

## Spinorphin, an Endogenous Inhibitor of Enkephalin-Degrading Enzymes, Potentiates Leu-Enkephalin-Induced Anti-allodynic and Antinociceptive Effects in Mice

Motoko Honda<sup>1,2</sup>, Hiroko Okutsu<sup>1</sup>, Tomoyuki Matsuura<sup>1</sup>, Tomohiro Miyagi<sup>1</sup>, Yukio Yamamoto<sup>3</sup>,  
Tadahiro Hazato<sup>3</sup> and Hideki Ono<sup>1,2,\*</sup>

<sup>1</sup>Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Science University of Tokyo,  
12 Ichigaya-Funagawara-machi, Shinjuku-ku, Tokyo 162-0826, Japan

<sup>2</sup>Laboratory of CNS Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University,  
3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan

<sup>3</sup>Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science,  
Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113-0021, Japan

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**ABSTRACT**—Spinorphin (LVVYPWT) has been isolated from the bovine spinal cord as an endogenous inhibitor of enkephalin-degrading enzymes. It has been reported that spinorphin has an antinociceptive effect, inhibitory effect on contraction of smooth muscle and anti-inflammatory effect. In the present study, the effects of leu-enkephalin and spinorphin on allodynia and mechanical and thermal nociceptions were examined in vivo using mice. Intrathecal (i.t.) administration of leu-enkephalin or spinorphin inhibited the allodynia induced by intrathecal nociceptin in a dose-dependent manner. Furthermore, spinorphin enhanced the inhibitory effect of enkephalin on allodynia induced by nociceptin. Naloxone antagonized both inhibitory effects of leu-enkephalin and spinorphin, suggesting that the endogenous opioidergic system can modulate allodynia. Intracerebroventricular (i.c.v.) administration of leu-enkephalin increased the nociceptive threshold of heat or mechanical stimulation to a mouse. Although i.c.v. administration of spinorphin had no effect on the threshold of heat or mechanical stimulation, spinorphin enhanced and prolonged the antinociceptive effect of leu-enkephalin. The enhancement of spinorphin on the antinociception produced by leu-enkephalin was reversed by pretreatment with naloxone. From these results, it is suggested that the effects of spinorphin on enkephalin-induced anti-allodynic and antinociceptive effects are due to inhibition of enkephalin-degrading enzymes.

**Keywords:** Spinorphin, Enkephalin-degrading enzyme, Antinociception, Allodynia

Enkephalin is an endogenous opioid peptide (1). The genuine effect of enkephalin has not been observed because it is hydrolyzed easily by its degrading enzymes in the body (2). Thus, a synthetic substance that inhibits enkephalin-degrading enzymes has been used (3–5). It has been shown that three peptidase inhibitors, amastatin, captopril and phosphoramidon, prevent the hydrolysis of one group of endogenous opioid peptides, including leu-enkephalin, and these peptidase inhibitors enhanced the effects of opioid peptides (3, 4, 6–10). Spinorphin (LVVYPWT) has been isolated from the bovine spinal cord as an endogenous inhibitor of enkephalin-degrading enzymes (11). Spinorphin

inhibits aminopeptidase, dipeptidyl aminopeptidase III, angiotensin-converting enzyme and enkephalinase. It has been reported that spinorphin has an antinociceptive effect at high intracerebroventricular (i.c.v.) dose, an inhibitory effect on contraction of smooth muscle and an anti-inflammatory effect (12, 13). However, it is unclear whether enkephalin is involved in these effects. Although we have shown that spinorphin potentiates the effect of enkephalin on synaptic transmission in isolated hippocampal slices of rats (14), the influence of spinorphin on the effect of enkephalin has not been examined in vivo.

In the present study, we examined the effects of spinorphin on anti-allodynic and antinociceptive effects of enkephalin in an attempt to evaluate the inhibitory effects of spinorphin on enkephalin degradation in vivo. Since

\*Corresponding author (affiliation #2). FAX: +81-52-836-3676  
E-mail: hiono@phar.nagoya-cu.ac.jp

different neurotransmitters were involved in mechanical and thermal nociception (15), tail pressure and tail-flick tests were employed to evaluate antinociception effects of enkephalin.

## MATERIALS AND METHODS

### *Animals*

All experimental protocols were approved by the Animal Care and Use Committee of Science University of Tokyo and were accordance with the guidelines of The Japanese Pharmacological Society.

Male ddY mice weighing 20–35 g were used. The animals were housed under conditions of a 12-h light-dark cycle and a constant temperature of 25°C and 60 ± 10% humidity. Animals had free access to food and water. The procedure for i.t. injections was adapted from the method of Hylden and Wilcox (16). For intrathecal (i.t.) administration, a 27-gauge stainless steel needle (0.35 mm o.d.) connected to a Hamilton microsyringe was inserted directly between the L5 and L6 spinal columns of a conscious mouse. For i.c.v. injection, a 27-gauge stainless steel needle (0.35 mm, o.d.) connected to a Hamilton microsyringe was inserted 2-mm caudal and 2-mm lateral to the bregma at a depth of 3.5 mm of a conscious mouse (17). Drugs in vehicle or vehicle (saline or distilled water) alone were injected slowly into the the subarachnoid or ventricular space of conscious mice.

### *Studies on allodynia*

Studies on allodynia were carried out essentially according to the method of Yaksh and Harty (18). After i.t. administration of drugs, mechanical allodynia was assessed once every 5 min over a 50-min period by light stroking of the flank of mice with a paintbrush (made from horse hair, SAKURA MR 6; Sakura Color Product Corporation, Osaka), and responses of mice, such as vocalization, biting, and escape from the probe, were observed. The allodynic response was ranked as follows: 0 = no response; 1 = mild squeaking with attempts to move away from the stroking probe; 2 = vigorous squeaking evoked by the stroking probe, biting at the probe, and strong efforts to escape.

### *Antinociceptive activity*

Antinociception was determined by the tail-flick and tail pressure tests. The tail-flick test was carried out using a Tail Flick Analgesia Meter (MK-330; Muromachi Kikai, Tokyo). An animal was gently restrained by hand, and radiant heat was directed onto an area 1.5 cm from the tip of the tail. The intensity of heat stimulus was adjusted so that the baseline latency was at 3- to 4-s and a 10-s cut-off time was imposed to avoid tissue damage. The tail pressure threshold was determined using a Randall-Selitto type

Analgesy Meter (Type 7200; Ugo Basile, Viale, Italy). The animal was gently restrained, and an incremental pressure was applied via a piston onto an area 0.5 cm from the base of the tail. The cut-off pressure was 200 g. Only mice responding behaviorally to pressure of 60–100 g were selected. Tail-flick and tail pressure measurements were taken twice at a 20-s interval and their mean was used for calculations.

Effects of i.c.v. administration of enkephalin and spinorphin on the nociceptive threshold were estimated in a time-course study (at time 0, a drug was administered). Antinociceptive activity for each animal was represented as percent of the pre-drug value.

### *Drugs*

Leu-enkephalin was purchased from Funakoshi (Tokyo) and nociceptin and naloxone hydrochloride were from Research Biochemical International (Natick, MA, USA). Spinorphin was synthesized by American Peptide Company Inc. (Sunnyvale, CA, USA). For tail-flick and tail pressure tests, leu-enkephalin and spinorphin were dissolved in distilled water due to low solubility in physiological saline while naloxone hydrochloride was in 0.9% w/v physiological saline. Leu-enkephalin was administered i.c.v. in doses of 1, 3 and 10 µg/5 µl. Spinorphin (5 µg/5 µl, i.c.v.) and naloxone hydrochloride (1 mg/kg, s.c.) were administered 15 min before injection of leu-enkephalin. For studies on allodynia, all drugs were dissolved in 0.9% w/v physiological saline. Nociceptin (50 pg/mouse), leu-enkephalin (0.1, 0.3 and 1 µg/mouse), spinorphin (0.3, 1 and 5 µg/mouse) and naloxone hydrochloride (0.3 µg/mouse) were administered i.t. The maximum volume of injection was 10 µl. Control mice received vehicle (saline: i.t., distilled water: i.c.v.).

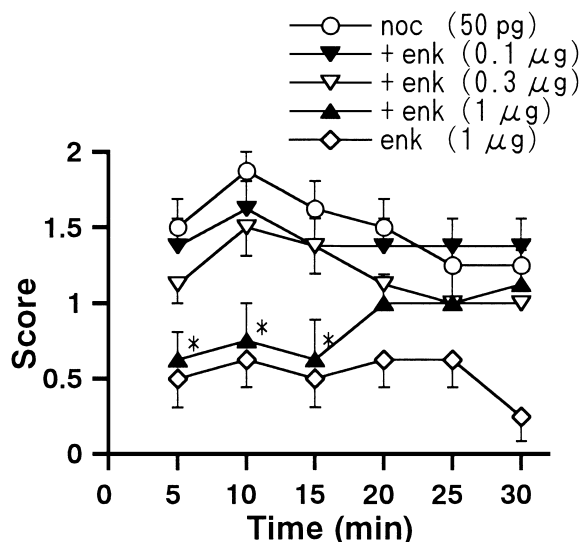
### *Statistical analyses*

The data are expressed as the mean ± S.E.M. For studies on allodynia, the two-tailed Mann-Whitney's U-test was used to compare the data between two groups, while two-tailed non-parametric Bonferroni type multiple comparison following Kruskal-Wallis test was used for multiple comparisons of the control and treated groups (19). For the tail-flick and tail pressure tests, Student's *t*-test or Welch's procedure was used to compare the data between two groups, while two-tailed parametric Bonferroni type multiple *t*-test following ANOVA was used for multiple comparisons in more than 3 groups (20). Differences at *P* < 0.05 (two-tailed) were considered significant.

## RESULTS

### *The effect of leu-enkephalin and spinorphin on allodynia*

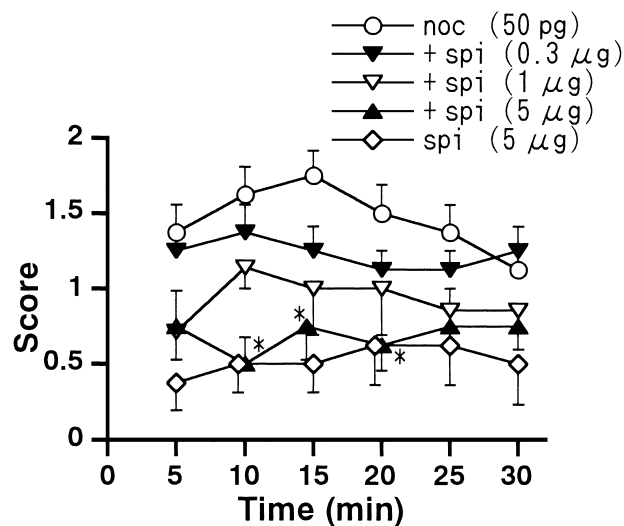
The i.t. administration of nociceptin (50 pg/mouse)



**Fig. 1.** Effect of leu-enkephalin on the nociceptin-induced allodynia. Nociceptin (noc, 50 pg/mouse) was injected simultaneously with leu-enkephalin (+enk; 0.1, 0.3 and 1  $\mu\text{g}$  ( $1.8 \times 10^{-10}$ ,  $5.4 \times 10^{-10}$  and  $1.8 \times 10^{-9}$  mol)/mouse) into the subarachnoid space of conscious mice. Each point represents the score of allodynia as the mean  $\pm$  S.E.M. of 8 mice in each group. Leu-enkephalin alone (enk, 1  $\mu\text{g}$ /mouse) had no effect. The significance of the differences between nociceptin (50 pg/mouse) and leu-enkephalin (0.1, 0.3 and 1  $\mu\text{g}$ /mouse) was determined by the non-parametric Bonferroni type multiple comparison: \* $P < 0.05$ .

induced prominent responses such as vocalization, biting, and escape from the probe following tactile stimuli applied to the flank. The allodynia was evoked by the first stimulus 5 min after i.t. injection; the maximal response was observed at 10–15 min and the maximal score of allodynia was  $1.78 \pm 0.07$  ( $n = 32$ ). Because at 35 min after i.t. injection of nociceptin, the mean score of allodynia returned to about 1 (data not shown), we presented the change of score until 30 min after the injection. The i.t. administration of leu-enkephalin (0.1, 0.3 and 1  $\mu\text{g}$ /mouse) inhibited the allodynia induced by nociceptin in a dose-dependent manner: leu-enkephalin inhibited nociceptin-induced allodynia significantly at 1  $\mu\text{g}$ /mouse, with the mean score of allodynia being  $0.75 \pm 0.25$  (Fig. 1). Leu-enkephalin (1  $\mu\text{g}$ /mouse, i.t.) did not induce allodynia by itself (Fig. 1).

Spinorphin (0.3, 1 and 5  $\mu\text{g}$ /mouse, i.t.) also inhibited nociceptin-induced allodynia dose-dependently, and the inhibition produced by spinorphin was significant at 5  $\mu\text{g}$ /mouse of spinorphin; the mean score of allodynia decreased to  $0.5 \pm 0.15$  at 10 min after the injection (Fig. 2). Spinorphin (5  $\mu\text{g}$ /mouse, i.t.) did not induce allodynia by itself (Fig. 2). The co-administration of naloxone hydrochloride (0.3  $\mu\text{g}$ /mouse, i.t.) attenuated the inhibitory effect of leu-enkephalin or spinorphin on allodynia induced by nociceptin (Fig. 3). The effect of spinorphin (5  $\mu\text{g}$



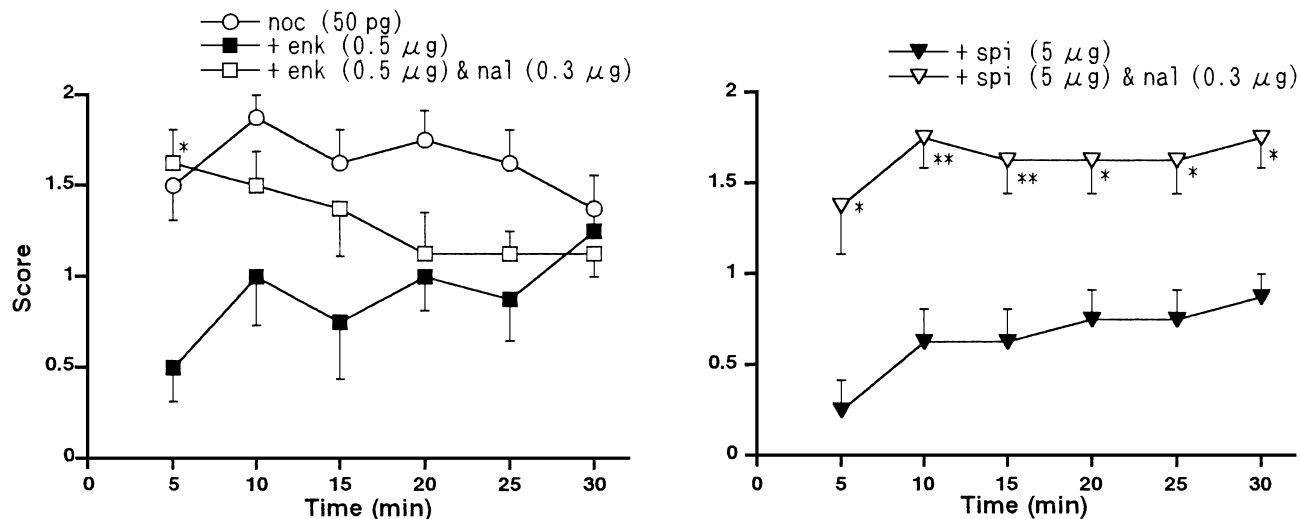
**Fig. 2.** Effect of spinorphin on the nociceptin-induced allodynia. Nociceptin (noc, 50 pg/mouse) was injected simultaneously with spinorphin (+spi; 0.3, 1 and 5  $\mu\text{g}$  ( $3.42 \times 10^{-10}$ ,  $1.14 \times 10^{-9}$  and  $5.7 \times 10^{-9}$  mol)/mouse) into the subarachnoid space of conscious mice. Each point represents the score of allodynia as the mean  $\pm$  S.E.M. of 8 mice in each group. Spinorphin alone (spi, 5  $\mu\text{g}$ /mouse) had no effect. The significance of the differences between nociceptin (50 pg/mouse) and spinorphin (0.3, 1 and 5  $\mu\text{g}$ /mouse) was determined by the non-parametric Bonferroni type multiple comparison: \* $P < 0.05$ .

/mouse, i.t.) was antagonized completely at all the time of measurement.

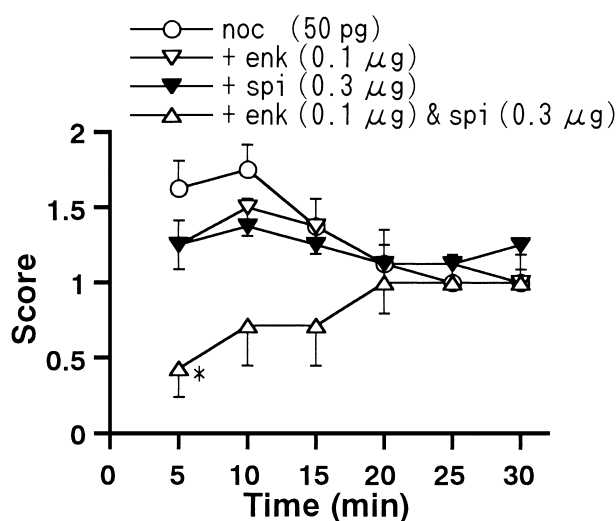
Although a weak inhibition of the allodynia induced by nociceptin was observed after the administration of leu-enkephalin (0.1  $\mu\text{g}$ /mouse, i.t.) or spinorphin (0.3  $\mu\text{g}$ /mouse, i.t.), the co-administration of spinorphin and leu-enkephalin enhanced the inhibition of allodynia more than the effect of spinorphin or leu-enkephalin alone and this inhibition was significant at 5 min after the injection (Fig. 4).

#### *The antinociceptive effect of leu-enkephalin and spinorphin in tail-flick and tail pressure test*

Leu-enkephalin (1, 3 and 10  $\mu\text{g}$ /mouse, i.c.v.) increased the tail-flick threshold (Fig. 5). The most potent effect was observed at 5 and 10 min after the injection. At a dose of 10  $\mu\text{g}$ /mouse, the tail-flick latency increased to  $133.6 \pm 5.9\%$  and  $138.7 \pm 4.4\%$  at 5 and 10 min after the injection of leu-enkephalin, respectively. The tail pressure threshold was significantly increased at 5 and 10 min after the injection of a high dose of leu-enkephalin: at 10 min after the injection of 3 and 10  $\mu\text{g}$ /mouse, i.c.v., the tail pressure threshold was increased to  $121.5 \pm 6.3\%$  and  $117.8 \pm 2.7\%$ , respectively. The antinociceptive effect of leu-enkephalin (3  $\mu\text{g}$ /mouse, i.c.v.) was antagonized by naloxone (1 mg/kg, s.c.). Spinorphin alone (5  $\mu\text{g}$ /mouse,



**Fig. 3.** Effect of naloxone on the inhibitory effect of leu-enkephalin (left) or spinorphin (right) on the nociceptin-induced allodynia. Nociceptin (50 pg/mouse) was injected simultaneously with leu-enkephalin (+enk, 0.5  $\mu$ g ( $0.9 \times 10^{-9}$  mol)/mouse) or spinorphin (+spi, 5  $\mu$ g ( $5.7 \times 10^{-9}$  mol)/mouse) and naloxone hydrochloride (nal, 0.3  $\mu$ g/mouse) into the subarachnoid space of conscious mice. Each point represents the score of allodynia as the mean  $\pm$  S.E.M. of 8 mice in each group. The significance of the differences between leu-enkephalin (0.5  $\mu$ g/mouse) and leu-enkephalin (0.5  $\mu$ g/mouse) + naloxone hydrochloride (0.3  $\mu$ g/mouse) or spinorphin (5  $\mu$ g/mouse) and spinorphin (5  $\mu$ g/mouse) + naloxone hydrochloride (0.3  $\mu$ g/mouse) was determined by Mann-Whitney's U-test: \* $P < 0.05$  and \*\* $P < 0.01$ .



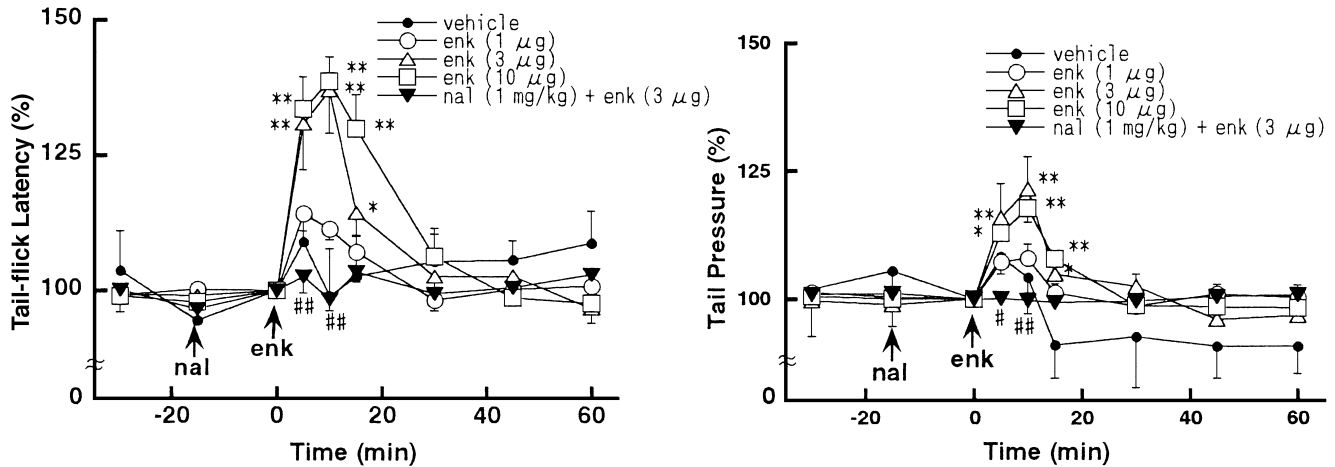
**Fig. 4.** Effect of spinorphin and leu-enkephalin on the nociceptin-induced allodynia. Nociceptin (noc, 50 pg/mouse) was injected simultaneously with spinorphin (+spi, 0.3  $\mu$ g ( $3.42 \times 10^{-10}$  mol)/mouse) and leu-enkephalin (+enk, 0.1  $\mu$ g ( $1.8 \times 10^{-10}$  mol)/mouse) into the subarachnoid space of conscious mice. Each point represents the score of allodynia as the mean  $\pm$  S.E.M. of 8 mice in each group. The significance of the differences between leu-enkephalin (0.1  $\mu$ g/mouse) and spinorphin (0.3  $\mu$ g/mouse) + leu-enkephalin (0.1  $\mu$ g/mouse) was determined by Mann-Whitney's U-test: \* $P < 0.05$ .

i.c.v.) had no effect on the threshold of tail-flick and tail pressure tests (Fig. 6). On the other hand, spinorphin (5  $\mu$ g/mouse, i.c.v.) significantly enhanced the antinociceptive

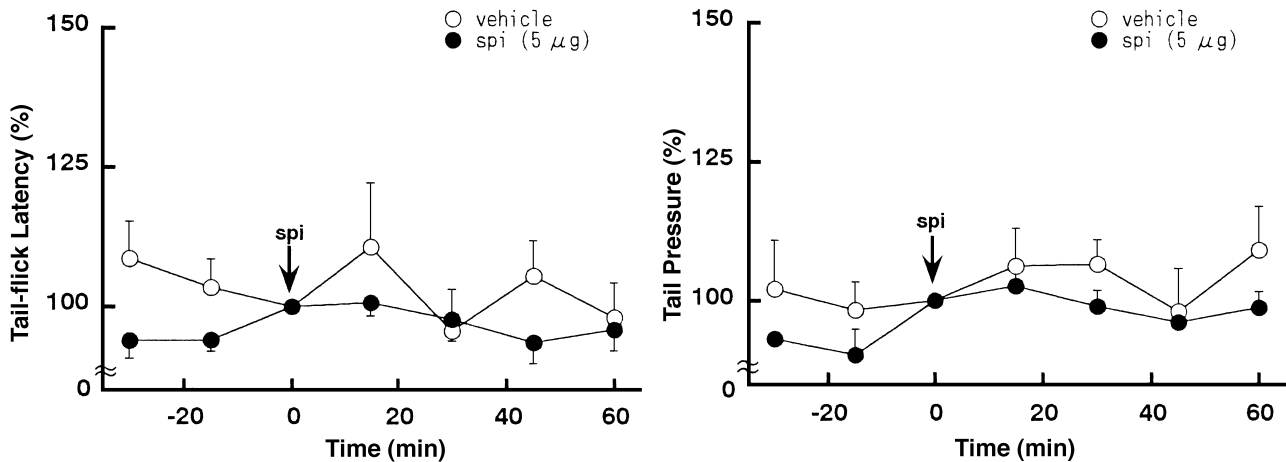
effect of leu-enkephalin (1  $\mu$ g/mouse, i.c.v., Fig. 7). Although the antinociceptive effect of leu-enkephalin alone continued 15 min after the injection, spinorphin prolonged the effect of leu-enkephalin until 30 min after the injection (Fig. 7).

## DISCUSSION

Although the transmitter systems underlying the allodynia are not known, some possible mechanisms have been shown. Some studies have shown that allodynia is induced by i.t. administration of a glycine-receptor antagonist, a GABA<sub>A</sub>-receptor antagonist and L-glutamate (21–24). Minami et al. have shown that hyperalgesia induced by nociceptin was mediated by substance P and nociceptin-induced allodynia was mediated by glutamate through the N-methyl-D-aspartate receptor (25). It was shown that leu-enkephalin mediates antinociception supraspinally and spinally by interacting with  $\delta_1$ - and  $\delta_2$ -opioid receptors, respectively (26). Thus, we examined the possibility that leu-enkephalin can affect allodynia induced by intrathecal nociceptin at the spinal cord level. In the present study, we used a dose of 50 pg/mouse of nociceptin to induce allodynia. Hara et al. showed that i.t. injection of nociceptin at 2.5 ng/kg induced the maximal allodynia in mice (27) and this dose corresponded to 50 pg/mouse (i.t.) which was used in this study. Also in our preliminary experiments, nociceptin induced allodynia peaked at 50 pg/mouse (i.t.). In the present study, the allodynia induced



**Fig. 5.** The antinociceptive effect of leu-enkephalin (enk, 1, 3 and 10  $\mu\text{g}$  ( $1.8 \times 10^{-9}$ ,  $5.4 \times 10^{-9}$  and  $1.8 \times 10^{-8}$  mol)/mouse, i.c.v.) on the nociceptive threshold measured by tail-flick (left) and tail pressure tests (right) in mice. Each point represents the mean  $\pm$  S.E.M. of 5–6 mice. Ordinates: mean thresholds expressed as percentages of the corresponding values at time 0. Abscissae: time in minutes after the injection of leu-enkephalin. The significance of the differences between vehicle and leu-enkephalin (1, 3 and 10  $\mu\text{g}$ /mouse) or leu-enkephalin (3  $\mu\text{g}$ ) and naloxone hydrochloride (nal, 1 mg/kg) + leu-enkephalin (3  $\mu\text{g}$ ) was determined by the Bonferroni type multiple comparison following ANOVA, 4 comparisons in 5 groups: \* $P < 0.05$  and \*\* $P < 0.01$  vs vehicle, # $P < 0.05$  and ## $P < 0.01$  vs enk (3  $\mu\text{g}$ /mouse).

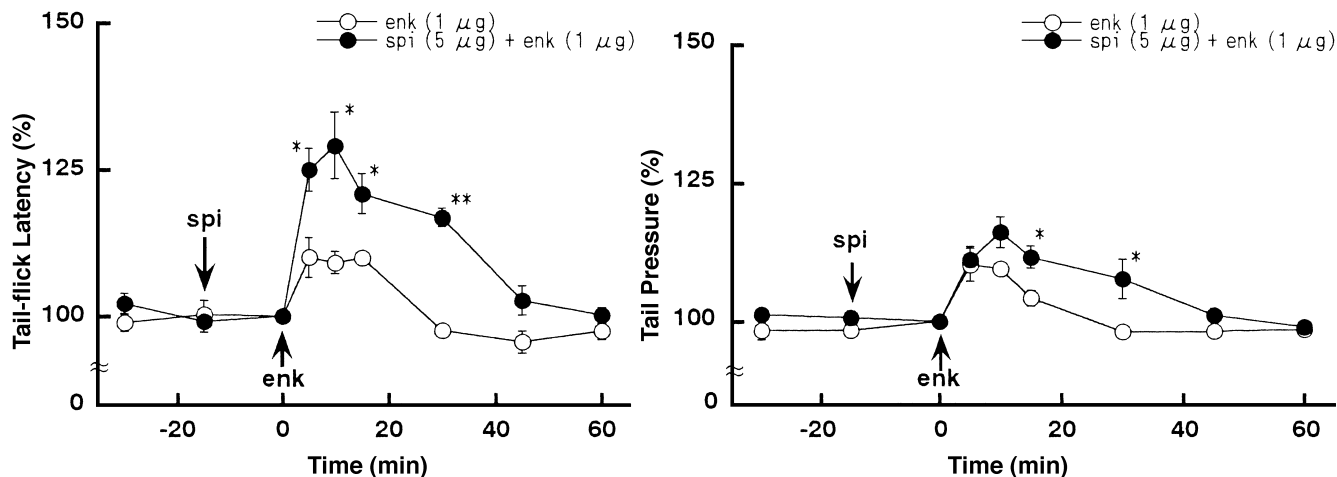


**Fig. 6.** The effect of spinorphin (spi, 5  $\mu\text{g}$  ( $5.7 \times 10^{-9}$  mol)/mouse, i.c.v.) on the nociceptive threshold measured by tail-flick (left) and tail pressure tests (right) in mice. Each point represents the mean  $\pm$  S.E.M. of 5–6 mice. Ordinates: mean thresholds expressed as percentages of the corresponding values at time 0. Abscissae: time in minutes after the injection of spinorphin.

by nociceptin peaked at 10–15 min after the injection and it was in agreement with the results of Hara et al. (Figs. 1–4, noc). I.t. administration of leu-enkephalin or spinorphin inhibited the allodynia induced by nociceptin in a dose-dependent manner (Figs. 1 and 2). Furthermore, spinorphin enhanced the inhibition produced by enkephalin (Fig. 4). The significant inhibition of allodynia was observed at a different time for leu-enkephalin (Fig. 1), spinorphin (Fig. 2), or leu-enkephalin + spinorphin (Fig. 4): 5–15 min, 10–20 min, or 5 min after the injection, respectively. A tendency of inhibition was observed at 10 and 15 min after co-injection of leu-enkephalin (0.1  $\mu\text{g}$ /mouse)

and spinorphin (0.3  $\mu\text{g}$ /mouse). Thus, spinorphin might enhance the effect of leu-enkephalin through inhibition of degradation of enkephalin. Naloxone antagonized both inhibitory effects of leu-enkephalin and spinorphin (Fig. 3), suggesting that increase of enkephalin concentration within the spinal cord can inhibit allodynia induced by nociceptin. The effect of spinorphin lasted longer than that of leu-enkephalin. This may be because spinorphin is not degraded as easily as leu-enkephalin. Therefore, it is suggested that endogenous opioidergic systems can modulate allodynia induced by nociceptin.

I.c.v. administration of leu-enkephalin increased the



**Fig. 7.** The antinociceptive effect of leu-enkephalin (enk,  $1 \mu\text{g}$  ( $1.8 \times 10^{-9}$  mol)/mouse, i.c.v.) and spinorphin (spi,  $5 \mu\text{g}$  ( $5.7 \times 10^{-9}$  mol)/mouse, i.c.v.) + leu-enkephalin ( $1 \mu\text{g}$ /mouse, i.c.v.) on the nociceptive threshold measured by tail-flick (left) and tail pressure tests (right) in mice. Each point represents the mean  $\pm$  S.E.M. of 6 mice. Ordinates: mean thresholds expressed as percentages of the corresponding values at time 0. Abscissae: time in minutes after the injection of leu-enkephalin. The significance of the differences between leu-enkephalin ( $1 \mu\text{g}$ /mouse) and spinorphin ( $5 \mu\text{g}$ /mouse) + leu-enkephalin ( $1 \mu\text{g}$ /mouse) was determined by the two-tailed Student's *t*-test or Welch's procedure: \* $P < 0.05$  and \*\* $P < 0.01$ .

nociceptive threshold of tail-flick and tail pressure and naloxone antagonized these effects (Fig. 5). These effects of leu-enkephalin were transient and the results were in accordance with those of Belluzzi et al. (28). I.c.v. leu-enkephalin was suggested to be effective at the supraspinal structure because the degradation of leu-enkephalin was very rapid. Although the precise mechanisms of antinociceptive effects of i.c.v. leu-enkephalin are not known, the descending noradrenergic and serotonergic inhibitory systems may be involved as in the case of morphine-induced analgesia (15, 29). Although i.c.v. administration of spinorphin alone had no effect on the nociceptive threshold of tail-flick or tail pressure (Fig. 6), the same dose of spinorphin enhanced and prolonged the antinociceptive effect of leu-enkephalin (Fig. 7). Furthermore, the enhancement of spinorphin on the antinociception produced by leu-enkephalin was reversed by pretreatment with naloxone (not shown). However, it is unclear whether i.c.v. administered leu-enkephalin + spinorphin are effective at the supraspinal level because we could not exclude the possibility that leu-enkephalin, which was not degraded by enzymes, diffused to the spinal level. From these results, it is suggested that spinorphin enhanced the antinociceptive effect of leu-enkephalin through inhibition of enkephalin-degrading enzymes.

In conclusion, both of leu-enkephalin and spinorphin antagonized allodynia induced by nociceptin and the effect of leu-enkephalin was enhanced by spinorphin, an endogenous inhibitor of enkephalin-degrading enzymes. Leu-enkephalin inhibited nociceptive responses of mice and the effect was enhanced by spinorphin. Thus, it is suggested

that the effect of spinorphin was due to inhibition of enkephalin-degrading enzymes and that spinorphin inhibited enkephalin degrading enzymes in vivo. Further studies are needed to study physiological role of spinorphin in the brain and the spinal cord.

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