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Degradation of nociceptin (orphanin FQ) by mouse spinal cord synaptic membranes is triggered by endopeptidase-24.11: an *in vitro* and *in vivo* study

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Abstract

We analyzed spinal metabolic pathway of nociceptin/orphanin FQ related to pain-transmission or modulation in the both *in vitro* and *in vivo* experiments. Nociceptin was degraded by spinal synaptic membranes. Major metabolites of nociceptin were free phenylalanine, nociceptin (1–13) and nociceptin (14–17). Both the degradation of nociceptin and the accumulation of the major cleavage metabolites, nociceptin (1–13) and nociceptin (14–17), were strongly inhibited by a metal chelator and also by specific inhibitors of endopeptidase-24.11, thiorphan and phosphoramidon. Furthermore, purified endopeptidase-24.11 hydrolyzed nociceptin at the cleavage site (Lys^{13}–Leu^{14} bond) identical to that by spinal synaptic membranes. Recently, we have found that nociceptin, injected intrathecally at small doses (fmol order) elicits a behavioral response consisting of scratching, biting and licking in mice. In the present study, we have examined the effect of peptidase inhibitors on the behavioral response elicited by intrathecal injection of nociceptin in mice. Phosphoramidon simultaneously injected with nociceptin additively enhanced nociceptin-induced behavioral response, whereas the nociceptin-induced behavioral response was unaffected by either bestatin, an aminopeptidase inhibitor or captopril, an angiotensin-converting enzyme inhibitor. However, the nociceptin effect was potentiated by combined injection of phosphoramidon and bestatin, indicating that inhibition of aminopeptidase may also contribute to inducing the behavioral response to nociceptin. These data suggest that endopeptidase-24.11 plays a major role in initial stage of nociceptin metabolism at the spinal cord level in mice.

Keywords: Metabolic pathway; Neprilysin; Neutral endopeptidase; Synaptic membranes; Intrathecal injection; Nociceptin-induced behavioral response

1. Introduction

Nociceptin [1], also referred to as orphanin FQ [2], a heptadecapeptide with a structure of Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln, was discovered as the endogenous ligand for the opioid receptor like-1 (ORL-1) receptor, recently named OP_4 receptor in accord with the proposal by Hamon [3]. Although nociceptin shows homology with dynorphin A, an endogenous κ-opioid receptor agonist, it lacks affinity for traditional opioid receptors (μ, δ and κ) while dynorphin A does not bind ORL-1 (OP_4) receptor. Nociceptin has a multiple physiological function in the central nervous system, including modulation of pain [4] and learning [5]. Immunohistological [6] and *in situ* hybridization [7,8], studies have shown that expressions of immunoreactivity and mRNA of ORL-1 (OP_4) receptor are densely distributed in the periaqueductal gray, dorsal raphe, locus coeruleus and the spinal dorsal horn. These areas are involved in the processing and modulation of nociceptive transmission. In addition, pronociceptin gene transcript [9,10] and the precursor protein of nociceptin [11–14] are highly expressed in the dorsal horn of the spinal cord and medulla, particularly in the superficial laminae. The dorsal horn of the spinal cord is considered to be an important site for

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Abbreviations: CSF, cerebrospinal fluid; GEMSA, guanidinoethyl mercaptosuccinic acid; HPLC, high performance liquid chromatography; i.t., intrathecally; ORL-1, opioid receptor like-1; PCMBS, *p*-chloromercuribenzenesulfonic acid; PHMB, *p*-hydroxy mercuribenzoate; PMSF, phenylmethylsufonyl fluoride; TFA, trifluoroacetic acid.

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nociceptive transmission [15] and many neurotransmitters such as substance P [16–18] and glutamate [19,20] are involved in the modulation of afferent nociceptive informations. In the behavioral studies, intracerebroventricularly (i.c.v) or intrathecally (i.t.) administered nociceptin in mice and rats induces hyperalgesia, analgesia, hyperalgesia followed by analgesia, and reversal of opioid-or stress-induced analgesia [21–30]. Recently, we have found that nociceptin at low doses (fmol order) injected i.t., can elicit a characteristic behavioral response of hindlimb scratching, biting and licking of hindpaw in conscious mice similar to that seen after i.t. injection of substance P [31]. Thus, these findings suggest that nociceptin plays a physiological role in the modulating nociceptive information in the spinal cord. The physiological action of nociceptin as a neurotransmitter or modulator in the central nervous system may be related to degradation of nociceptin by peptidase, as for other neuropeptides. There are some reports concerning the metabolism of nociceptin in the central nervous system [32]. These reports are clearly different from one another whether experimental studies are performed *in vivo* or *in vitro* with brain slices, dissociated neurons or subcellular fractions of brain tissue. The physiological effect of nociceptin is probably terminated by a membrane-bound, extracellularly oriented peptidase (ectoenzyme) capable of degrading nociceptin in the synaptic region, by analogy with membrane-bound acetylcholinesterase functioning in acetylcholine degradation in the synapse, although one cannot exclude an intracellular cleavage of the peptide after internalization. For instance, Met- and Leu-enkephalins are metabolized by membrane-bound peptidases, endopeptidase-24.11 (neprilysin) and aminopeptidase [33]. Inhibitors of endopeptidase-24.11 such as thiorphan and acetorphan slow the degradation of enkephalins *in vivo* and possess antinociceptive properties [33]. However, inactivation of nociceptin in the spinal synaptic region has not been well elucidated. Recently, we have reported that N-terminal fragments of nociceptin, nociceptin (1–7), nociceptin (1–9) and nociceptin (1–13) antagonize nociceptin-induced scratching, biting and licking in mice when co-injected i.t., whereas these nociceptin N-terminal fragments, injected alone, are inactive [34]. These results suggest that nociceptin N-terminal fragments are not simply inactive substances degraded by enzymatic processes. In this study, we analyzed spinal metabolic pathway of nociceptin in the both *in vitro* and *in vivo* experiments to elucidate physiological functions of nociceptin as a pain-transmitter or modulator.

2. Materials and methods

2.1. Animals

Male mice of ddY strain weighing 20–25 g were purchased from Shizuoka Laboratory Center. They were housed in cages of 15–20 animals matched for weight and placed in a colony room. Animals were housed with free access to standard food (Clea Japan, Inc.) and tap water in an air-conditioned room under a constant 12-hr light:12-hr dark cycle (light on 08:00 hr) at a temperature of 22–24° and 60–70% relative humidity.

All animal experiments were used only for a single experiment. This study was carried out in accordance with the approval of the Ethics Committee of Animal Experiment in Tohoku Pharmaceutical University and the guidelines of the ethics of the International Association for the Study of Pain [35], and efforts were made to minimize suffering and to reduce the number of animals used.

2.2. Chemicals and reagents

Nociceptin and its fragments were a generous gift from Dr. Jun Sasaki and Dr. Yasushi Matsumura of Asahi Glass Co. The commercial reagents used were: *p*-chloromercuribenzenesulfonic acid (PCMBS), enalapril maleate, thiorphan, bovine serum albumin (Sigma Chemical Co), phosphoramidon, pepstatin, leupeptin (Peptide Institute, Inc.), phenyl isothiocyanate, amino acids standard solution Type H (Wako Pure Chemical Industries Ltd.), guanidinoethylmercaptosuccinic acid (GEMSA) (Calbiochem Co), constant boiling hydrochloric acid (Pierce Chemical Co), neutral endopeptidase (endopeptidase-24.11) (Elastin Products Co, Inc.), bestatin, captopril, *p*-hydroxy mercuribenzeoate (PHMB) and phenylmethylsulfonyl fluoride (PMSF) (Nacalai tesque, Inc.).

2.3. Preparation of synaptic membranes from mouse spinal cord

Mouse spinal cord was washed with 10 mM Tris–HCl (pH 7.5) containing 155 mM NaCl, suspended in a 10-fold volume of 10% (w/w) sucrose, and homogenized with a Teflon homogenizer. The homogenate was centrifuged at 800 *g* for 20 min and then at 9000 *g* for 20 min. The second pellet was suspended in about a 5-fold volume of 5 mM Tris–HCl (pH 8.1). Then the suspension was incubated at 0° for 30 min for lysis of the pellet and subjected to discontinuous sucrose density gradient centrifugation according to the method of Jones and Matus [36]. Particle materials present at the interface between the layer containing 28.5% sucrose and that containing 34% sucrose were taken, diluted with 5 mM Tris–HCl (pH 8.1), and centrifuged at 100,000 *g* for 1 hr. The resulting pellet was suspended in 25 mM Tris–HCl (pH 8.1) and stored at −80°. Protein was determined by the method of Bradford [37] using bovine serum albumin as a standard.

2.4. HPLC analysis of the degradation of nociceptin

The degradation of nociceptin by synaptic membranes of mouse spinal cord was carried out in a mixture (0.1 mL) containing 25 mM Tris–HCl (pH 7.4), 155 mM NaCl,

50 μM nociceptin and 25 μg membrane protein. Samples of purified endopeptidase-24.11 from porcine kidney [38] were incubated with a mixture (0.1 mL) comprising 25 mM Tris–HCl (pH 7.4), 155 mM NaCl, 0.1% Triton-X 100, 50 μM nociceptin and 100 ng of the enzyme.

In each case, the reaction was allowed to proceed at 37° for an appropriate time and terminated at 100° for 10 min. The reaction mixture was centrifuged, filtered through membrane filter (Cosmonice Filter; pore size, 450 nm) and subjected to HPLC on a reversed phase column (4.6 mm × 150 mm) of SYMMETRY^{TM}C18 (Nihhon Waters K.K.), which had previously been equilibrated with 1.0% (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid (TFA). Elution was carried out at room temperature with a 60 min linear gradient of 1–65% acetonitrile in 0.05% TFA at a flow rate of 1.0 mL/min. The peptide fragments eluted were detected by monitoring the absorbance at 210 nm.

2.5. Identification of nociceptin fragments

The cleavage products separated by HPLC were manually collected, lyophilized and hydrolyzed with 6 M hydrochloric acid at 150° for 1 hr. Hydrolyzed samples were dried under vacuum. Individual samples were dried again after adding 20 μL of methanol–water–triethylamine (2:2:1). A phenylthiocarbamyl derivative of amino acids was formed by adding 20 μL of the derivatization reagent consisted of methanol–triethylamine–water–phenylthiocyanate (7:1:1:1) to dried sample and sealing for 20 min at room temperature. The reagents were then removed under vacuum. Individual samples were dissolved in 200 μL of PICO-TAG^{TM} Diluents (Nihhon Waters K.K.). A total of 20 μL aliquots of the dissolved samples were analyzed by HPLC on PICO-TAG Column (3.9 mm × 300 mm, Nihhon Waters K.K.). Elution was carried out according to method of Bidlingmeyer *et al.* [39]. Phenylthiocarbamyl derivatives of amino acids eluted were detected by monitoring the absorbance at 254 nm. Quantitation of amino acids was done by calculating the area under each integrated peak in chromatogram and comparing it with a standard amino acid at various concentrations. The reproducibility of these data was confirmed by value for triplicate.

2.6. Intrathecal injection procedure

The i.t. injection procedure was adapted from the method of Hylden and Wilcox [40]. A 28 gauge stainless-steel needle attached to a 50 μL Hamilton microsyringe was inserted between lumbar 5 and 6 in unanesthetized mice, and drugs were given slowly in a volume of 5 μL. The accurate placement of the administration was indicated by a quick flick of mouse's tail. The effect of peptidase inhibitors on nociceptin-induced behavioral response was determined by co-administration of various peptidase inhibitors and nociceptin. All compounds used were dissolved in artificial cerebrospinal fluid (CSF). The composition of artificial CSF in mM was: NaCl (7.4), KCl (0.19), $MgCl_2$ (0.19) and $CaCl_2$ (0.14).

2.7. Behavioral observation

One hour prior to i.t. injection, animals were adapted to an individual plastic cage (22.0 cm × 15.0 cm × 12.5 cm), which also served as the observation chamber. Immediately following i.t. injection of nociceptin (3.0 fmol), each mouse was replaced into the transparent cage and behavioral testing was begun. The mice were observed for beginning immediately after i.t. injection of nociceptin. The total response time(s) of behaviors was measured in 5 min intervals for 30 min after nociceptin as described above. These behaviors included caudally directed biting and licking along with reciprocal hindlimb scratching.

2.8. Analyses of data

Statistical analyses of the results were performed using the Dunnett's test for multiple comparisons, after ANOVA. Differences were considered to be significant if $P < 0.05$. All values are expressed as means ± SEM.

3. Results

3.1. Degradation of nociceptin by synaptic membranes of mouse spinal cord

We examined the degradation of substance P and Leu-enkephalin, a well-known neurotransmitter or modulator in spinal synaptic region, by the spinal synaptic membrane, in order to compare the time course of hydrolysis of substance P and Leu-enkephalin with that of nociceptin. Degradation products of nociceptin, substance P and Leu-enkephalin by the membrane preparation of mouse spinal cord were initially analyzed by reversed phase HPLC under conditions of isocratic elution. The chromatographic peaks corresponding to the original substrates, nociceptin, substance P and Leu-enkephalin were found to decrease as a function time (Fig. 1). The half-life for hydrolysis of 5.0 nmol of nociceptin, substance P, Leu-enkephalin and nociceptin by 25 μg membrane protein was 103, 55 and 46 min, respectively. Degradation of nociceptin by synaptic membranes of mouse spinal cord was analyzed by reversed phase HPLC under gradient elution of acetonitrile (Fig. 2). Thirteen peaks were separated and named alphabetically from a to l according to the increasing order of their retention times. When only the membrane was incubated in the absence of nociceptin or only nociceptin was incubated, newly formed peaks were not detected at all. The position of peak N was identical to that of the substrate, nociceptin, and the area of this peak decreased as a function of time, whereas those of other peaks increased. The results of analyses of amino acid composition for

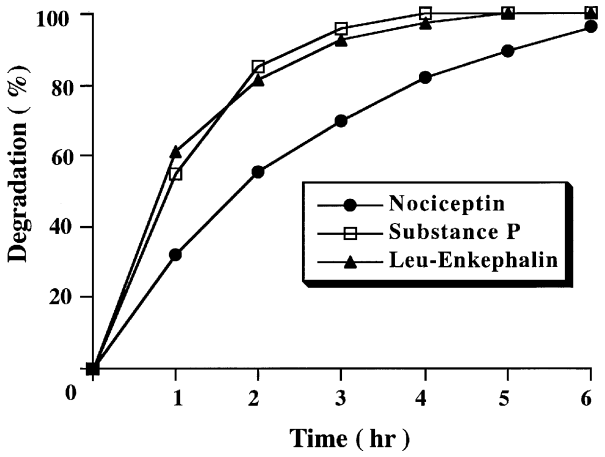

Fig. 1. Time courses of nociceptin, substance P and Leu-enkephalin degradation by mouse spinal cord synaptic membranes. The reaction mixture (0.1 mL, pH 7.4) containing 25 μg protein of the membrane preparation, was incubated at 37° for 0–6 hr. After heating at 100° for 10 min, 10 μL aliquots of the mixture were analyzed by HPLC on a reversed phase column (4.6 mm × 150 mm) of Symmetry C18. The results are the mean of triplicate determinations.

cleavage products separated by HPLC allowed their assignment (Table 1). Fragments b, c, g, h and l were assignable as nociceptin (13–17), free phenylalanine, nociceptin (2–17), nociceptin (1–13), and nociceptin (1–7), respectively, by comparing their retention times in HPLC. The yield of peaks (a–l) was calculated on the basis of nociceptin degraded by using procedure of quantitative amino acid analysis. Thus, major metabolites of nociceptin were free phenylalanine, nociceptin (1–13) and nociceptin (14–17). Time course of formation of representative cleavage products by HPLC is shown in Fig. 3. In particular, peak h corresponding to nociceptin (1–13), increased in amount only during a short incubation time (4 hr) and then decreased afterward, a finding suggesting a subsequent degradation of this fragment.

3.2. Effect of peptidase inhibitors on the degradation of nociceptin by mouse spinal cord synaptic membranes

The effect of various peptidase inhibitors on the degradation of nociceptin by mouse spinal synaptic membranes is summarized in Table 2. The effect of peptidase inhibitors on the initial cleavage rate of nociceptin was analyzed by measuring the effects on decrease of the HPLC peak for nociceptin. A metal chelator, *o*-phenanthroline and specific inhibitors for endopeptidase-24.11, phosphoramidon and thiorphan, inhibited nociceptin degradation by synaptic membranes. Other inhibitors, including angiotensin-converting enzyme inhibitors (captopril and enalapril), an aminopeptidase inhibitor (bestatin), a basic carboxypeptidase inhibitor (GEMSA), and inhibitors for serine (PMSF and leupeptin), cysteine (PCMBS) and acid protease (pepstatin) inhibitors had little inhibitory effects on nociceptin degradation.

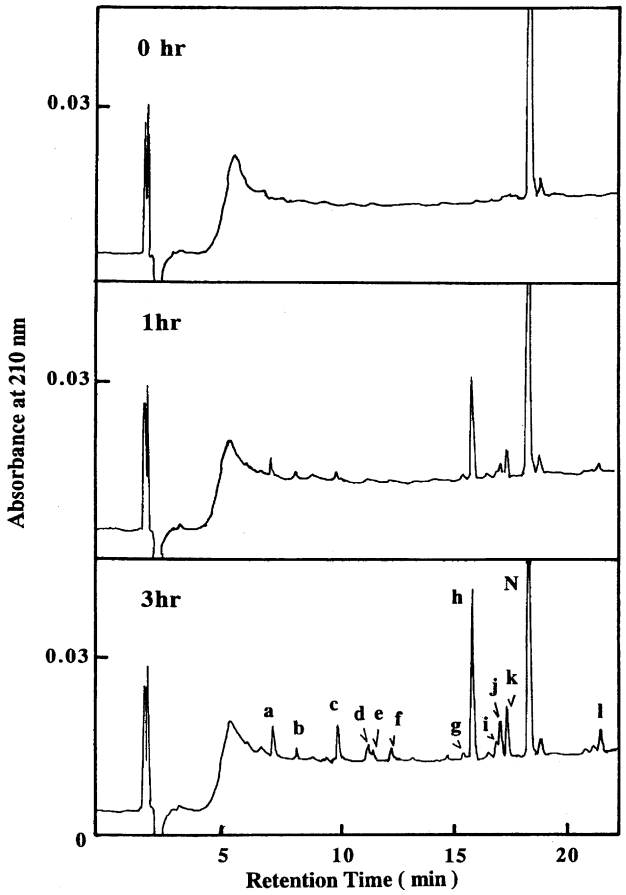

Fig. 2. Degradation of nociceptin by mouse spinal cord synaptic membranes. The reaction mixture (0.1 mL, pH 7.4) containing 25 μg protein of the membrane preparation, was incubated at 37° for 0, 1 and 3 hr. After heating at 100° for 10 min, 10 μL aliquots of the mixture were analyzed by HPLC on a reversed phase column (4.6 mm × 150 mm) of Symmetry C18, which had been equilibrated with 1.0% acetonitrile in 0.05% TFA. Elution was carried out at room temperature with a 60 min linear gradient of 1.0–65% acetonitrile in 0.05% TFA at a flow rate of 1.0 mL/min. The absorbance at 210 nm was monitored. (a) Nociceptin (14–17); (b) nociceptin (13–17); (c) phenylalanine; (d) nociceptin (2–13); (e) nociceptin (2–9); (f) nociceptin (2–11); (g) nociceptin (2–17); (h) nociceptin (1–13); (i) nociceptin (1–10); (j) nociceptin (1–9); (k) nociceptin (1–11); (N) nociceptin; (l) nociceptin (1–7).

The effect of peptidase inhibitors on the generation of cleavage products of nociceptin separated HPLC was also analyzed (Fig. 4). The generation of major metabolites, nociceptin (1–13) and (14–17) was strongly inhibited by the addition of phosphoramidon and thiorphan, whereas the generation of nociceptin (1–11) and (13–17) was not affected (Fig. 4 and Table 3). Enalapril exerted little inhibitory effect on the generation of almost all of the peaks. In the presence of bestatin, appearance of phenylalanine was strongly inhibited (Table 3).

3.3. Degradation of nociceptin by purified endopeptidase-24.11

Further experiments were done to ascertain whether a purified preparation of endopeptidase-24.11 could hydro-

Table 1
Amino acid composition of fragments of nociceptin[a] produced through the action of mouse spinal cord membrane preparation

Peak	Amino acid (mol%)										Fragment identified	Yield (%)[b]
	Phe	Gly	Thr	Ala	Arg	Lys	Ser	Leu	Asp	Glu		
a	0	0	0	20	0	0	0	33	27	20	14–17	22
b	0	0	0	20	0	19	0	24	18	19	13–17	11
c	81	0	0	5	5	2	0	3	0	4	Phe	22
d	11	26	6	14	18	16	9	0	0	0	2–13	2
e	13	32	17	12	12	14	0	0	0	0	2–9	2
f	11	27	10	18	12	12	10	0	0	0	2–11	2
g	7	17	6	17	14	12	7	7	6	7	2–17	2
h	17	24	8	16	14	16	5	0	0	0	1–13	22
i	22	29	10	9	12	10	8	0	0	0	1–10	2
j	24	30	12	10	11	13	0	0	0	0	1–9	5
k	20	25	10	17	10	11	7	0	0	0	1–11	6
N[c]	15	17	6	17	10	15	4	6	5	5	Complete	
l	32	39	16	13	0	0	0	0	0	0	1–7	5

[a] The sequence, Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln.
[b] Yield (%) was determined on the basis of nociceptin degraded.
[c] The extent of degradation of nociceptin was 59%.

lyze nociceptin. Nociceptin was hydrolyzed by the purified endopeptidase-24.11 and two major peaks of nociceptin (14–17) and nociceptin (1–13) were detected as a and h, respectively (Fig. 5). These fragments were identical with those detected as major peaks in the case of nociceptin degradation by spinal synaptic membranes. In the presence of phosphoramidon, the generation of their peaks was strongly suppressed (Fig. 5).

3.4. Effect of peptidase inhibitors on the behavioral response induced by i.t. injection of nociceptin

Our recent behavioral data [30] show that i.t. injection of nociceptin at small doses (fmol order) elicits a scratching, biting and licking response resembling that of i.t. substance P in mice. This characteristic behavior induced by nociceptin (3.0 fmol) was not significantly affected by PHMB (0.25 and 1.0 nmol), a thiol protease inhibitor, captopril (4.0 nmol), an angiotensin-converting enzyme inhibitor, and bestatin (0.25, 0.5, 2.0 and 4.0 nmol), an aminopeptidase inhibitor (data not shown). Each peptidase inhibitor alone except for phosphoramidon showed no notable behavioral changes, which was approximately the same value with artificial CSF control (40.0 ± 1.9 s/30 min) (data not shown). The i.t. injected phosophoramidon (2.0 pmol) alone enhanced the behavioral response when compared to CSF control (Fig. 6). During the 30 min observation, total response time of nociceptin (3.0 fmol) was additively elevated by co-administration of phosphoramidon (2.0 pmol), a specific inhibitor of endopeptidase-24.11 (Fig. 6). Nociceptin-induced behavioral response was potentiated by combined injection of phosphoramidon (2.0 pmol) and bestatin (2.0 nmol) (Fig. 6).

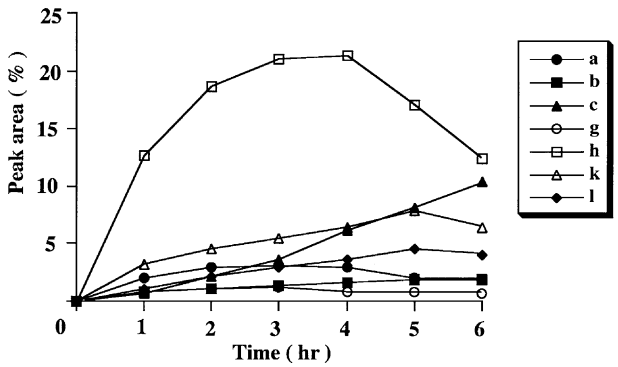

Fig. 3. Time course of nociceptin degradation products produced through the action of mouse spinal cord synaptic membranes. The reaction mixture was incubated and treated with the same procedure as described in Fig. 1. Cleavage products were separated by HPLC as described in Fig. 2 and their peaks areas traced on the chart were measured. The vertical coordinate indicates the relative value of the peak area calculated on the basis of that of the substrate, nociceptin at 0 hr. The results are the mean of triplicate determinations. (a) Nociceptin (14–17); (b) nociceptin (13–17); (c) phenylalanine; (g) nociceptin (2–17); (h) nociceptin (1–13); (k) nociceptin (1–11); (l) nociceptin (1–7).

Table 2
Effect of peptidase inhibitors on the degradation of nociceptin by mouse spinal cord synaptic membranes[a]

Inhibitor	Concentration (mM)	Inhibition (%)
o-Phenanthroline	1.0	81
Phosphoramidon	0.1	50
Thiorphan	0.1	52
Captopril	0.1	0
Enalapril	0.1	0
Bestatin	0.1	11
PMSF	1.0	13
Leupeptin	0.1	0
PCMBS	1.0	19
GEMSA	0.1	0
Pepstatin	0.1	0

[a] The activity was measured on the basis of the disappearance of nociceptin, as detected by HPLC with isocratic elution.

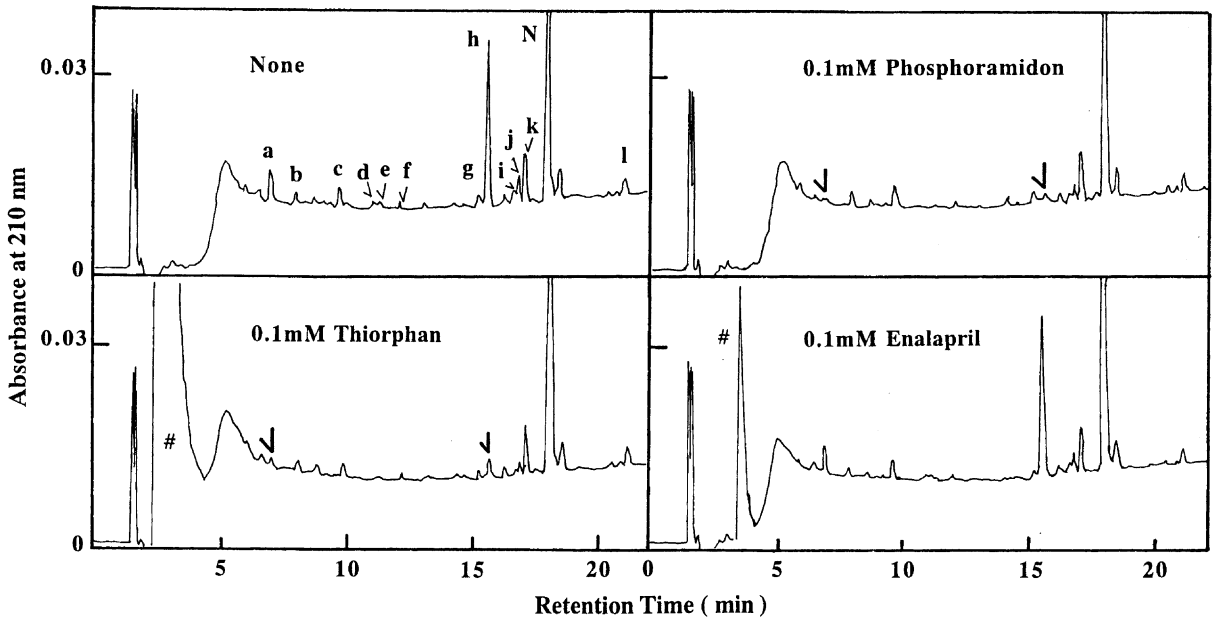

Fig. 4. Effect of peptidase inhibitors on the degradation of nociceptin by mouse spinal cord synaptic membranes. The reaction mixture (0.1 mL, pH 7.4) containing 25 μg protein of the membrane preparation, was incubated at 37° for 3 hr in the absence or presence of 0.1 mM phosphoramidon, 0.1 mM thiorphan, or 0.1 mM enalapril. After heating at 100° for 10 min, 10 μL aliquots of the mixture were analyzed by HPLC as described in Fig. 2. The symbol '#' indicates the peak derived from the inhibitor added. (a) Nociceptin (14–17); (b) nociceptin (13–17); (c) phenylalanine; (d) nociceptin (2–3); (e) nociceptin (2–9); (f) nociceptin (2–11); (g) nociceptin (2–17); (h) nociceptin (1–13); (i) nociceptin (1–10); (j) nociceptin (1–9); (k) nociceptin (1–11); (N) nociceptin; (l) nociceptin (1–7).

Table 3
Effect of peptidase inhibitors on the formation of nociceptin metabolites following incubations with the mouse spinal cord synaptic membrane[a]

Nociceptin metabolite	Inhibition (%)			
	Phosphoramidon (0.1 mM)	Thiorphan (0.1 mM)	Enalapril (0.1 mM)	Bestatin (0.1 mM)
14–17	89	72	12	21
13–17	0	0	21	22
Phenylalanine	0	4	10	88
1–13	93	87	1	15
1–11	15	14	1	0

[a] The extent of inhibition was determined by measuring the decrease of major peaks derived from nociceptin degraded.

4. Discussion

4.1. Involvement of endopeptidse-24.11 in the degradation of nociceptin by spinal synaptic membranes

In this study, we focused our attention on the membrane-bound peptidase (ectoenzyme), because it is thought that nociceptin functioning as neuro-transmitters or modulators could be degraded at the plasma membrane of neuronal cells to abolish their physiological function. To our knowledge, the concurrent monitoring of nociceptin and its metabolic fragments by synaptic membranes of mouse spinal cord have not yet been explored. It can be assumed that spinal synaptic membranes degrade nociceptin as efficiently as substance P and Leu-enkephalin, since half-life for hydrolysis of nociceptin was 1.9- and 2.2-fold longer, respectively than that in case of substance P and Leu-enkephalin. The present results show that nociceptin was readily cleaved into nociceptin (1–13), nociceptin (14–17) and free phenylalanine as major products when incubated with synaptic membranes of mouse spinal cord. Small amounts of other fragments were also found, indicating nociceptin (1–7), (1–9), (1–10), (1–11), (2–9), (2–11), (2–13), (2–17) and (13–17). We have presented several lines of evidence to suggest that the degradation of nociceptin by mouse spinal cord synaptic membranes may be initially triggered by the action of endopeptidase-24.11. Both the degradation of nociceptin and accumulation of major metabolites produced from the cleavage of Lys^{13}–Leu^{14} bond were suppressed by the presence of inhibitor of endopeptidase-24.11 such as phosphoramidon and thiorphan. The fact that purified endopeptidase-24.11 hydrolyzed nociceptin at the same site as mouse spinal cord synaptic membranes and i.t. co-administration of phosphoramidon enhanced nociceptin-induced behavioral response *in vivo* experiment supports the above assump-

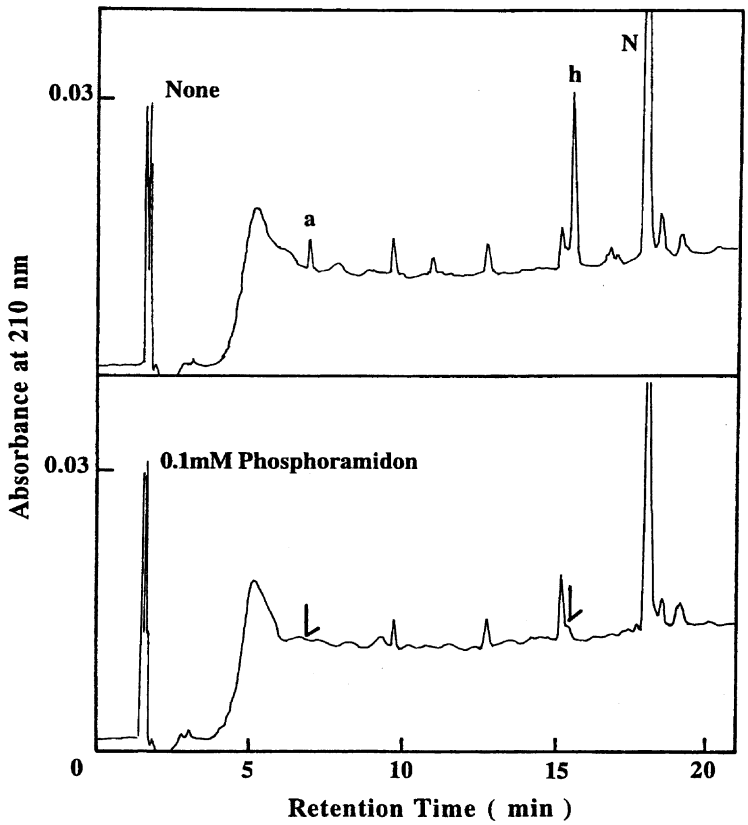

Fig. 5. Degradation of nociceptin by endopeptidase-24.11. The reaction mixture (pH 7.4) containing 100 ng of purified endopeptidase-24.11 from porcine kidney, was incubated at 37° for 2 hr in the absence of an inhibitor (upper panel), or in the presence of 0.1 mM phosphoramidon (lower panel). After heating at 100° for 10 min, 10 μL aliquots of the mixture were analyzed by HPLC as described in Fig. 2. (a) Nociceptin (14–17); (h) nociceptin (1–13); (N) nociceptin.

tion. Neuronal localization of endopeptidase-24.11 has been demonstrated by immunohistochemical technique. The highest density of endopeptidase-24.11 immunoreactivity was observed in areas involved in pain control mechanism (superficial layers of the spinal nucleus of the trigeminal nerve of the dorsal horn of the spinal cord), which also display the highest immunoreactive for both enkephalins and substance P [41]. These results suggest that endopeptidase-24.11, present in spinal synaptic membrane, may play a major role in initial stage of nociceptin metabolism at the spinal cord level in mice. The present results that the generation of free phenylalanine was strongly inhibited by bestatin, an aminopeptidase inhibitor, which had little effect on the degradation of nociceptin may imply the involvement of aminopeptidase in secondary cleavage of the initially formed cleavage products by the action of endopeptidase-24.11.

Although there have been a few reports describing metabolism of nociceptin in the central nervous system [32], these results are inconsistent with our results. Montiel *et al*. [42] have reported that the nociceptin is metabolized by endopeptidase-24.15 and aminopeptidase N in mouse brain cortical slice into four main metabolites, nociceptin (1–7), (12–17), (13–17) and (2–17), whereas endopeptidase-24.11 and endopeptidase-24.16 may not be involved in nociceptin metabolism to any great extent. However, we can exclude the involvement of endopeptidase-24.15 which is known as a thiol-sensitive metallo-endopeptidase [43,44] on nociceptin metabolism in the mouse spinal cord, since SH-reagents such as PCMBS and PHMB had little effects on nociceptin metabolism in our both *in vitro* and *in vivo* experiments. Sandin *et al*. [45] have reported that the *in vivo* metabolism of nociceptin in rat hippocampus occurred in two steps, formation of nociceptin (1–13) followed by nociceptin (1–9) although susceptibility of peptidase involved in nociceptin degradation to peptidase inhibitors have not been described. Subsequent work by the same group showed metabolism of nociceptin by soluble enzyme fraction of rat brain primary cortical cells, human neuroblastoma cells and small cell lung carcinoma cells [46]. The fragmentation of nociceptin was qualitatively the same in all cell lines, with nociceptin (1–13) and (1–9) as major metabolites. The cleavage was inhibited by metals and SH-reagents but not by phosphoramidon and thiorphan suggesting that the enzymatic activity was phosphoramidon and thiorphan-insensitive metalloendopeptidase. Moreover, the metabolism of nociceptin in rat spinal cord homogenate has been studied using mass spectrometry [47]. Nociceptin was degraded mainly into nociceptin (1–11), which was further truncated to nociceptin (1–6)

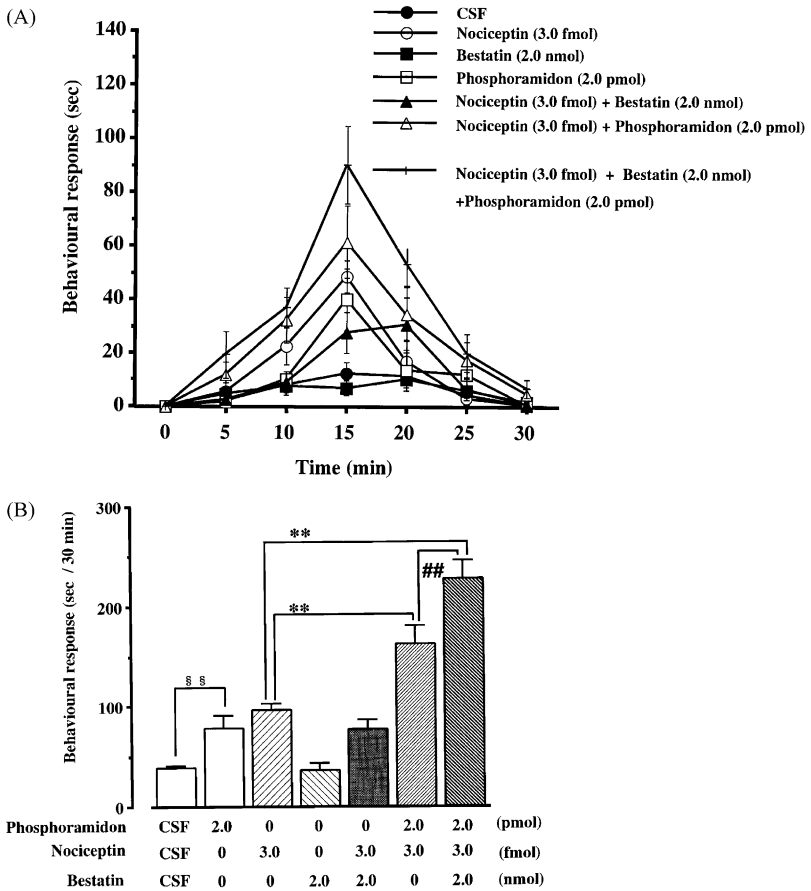

Fig. 6. Effect of phosphoramidon and/or bestatin on nociceptin-induced behavioral response in mice. (A) Time course effect of phosphoramidon (4.0 pmol) and/or bestatin (2.0 nmol) on nociceptin-induced behavioral response. (B) Effect of phosphoramidon and/or bestatin on nociceptin-induced behavioral response. The duration of scratching, biting and licking induced by nociceptin (3.0 fmol) was determined over a 30 min period starting immediately after injection. Mice were co-administered phosphoramidon (4.0 pmol) and/or bestatin (2.0 nmol) in combination with nociceptin (3.0 fmol). The data are given as the standard ± SEM for groups of 10 mice. (§§) $P < 0.01$ when compared to CSF alone. (**) $P < 0.01$ when compared to nociceptin alone. (##) $P < 0.01$ when compared to the group receiving administration of phosphoramidon and nociceptin.

as a final product. The enzyme responsible appeared to be soluble, neutral serine endopeptidase, dependent on metal ions. Although the reason for this discrepancy remains unknown, these differences of nociceptin metabolism in the central nervous system may arise from a difference of regional distribution of the enzymes and subcellular fractions of brain and spinal cord.

4.2. Endopeptidase-24.11 may be a key enzyme responsible for termination of nociceptin-induced behavioral response at spinal cord level

The present behavioral results confirm our earlier study showing that i.t. injection of nociceptin induces a behavioral syndrome characterized by a scratching, biting and licking episode in mice [31]. This spinally-mediated behavioral response was enhanced by co-administration of phosphoramidon, an inhibitor of endopeptidase-24.11. The behavioral data supports the involvement of endopeptidase-24.11 as an enzyme that is important in the spinal catabolism of nociceptin. The behavioral response to nociceptin was not significantly changed by co-administration of either, bestatin or captopril. Therefore, it seems likely that aminopeptidase and angiotensin-converting enzyme do not play a predominant role in modulating the spinal action of nociceptin. It is of importance to note that truncating the N-terminal Phe residue to generate nociceptin (2–17) leads to a drastic decrease of affinity for ORL-1 (OP_4) receptor [48]. In the present *in vivo* study, simultaneous i.t. injection of phosphoramidon and bestatin potentiated the behavioral response to nociceptin. It is therefore reasonable to suggest that inhibition of endopeptidase-24.11 and aminopeptidase may be required to exhibit full behavioral expression of nociceptin in the spinal cord. Several biochemical data have revealed that purified endopeptidase-24.11 can hydrolyze a variety of neuropeptides such as substance P [49], neurokinin A [50], somatostatin [51], neurotensin [49], cholecytokinin [52] and enkephalin [49]. In fact, the scratching, biting and licking response induced by i.t. injection of substance P or neurokinin A can be markedly potentiated and prolonged by co-administration of phosphoramidon [53]. Recently, we have suggested in the behavioral experiment with antiserum to substance P that nociceptin-induced nociceptive

response may be mediated *via* the release of substance P from the primary afferents [29,31]. Taking account of these reported results, it is speculated that endopeptidase-24.11 may be a major enzyme for degrading not only nociceptin, but also substance P released by exogenously given nociceptin into the spinal cord.

4.3. Pharmacological activity of nociceptin N-terminal fragments

The present data demonstrate that spinal synaptic membranes can hydrolyze nociceptin *in vitro* essentially at the Lys^{13}–Leu^{14} bond, in addition to the Ala^{7}–Arg^{8} and Lys^{9}–Ser^{10} bonds, generating the metabolites, (1–13), (1–7) and (1–9). These shorter N-terminal fragments of nociceptin have been shown to possess biological activity [30,34,47,54–57], indicating that they are not simply inactive substances degraded by enzymatic processes. Recently, we have found that nociceptin, given i.t., induces hyperalgesia in mice, which is blocked by co-injection of nociceptin (1–7) as assayed by the tail-flick and paw-withdrawal tests [30]. Furthermore, N-terminal fragments of nociceptin (1–7), (1–9) and (1–13) have been found to antagonize the scratching, biting and licking response induced by nociceptin [34]. It should be mentioned that nociceptin (1–13) is effective in antagonizing nociceptin-induced response at an equimolar dose and has much more potent antagonizing action than nociceptin (1–7) and (1–9). Thus, it seems evident that there is a possible functional mechanism between nociceptin and its N-terminal fragments in the spinal cord. However, the N-terminal fragments do not act as a competitive antagonist against nociceptin, since nociceptin (1–7), (1–11) and (1–13) have poor affinity for ORL-1 (OP_4) receptor [48,58]. Furthermore, nociceptin (1–7) and (1–11) have not been reported to have affinity for traditional three types of opioid receptors (μ, δ and κ), ruling out an opioid receptor interaction [59]. Recently, Mathis *et al*. [60] have shown that there is a high affinity nociceptin (1–11) binding site with a selectivity profile distinct from ORL-1 (OP_4) receptor in mouse brain. In addition, Xie *et al*. [61] have reported that ORL-1 (OP_4) receptor has two alternative spliced isoforms consisted of full-length ORL-1 (OP_4) receptor and truncated ORL-1 (OP_4) receptor in the rat. Biochemical studies used two different type recombinant receptors expressed in mammalian cells have demonstrated that profiles of nociceptin-binding and inhibitory effects on cyclic AMP production for two type receptors were completely different. The mechanism underlying the antagonizing effect of N-terminal fragments of nociceptin may be explained by nociceptin (1–11) binding sites or alternative splice form receptor in the mouse spinal cord. As described in Section 1, the pharmacology of nociceptin is quite complex. Despite its nociceptive action in small doses, higher nociceptin doses are analgesic, both peripherally and centrally [22,31,54,55,62,63]. In our previous study [30], i.t. nociceptin in high doses ranging from 300 to 1200 pmol reduced the tail-flick and paw-withdrawal responses (analgesia), as opposed to the hyperalgesia at lower doses (1.5–30 fmol). We would expect that the antinociceptive effect induced by nociceptin in high doses may in part be arised from N-terminal fragment (1–13) of nociceptin formed by endopeptidase-24.11 in the spinal cord. This speculation is supported by behavioral data that nociceptin (1–7) and (1–11), administered i.c.v., produces an antinociceptive response without inducing hyperalgesia in the tail-flick assay [55], and i.t. injected nociceptin (1–13) is antinociceptive in the capsaicin test (unpublished observations). A similar phenomenon that metabolites derived from enzymatic metabolism by membrane-bound peptidase have a biological activity was seen in the case of substance P. In our previous reports [64–66] we have found that substance P (1–7) fragment produced by spinal endopeptidase-24.11 has an antagonistic effect on the substance P-induced behavioral response when co-injected i.t., whereas substance P (1–7), injected i.t. alone, is inactive.

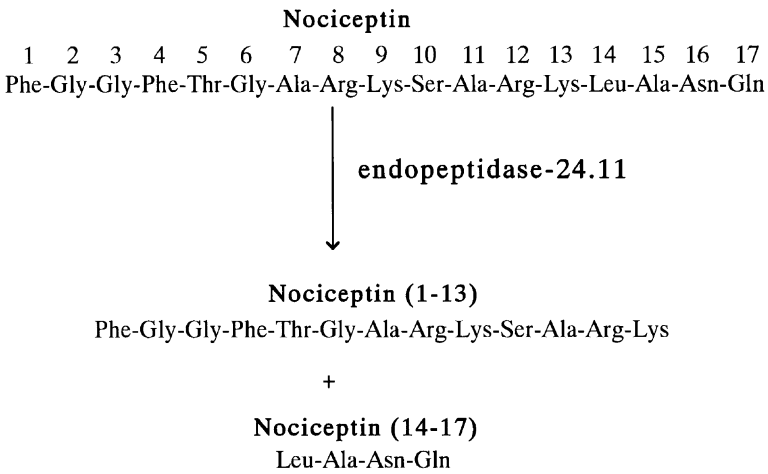

Fig. 7. Scheme for metabolism of nociceptin by synaptic membranes of mouse spinal cord.

Furthermore, the effect of substance P (1–7) may not be mediated by an action at NK_1 receptor but by an interaction with substance P (1–7) binding site or its own receptor [67], since substance P (1–7) has no binding for NK_1 receptors in the spinal cord [68–70].

In conclusion, a physiological role for the degradation of nociceptin by endopeptidase-24.11 in the spinal cord (Fig. 7) is not only responsible for inactivation of nociceptin, but may also lead to formation of N-terminal nociceptin fragments which are shown to display a different physiological function.

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