are means ± SEM of at least five experiments.

decreased as the chain length increased. Here, it should be noted that Hex-RYYRIK-NH_2 having a hexanoyl ($CH_3CH_2CH_2CH_2CH_2CO$ = $C_5H_{11}CO$) group is clearly stronger than Va-RYYRIK-NH_2 having a valeryl ($CH_3CH_2CH_2CH_2CO$ = C_4H_9CO) group.

2.4. Activities of acyl-RYYRIK-NH_2 peptides with branched acyl–alkyl groups

In the [^{35}S]GTPγS binding assay (valeryl=)$CH_3CH_2CH_2CH_2CO$-RYYRIK-NH_2 was most active among acyl-RYYRIK-NH_2 peptides. We next examined a series of acyl-RYYRIK-NH_2, which have the acyl group (C_nH_{2n+1}-CO, n = 3–5) with eight different branched alkyl groups. Those include the acyl groups such as isobutyryl (isoBu, $(CH_3)_2CHCO$–), isovaleryl (isoVa, $(CH_3)_2CHCH_2CO$–), 2-methylbutanoyl (MeBut, $CH_3CHCH(CH_3)CO$–), pivaloyl (Piv, $(CH_3)_3CCO$–), 2-methylpentanoyl (2-MePen, $CH_3CH_2CH_2CH(CH_3)CO$–), *tert*-butylacetyl (*t*-BuAc, $(CH_3)_3CCH_2CO$–), 2,2-dimethylbutanoyl (2,2-diMeBut, $CH_3CH_2C(CH_3)_2CO$–), and 2-ethylbutanoyl (EtBut, $(CH_3CH_2)_2CHCO$–) (Table 1). The derivatives with the 3-methylpentanoyl ($CH_3CH_2CH(CH_3)CH_2CO$–) and 4-methylpentanoyl ($(CH_3)_2CHCH_2CH_2CO$–) groups were not prepared because their chlorides or acids were not commercially available. In contrast, a totally different type of acyl group, benzoyl (Bz, C_6H_5CO–) and adamantyl (Ada, $C_{10}H_{15}CO$–), was selected to assess the unique structural properties that contribute to receptor binding and activation.

When the binding ability of these acyl-substituted analogues was tested, it was found that the molecular size of the acyl–alkyl groups greatly affects the receptor-binding affinity (Table 2). The EtBut group induced the weakest activity, showing an approximately 120-fold decrease in binding affinity as compared with the parent compound Ac-RYYRIK-NH_2. Analogues having larger alkyl groups exhibited a weaker binding potency. It seems that the binding site for acyl–alkyl is not so large as to bind bulkier groups such as EtBut, 2,2-diMeBut, and 2-MePen.

Unexpectedly, the *tert*-butylacetyl (*t*-BuAc) derivative showed an affinity stronger than the compounds having acyl groups with the same molecular weight. This difference must be due to the very compact structure of the *t*-BuAc group. It was also found that Ada-RYYRIK-NH_2 is very potent (IC_{50} = 3.42 nM), despite the presence of the large acyl–alkyl $C_{10}H_{15}$. This unexpectedly high binding potency of Ada-RYYRIK-NH_2 must be due to the compactness of the adamantyl group. The π–electron rich benzoyl-protected compound Bz-RYYRIK-NH_2 also exhibits considerably strong binding potency (14.7 nM, Table 2). Collectively, the binding site for the acyl–alkyl group in the ORL-1 receptor appears to be the size of three carbons in the acyl-backone (4–5 Å) with several methyl groups.

In the [^{35}S]GTPγS binding assay, these analogues having bulky acyl groups still exhibited moderate receptor activation activity (EC_{50} = 20–50 nM and E_{max} = 10–40%, Table 2). Also, Bz-RYYRIK-NH_2 and Ada-RYYRIK-NH_2 showed an approximately 40% stimulatory response with EC_{50} of approximately 20 nM (Table 2). These results suggest that there is a space to capture

the bulky alkyl groups present at the N-terminus of R-CO-RYYRIK-NH_2, but their interaction with the ORL1 receptor is very subtle and vague, either stimulating or blocking the receptor activation.

To obstruct the receptor activation, the best-fitting alkyl group appears to be the isovaleryl (denoted as isoVa) group. The peptide isoVa-RYYRIK-NH_2 showed almost a negligible response at its 0.10–10 μM concentrations. Its E_{max} value at the 10 μM concentration was less than 5%, and thus it was impossible to estimate the EC_{50} value. It should be noted that branched isoVa-RYYRIK-NH_2 is clearly less potent than non-branched Va-RYYRIK-NH_2 in this [^{35}S]GTPγS binding assay.

2.5. Antagonist activity estimated by [^{35}S]GTPγS binding to human ORL1-Gα receptors

Because of a moderately high receptor-binding affinity (IC_{50} = 7.42 nM) and a low receptor activation efficacy, isoVa-RYYRIK-NH_2 appeared to be an efficient antagonist against nociceptin/ORL1. We then examined its competitive antagonism in the [^{35}S]GTPγS binding assay. For the Schild analysis, nociception was assayed in the presence of isoVa-RYYRIK-NH_2. Three series of dilutions of nociceptin were tested with different concentrations (10^{-8}, 10^{-7}, and 10^{-6} M concentrations, respectively) of isoVa-RYYRIK-NH_2. The assay solution was incubated for 60 min, with the expectation that competition would occur between nociceptin and isoVa-RYYRIK-NH_2 to reside in the ORL1 receptor.

As shown in Figure 3A, solo nociceptin demonstrates a superlative dose-dependent sigmoid curve. This nociceptin's concentration–response curve shifted rightward in the presence of isoVa-RYYRIK-NH_2, indicating that isoVa-RYYRIK-NH_2 occupies some population of ORL1 receptor. As the concentrations of isoVa-RYYRIK-NH_2 increased, the occupied population increased, resulting in a further rightward shift of the nociceptin curve (Fig. 3A). Eventually, the Schild analysis determined isoVa-RYYRIK-NH_2 as a potent competitive antagonist, with the pA_2 value calculated to be 8.80 (Fig. 3B). This analogue is three orders of magnitude more potent as an antagonist than [Nphe^1]nociceptin(1–13)–NH_2, and as potent as the nonpeptide J-113397,[30] the most potent antagonist reported to date.

We also examined the competitive antagonism of Va-RYYRIK-NH_2 in the [^{35}S]GTPγS binding assay. This compound shifted the nociceptin curve similar to that of isoVa-RYYRIK-NH_2, and its pA_2 value was calculated to be 8.51. Thus, together with the fact that Va-RYYRIK-NH_2 retains a weak agonist activity in the [^{35}S]GTPγS binding assay in spite of almost the complete inactivity of isoVa-RYYRIK-NH_2, we concluded that isoVa-RYYRIK-NH_2 is slightly, but definitely, more potent as an antagonist than Va-RYYRIK-NH_2 in the [^{35}S]GTPγS binding assay. As a result, the present results indicate that isoVa-RYYRIK-NH_2 is the best antagonist among the N-terminal modified hexapeptides.

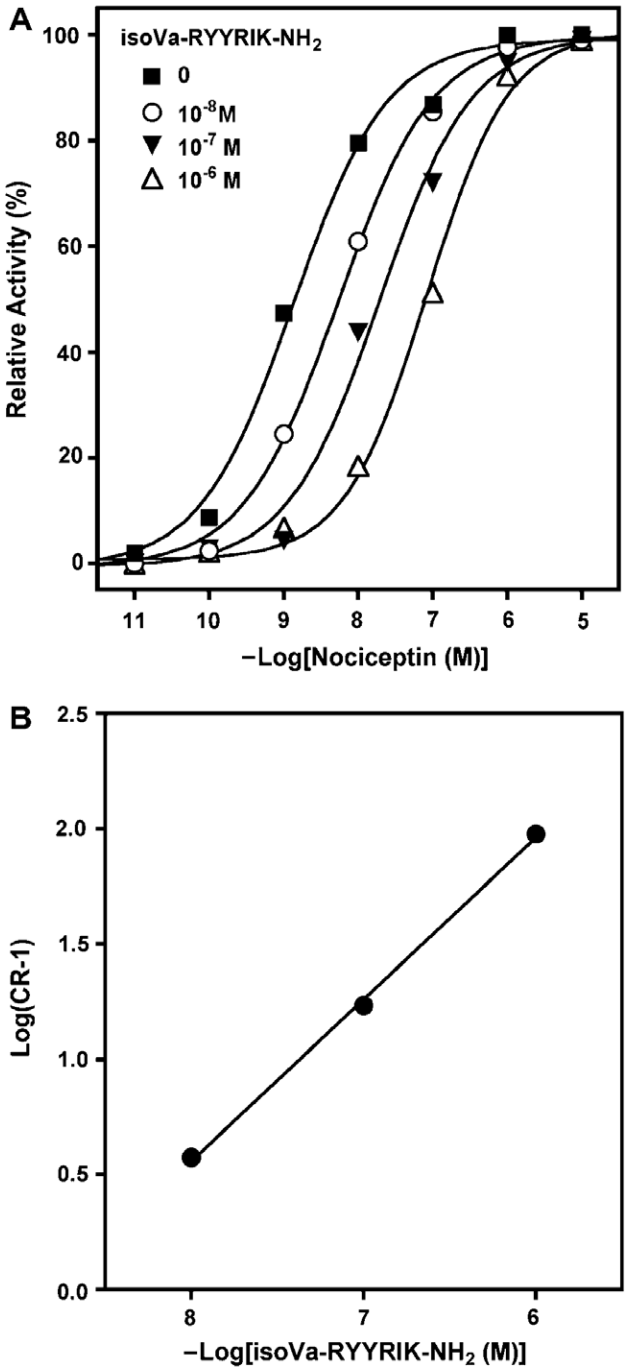

Figure 3. The antagonist activity isoVa-RYYRIK-NH_2 in the [^{35}S]GTPγS binding assay (A) and the Schild plot analysis of this assay (B). The extrapolated pA_2 value from the plot analysis was calculated to be 8.80 ± 0.20.

2.6. Antagonist activities of analogues in the mouse vas deferens

Nociceptin exerts inhibitory effects in electrically stimulated preparations such as the guinea pig ileum (GPI) and MVD.[31,32] In the present study, we established an assay system in which nociceptin inhibits the electrically evoked contractions of the MVD in a concentration-dependent manner. Its maximal effect was an approximately 80% reduction of the control contraction, and

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.

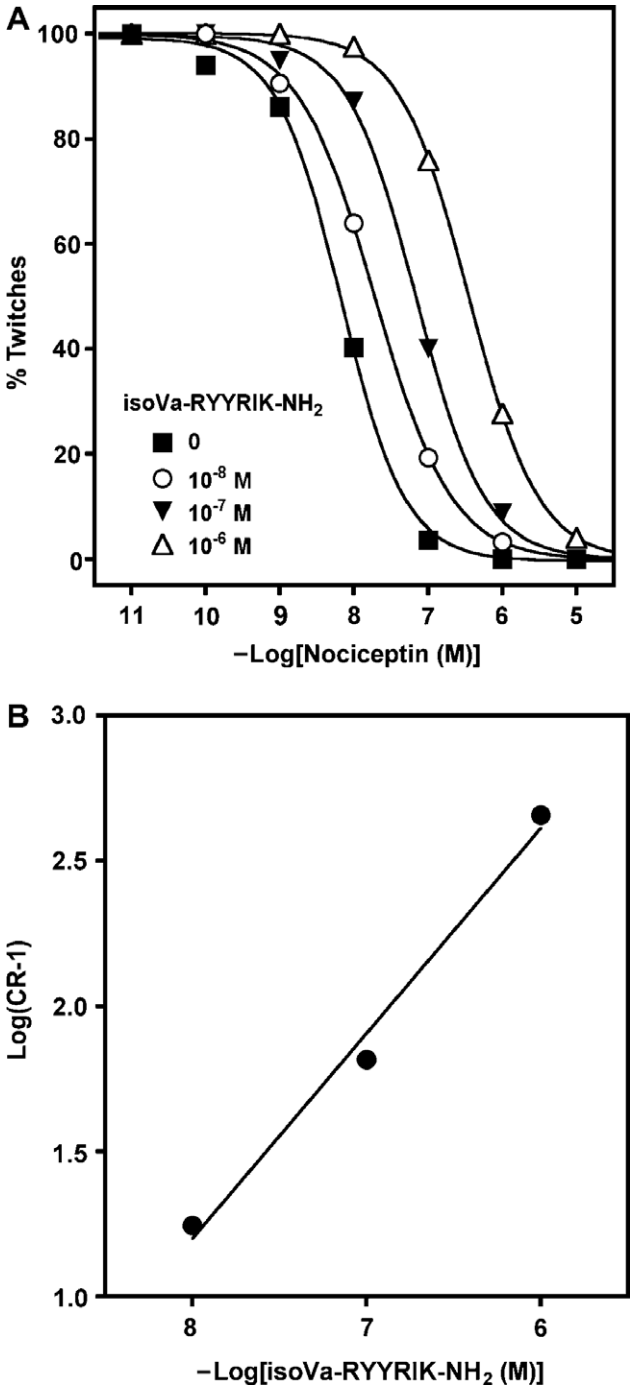

Figure 4. The antagonist activity isoVa-RYYRIK-NH_2 in the MVD muscle assay (A) and the Schild plot analysis of this assay (B). The extrapolated pA_2 value from the plot analysis was calculated to be 9.70 ± 0.32.

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.

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 [^{3}H]nociceptin in the human ORL1 receptor-binding assay.

Displacement of [^{3}H]nociceptin is feasible only when acyl-RYYRIK-NH_2 or acyl-RYYRWK-NH_2 occupies the same receptor site for [^{3}H]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.

Acknowledgments

This study was supported in part by Health and Labour Sciences Research Grants to Y.S., for Research on the Risk of Chemical Substances, from the Ministry of Health, Labor and Welfare of Japan. This work was also supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan to Y.S., and also in part by The Mochida Memorial Foundation for Medical and Pharmaceutical Research to A.M.

References and notes

1. Meunier, J. C.; Mollereau, C.; Toll, L.; Suaudeau, C.; Moisand, C.; Alvinerie, P.; Butour, J. L.; Guillemot, J. C.; Ferrara, P.; Monsarrat, B., et al. *Nature* **1995**, *377*, 532.
2. 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.; Civelli, O. *Science* **1995**, *270*, 792.
3. Topham, C. M.; Mouledous, L.; Poda, G.; Maigret, B.; Meunier, J. C. *Protein Eng.* **1998**, *11*, 1163.
4. Yamamoto, T.; Nozaki-Taguchi, N.; Sakashita, Y.; Kimura, S. *Prog. Neurobiol.* **1999**, *57*, 527.
5. Hao, J. X.; Xu, I. S.; Wiesenfeld-Hallin, Z.; Xu, X. J. *Pain* **1998**, *76*, 385.
6. Meunier, J. C. *Eur. J. Pharmacol.* **1997**, *340*, 1.
7. Sandin, J.; Georgieva, J.; Schott, P. A.; Ogren, S. O.; Terenius, L. *Eur. J. Neurosci.* **1997**, *9*, 194.
8. Manabe, T.; Noda, Y.; Mamiya, T.; Katagiri, H.; Houtani, T.; Nishi, M.; Noda, T.; Takahashi, T.; Sugimoto, T.; Nabeshima, T.; Takeshima, H. *Nature* **1998**, *394*, 577.
9. Mamiya, T.; Noda, Y.; Nishi, M.; Takeshima, H.; Nabeshima, T. *Brain Res.* **1998**, *783*, 236.
10. Zaveri, N. *Life Sci.* **2003**, *73*, 663.
11. Noda, Y.; Mamiya, T.; Nabeshima, T.; Nishi, M.; Higashioka, M.; Takeshima, H. *J. Biol. Chem.* **1998**, *273*, 18047.
12. Carpenter, K. J.; Dickenson, A. H. *Br. J. Pharmacol.* **1998**, *125*, 949.
13. Guerrini, R.; Calo, G.; Rizzi, A.; Bigoni, R.; Bianchi, C.; Salvadori, S.; Regoli, D. *Br. J. Pharmacol.* **1998**, *123*, 163.
14. Okawa, H.; Nicol, B.; Bigoni, R.; Hirst, R. A.; Calo, G.; Guerrini, R.; Rowbotham, D. J.; Smart, D.; McKnight, A. T.; Lambert, D. G. *Br. J. Pharmacol.* **1999**, *127*, 123.
15. Shah, S.; Page, C. P.; Spina, D. *Br. J. Pharmacol.* **1998**, *125*, 510.
16. Calo, G.; Guerrini, R.; Bigoni, R.; Rizzi, A.; Bianchi, C.; Regoli, D.; Salvadori, S. *J. Med. Chem.* **1998**, *41*, 3360.
17. Rizzi, A.; Bigoni, R.; Calo, G.; Guerrini, R.; Salvadori, S.; Regoli, D. *Eur. J. Pharmacol.* **1999**, *385*, R3.
18. Calo, G.; Guerrini, R.; Bigoni, R.; Rizzi, A.; Marzola, G.; Okawa, H.; Bianchi, C.; Lambert, D. G.; Salvadori, S.; Regoli, D. *Br. J. Pharmacol.* **2000**, *129*, 1183.
19. Allen, C. N.; Jiang, Z. G.; Teshima, K.; Darland, T.; Ikeda, M.; Nelson, C. S.; Quigley, D. I.; Yoshioka, T.;

Allen, R. G.; Rea, M. A.; Grandy, D. K. *J. Neurosci.* **1999**, *19*, 2152.
20. Chiou, L. C. *Br. J. Pharmacol.* **1999**, *128*, 103.
21. Emmerson, P. J.; Miller, R. J. *J. Physiol.* **1999**, *517*, 431.
22. Yakimova, K. S.; Pierau, F. K. *Neurosci. Lett.* **1999**, *274*, 87.
23. McDonald, J.; Calo, G.; Guerrini, R.; Lambert, D. G. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2003**, *367*, 183.
24. Okada, K.; Sujaku, T.; Chuman, Y.; Nakashima, R.; Nose, T.; Costa, T.; Yamada, Y.; Yokoyama, M.; Nagahisa, A.; Shimohigashi, Y. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 493.
25. Dooley, C. T.; Spaeth, C. G.; Berzetei-Gurske, I. P.; Craymer, K.; Adapa, I. D.; Brandt, S. R.; Houghten, R. A.; Toll, L. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 735.
26. Burnside, J. L.; Rodriguez, L.; Toll, L. *Peptides* **2000**, *21*, 1147.
27. Berger, H.; Bigoni, R.; Albrecht, E.; Richter, R. M.; Krause, E.; Bienert, M.; Calo, G. *Peptides* **2000**, *21*, 1131.
28. Kawano, C.; Okada, K.; Honda, T.; Nose, T.; Sakaguchi, K.; Costa, T.; Shimohigashi, Y. *J. Pept. Sci.* **2002**, *8*, 561.
29. Hildebrandt, J. D. *Mol. Pharmacol.* **2006**, *69*, 1079.
30. Kawamoto, H.; Ozaki, S.; Itoh, Y.; Miyaji, M.; Arai, S.; Nakashima, H.; Kato, T.; Ohta, H.; Iwasawa, Y. *J. Med. Chem.* **1999**, *42*, 5061.
31. Berzetei-Gurske, I. P.; Schwartz, R. W.; Toll, L. *Eur. J. Pharmacol.* **1996**, *302*, R1.
32. Calo, G.; Rizzi, A.; Bogoni, G.; Neugebauer, V.; Salvadori, S.; Guerrini, R.; Bianchi, C.; Regoli, D. *Eur. J. Pharmacol.* **1996**, *311*, R3.
33. Molinari, P.; Ambrosio, C.; Riitano, D.; Sbraccia, M.; Gro, M. C.; Costa, T. *J. Biol. Chem.* **2003**, *278*, 15778.
34. Judd, A. K.; Kaushanskaya, A.; Tuttle, D. J.; Sanchez, A.; Khroyan, T.; Polgar, W.; Toll, L. *J. Pept. Res.* **2003**, *62*, 191.
35. Judd, A. K.; Tuttle, D. J.; Jones, R. W.; Sanchez, A.; Polgar, W.; Berzetei-Gurske, I.; Toll, L. *J. Pept. Res.* **2004**, *64*, 87.
36. Bes, B.; Meunier, J. C. *Biochem. Biophys. Res. Commun.* **2003**, *310*, 992.
37. Moulédous, L.; Topham, C. M.; Mazarguil, H.; Meunier, J.-C. *J. Biol. Chem.* **2000**, *275*, 29268.
38. DeLean, A.; Munson, P. J.; Rodbard, D. *Am. J. Physiol.* **1978**, *235*, E97.
39. Arunlakshana, O.; Schild, H. O. *Br. J. Pharmacol. Chemother.* **1959**, *14*, 48.
40. Hughes, J.; Kosterlitz, H. W.; Leslie, F. M. *Br. J. Pharmacol.* **1975**, *53*, 371.