Bioorganic & Medicinal Chemistry 16 (2008) 9261–9267

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synergistic effect of basic residues at positions 14–15 of nociceptin on binding affinity and receptor activation

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

[a] Laboratory of Structure–Function Biochemistry, Department of Chemistry, Faculty of Sciences, Kyushu University, Fukuoka 812-8581, Japan
[b] Laboratorio di Farmacologia, Istituo Superiore di Sanatià, Viale Regina Elena 299, Roma, Italy

ARTICLE INFO

Article history:
Received 5 June 2008
Revised 30 August 2008
Accepted 5 September 2008
Available online 9 September 2008

Keywords:
Basic amino acids
Antagonist
Nociceptin
Synergistic potentiation
Superagonist

ABSTRACT

Nociceptin is an endogenous ligand that activates a G protein-coupled receptor ORL1 and contains two indispensable Arg-Lys (RK) dipeptide units at positions 8–9 and 12–13. By replacing an additional RK unit at positions 6–7, 10–11, 14–15, or 16–17, of the peptide we have identified the analog, [RK$^{14-15}$]nociceptin as a superagonist. In fact, this peptide exhibits 3-fold higher binding affinity and 17-fold greater potency in a functional GTPγS-binding assay compared to wild-type nociceptin. Here, we have further investigated the role of basic residues in position 14–15. The replacement of three other possible basic dipeptides, KR, RR, and KK, into nociceptin at positions 14–15 resulted in similar enhancements of binding affinity (3–5-fold) and biological potency (10–12-fold in the GTPγS assay). However, when only a single basic residue (Arg or Lys) was replaced in either position 14 or 15, all the resulting analogs showed moderate enhancements of binding and biological activity (2–4-fold in both). These results indicate that the addition of basic charges in positions 14 and 15 enhance in a synergistic fashion the interaction of nociceptin with the receptor and only the simultaneous presence of two adjacent basic residues yields an optimal effect. This suggests that specific electrostatic interactions between both amino acids present in 14–15 and corresponding residues in the receptor are responsible for the enhancement of nociceptin activity.

1. Introduction

Nociceptin,[1] also known as orphanin FQ,[2] is an endogenous ligand of the seven transmembrane receptor opioid receptor-like 1 (ORL1), the structure of which is similar to those of the three classical opioid receptors.[3–6] The ORL1 receptor has been identified and characterized in several mammals, including humans,[3] rats,[4,5] and mice.[6] Nociceptin is a heptadecapeptide, the amino acid sequence of which, FGGFTGARKSARKLANQ, has been found to be the same among various species[1,2,7–9] and to resemble the opioid peptide dynorphin. In spite of sequence homologies in both ligand and receptor, nociceptin does not interact with opioid receptors nor does ORL1 interact with opioid peptides.

Although nociceptin was originally reported to induce hyperalgesia[1], it has been subsequently shown to also participate in various physiological functions, such as locomotor activity,[10] spatial learning,[11] food intake, and neuropathic diseases including depression, stress, and anxiety.[12] For this reason, major efforts have been focused on the design of nociceptin antagonists as potentially useful analgesics and anti-neuropathy drugs. On the other hand, the synthesis of highly potent and selective ORL1 agonists is also important because it may not only facilitate the identification of receptor functional residues but may also result in ligands with enhanced desensitization and internalization of the receptor, thus providing a different kind of antagonistic response.[13,14]

Nociceptin contains two basic amino acid pairs, namely, Arg-Lys (RK) sequences at positions 8–9 and 12–13. These have been shown to be essential for both receptor recognition and activation.[15,16] This basic regions of nociceptin have been postulated to interact with a cluster of acidic amino acids in the second extracellular loop of the ORL1 receptor.[17–19] In our previous study, we synthesized a series of peptides in which an additional basic amino acid pair RK was placed adjacent to the original RK repeats.[20] By

Abbreviations: BAEE, benzoyl-L-arginine ethyl ester; BCA, bicinchoninic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Noc, nociceptin; RP-HPLC, reversed-phase HPLC; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

* Corresponding author. Tel./fax: +81 92 642 2584.
E-mail address: shimo@chem.kyushu-univ.jp (Y. Shimohigashi).
† These two authors contributed equally to this study.
‡ Present address: Mebiopharm Co., Ltd., Research and Development, Sumitomo Akasaka Building 5th Floor, 8-10-24, Akasaka, Minato-ku, Tokyo 107-0052, Japan.
§ Present address: NIH-NINDS-RBU, Bethesda, MD 20892, USA.

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.09.014

using human ORL1 expressed in HEK293 cells, we found that [RK$^{14-15}$]nociceptin ([RK$^{14-15}$]Noc) had 3-fold higher affinity and 17-fold greater biological activity than native nociceptin.

[RK$^{14-15}$]Noc is the first example of a functional superagonist for the ORL1 receptor. [RK$^{14-15}$]Noc elicits much more potentiated biological activity than expected based on the results for the receptor-binding affinity.[20] Since then, the RK$^{14-15}$ unit has been utilized by other groups as a building block to obtain nociceptin analogs with enhanced in vitro and in vivo activities.[21,22] Also, RK$^{14-15}$ has frequently been utilized as an essential building element to create more potent antagonists.[23–28]

In the present study, we synthesized a series of nociceptin analogs in which the amino acid residues at positions 14 and 15 were replaced by various amino acids, in order to elucidate whether or not both the residues of RK are necessary for activity enhancement and to obtain further insight into the molecular mechanism of nociceptin–receptor interactions. We evaluated the binding affinity of analogs by a radio-ligand receptor-binding assay using COS-7 cells expressing the rat ORL1 receptor and their biological potency in a [^{35}S]GTPγS-binding assay using. We also measured the susceptibility of the analogs to proteolysis using purified protease and rat brain membrane homogenates to assess whether enhanced resistance to proteolytic cleavage could account for the enhanced activity.

2. Results

2.1. Peptide syntheses

As shown in Table 1, nociceptin and its 22 analogs were evaluated for their activities against ORL1 receptors in this study. In addition to previously prepared analogs such as [RK$^{6-7}$]Noc (**1**), [RK$^{10-11}$]Noc (**2**), and [RK$^{14-15}$]Noc (**3**),[20] we tested two newly synthesized RK-containing analogs—i.e., single RK-inserted [RK$^{16-17}$]Noc (**4**) and double RK-inserted [RK$^{14-15,16-17}$]Noc (**5**). In one series of analogs, the original Leu^{14}–Ala^{15} was replace with three other kinds of basic dipeptides: [KR$^{14-15}$]Noc (**6**), [RR$^{14-15}$]Noc (**7**), and [KK$^{14-15}$]Noc (**8**). In another series of analogs, Arg and Lys were incorporated at either position 14 or 15 to obtain [R^{14}]Noc (**9**), [K^{14}]Noc (**10**), [R^{15}]Noc (**16**), and [K^{15}]Noc (**17**).

Various hydrophobic amino acids were further substituted for the residues at positions 14 and 15. The analogs containing aliphatic amino acids (Ala, Val, and Leu) are [A^{14}]Noc (**11**), [V^{14}]Noc (**12**) [L^{15}]Noc (**18**), and [V^{15}]Noc (**19**), while those containing aromatic amino acids (Phe, Tyr, and Trp) are [F^{14}]Noc (**13**), [Y^{14}]Noc (**14**), [W^{14}]Noc (**15**), [F^{15}]Noc (**20**), [Y^{15}]Noc (**21**), and [W^{15}]Noc (**22**).

All such analogs were synthesized by the Fmoc solid-phase methodology. Peptides were obtained with an average yield of approximately 50%. The purity was verified by analytical HPLC, in which all peptides emerged as a single peak. Synthesized peptides containing 5–8 basic amino acids (Arg and Lys) were eluted at 13.0–16.2 min of retention time in this reversed-phase (RP)-HPLC (Table 2), while native nociceptin having 4 basic amino acids was eluted at 16.4 min. There was a slight tendency for nociceptin analogs having more than 5 basic amino acids to be eluted faster than nociceptin under the same elution conditions. The measured mass numbers were coincident with the calculated values (Table 2).

2.2. Activities of nociceptin for the rat ORL1 receptor expressed in COS-7 cells

A saturation binding assay followed by Scatchard analysis was used to determine the dissociation constant of [^{3}H]nociceptin against the rat ORL1 receptor expressed in COS-7 cells. The K_d value (92 pM) was almost identical to that obtained in the assay using human ORL1 receptors expressed in HEK293 cells.[20] [^{3}H]nociceptin yielded an appropriate specific-binding curve (data not shown). The Scatchard analysis of the data showed a linear straight line, indicating the presence of a homogenous population of ORL1 (B_{max} = 700 fmol/mg protein). In the competitive receptor-binding assay, nociceptin showed very potent binding to the ORL1 expressed in COS-7 cells. The half-maximal concentration (IC_{50}) for inhibition of the binding of [^{3}H]nociceptin was calculated to be 0.73 nM (Table 3).

Table 1
The list of a series of RK-inserted nociceptin analogs and [Leu^{14} and/or Ala^{15}]-substituted nociceptin analogs

Peptide No.	Peptides	Sequences	Number of basic amino acids
	Nociceptin (Noc)	FGGFTGARKSARKLANQ	4
1	[RK$^{6-7}$]Noc	FGGFT**RK**RKSARKLANQ	6
2	[RK$^{10-11}$]Noc	FGGFTGARK**RK**RKLANQ	6
3	[RK$^{14-15}$]Noc	FGGFTGARKSARK**RK**NQ	6
4	[RK$^{16-17}$]Noc	FGGFTGARKSARKLA**RK**	6
5	[RK$^{14-15,16-17}$]Noc	FGGFTGARKSARK**RKRK**	8
6	[KR$^{14-15}$]Noc	FGGFTGARKSARK**KR**NQ	6
7	[RR$^{14-15}$]Noc	FGGFTGARKSARK**RR**NQ	6
8	[KK$^{14-15}$]Noc	FGGFTGARKSARK**KK**NQ	6
9	[R^{14}]Noc	FGGFTGARKSARK**R**ANQ	5
10	[K^{14}]Noc	FGGFTGARKSARKKANQ	5
11	[A^{14}]Noc	FGGFTGARKSARK**A**ANQ	4
12	[V^{14}]Noc	FGGFTGARKSARK**V**ANQ	4
13	[F^{14}]Noc	FGGFTGARKSARK**F**ANQ	4
14	[Y^{14}]Noc	FGGFTGARKSARK**Y**ANQ	4
15	[W^{14}]Noc	FGGFTGARKSARKWANQ	4
16	[R^{15}]Noc	FGGFTGARKSARKL**R**NQ	5
17	[K^{15}]Noc	FGGFTGARKSARKL**K**NQ	5
18	[L^{15}]Noc	FGGFTGARKSARKLLNQ	4
19	[V^{15}]Noc	FGGFTGARKSARKLVNQ	4
20	[F^{15}]Noc	FGGFTGARKSARKL**F**NQ	4
21	[Y^{15}]Noc	FGGFTGARKSARKL**Y**NQ	4
22	[W^{15}]Noc	FGGFTGARKSARKL**W**NQ	4

Bold letters indicate the amino acids replaced. The amino acid residues underlined are basic amino acids Arg(=R) and Lys(=K).

Table 2
Synthetic yield and analytical data of a series of RK-inserted and [Leu^{14} and/or Ala^{15}]-substituted nociceptin analogs

	Peptides	Yield (%)	RP-HPLC Retention time (min)	MALDI-TOF MS	
				Found (m/z)	Calcd (m+H$^+$)
4	[RK$^{16-17}$]Noc	50	16.0	1851.8	1852.2
5	[RK$^{14-15,16-17}$]Noc	30	13.0	1951.0	1951.3
6	[KR$^{14-15}$]Noc	65	16.2	1910.0	1910.2
7	[RR$^{14-15}$]Noc	53	15.8	1937.4	1938.2
8	[KK$^{14-15}$]Noc	56	15.8	1881.5	1882.2
9	[R^{14}]Noc	55	15.3	1852.3	1853.1
10	[K^{14}]Noc	74	14.0	1824.2	1825.1
11	[A^{14}]Noc	64	14.5	1767.0	1768.0
12	[V^{14}]Noc	60	15.5	1796.3	1796.1
13	[F^{14}]Noc	43	16.1	1842.7	1844.1
14	[Y^{14}]Noc	68	16.8	1859.7	1860.1
15	[W^{14}]Noc	30	21.9	1882.4	1883.1
16	[R^{15}]Noc	38	15.7	1894.2	1895.2
17	[K^{15}]Noc	17	15.2	1866.9	1867.2
18	[L^{15}]Noc	60	19.1	1851.0	1852.1
19	[V^{15}]Noc	50	16.5	1837.5	1838.1
20	[F^{15}]Noc	55	17.4	1885.5	1886.2
21	[Y^{15}]Noc	34	16.3	1901.5	1902.2
22	[W^{15}]Noc	32	25.9	1924.9	1925.2

Table 3
Binding potency and biological activity of nociceptin and synthetic analogue peptides to rat ORL1 receptors

	Nociceptin analogs	Binding Potency IC_{50} (nM)	GTPγS-binding activity EC_{50} (nM)
	Nociceptin	0.73 ± 0.24	13 ± 2.4
1	[RK$^{6-7}$]Noc	13 ± 5.9	870 ± 220
2	[RK$^{10-11}$]Noc	0.94 ± 0.55	86 ± 17
3	[RK$^{14-15}$]Noc	0.21 ± 0.12	1.1 ± 0.18
4	[RK$^{16-17}$]Noc	0.54 ± 0.16	2.7 ± 0.12
5	[RK$^{14-15,16-17}$]Noc	0.42 ± 0.21	2.3 ± 0.13
6	[KR$^{14-15}$]Noc	0.21 ± 0.15	1.3 ± 0.41
7	[RR$^{14-15}$]Noc	0.27 ± 0.17	0.98 ± 0.19
8	[KK$^{14-15}$]Noc	0.16 ± 0.09	1.1 ± 0.06
9	[R^{14}]Noc	0.33 ± .14	4.0 ± 0.82
10	[K^{14}]Noc	0.41 ± 0.23	4.1 ± 1.7
11	[A^{14}]Noc	1.5 ± 0.25	11 ± 1.4
12	[V^{14}]Noc	1.1 ± 0.15	15 ± 2.9
13	[F^{14}]Noc	1.1 ± 0.22	9.0 ± 4.8
14	[Y^{14}]Noc	1.1 ± 0.12	11 ± 2.5
15	[W^{14}]Noc[a)]	0.41 ± 0.24	5.8 ± 2.3
16	[R^{15}]Noc	0.31 ± 0.14	3.2 ± 1.1
17	[K^{15}]Noc	0.41 ± 0.24	5.8 ± 2.3
18	[L^{15}]Noc	0.48 ± 0.13	10 ± 3.3
19	[V^{15}]Noc	2.2 ± 0.77	30 ± 7.5
20	[F^{15}]Noc	1.0 ± 0.40	12 ± 4.1
21	[Y^{15}]Noc	1.0 ± 0.37	33 ± 6.5
22	[W^{15}]Noc[a]	2.1 ± 0.64	50 ± 7.9

Data are mean ± SEM of at least three experiments (n = 3–5).
[a] Analogs **15** and **22** were also reported previously by us in the literature: *Bull. Chem. Soc. Jpn.*, **72**, 1898 (1999).

The biological activity of nociceptin was assessed in the [^{35}S]GTPγS-binding assay using membranes prepared from COS-7 cells expressing rat ORL1 receptor. The concentration of nociceptin inducing 50% of the maximal stimulation (EC_{50}) was estimated to be 13 nM (Table 3).

2.3. Structure–activity relationships of RK-incorporating nociceptin analogs

Using the competitive receptor-binding assay in COS-7 cells expressing rat ORL1, [RK$^{14-15}$]Noc (**3**) was very potent (IC_{50}, 0.21 nM), displaying a 4-fold enhancement of binding affinity compared to nociceptin (Table 3). Analog **3** was the most potent (EC_{50}, 1.1 nM) in the GTPγS functional assay, where it was about 12-fold more potent than nociceptin (Table 3). These results clearly indicate that [RK$^{14-15}$]Noc exhibits much more enhanced biological activity than expected from the receptor-binding assay, as reported previously.[20]

The peptides had relatively minor effects on the maximal stimulation of GTPγS-binding compared with nociceptin. For example, [RK^{14,15}]Noc exhibited a slight enhancement of maximal response (approximately 10% greater than nociceptin as shown in Figure 1). The only exception is [RK$^{6-7}$]Noc (**1**), which exhibited a significantly reduced maximal response (approximately 60%) (Fig. 1). This peptide was also very weak in both the receptor-binding and GTPγS-binding assays (Table 1). [RK$^{10-11}$]Noc (**2**) showed almost the same receptor affinity as nociceptin, and approximately a 7-fold reduction of potency in the GTPγS functional assay (Table 1). These results suggested that **2** might have antagonist characteristics, although it exhibited >90% of the maximum nociceptin response in the GTPγS-binding assay (Fig. 1).

In the attempt to obtain analogs more potent than [RK$^{14-15}$]Noc, [RK$^{16-17}$]Noc (**4**) and [RK$^{14-15,16-17}$]Noc (**5**) were synthesized. [RK$^{16-17}$]Noc was very potent in the receptor-binding assay and functional assay (IC_{50} = 0.54 nM and EC_{50} = 2.7 nM). However, this analog **4** was about two-fold less potent than [RK$^{14-15}$]Noc (Table 3).

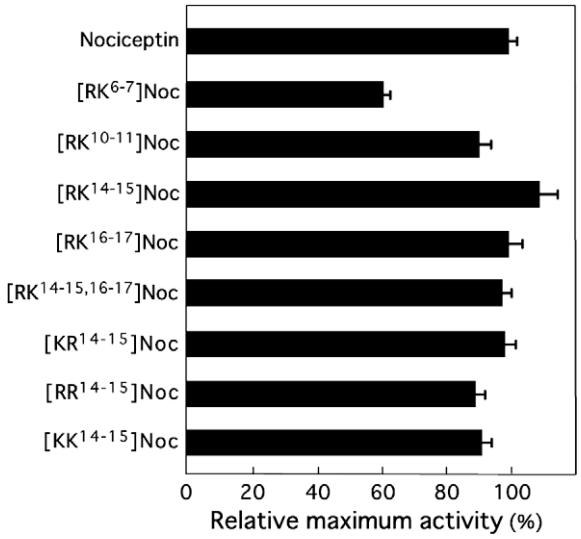

Figure 1. The maximum responses of rat ORL1 receptor stimulated by dual basic peptide unit-incorporated analogs of nociceptin in the GTPγS functional assay. Data are mean ± SEM of at least three experiments (n = 3–5).

[RK$^{14-15,16-17}$]Noc was as potent as [RK$^{16-17}$]Noc (IC_{50} = 0.42 nM and EC_{50} = 2.3 nM). All these results suggest that the activity enhancement effect by RK-substitution at the positions 15–16 dominates over that at the positions 14–15, and indicate that [RK$^{14-15}$]Noc is the analog with the optimal enhancement of affinity and bioactivity.

2.4. Dibasic RK$^{14-15}$ replaceable with KR, RR, or KK

To evaluate if the increase in nociceptin activity might be critically dependent on the type of basic residues replaced in positions 14–15, we compared the activities of the analogs [KR$^{14-15}$]Noc (**6**), [RR$^{14-15}$]Noc (**7**), and [KK$^{14-15}$]Noc (**8**) with that of [RK$^{14-15}$]Noc. As shown in Table 3, all three peptides [KR$^{14-15}$]Noc, [RR$^{14-15}$]Noc, and [KK$^{14-15}$]Noc showed almost the same levels of activity as [RK$^{14-15}$]Noc. These results clearly indicate that very similar enhancements of activity can be achieved as long as two basic charges are present in positions 14–15, regardless of the nature of the amino acid that carries them.

2.5. The importance of a basic dipeptide unit at the consecutive positions 14–15

To evaluate the importance of dual versus single basic residue replacement in enhancing nociceptin activity, we synthesized analogs containing Arg and Lys at either position 14 or 15. Peptides incorporating a single basic amino acid, namely, [R^{14}]Noc (**9**), [K^{14}]Noc (**10**), [R^{15}]Noc (**16**), and [K^{15}]Noc (**17**), were examined in the receptor-binding and GTPγS assays. All these analogs displayed a binding affinity that was approximately twice greater than nociceptin (Table 3), but slightly smaller than the corresponding peptides having the same basic amino acids at both positions 14 and 15 (i.e., [RK$^{14-15}$]Noc (**3**), [KR$^{14-15}$]Noc (**6**), [RR$^{14-15}$]Noc (**7**), and [KK$^{14-15}$]Noc (**8**)).

The EC_{50} values of [R^{14} or K^{14}]Noc (**9**, **10**) and [R^{15} or K^{15}]Noc (**16**, **17**) in the GTPγS assay ranged between 3.2 and 5.8 nM. Thus, they were 2–4 times more potent than nociceptin, but 4 times less potent than the analogs incorporating two basic amino acids. Consequently, the introduction of two basic residues at positions 14–15 appears to increase in an additive fashion the activity of nociceptin.

2.6. The effect of aliphatic and aromatic residues at position 14 or 15

The original amino acids at positions 14 and 15 are Leu and Ala, respectively. Replacement of these amino acids with the basic amino acids Arg and Lys greatly potentiated nociceptin to interact with ORL1. We next replaced Leu^{14} and Ala^{15} with other amino acids to examine whether or not amino acids other than Arg and Lys can potentiate nociceptin activity. When Leu^{14} was replaced with Ala, Val, Phe, Tyr, and Trp, only $[W^{14}]$Noc exhibited a binding affinity (0.41 nM) and a biological activity comparable to those of the positive charge carrying analogs $[R^{14}]$Noc and $[K^{14}]$Noc (Table 3). Clearly, Trp can mimic the effect of Arg and Lys at position 14 of nociceptin. All other analogs, $[A^{14}]$Noc, $[V^{14}]$Noc, $[F^{14}]$Noc, and $[Y^{14}]$Noc maintained the activity of nociceptin but did not exhibit any enhancement.

In contrast, when Ala^{15} was replaced with Leu, Val, Phe, Tyr, and Trp, no substitution produced an enhancement of activity comparable to that observed with $[R^{15}]$Noc and $[K^{15}]$Noc (Table 3). In the receptor-binding assay, $[L^{15}]$Noc was as potent as $[R^{15}]$Noc and $[K^{15}]$Noc as shown in Table 3. Apparently, these three nociceptin analogs, $[L^{15}]$Noc, $[R^{15}]$Noc, and $[K^{15}]$Noc, are a few times more potent than wild-type nociceptin. On the other hand, $[L^{15}]$Noc was almost as active as wild-type nociceptin in the GTPγS-binding biological assay. This made $[L^{15}]$Noc be a few times less potent than both $[R^{15}]$Noc and $[K^{15}]$Noc. Obviously, the activity enhancement of $[L^{15}]$Noc in the receptor-binding assay is due to the reason different from that by $[R^{15}]$Noc and $[K^{15}]$Noc.

2.7. Enzymatic digestion assay

In principle, the enhanced potency of nociceptin analogs may result from increased resistance to endogenous proteases, which are usually present in the membrane preparations that are used to measure the binding affinity and the GTPγS functional activity of the peptides. To evaluate such possibility, we first investigated the hydrolysis of nociceptin and its analogs by the serine protease trypsin, which cleaves the peptide bond at the carboxyl side of basic amino acids Arg and Lys. The 17-mer native nociceptin peptide, (denoted here as nociceptin-(1–17)), was completely degraded in 2 h by trypsin to afford smaller peptide fragments such as nociceptin-(1–8) (FGGFTGAR) and nociceptin-(1–9) (FGGFTGARK). Other potential fragments, such as nociceptin-(1–12) (FGGFTGARKSAR) and nociceptin-(1–13) (FGGFTGARKSARK) were not detected. The identity of cleaved fragments were established by the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of HPLC eluates. After 4 h, only nociceptin-(1–8) and nociceptin-(1–9) were still detectable in HPLC.

$[RK^{14-15}]$Noc was hydrolysed by trypsin to mainly yield nociceptin-(1–8), nociceptin-(1–9), and nociceptin-(1–12) in the first 30 min. Other possible fragments, such as nociceptin-(1–13) (FGGFTGARKSARK), nociceptin-(1–14) (FGGFTGARKSARKR), and nociceptin-(1–15) (FGGFTGARKSARKRK), were not detected at all. After 3 h, $[RK^{14-15}]$Noc disappeared entirely, and only nociceptin-(1–8) and nociceptin-(1–9) remained detectable.

Figure 2A depicts the trypsin hydrolysis of nociceptin and its analogs as a function of time. The half-life times ($t_{1/2}$) estimated from such experiments were 13 min for nociceptin and 30 min for $[RK^{14-15}]$Noc (Fig. 2A), indicating that native nociceptin is degraded by trypsin at a 2.3-fold faster rate than $[RK^{14-15}]$Noc. Single basic residue-carrying analogs such as $[R^{14}]$Noc and $[K^{15}]$Noc displayed rates of hydrolysis ($t_{1/2}$, 23 and 20 min, respectively) intermediate between those of nociceptin and $[RK^{14-15}]$Noc. Thus, the incorporation of basic amino acids at positions 14 and 15 can increase resistance to trypsin hydrolysis.

The HPLC degradation profiles of nociceptin and $[RK^{14-15}]$Noc in rat brain homogenate were much more complex, showing multiple minor peaks (Fig. 2C). Since brain homogenate contains various types of membrane-bound proteases including trypsin-like enzymes, peptide bonds other than Arg-Lys-Xaa can be cleaved to provide many additional peptide fragments. For instance, we could detect peaks such as nociceptin-(3–16) (GFTGARKSARKL) and nociceptin-(4–17) (FTGARKSARKLANQ) in nociceptin digestion (Fig. 2C). Both nociceptin and $[RK^{14-15}]$Noc were degraded completely in 48 h (Fig. 2B and C). The $t_{1/2}$ values of nociceptin and $[RK^{14-15}]$Noc were only slightly different (8 and 10 h, respectively) (Fig. 2B). It should be noted, however, that during the first 60 min of incubation both nociceptin and $[RK^{14-15}]$Noc were degraded only by less than 10%. These degradations were almost abolished in the presence of the enzyme inhibitor bacitracin (data not shown). Moreover, in membrane preparations of COS-7 cells used as suspensions identical to those employed for the binding assays, but with no expression of the receptor, we could not detect any significant degradation of nociceptin or $[RK^{14-15}]$Noc (data not shown).

Taken collectively, such results indicate that although basic residues substitutions can enhance the resistance of nociceptin to trypsin hydrolysis, the differences in binding affinity and bioactivity cannot be accounted for by altered protease stability during the assays.

3. Discussion

We have shown previously and confirmed here that $[RK^{14-15}]$Noc is a potent analog of nociceptin both in the receptor-binding assay and in the GTPγS assay. In the present study, we show that such enhancement of nociceptin activity can be achieved by any combination of basic residues pairs that are replaced in positions 14–15. In fact, the binding affinity and biological potencies of $[KR^{14-15}]$Noc, $[RR^{14-15}]$Noc, and $[KK^{14-15}]$Noc were not significantly different from those of $[RK^{14-15}]$Noc. In contrast, the single replacement of Leu^{14} or Ala^{15} with Arg or Lys generated analogs exhibiting smaller enhancement of binding affinity and bioactivity. All these results indicate that the optimal effect in enhancing the binding affinity and receptor activation of nociceptin requires that two positively charged amino acids are simultaneously incorporated into positions 14–15. The highly enhanced biological activity of $[RK^{14-15}]$Noc and similar substituted analogs results from a more favourable interaction with the receptor and cannot be explained by increased resistance to proteolysis in the binding assays.

In the present study, we obtained additional information on the structure–activity requirements of positions 14–15 in nociceptin. First, it was found that the activity enhancement induced by the presence of a basic amino acid at position 14, could be mimicked with the aromatic amino acid Trp. The only structural element that Lys, Arg, and Trp have in common is that they can potentially interact with aromatic residues such as Tyr, Trp, His, and Phe. In fact, Lys and Arg interact with these aromatic amino acids by a cation/π interaction,[29] while Trp interacts with them via π/π hydrophobic interaction.[30] Among the aromatic amino acids, only Trp can be used as a substitute for Arg and Lys at position 14 of nociceptin to reinforce the activities. Although the reason for this finding is not clear, it may be attributable to Trp possessing a larger aromatic ring than any of these other aromatic amino acids.

Unlike position 14, position 15 did not tolerate any aromatic substitution and only Arg^{15} or Lys^{15} produced enhancements of nociceptin activity. Therefore, the requirement for a favourable electrostatic interaction is more stringent in position 15 than in position 14.

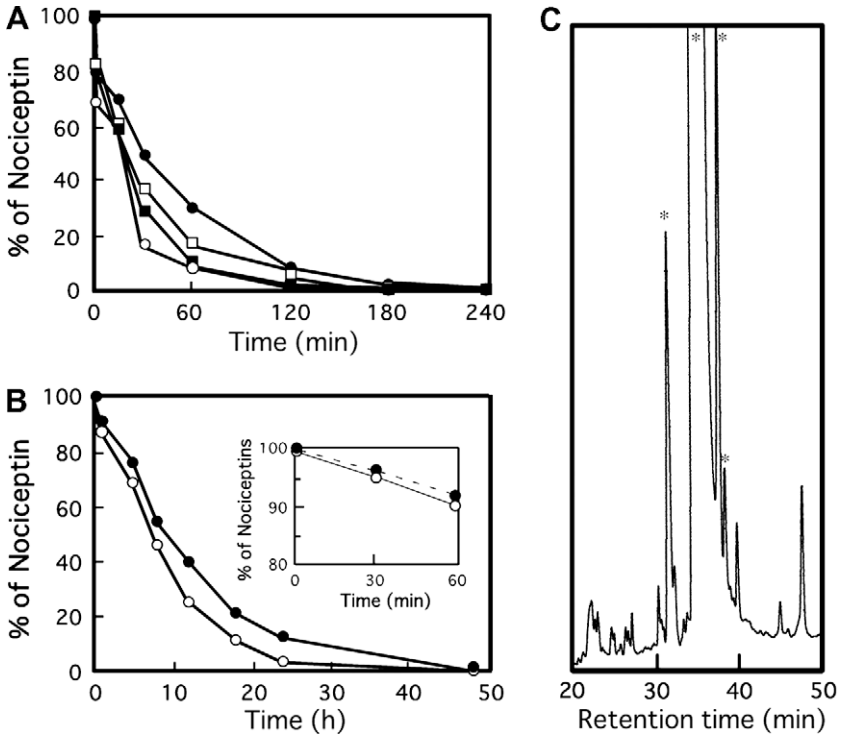

Figure 2. The profiles of enzymatic digestion of nociceptin and its analogs. (A) Digestion with purified enzyme trypsin: ○, nociceptin; □, [R^{14}]Noc; ■, [K^{15}]Noc; and ●, [RK^{14-15}]Noc. (B) Digestion with rat brain homogenate: ○, nociceptin; and ●, [RK^{14-15}]Noc. (**C**) HPLC profile of nociceptin digestion with rat brain homogenate after 60 min. Peaks marked (*) are of rat brain homogenate. Identified peaks: 32.01 min for nociceptin in conjunction with materials from the rat brain homogenate; 40.33 min for nociceptin-(3–16); and 48.19 min for nociceptin-(4–17). Nociceptin and its derivatives were degraded completely after incubation for many hours, but almost no degradation was observed when treated with enzyme inhibitor bacitracin.

Based on studies examining a series of chimeric receptors and computational modelling, it has been suggested that nociceptin interacts with ORL1 by electrostatic interactions between its two native RK repeats (RK^{8-9} and RK^{12-13}) and a receptor acidic region present in the second extracellular loop.[17,18,31] It is thus possible that the additional RK repeat incorporated in 14–15 may contribute to enlarge the positive electrostatic field that links nociceptin to the acidic cluster on the receptor. It is also possible, however, that the newly introduced dipeptide allows nociceptin to establish additional interactions, perhaps via π/cation bonds with nearby aromatic residues present in the receptor, as discussed above. These extra interactions may stabilize the total ligand/receptor interaction thus improving both ligand affinity and receptor activation. Identification of the receptor-binding site for RK^{14-15} might afford important structural information regarding the design of receptor agonists and antagonists. A site-directed receptor mutagenesis study to identify residues responsible for the enhanced potency of RK^{14-15} is currently underway in our laboratory.

4. Conclusion

The amino acid sequence of hyperalgesic neuropeptide nociceptin consists of two repeated RK units at positions 8–9 and 12–13. By placing another RK, KR, RR, or KK at position 14–15, where Leu-Ala is originally present, highly potent analogs were obtained. The resulting [(R, or K)^{14}, (R, or K)^{15}]Noc exhibited much more enhanced biological activity than expected from its receptor-binding activity. It was found that this synergistic enhancement in biological activity occurs only when basic amino acids are simultaneously incorporated at consecutive positions 14–15, regardless of the sequence. In particular, the basic amino acid at position 15 is indispensable, whereas the basic amino acid at position 14 is replaceable with Trp.

5. Experimental

5.1. Peptide syntheses

All the peptides were synthesized by manual solid peptide syntheses or an automated peptide synthesizer ABI 433A (Applied Biosystems Inc., Foster City, CA) with the Fmoc synthetic strategy (0.1 mmol scale). Peptides were liberated from the resin using Reagent K (82.5% trifluoroacetic acid, 5% water, 5% thioanisole, 5% phenol, and 2.5% 1,2-ethanedithiol) at room temperature. After 3 h, the reaction was terminated with diethyl ether. The purification was carried out by RP-HPLC on a preparative column (25 × 250 mm, Cica-Merck LiChrospher RP-18 (e), 5 μm) with a linear gradient of 0.1% trifluoroacetic acid (TFA) and 80% acetonitrile containing 0.1% TFA. Detection was carried out at 230 nm. The fractions containing pure peptides were lyophilized, and the purity was verified by analytical RP-HPLC (4 × 250 mm, Cica-Merck LiChrospher 100 RP-18, 5 μm). Mass spectra of peptides were measured on a mass spectrometer Voyager™ DE-PRO (PerSeptive Biosystems Inc., Framingham, MA) with the method of MALDI-TOF.

5.2. Expression plasmid, cell culture, transfection, and membrane preparation

The cDNA clone of the rat ORL1 receptor was inserted into a pcDNA3 expression vector (Invitrogen, San Diego, CA) at the NotI/XbaI sites. COS-7 cells were maintained in Dulbecco's modi-

fied Eagle's medium supplemented with 10% fetal bovine serum and suitable antibiotics in a 5% CO_2 atmosphere at 37 °C. Plasmid DNA (30 μg) was transiently transfected into confluent COS-7 cells on a 150-cm^2-culture plate using the DEAE-dextran method. After 48 h, cells were harvested and centrifuged for 10 min at 500*g* (4 °C). Cells were resuspended in 5 mM Tris buffer containing 1 mM EGTA and 11% saccharose (pH 7.4), and homogenized with the Teflon tissue homogenizer. The homogenate was centrifuged for 10 min at 1000*g* (4 °C). The supernatant was recentrifuged for 20 min at 40,000 *g* (4 °C), and the pellet was washed with 5 mM Tris buffer containing 1 mM EGTA (pH 7.4). The concentration of membrane protein was estimated by the BCA protein assay method using bicinchoninic acid (Pierce, Rockford, IL). The prepared membrane was frozen at –80 °C until use.

5.3. Radio-ligand receptor-binding assay

For the saturation binding assay, a series of reaction mixtures containing 5 μg/ml membrane protein were incubated with increasing concentrations of [^{3}H]nociceptin (158 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK) (0.05–2 nM) for 90 min at 25 °C in 50 mM HEPES–Tris buffer (pH 7.4) containing 0.1% BSA. Bacitracin (100 μg/ml) was added as an enzyme inhibitor. After incubation, each incubation mixture (500 μl) was filtered through glass fiber filters (GF/B; Whatman Inc., Clifton, NJ), which were pre-steeped with 0.5% ethylene imine polymer for 1 h, and rinsed twice with 50 mM Tris–HCl buffer (pH 7.4). Non-specific binding was determined in the presence of 10 μM nociceptin. The receptor-binding potencies of the synthetic peptides were assessed by competitive binding assay. Briefly, membranes (5 μg/ml), serial concentrations of synthetic peptide, and 0.05 nM [^{3}H]nociceptin were incubated for 90 min at 25 °C in the same buffer (2 ml in each tube). The computer program ALLFIT[32] 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. In vitro [^{35}S]GTPγS-binding assays

The in vitro biological activities of the synthetic peptides were appraised by [^{35}S]GTPγS-binding assay. The membranes (5 μg) were suspended in 50 mM HEPES–Tris buffer (pH 7.4) containing 100 mM NaCl, 10 mM $MgCl_2$, 200 mM EGTA, and 200 μM dithiothreitol. Bacitracin (100 μg/ml) was added as an enzyme inhibitor. Each tube (100 μl) was incubated for 60 min at 25 °C with the appropriate concentration of peptides in the presence of 3 μM GDP and 100 pM of [^{35}S]GTPγS (1,000 Ci/mmol; Amersham Pharmacia Biotech). Non-specific binding was determined in the presence of 10 μM GTPγS. After incubation, each mixture was filtered through glass fiber filters (GF/B; Whatman) and rinsed in the same manner as described for the radio-ligand receptor-binding assay. The functional activity was estimated as EC_{50}, i.e., the peptide concentration generating 50% of the maximal observed stimulation of GTPγS-binding.

5.5. Enzymatic digestion assay

In the trypsin-digestion assay, the reactions were carried out in 50 mM Tris–HCl buffer (pH 8.0) at 25 °C. The reaction mixture (500 μl) including 100 μM substrate peptide was digested with 2 nM TPCK-treated bovine pancreas trypsin (12,000 BAEE units/mg). The digestion was terminated at different reaction times (0, 1, 4, 16, 32, 64 min, 2, and 4 h) by adding 100 μl of 5 M AcOH. The aliquots were analyzed on RP-HPLC with a linear gradient of 0.1% TFA and 80% acetonitrile containing 0.1% TFA. The masses of the major isolated peaks were determined using a MALDI-TOF mass spectrometer.

In the digestion assay with rat brain homogenate, rat brain was homogenized by a Polytron tissue homogenizer in 10 mM Tris–HCl buffer (pH 7.4), and the homogenate was centrifuged for 15 min at 40,000 *g* (4 °C). This operation was repeated twice more with the same buffer and the final mixture was added to each reaction solution. The reaction was terminated at different times (0, 0.5, 1, 2, 4, 8, 12, 16, 24, and 48 h) by adding trichloroacetic acid for denaturation of enzyme proteins. Other conditions were the same as for the trypsin-digestion assay.

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.

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. *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. Mollereau, C.; Parmentier, M.; Mailleux, P.; Butour, J. L.; Moisand, C.; Chalon, P.; Caput, D.; Vassart, G.; Meunier, J. C. *FEBS Lett.* **1994**, *341*, 33.
4. Chen, Y.; Fan, Y.; Liu, J.; Mestek, A.; Tian, M.; Kozak, C. A.; Yu, L. *FEBS Lett.* **1994**, *347*, 279.
5. Wang, J. B.; Johnson, P. S.; Imai, Y.; Persico, A. M.; Ozenberger, B. A.; Eppler, C. M.; Uhl, G. R. *FEBS Lett.* **1994**, *348*, 75.
6. Nishi, M.; Takeshima, H.; Mori, M.; Nakagawara, K.; Takeuchi, T. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1353.
7. Mollereau, C.; Simons, M. J.; Soularue, P.; Liners, F.; Vassart, G.; Meunier, J. C.; Parmentier, M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8666.
8. Okuda-Ashitaka, E.; Tachibana, S.; Houtani, T.; Minami, T.; Masu, Y.; Nishi, M.; Takeshima, H.; Sugimoto, T.; Ito, S. *Brain Res. Mol. Brain Res.* **1996**, *43*, 96.
9. Pan, Y. X.; Xu, J.; Pasternak, G. W. *Biochem. J.* **1996**, *315*, 11.
10. Sandin, J.; Georgieva, J.; Schott, P. A.; Ogren, S. O.; Terenius, L. *Eur. J. Neurosci.* **1997**, *9*, 194.
11. 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.
12. Jenck, F.; Moreau, J. L.; Martin, J. R.; Kilpatrick, G. J.; Reinscheid, R. K.; Monsma, F. J., Jr.; Nothacker, H. P.; Civelli, O. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14854.
13. Thomsen, C.; Hohlweg, R. *Br. J. Pharmacol.* **2000**, *131*, 903.
14. Wichmann, J.; Adam, G.; Rover, S.; Hennig, M.; Scalone, M.; Cesura, A. M.; Dautzenberg, F. M.; Jenck, F. *Eur. J. Med. Chem.* **2000**, *35*, 839.
15. Dooley, C. T.; Houghten, R. A. *Life Sci.* **1996**, *59*, PL23.
16. Reinscheid, R. K.; Ardati, A.; Monsma, F. J., Jr.; Civelli, O. *J. Biol. Chem.* **1996**, *271*, 14163.
17. Topham, C. M.; Mouledous, L.; Poda, G.; Maigret, B.; Meunier, J. C. *Protein Eng.* **1998**, *11*, 1163.
18. Mollereau, C.; Mouledous, L.; Lapalu, S.; Cambois, G.; Moisand, C.; Butour, J. L.; Meunier, J. C. *Mol. Pharmacol.* **1999**, *55*, 324.
19. Zhang, C.; Miller, W.; Valenzano, K. J.; Kyle, D. J. *J. Med. Chem.* **2002**, *45*, 5280.
20. 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.
21. Rizzi, D.; Rizzi, A.; Bigoni, R.; Camarda, V.; Marzola, G.; Guerrini, R.; De Risi, C.; Regoli, D.; Calo, G. *J. Pharmacol. Exp. Ther.* **2002**, *300*, 57.
22. Broccardo, M.; Linari, G.; Guerrini, R.; Agostini, S.; Petrella, C.; Improta, G. *Peptides* **2005**, *26*, 1590.
23. Calo, G.; Rizzi, A.; Rizzi, D.; Bigoni, R.; Guerrini, R.; Marzola, G.; Marti, M.; McDonald, J.; Morari, M.; Lambert, D. G.; Salvadori, S.; Regoli, D. *Br. J. Pharmacol.* **2002**, *136*, 303.
24. McDonald, J.; Calo, G.; Guerrini, R.; Lambert, D. G. *Arch. Pharmacol.* **2003**, *367*, 183.
25. Carra, G.; Rizzi, A.; Guerrini, R.; Barnes, T. A.; McDonald, J.; Hebbes, C. P.; Mela, F.; Kenigs, V. A.; Marzola, G.; Rizzi, D.; Gavioli, E.; Zucchini, S.; Regoli, D.; Morari, M.; Salvadori, S.; Rowbotham, D. J.; Lambert, D. G.; Kapusta, D. R.; Calo, G. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 1114.
26. Calo, G.; Guerrini, R.; Rizzi, A.; Salvadori, S.; Burmeister, M.; Kapusta, D. R.; Lambert, D. G.; Regoli, D. *CNS Drug Rev.* **2005**, *11*, 97.
27. Chiou, L. C.; Liao, Y. Y.; Guerrini, R.; Calo, G. *Eur. J. Pharmacol.* **2005**, *515*, 47.

28. Peng, Y. L.; Chang, M.; Dong, S. L.; Li, W.; Han, R. W.; Fu, G. X.; Chen, Q.; Wang, R. *Regul. Pept.* **2006**, *134*, 75.
29. Dougherty, D. A. *Science* **1996**, *271*, 163.
30. Mitchell, J. B. O.; Nandi, C. L.; McDonald, I. K.; Thornton, J. M. *J. Mol. Biol.* **1994**, *239*, 315.
31. 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.
32. DeLean, A.; Munson, P. J.; Rodbard, D. *Am. J. Physiol.* **1978**, *235*, E97.