Major Metabolites of Substance P Degraded by Spinal Synaptic Membranes Antagonize the Behavioral Response to Substance P in Rats

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Abstract □ Substance P (SP) was degraded by synaptic membranes of rat spinal cord. Cleavage products were separated by reversed phase high performance liquid chromatography and identified by amino acid composition analyses. Major products of SP were phenylalanine, SP(1–4), SP(1–6), SP(1–7), SP(10–11), and SP(8–9). Both the degradation of SP and the accumulation of the major cleavage products were strongly inhibited by a metal chelator, *o*-phenanthroline, and also by specific inhibitors of endopeptidase-24.11, thiorphan, and phosphoramidon. Thus, endopeptidase-24.11 plays a major role in SP degradation in the rat spinal cord. N-Terminal fragments, SP(1–7) and SP(1–4), detected after incubation with spinal synaptic membranes were examined in vivo for antagonism against the scratching, biting, and licking response induced by intrathecal (IT) injection of SP (3.0 nmol) in rats. When IT coadministered with SP, SP(1–7) and SP(1–4) produced a significant inhibition of behavioral response to SP with ED_{50} of 135.0 pmol and 6.2 nmol, respectively. These results suggest that the degradation of SP in the spinal cord is not only responsible for inactivation of parent peptide, but may also lead to the formation of N-terminal SP-fragments which are shown to display a novel physiological function.

Introduction

Substance P (SP) is an undecapeptide which is widely distributed in the central nervous system and peripheral tissues.[1] This peptide is thought to function as a neurotransmitter or neuromodulator after secretion into synapse.[2,3] A member of tachykinins, SP is the endogenous ligand for the tachykinin NK_1 receptor, while neurokinin A and neurokinin B are endogenous ligands for NK_2 and NK_3 receptors, respectively.[4,5]

SP is believed to play an important role as an excitatory peptide in the spinal cord. Recently, two groups have reported that mice in which the preprotachykinin A gene was disrupted showed reduced responses to pain stimuli. Zimmer et al.[6] indicated that knockout mice which cannot produce SP and neurokinin A display no significant pain responses following hindpaw formalin injection. In addition, the animals have an increased pain threshold in the hot-plate test. Cao et al.[7] also reported that nociceptive response evoked by a hindpaw injection of capsaicin, an intensely noxious chemical stimulus that directly activated C-fibers, was significantly reduced in the knockout mice. They also found decreased nociceptive response of the knockout mice in the model of acute visceral pain by using the procedure of the acetic acid- and $MgSO_4$-induced writhing assay.

The physiological action of SP is probably terminated by a membrane-bound protease capable of degrading SP in the synaptic region, by analogy with membrane-bound acetylcholinesterase functioning in acetylcholine degradation in the synapse. Several membrane-bound neuropeptidases, endopeptidase-24.11,[8−10] SP-degrading enzyme from human brain,[11] SP-degrading endopeptidase from rat brain,[12] post-proline dipeptidyl-aminopeptidase,[13] and angiotensin-converting enzyme,[10,14−16] have been reported to be involved in the degradation of SP. SP is degraded into N- and C-terminal fragments by endogenous neuropeptidase. However, the physiological function of these neuropeptidases from a membrane fraction of rat spinal cord has still remained obscure. It has been reported that some of C-terminal SP-fragments exert actions within the central nervous system similar to those observed with SP, whereas N-terminal fragments have opposite or antagonizing effects on SP action.[17]

In our previous study,[18] SP(1−7) and SP(1−8) in the low picomole range antagonize the scratching, biting, and licking response induced by IT co-administration of SP in mice. In this study, we examined the degradation of SP by synaptic membranes of rat spinal cord. Additional in vivo experiments were performed to investigate the antagonizing effect of N-terminal SP-metabolites, SP(1−7) and SP-(1−4), on the characteristic behavioral response elicited by IT administration of SP in the rat.

Experimental Section

Animals—Male Wistar rats (Shizuoka Laboratory Center, Japan), weighing 250−300 g at the time of surgery, served as subjects. Animals were housed in a temperature (22 ± 24 °C)- and humidity (60−70%)-controlled room illuminated on a 12-h light and 12-h dark cycle. Food (Clea Japan, Inc., Osaka, Japan) and water were provided ad libitum throughout the course of the study.

Peptides and Chemicals—The commercial drugs used were: SP, phosphoramidon, chymostatin, leupeptin (Peptide Institute, Inc., Osaka, Japan), SP (1−4), SP(1−7), SP(1−9), SP(2−11), SP-(3−11), SP(4−11), SP(5−11), SP(8−11), SP(9−11), SP(6−11), SP-(7−11), *p*-chloromercuribenzenesulfonic acid (PCMBS), thiorphan (Sigma Chemical Co., St. Louis, MO), constant boiling hydrochloric acid (Pierce Chemical Co., Rockford, IL), captopril and phenylmethyl sulfonyl fluoride (PMSF) (Nacalai tesque, INC, Kyoto, Japan). Z-321 was obtained through courtesy of Zeria Pharmaceutical Co., Ltd., Saitama, Japan.

Preparation of Synaptic Membranes from Rat Spinal Cord—Rat spinal cord was washed with 10 mM Tris-HCl (pH 7.5)

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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-HCI (pH 8. 1). Then the suspension was incubated at 0 °C for 30 min for lysis of the pellet and subjected to discontinuous sucrose density gradient centrifugation according to the method of Jones and Matus.[19] 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-HCI (pH 8.1), and centrifuged at 100 000*g* for 1 h. The resulting pellet was suspended in 10 mM [2-[4-(2-hydroxyethyl)1-piperazinyl]ethanesulfonic acid (HEPES)−NaOH (pH 7.5) containing 155 mM NaCl and stored at −80 °C. Protein was determined by the method of Bradford[20] using bovine serum albumin (Bio-Rad) as a standard.

Degradation of Substance P by Synaptic Membranes of Rat Spinal Cord—The degradation of SP by synaptic membranes of rat spinal cord was carried out at 37 °C in a mixture (0.1 mL) containing of 10 mM HEPES−NaOH (pH 7.4), 80 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 50 μM SP, and 50 μg protein of the membrane. The reaction was terminated by heating at 100 °C for 10 min. The reaction mixture was centrifuged, filtered through a membrane filter (Cosmonice Filter; pore size, 450 nm), and subjected to high performance liquid chromatography (HPLC) on a reversed phase column (4.6 × 150 mm) of SYMMETRYC18 (Waters) which had previously been equilibrated with l % (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid (TFA). Elution was carried out at room temperature with a 32 min linear gradient of 1−65% acetonitrile in 0.05% TFA at a flow rate of 1 mL/min. The peptide fragments eluted were detected by monitoring the absorbance at 210 nm.

Identification of Substance P Fragments—The cleavage products separated by HPLC were lyophilized and hydrolyzed with 6 M hydrochloric acid containing 0.1% mercaptoethanol at 150 °C for 1 h. Their amino acid compositions were determined as phenylthiocarbamoyl amino acid derivatives by the reversed phase HPLC.[21]

Intrathecal Injection—To permit application of peptides directly into the spinal subarachnoid space, rats were anesthetized with pentobarbital sodium (50 mg/kg, ip) and chronically implanted with IT catheters.[22,23] Briefly, this involved inserting a length of polyethylene tubing (PE-10) following laminectomy between L3 and L4 and careful placing the catheter tip in the subarachnoid space of L5 and L6. The rats were allowed to recover over 7 days following implantation of the catheter. The catheter was filled with sterile artificial cerebrospinal fluid (CSF) containing (in g/L) NaCl 7.4, KCl 0.19, $MgCl_2$ 0.19, $CaCl_2$ 0.14. Peptides used in the experiments were administered in volumes of 10 μL followed by 15 μL of artificial CSF to ensure that peptides reached the spinal cord. SP(1−7) or SP(1−4) was mixed and coadministered IT with SP such that all doses were delivered in a total volume of 10 μL followed by 15 μL of artificial CSF to flush the catheter.

Behavioral Observation—One hour prior to IT injection, animals were adapted to an individual plastic cage (34 × 30 × 17 cm) which also served as the observation chamber. Immediately following IT injection of SP, each rat was replaced into the transparent cage, and behavioral testing was begun. The total respose time(s) of behaviors was measured for 5 min beginning immediately after IT injection of SP. These behaviors included hindlimb scratching directed toward the flank, biting, or licking of the hindpaw. All these different behaviors were pooled as a single value for each animal. Studies on the behavioral experiments were performed with the approval of the Ethics Committee of Animal Experiment in Tohoku Pharmaceutical University.

Calculations of ED_{50} and Data Analysis—Statistical analyses of the results were performed using the Dunnett's test for multiple comparisons, after analysis of variance (ANOVA). Differences were considered to be significant if $P < 0.05$. All values are expressed as means ±SEM.

Results

Degradation of SP by Synaptic Membranes of Rat Spinal Cord—Degradation of SP by synaptic membranes of rat spinal cord was analyzed by HPLC (Figure 1). About

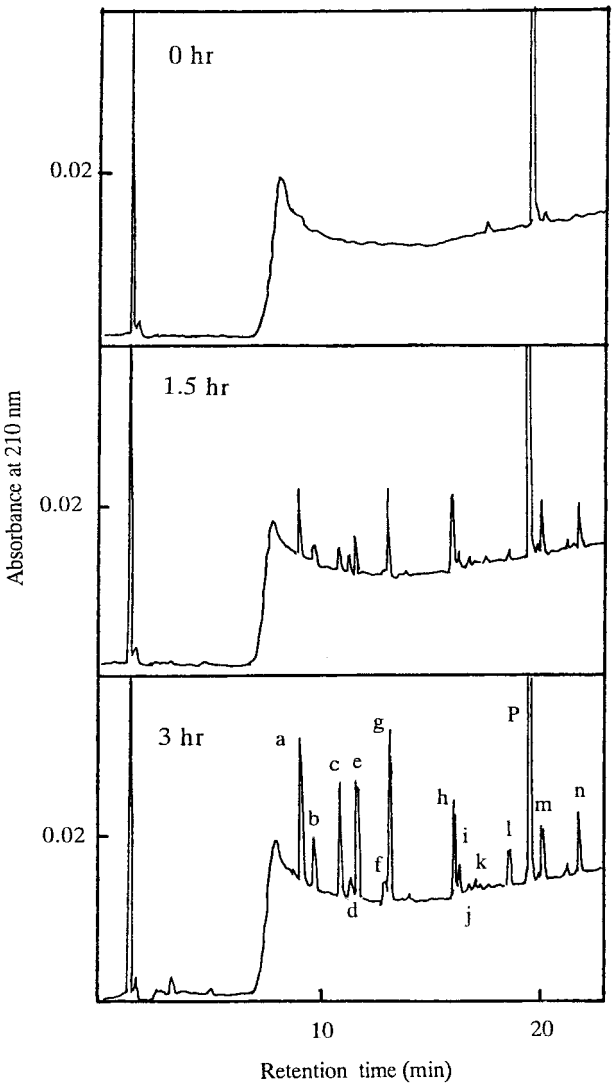

Figure 1—Degradation of substance P by synaptic membranes of rat spinal cord. The reaction mixture (0.5 mL, pH 7.5) containing 150 μg of the membrane preparation was incubated 37 °C for 0, 1.5, and 3.0 h. After heating at 100 °C for 5 min, 30 μL aliquots of the mixture were analyzed by HPLC on a reversed-phase column (4.6 × 150 mm) of SYMMETRYC18, which had been equilibrated with 1% acetonitrile in 0.05% trifluoroacetic acid. Elution was carried out at the room temperature with a 32-min linear gradient of 1–65% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1 mL/min. The absorbance at 210 nm was monitored.

14 peaks were separated and named alphabetically from a to n according to the increasing order of their retention times. When only the membrane was incubated in the absence of SP or only SP was incubated, newly formed peaks were not detected at all. The position of peak P was identical to that of the substrate, SP, and the area of this peak decreased as a function of time (i.e., 15% and 32% of SP were degraded 1.5 h and 3 h after incubation, respectively), whereas those of other peaks increased. The results of analyses of amino acid compositions for the cleavage products separated by HPLC allowed their assignment (Table 1). Fragments b, c, g, h, l, m, and n were assignable as SP(1−4), free phenylalanine, SP(1−7), SP(1−9), SP(8−11), SP(3−11), and SP(5−11), respectively, by comparing their retention times in HPLC. The yield of peaks (a to n) was calculated on the basis of SP degraded by using procedure of quantitative amino acid composition analysis. Thus, N-terminal SP-fragments, SP(1−4), SP(1−6), SP(1−7), and SP(1−9), were found as major products in addition to free phenylalanine, SP(8−9), and SP(10−11).

Table 1–Amino Acid Composition of Fragments of SP[a] Produced through the Action of Synaptic Membranes of Rat Spinal Cord

peak	amino acid (mol %)								fragment identified	yield[b] (%)
	Arg	Pro	Lys	Glu	Phe	Gly	Leu	Met		
a	16	32	20	32	0	0	0	0	1–6	13
b	25	48	26	0	0	0	0	0	1–4	11
c	0	0	0	0	100	0	0	0	Phe	45
d	0	0	0	0	0	0	66	32	10–11	15
e	0	0	0	0	52	48	0	0	8–9	38
f	0	12	20	35	29	0	4	0	3–8	3
g	13	24	15	32	15	0	0	0	1–7	18
h	9	19	14	22	25	11	0	0	1–9	5
i	0	7	9	17	44	20	2	0	nd	
j	0	13	13	20	36	6	11	0	3–10	1
k	0	2	3	32	38	20	0	6	5–9	2
l	0	0	0	1	33	27	29	8	8–11	6
m	0	10	14	19	27	12	11	6	3–11	3
n	0	0	0	27	36	16	13	7	5–11	2
P[c]	8	14	11	16	22	10	13	6	complete	

[a] The sequence, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH_2. [b] Yield was determined on the basis of SP degraded. [c] The extent of degradation of SP was 68%. n.d., not determined.

Table 2–Effect of Protease Inhibitors on the Degradation of SP by Synaptic Membranes of Rat Spinal Cord[a]

inhibitor	concentration (mM)	inhibition (%)
o-phenanthroline	1.0	94
phosphoramidon	0.1	78
thiorphan	0.1	80
PCMBS	1.0	18
PMSF	1.0	24
captopril	0.1	0
bestatin	0.1	0
leupeptin	0.1	0
chymostatin	0.1	28
Z-321	0.1	3

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

Next, we examined the effects of various protease inhibitors on the degradation of SP by synaptic membranes of rat spinal cord. First, the inhibitory effects on the initial cleavage rate of SP were analyzed by measuring the effects on decrease of the HPLC peak for SP (peak P). The result was shown in Table 2. A metal chelator, *o*-phenanthroline and specific inhibitors for endopeptidase-24.11, phosphoramidon and thiorphan, inhibited SP degradation by synaptic membranes. Other inhibitors, including an angiotensin-converting enzyme inhibitor (captopril), a prolylendopeptidase inhibitor (Z-321),[24] an aminopeptidase inhibitor (bestatin), and inhibitors (PCMBS, PMSF, leupeptin, and chymostatin) for serine and cysteine proteases, had little inhibitory effects on SP degradation.

Second, the inhibitory effects on the generation of cleavage products of SP separated by HPLC were analyzed (Figure 2). The generation of all of the peaks including major peaks of N-terminal SP-fragments was strongly inhibited by the addition of thiorphan. Phosphoramidon and *o*-phenanthroline also showed strong inhibition, while other inhibitors exerted little inhibitory effect on the generation of almost all of the peaks (data not shown).

Effect of SP(1−7) and SP(1−4) on SP-Induced Behavioral Response—IT injections of SP (3.0 nmol) produced an immediate behavioral syndrome characterized by reciprocal hindlimb scratching, caudally directed biting, and licking. The intensity of the behavior peaked at 0−5 min, declined gradually at 5−10 min, and was no longer apparent at 15 min postinjection. In subsequent experiments, therefore, the animals were observed for 10 min following IT administration. In the in vivo experiments, N-terminal SP-fragments were tested for antagonism against the behavioral response induced by IT administration of SP. When coadministered IT with SP, SP(1−7) at doses of 40−400 pmol reduced the SP-induced behavioral response consisting of scratching, biting, and licking in a dose-dependent way (Figure 3). The SP-induced response was also dose-dependently decreased by IT coadministration of SP(1−4) (0.6−38.4 nmol) (Figure 3). The ED_{50} for SP(1−7) and SP(1−4) was 135.0 pmol and 6.2 nmol against SP, respectively. SP(1−7) and SP(1−4) in the dose-range tested did not produce SP-like behavioral response or any other motor disturbance.

Discussion

Although a number of laboratories have demonstrated that SP is extracellularly hydrolyzed by cell-surface endopeptidase, within the central nervous system,[8−16] the concurrent monitoring of SP and its metabolic fragments by synaptic membranes of rat spinal cord have not yet been explored. It is extremely of importance to note metabolic routes of SP in the spinal cord, since spinal dorsal horn is regarded as an important site for the processing of information related to the transmission and/or modulation of sensory signals including pain. The present findings show that SP was readily cleaved into SP(1−7) as a major product when incubated with synaptic membranes of rat spinal cord. Other N-terminal SP-fragments were also found, including SP(1−4), SP(1−6), and SP(1−9). It is, therefore, likely that the C-terminal SP-fragments released through the initial cleavage of SP may be susceptible readily to the action of neuropeptidases, since recoveries of C-terminal fragments were low compared with those of N-terminal fragments. These results are in accordance with our previous data that SP(1−7) is the main metabolite after endogenous cleavage of SP; in the dorsal spinal cord of rats the levels of SP(1−7) were about 20% those of SP, though in most of areas of the CNS the concentrations of SP(1−7) were about 10% those of SP.[25,26] Both the degradation of SP and the accumulation of the major cleavage products were strongly inhibited by a metal chelator, *o*-phenanthroline, and also by specific inhibitors of endopeptidase-24.11, thiorphan and phosphoramidon. Formation of the fragment SP(1−4) was also inhibited by the addition of thiorphan and phosphoramidon to the reaction medium. Since fragment SP(1−4) has not been reported to be formed directly by the action of endopeptidase-24.11 on SP, it may arise from the action of some other protease on the initial cleavage products formed by endopeptidase-24.11. Other peptide fragments were those formed by the cleavage between the Gln^6-Phe^7, Phe^7-Phe^8, Gly^9-Leu^{10} bonds of SP, all of which have been reported to be the sites of cleavage by endopeptidase-24.11 obtained from various synaptic membranes of pig caudate,[8] pig kidney,[9] human kidney,[10] rat substantia nigra,[27] glioma C6 cells,[28] and glial cells cultured from rat fetal brain.[29] In our previous report,[30] we have examined the effect of protease inhibitors on the scratching, biting, and licking response elicited by IT injection of SP in mice. Phosphoramidon simultaneously injected with SP remarkably enhanced and prolonged SP-induced behavioral response in a dose-dependent manner. SP-induced behavioral response was not enhanced by bestatin, an aminopeptidase inhibitor, and captopril, an angiotensin-converting enzyme inhibitor. Taking account of these behavioral observations, the present results suggest that thiorphan-sensitive metalloendopeptidse, probably endopeptidase-24.11, would play a critical role in the initial stage of cleavage of SP by synaptic membranes of rat spinal cord.

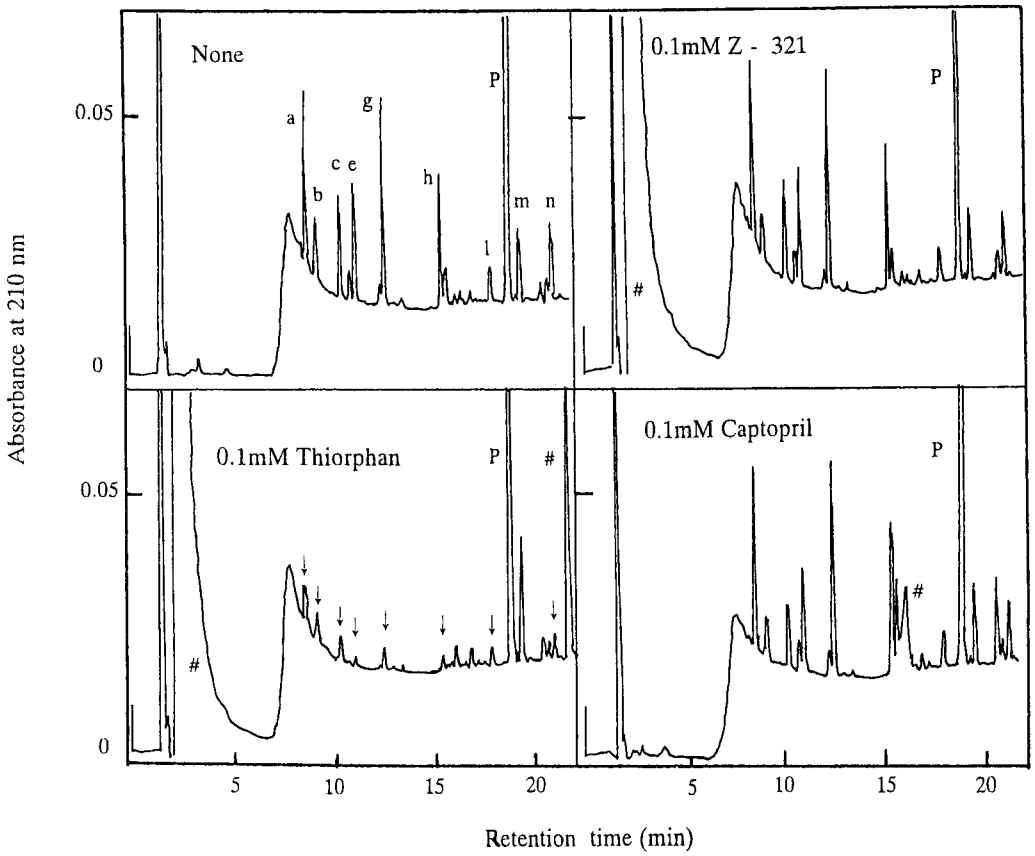

Figure 2—Effect of protease inhibitors on the degradation of substance P by synaptic membranes of rat spinal cord. The reaction mixture (0.5 mL, pH 7.5) containing 50 μg of the membrane preparation was incubated 37 °C for 6 h in the absence or the presence of 0.1 mM thiorpan, 0.1 mM Z-321, or 0.1 mM captopril. After heating at 100 °C for 5 min to stop the reaction, 20 μL aliquots of the sample were analyzed by HPLC as in Figure 1. # indicates the peak derived from the inhibitor added.

There are some reports related to SP degradation in rat spinal cord. Mauborgne et al.[31] have previously examined the effect of peptidase inhibitors on SP-like immunoreactive materials released by K^+-induced depolarization from slices of the rat spinal cord. They indicated that bacitracin was the most potent agent to protect SP-like immunoreactive materials from degradation, and captopril and thiorphan also protected it from degradation. Suzuki et al.[32] have reported the effects of peptidase inhibitors on the enzymatic degradation of SP by synaptic membranes of neonatal rat spinal cord. While they did not analyze and characterize the cleavage products of SP, they have presented that thiorphan, actinonin for an aminopeptidase N inhibitor, and captopril inhibited SP degradation. The inhibition potency of each peptidase inhibitor was lower than that of the mixture. They have also reported in electrophysiological studies that the magnitude of SP-evoked depolarization of a lumbar ventral root in the isolated spinal cord preparation was increased by a mixture of peptidase inhibitors, consisting of actinonin for an aminopeptidase N inhibitor, arphamenine B for an aminopeptidase B inhibitor, bestatin for an aminopeptidase B and M inhibitor, captopril, and thiorphan. These results are inconsistent with our results. Although the reason for this discrepancy remains unknown, in all cases, however, susceptibilities of SP degradation to thiorphan are consistent with each other. Recent biochemical data show that two enzymes with SP-degrading activity from the membrane bound fraction of the rat spinal cord are separated,[33] one enzyme exhibited similarity to endopeptidase-24.11, while the other resembled a substance P converting endopeptidase which has been identified and purified from human cerebrospinal fluid. Accordingly, SP(1−7), SP(1−8), SP(8−11), and SP(9−11) were detected after incubation with both enzyme. The former enzyme preparation also gave SP(1−6) as a major product. It is still unknown whether these enzymes play critical roles in SP degradation in rat spinal cord synapses.

Thus, SP is enzymatically cleaved into several C-terminal and N-terminal fragments, which have biological activity in the CNS sensory system. Some of C-terminal fragments, injected IT into mice, elicit scratching, biting, and licking response similar to that observed with SP, whereas N-terminal fragments have antagonizing effects on SP-induced behavioral response.[34] In addition, central actions of N-terminal fragments have previously been found to produce effects opposite to those of C-terminal fragments in mice. For example, the N-terminal heptapeptide inhibited grooming and increased rearing, whereas the C-terminal hexapeptide had the opposite effect.[17,35] Studies with microinjection into the substantia nigra demonstrate that SP induces dopamine release in the ipsilateral striatum and produces contralateral rotation in a dose-dependent manner.[36] In addition, intranigrally injected SP(1−7) produces an antagonistic action on the SP-induced response. In agreement with these previous findings, SP(1−7), injected into conscious rats produced a dose-dependent antagonizing effect on SP-induced behavioral response. Similar effects were obtained by coadministration of SP-(1−4), though SP(1−4) is approximately 45 times less potent than SP(1−7) in antagonizing the response to SP. These results are in line with our recent data in mice (unpublished data) that SP(1−7) is much more potent than SP(1−4) as assayed by the spinally mediated SP response. Taken together, our biochemical and pharmacological results suggest that endogenously formed SP(1−7) may be a main modulator of SP actions in the rat spinal cord.

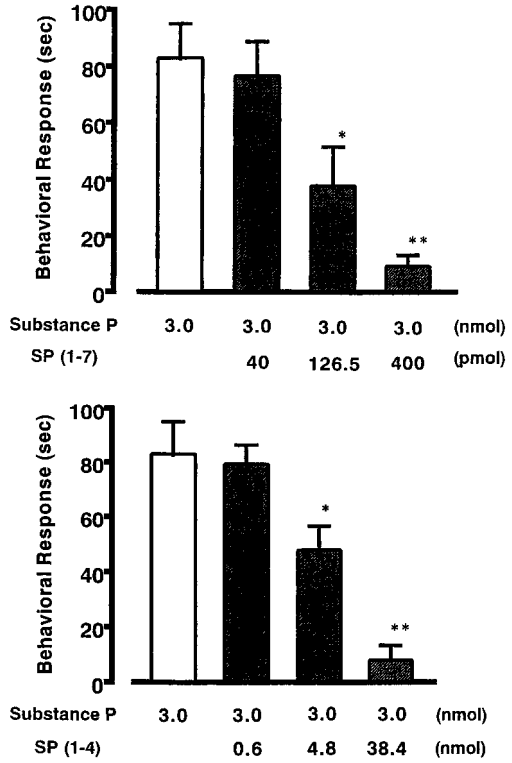

Figure 3—The effectiveness of substance P(1–7) (upper panel) and substance P(1–4) (lower panel) in antagonizing the scratching, biting, and licking behavior induced by substance P in rats. Substance P (3.0 nmol) was coadministered intrathecally with substance P(1–7) or substance P(1–4). The duration of the behavioral response induced by substance P was determined over a 10 min period starting immediately after intrathecal injection. The data are given as means ±SEM of six rats in each group. $^{**}P < 0.01$, $^{*}P < 0.05$ when compared to substance P alone.

Mechanism of spinal actions of N-terminal SP-fragments is still unclear. In the present study, there was no significant difference of potency in the antagonizing effect of SP(1−7) on SP-induced behavioral response between rats and mice, when comparing the ED_{50} value of SP(1−7) (135.0 pmol) against SP (3.0 nmol) in rats with that of SP(1−7) (2.9 pmol) against SP (100 pmol) in mice.[34] These data suggest that SP(1−7) may inhibit binding of [^{3}H]-SP(1−7) to rat and mouse spinal cord membranes with similar potency.[37] It is noteworthy that similar phenomena are seen in affinities of CP-96,345 for tachykinin NK_1 receptor; the nonpeptide tachykinin NK_1 receptor antagonist, CP-96,345, was much less active at NK_1 receptors in rat and mouse than the other mammalian species including human, whereas there was no significant difference in affinity of CP-96,345 for NK_1 receptors between rat and mouse.[38,39]

It is of interest to note that the antagonistic effect of SP-(1−7) on SP-induced behavioral response in mice was inhibited by naloxone, an opioid receptor antagonist, and [D-Pro^2, D-Phe^9] SP(1−7), an inhibitor of [^{3}H]-SP(1−7) binding, but not by selective antagonists of μ-, δ-, and κ-opioid receptors.[40] One could speculate that a physiological action of SP(1−7) in the spinal cord may not be mediated by an action at a typical opioid receptor but by an action at SP(1−7) binding site or its own receptor.

In conclusion, the degradation of SP by endopeptidase-24.11 in the spinal cord is not only responsible for inactivation of SP, but may also lead to the formation of N-terminal fragments which are shown to display a novel physiological function as a result of a shift to receptor specificity.

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