Effects of a Mixture of Peptidase Inhibitors (Amastatin, Captopril and Phosphoramidon) on Met-Enkephalin-, β-Endorphin-, Dynorphin-(1–13)- and Electroacupuncture-Induced Antinociception in Rats

Shiroh Kishioka, Yoshiyuki Miyamoto, Yuko Fukunaga, Shigeru Nishida and Hiroyuki Yamamoto

Department of Pharmacology, Wakayama Medical College, Wakayama 640, Japan

ABSTRACT—The effects of a mixture of three peptidase inhibitors (PIs), amastatin, captopril and phosphoramidon, on methionine-enkephalin (Met-enk), β-endorphin (β-end), dynorphin-(1–13) (Dyn) and electroacupuncture (EA)-induced antinociception were compared in rats. EA was performed by passing electric pulses (3 Hz, 0.1-msec duration, for 45 min) through acupuncture needles inserted into the Hoku-point. The antinociceptive effect was estimated by the hind paw pressure test. The antinociceptive effects of Met-enk and β-end injected i.c.v. or i.t. and of Dyn injected i.t. were clearly potentiated by the PIs pretreated by the same administration routes as used for the injection of opioid peptides. The antinociceptive effects of Met-enk, β-end and Dyn injected i.c.v. were also potentiated significantly by i.t.-PIs. PIs injected into the periaqueductal gray (PAG) potentiated EA antinociception. However, the EA effect was not affected by i.t.-PIs and was rather attenuated by i.c.v.-PIs. These results suggest that: i) Met-enk hydrolyzing enzymes are involved in the degradation of not only Met-enk but also β-end and Dyn in the rat central nervous system; ii) Met-enk and β-end act on both supraspinal and spinal sites, while Dyn acts only on the spinal site; iii) EA antinociception is mediated by supraspinal Met-enk and/or β-end; and iv) an anti-opiate peptide system may be activated by EA stimulation, being susceptible to Met-enk hydrolyzing enzymes.

Keywords: Electroacupuncture, Endogenous opioid peptide, Peptidase inhibitor, Intracerebroventricular and/or intrathecal, Periaqueductal gray

Three distinct families of endogenous opioid peptides have been identified: opioid peptides derived from proenkephalin A, proenkephalin B and proopiomelanocortin. The antinociceptive effect of these peptides administered exogenously has been reported, and the physiological role of endogenous opioid peptides in the processes of nociceptive perception has been suggested (1). The antinociceptive effect of methionine-enkephalin (Met-enk) was demonstrated by intracerebroventricular (i.c.v.) (2) and intrathecal (i.t.) (3) administration and intracerebral microinjection into the periaqueductal gray (PAG) (4). The antinociceptive effect of β-endorphin (β-end) (5) was induced by i.c.v. and i.t. administration. The antinociceptive effect of dynorphin-(1–13) (Dyn) was induced by i.t. administration (6), but little or no effect was detected after i.c.v. administration (6–8) or microinjection into the PAG (8). The routes by which the opioid peptides induce their antinociceptive effects may suggest the regions of the central nervous system (CNS) in which endogenous opioid peptides exert their effects on nociceptive perception.

The involvement of endogenous opioid peptides in electroacupuncture (EA)-induced antinociception has been demonstrated through several lines of evidence. EA-antinociception can be blocked by μ-, δ- and κ-opioid-receptor antagonists (i.e., β-funaltrexamine, ICI 174,864 and norbinaltorphimine, respectively) (9) and antibodies against Met-enk (into PAG) (10), β-end (into PAG) (10) and Dyn (into i.t.) (11).

Three distinct peptidases, aminopeptidase (12), peptidyl dipeptidase A (13) and enkephalinase (14), are known to catalyze the enzymatic degradation of Met-enk. A mixture of an aminopeptidase inhibitor, a peptidyl dipeptidase A inhibitor and an enkephalinase inhibitor has been shown to completely protect Met-enk from degradation produced by incubation with either an ileal
or a striatal membrane fraction from guinea pigs (15). Also, it has been shown that the effects of Met-enk in vivo and in vitro were potentiated by the mixture of peptidase inhibitors; the loss of the righting reflex and the inhibition of the tail-flick response were produced by Met-enk administered s.c. in infant rats pretreated with the mixture of three peptidase inhibitors (16), and the inhibitory effect of Met-enk on the contraction of isolated ileum (17, 18) and vas deferens (17, 19, 20) were increased by the mixture of peptidase inhibitors. However, the enzymatic degradation of β-end and Dyn is not fully understood, and the involvement of the Met-enk hydrolyzing enzymes in the degradation of β-end and Dyn in CNS is not clear.

The first purpose of the present experiment was to explore the effect of a mixture of three peptidase inhibitors (amastatin, captopril and phosphoramidon: PIs) on Met-enk-, β-end- and Dyn-induced antinociception in order to study the site of action (spinal or supraspinal) of these opioid peptides and to evaluate the possible roles of the PIs in the degradation of β-end and Dyn by the combination of administration routes (i.c.v. and i.t.) of these peptides and PIs. Secondarily, the effect of the PIs (i.c.v. and/or i.t., PAG) on EA-antinociception was studied to elucidate the participation of the opioid peptides in EA-antinociception and the site of the CNS at which the opioid peptides are involved in EA-antinociception.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Clea, Osaka), weighing 250–350 g, were used. The animals were housed two to a hanging wire cage in an animal room with controlled temperature (22–24°C), humidity (60–70%) and lighting (7:00–19:00) for at least 2 weeks before the experiment. Food (MF; Oriental Yeast Co., Tokyo) and water were provided ad libitum.

Electroacupuncture

Stainless steel acupuncture needles (No. 5; Igarashi Ika Kogyo Co., Ltd., Tokyo) were inserted at acupuncture points, so called “Hoku-points”, of both forelegs, which were located between the first and second metacarpal bones. Electric current (3 Hz, 0.1-msec duration) was applied via needles by an electrical stimulator (Tokki-Model II, Igarashi Ika Kogyo Co., Ltd.) for 45 min. Maximum intensity of stimulation was selected to induce twitching of both forelegs corresponding to the stimulation frequency, but not strong enough to exhibit the flight reaction (struggle) or squeaking. During the stimulation, the rats were held loosely in both hands to avoid the stress of restraint.

Electroacupuncture

Stainless steel acupuncture needles (No. 5; Igarashi Ika Kogyo Co., Ltd., Tokyo) were inserted at acupuncture points, so called “Hoku-points”, of both forelegs. Electric current (3 Hz, 0.1-msec duration) was applied via needles by an electrical stimulator (Tokki-Model II, Igarashi Ika Kogyo Co., Ltd.) for 45 min. Maximum intensity of stimulation was selected to induce twitching of both forelegs corresponding to the stimulation frequency, but not strong enough to exhibit the flight reaction (struggle) or squeaking. During the stimulation, the rats were held loosely in both hands to avoid the stress of restraint.

Surgical procedures

For i.c.v.-injection, rats were implanted with a 20-gauge guide cannula in the skull, as previously described (2). The coordinates were: 2 mm left lateral to the sagittal suture and 1 mm caudal to the coronal suture. A dummy stylet was left in the guide cannula until drug administration. For i.t.-injection, rats were implanted with spinal catheters as follows: A midline dorsal incision was made, and the lumbar vertebrae were exposed unilaterally. Then, an intervertebral puncture between L2 and L3 was made with a 21-gauge needle, and a PE-10 polyethylene tube filled with sterile saline was inserted 2 cm rostrally to bring its tip to Th12. The other end of the catheter was passed subcutaneously and was taken out between the scapulae. The guide cannula and spinal catheter were implanted at the same time.

The guide cannula for PAG-injection, which was the same as used for i.c.v.-injection, was implanted on the left lateral skull. The stereotaxic coordinates of PAG were: AP, +1.7; lateral, 0.7; and vertical, 4.0 mm above the interaural line, according to the stereotaxic atlas of Paxinos and Watson (21).

After surgery, the rats were allowed at least 7 days of postoperative recovery before testing.

Microinjection procedures

The i.c.v.-injection was made in a volume of 10 μl over a 30-sec period using a 29-gauge injection needle, which was inserted 5 mm beyond the surface of the skull to bring its tip into the left lateral ventricle. The i.t.-injection was made in a volume of 15 μl (5 μl of drug solution and an additional 10 μl of saline for flushing the catheter) over a 30-sec period.

For the PAG-injection, a 29-gauge injection needle was inserted 6.0 mm from the surface of the skull into the PAG, and 0.5 μl of solution was delivered through the injection needle over a 60-sec period.

Measurements of nociceptive thresholds

The non-inflamed hind paw pressure method (2) with an analgesy meter (Ugo Basile, Milan, Italy) was used to estimate the nociceptive threshold. The basal nociceptive thresholds were measured twice, 15 min apart, just before EA or opioid peptide injection and then every 15 min for 120 min. A cut-off loading pressure of 1500 g was used to prevent tissue damage, and the nociceptive threshold was considered to be 1500 g. The EA- and the opioid peptides-induced antinociceptions were evaluated by the maximum increase in the nociceptive thresholds (MAX, g) and area under the antinociceptive curve (AUC, kg × min).

Location of site of injection

At the end of the experiment, india ink was injected by
the same manipulations as the drug solution. The sites of i.c.v.- and i.t.-injection were verified by gross examination. The site of PAG-injection was identified in serial frozen sections of 20-μm thickness.

**Drugs**

Methionine-enkephalin (Met-enk; Peptide Institute, Inc., Minoh), human β-endorphin (β-end; Peninsula Labo., Inc., Belmont, CA, USA), dynorphin-(1–13) (Dyn, Peninsula Labo., Inc.), amastatin (Peptide Institute, Inc.), captopril (Sankyo Company, Tokyo) and phosphoramidon (Peptide Institute, Inc.) were used.

Doses of Met-enk, β-end and Dyn were 50, 5 and 15 μg/rat, i.c.v. or 50, 5 and 10 μg/rat, i.t., respectively. A mixture (PIs) of amastatin, captopril and phosphoramidon was prepared in a concentration of $1.8 \times 10^{-4}$ M for i.c.v. and/or i.t. injection and a concentration of $10^{-3}$ M for PAG injection. PIs were administered 10 min before the injection of opioid peptides or the start of EA stimulation.

Opioid peptides were dissolved in saline. PIs were dissolved in distilled water in a concentration of $10^{-3}$ M and then were diluted with saline. All drugs were stored at $-80°C$ until used.

**Statistical analyses**

Values are presented as the means±S.E.M. In analyzing the data, comparisons of the values with the respective controls were done by Student's paired t-test or one-way analysis of variance (ANOVA) followed by the Newman-Keuls test as appropriate (22). Differences with a P value less than 0.05 were considered significant.
RESULTS

Effects of PIs (i.c.v. or i.t.) on Met-enk (i.c.v. or i.t.)-induced antinociception

Met-enk i.c.v. in a dose of 50 μg/rat did not induce a significant increase in nociceptive thresholds (Student’s paired t-test: Fig. 1A, upper panel), whereas an apparent increase in nociceptive thresholds was produced by Met-enk i.c.v. after i.c.v.- and i.t.-administration of PIs, both antinociceptive effects attaining maximum at 15 min after Met-enk (Fig. 1A, upper panel). The Met-enk i.c.v.-induced effect after i.c.v.-PIs was more prominent than that after i.t.-PIs, and the MAX of Met-enk i.c.v.-induced antinociception after i.c.v.-PIs was twice of that after i.t.-PIs. The MAX of Met-enk i.c.v.-induced antinociception after i.c.v.-PIs was the same as that after i.t.-PIs, indicating that the antinociceptive effect of Met-enk i.c.v. after i.t.-PIs lasted longer than that after i.c.v.-PIs (Fig. 1A, lower panels).

Met-enk injected i.t. in a dose of 50 μg/rat did not induce a significant increase in nociceptive thresholds (Student’s paired t-test: Fig. 1B, upper panel) with or without i.c.v.-PIs, but produced a slight, but significant increase in nociceptive threshold after i.t.-PIs, reaching maximum at 30 min with restoration at 60 min (Fig. 1B, upper panel). Met-enk i.t.-induced antinociception after i.t.-PIs was also shown by a significant increase in MAX and AUC\text{0-120 min} (Fig. 1B, lower panels).

Effects of PIs (i.c.v. or i.t.) on β-end (i.c.v. or i.t.)-induced antinociception

As indicated in Fig. 2A (upper panel), β-end i.c.v. in a dose of 5 μg/rat produced a moderate antinociception (0 min vs 15 min and 30 min, \text{P}<0.01; 0 min vs 45 min, \text{P}<0.05)
P < 0.05: Student’s paired t-test), attaining maximum at 15–30 min with restoration to the pre-drug nociceptive threshold at 60 min after β-end. Both i.c.v.- and i.t.-PIs enhanced the maximum effect and prolonged the duration of β-end i.c.v.-induced antinociception. The potentiation of β-end i.c.v.-induced antinociception after i.c.v.-PIs was more prominent than that after i.t.-PIs and β-end i.c.v.-induced antinociception after i.c.v.-PIs was still evident at 120 min after β-end. AUCO–120 min of β-end i.c.v.-induced antinociception after i.c.v.-PIs was more than twice that after i.t.-PIs, although the MAX after i.c.v.-PIs was the same as that after i.t.-PIs (Fig. 2A, upper and lower panels).

A slight increase in nociceptive threshold was induced by β-end i.t. in a dose of 5 μg/rat (0 min vs 45 min, P < 0.01; 0 min vs 30 min, 60 min, 75 min and 90 min, P < 0.05: Student’s paired t-test: Fig. 2B, upper panel), reaching maximum at 30 min with restoration to the pre-drug nociceptive threshold at 105 min. Antinociception induced by β-end i.t. was not affected by i.c.v.-PIs, but was clearly potentiated by i.t.-PIs, as evidenced by the increases in both MAX and AUCO–120 min (Fig. 2B, upper and lower panels).

Effects of PIs (i.c.v. or i.t.) on Dyn (i.c.v. or i.t.)-induced antinociception

A dose of 15 μg/rat, i.c.v. and 10 μg/rat, i.t. of Dyn did not elicit any walking dysfunction that might interfere with the analgesic test.

An increase in nociceptive threshold, although very slight, was induced by i.c.v.-Dyn in a dose of 15 μg/rat (0 min vs 15 min and 30 min, P < 0.01; 0 min vs 45 min, 60 min and 75 min, P < 0.05: Student’s paired t-test: Fig. 3A, upper panel). Neither MAX nor AUCO–120 min of

---

Fig. 3. Effects of PIs administered i.c.v. or i.t. on Dyn-induced antinociception estimated by the hind-paw pressure test. A: i.c.v.-Dyn (15 μg/rat)-induced antinociception. B: i.t.-Dyn (10 μg/rat)-induced antinociception. The upper panel indicates the time course of Dyn antinociception. Lower panels indicate MAX and AUCO–120 min. Doses of PIs given i.c.v. and i.t. were 1.8 nmol and 0.9 nmol, respectively. PIs were given 10 min before Dyn. In the lower panels, + and − indicate pretreatment with PIs and with vehicle (Veh: saline), respectively. ●: i.c.v.-Veh+i.t.-Veh, ▲: i.c.v.-PIs+i.t.-Veh, ■: i.c.v.-Veh+i.t.-PIs. Each value represents the mean and vertical bars indicate the S.E.M. of 12–15 rats (i.c.v.-Dyn) or of 9–10 rats (i.t.-Dyn). Statistical differences were analyzed by one way ANOVA followed by the Newman-Keuls test. **P < 0.01, *P < 0.05, for the difference from i.c.v.-Veh+i.t.-Veh; **P < 0.01, for the difference from i.c.v.-PIs+i.t.-Veh. Statistical difference between groups: *P < 0.01, **P < 0.05.
i.c.v.-Dyn induced antinociception were affected by i.c.v.-PIs, but both MAX and AUC\textsubscript{0-120 min} were potentiated by i.t.-PIs (Fig. 3A, lower panels).

Dyn given i.t. in a dose of 10 \( \mu \text{g/rat} \) did not show an antinociceptive effect (Student’s paired \( t \)-test: Fig. 3B, upper panel) with or without i.c.v.-PIs. Apparent antinociception, however, was produced by i.t.-Dyn after i.t.-PIs, and it reached maximum at 15–45 min, but was not restored to the pre-drug nociceptive threshold at 120 min. The i.t.-Dyn induced antinociception after i.t.-PIs was indicated as a significant increase in MAX and AUC\textsubscript{0-120 min} (Fig. 3B, upper and lower panels).

**Fig. 4. Effects of PIs given i.c.v. and/or i.t. on EA-induced antinociception.** The nociceptive thresholds were estimated by the hind paw pressure test. EA was performed by stimulation of the Hoku-points (3 Hz, 0.1-msec duration, for 45 min). The upper panel indicates the time course of EA antinociception. Lower panels indicate MAX and AUC\textsubscript{0-90 min}. Doses of PIs given i.c.v. and i.t. were 1.8 nmol and 0.9 nmol, respectively. PIs were given 10 min before the start of EA stimulation. In the lower panels, + indicates EA stimulation or pretreatment with PIs, and − indicates no EA stimulation or pretreatment with vehicle (Veh: saline). ●: i.c.v.-Veh + i.t.-Veh + EA (n=15), ▲: i.c.v.-PIs + i.t.-Veh + EA (n=14), ■: i.c.v.-Veh + i.c.v.-PIs + i.t.-PIs + EA (n=12), ○: i.c.v.-PIs + i.t.-PIs + Veh + EA (n=12), △: i.c.v.-PIs + i.t.-PIs and without EA (n=11). Each value represents the mean and vertical bars indicate the S.E.M. Statistical differences were analyzed by one way ANOVA followed by the Newman-Keuls test. \( ^{\ddagger}P<0.05 \), for the difference from i.c.v.-Veh + i.t.-Veh with EA stimulation. \( ^{\ddagger\ddagger}P<0.01 \), for the difference from i.c.v.-PIs + i.t.-PIs without EA stimulation. Statistical difference between groups: \( ^{\ddagger}P<0.05. \)

**Fig. 5. Potentiating effect of PIs injected into PAG on EA-induced antinociception.** The nociceptive thresholds were estimated by the hind paw pressure test. EA was performed by stimulation of the Hoku-points (3 Hz, 0.1-msec duration, for 45 min). The upper panel indicates the time course of EA antinociception. Lower panels indicate MAX and AUC\textsubscript{0-90 min}. PIs (0.5 nmol) were injected into the PAG 10 min before the start of EA stimulation. In the lower panels, + indicates EA stimulation or pretreatment of the PAG with PIs (PAG-PIs), and − indicates no EA stimulation or pretreatment with vehicle (Veh: distilled water). ●: Veh + EA (n=7), ▲: PIs + EA (n=8), ○: Veh without EA (n=5), △: PIs without EA (n=5). Each value represents the mean and vertical bars indicate the S.E.M. Statistical differences were analyzed by one way ANOVA followed by the Newman-Keuls test. **\( ^{\ddagger\ddagger}P<0.01 \), for the difference from PAG-Veh with EA stimulation. \( ^{\ddagger\ddagger}P<0.01 \), for the difference from PAG-Veh without EA stimulation. \( ^{\ddagger}P<0.05 \), for the difference from PAG-PIs without EA stimulation. Statistical difference between groups: \( ^{\ddagger}P<0.05. \)

Effects of PIs (i.c.v. and/or i.t.) on EA-induced antinociception

The nociceptive threshold was increased progressively during EA for 45 min and rapidly restored to the pre-EA nociceptive threshold after termination of EA (0 min vs 15 min, 30 min, 45 min and 60 min, \( P<0.01;0 \text{ min vs } 75 \text{ min, } P<0.05: \) Student’s paired \( t \)-test: Fig. 4, upper panel).

PIs administered i.c.v. + i.t. did not induce any change of nociceptive threshold (Fig. 4, upper panel).

EA-induced antinociception was not affected by i.t.-PIs, but the nociceptive threshold at 30 min of EA and MAX of EA antinociception after i.c.v.- and i.c.v. + i.t.-
Pis were lower than those after i.c.v.+i.t.-vehicle and i.t.-Pis (Fig. 4, upper and lower panels); i.e., EA-induced antinociception was not enhanced and rather attenuated by i.c.v.-Pis.

**Effects of PIs into the PAG on EA-induced antinociception**

Data from rats with injection sites within the PAG were used. Intra-PAG injections of vehicle and PIs by themselves elicited no change of nociceptive threshold (Student’s paired t-test: Fig. 5, upper and lower panels).

EA-induced antinociception was potentiated by PIs into the PAG; i.e., the nociceptive threshold at 30 min of EA and both the MAX and AUC\textsubscript{90 min} of the EA-induced antinociception after PIs into the PAG were increased as compared with those after the vehicle into the PAG (Fig. 5, upper and lower panels).

**DISCUSSION**

The PIs administered i.c.v.+i.t. or into PAG with the dose used in the present experiment did not affect the nociceptive threshold estimated by the hind paw pressure test in rats (Figs. 4 and 5, upper panel).

Although i.c.v.-Met-enk (50 μg/rat) alone did not induce any change of the nociceptive threshold, a profound antinociception was produced by this dose of i.c.v.-Met-enk after i.c.v.-Pis. Met-enk given i.t. (50 μg/rat) alone induced no antinociception, but induced an apparent antinociception after i.t.-Pis (Fig. 1). It has been shown previously that the inhibition of enzymatic degradation of Met-enk results in the potentiation of the effects of Met-enk administered exogenously (17, 23). Accordingly, the present results suggest that the dose of i.c.v.- and i.t.- PIs used in this study was sufficient to inhibit the enzymatic breakdown of Met-enk injected i.c.v. and i.t., respectively, and are in line with previous reports that Met-enk acts on both spinal and supraspinal sites to produce antinociception (2, 3).

β-End (5 μg/rat, i.c.v. or i.t.) produced antinociception, being consistent with the previous report that supraspinal or spinal action of β-end results in antinociception (5). β-End i.c.v.- and i.t.-induced antinociception was increased by i.c.v.- and i.t.-Pis, respectively (Fig. 2). It was reported that Met-enk hydrolyzing enzymes degraded the shorter fragments of β-end less than 19 residues, and the effects of opioid peptides were increased by peptidase inhibition (24). Kuno et al. (17) reported that the same PIs as used in the present experiment augmented the β-end-induced inhibition of electrical twitch responses of mouse vas deferens, suggesting that three enkephalin-hydrolyzing peptidases might be responsible for the enzymatic inactivation of β-end.

Thus, the potentiation of β-end-induced antinociception after PIs may result from the inhibition of enzymatic degradation of β-end, and it is suggested that Met-enk hydrolyzing enzymes may be involved in the degradation of β-end administered exogenously.

As shown in Fig. 3, no antinociceptive effect of Dyn at 15 μg/rat, i.c.v. or 10 μg/rat, i.t. alone was observed. I.c.v.-Dyn after i.c.v.-Pis did not produce any antinociceptive effect but i.t.-Dyn after i.t.-Pis elicited apparent antinociception. It has been reported (7, 25, 26) that the inhibitors of Met-enk hydrolyzing enzyme protected against the degradation of dynorphins administered i.c.v. and potentiated the effect of dynorphins administered i.c.v. Therefore, i.t.-Dyn induced antinociception after i.t.-Pis may be the result of inhibition of degradation of i.t.-Dyn by i.t.-Pis. This is consistent with the previous report that Dyn exerts its antinociceptive effect through spinal action (6).

Interestingly, the effects of i.c.v.-injected Met-enk, β-end and Dyn were potentiated by i.t.-administration of PIs (Figs. 1, 2 and 3). Two possibilities can be considered to account for the phenomena: i) The opioid peptides injected i.c.v. stimulate the descending nervous system, which releases endogenous opioid peptide in the spinal cord, and the antinociceptive effect of the released opioid peptide is potentiated by i.t.-Pis. ii) The opioid peptides injected i.c.v. flow into the spinal site through the CSF, and the antinociceptive effect of the leaked opioid peptide is potentiated by i.t.-Pis.

With regard to the first possibility, it was demonstrated that i.c.v.-administered β-end released Met-enk in the spinal cord by activating the descending nervous system in rats (27). We can thus postulate that the potentiation of i.c.v.-β-end induced antinociception after i.t.-Pis may be result from the potentiation of the released spinal Met-enk-induced antinociception after i.t.-Pis, because the effect of i.t.-Met-enk was potentiated by i.t.-Pis (Fig. 1). At present, however, there is no information on the interaction between the other supraspinal opioid peptides (Met-enk and Dyn) and the descending spinal opioidergic system. As to the second possibility, Miyamoto et al. (28) reported from our laboratory that the morphine content in the spinal cord was about 1/145 of that in the hypothalamus + midbrain at 45 min after i.c.v. injection of morphine. So, we can not rule out the possibility that i.c.v.-injected opioid peptides leaked into the spinal subarachnoid space.

The antinociceptive effects of Met-enk and β-end injected i.c.v. were potentiated by i.c.v. and i.t. administration of PIs, and the effects of Met-enk and β-end injected i.t. were potentiated by i.t.-Pis. The effects of Dyn injected i.c.v. and i.t. were potentiated by i.t.-Pis. So, if some of the endogenous opioid peptides are released supraspinally...
and/or spinally by EA stimulation, EA-induced antinociception could be potentiated by i.c.v., i.t. or i.c.v. +i.t. administration of PIs. Unexpectedly, EA antinociception was not affected by i.t.-PIs and was rather attenuated by i.c.v.- or i.c.v.+i.t.-PIs (Fig. 4). The antinociceptive effects of i.t.-injected Met-enk, β-end and Dyn were increased only after i.t.-PIs (Figs. 1, 2 and 3). The dynorphin A in the CSF has been shown to be increased by EA stimulation of high frequency, while we employed a stimulation of low frequency which might not have increased the release of dynorphins (29). Although the antinociceptive effects of i.c.v.-injected Met-enk and β-end were potentiated after both i.c.v.- and i.t.-PIs, the potentiation after i.c.v.-PIs was more prominent than that after i.t.-PIs (Figs. 1 and 2). We can then assume that the non-effect of i.t.-PIs on EA antinociception indicates little or no involvement of spinal Met-enk and β-end released by EA stimulation of low frequency. The EA-induced antinociception was, however, potentiated by PIs injected into the PAG (Fig. 5), and this was in line with previous reports that EA antinociception was potentiated by captopril injected into the PAG (30) and by bestatin and thiorphan injected into the nucleus accumbens (31) in rabbits.

This potentiation of EA antinociception by PIs injected into the PAG (Fig. 5) is also consistent with the previous finding on the involvement of Met-enk and β-end of PAG in EA antinociception using antibodies against Met-enk and β-end (10). In fact, the antinociceptive effects of i.c.v.-injected Met-enk and β-end were dramatically potentiated by i.c.v.-PIs (Figs. 1 and 2), but the EA antinociception was decreased after i.c.v.-PIs (Fig. 4). The existence of anti-opiate peptides has been reported (32). Cholecystokinin octapeptide (CCK-8) and Phe-Met-Arg-Phe (FMRF) amide were shown to be released by β-end (33) and morphine (34), respectively; and the regional distribution of these anti-opiate peptides in the brain was indicated to be different from that of Met-enk (35, 36). It was reported that CCK-8 was inactivated exclusively by aminopeptidase and enkephalinase (37), and the degradation of CCK-8 was reduced by bacitracin (38, 39) and bestatin + phosphoramidon (40). The release of the C-terminal phenylalanilamidé of FMRF amide was induced by enkephalinase, and this hydrolysis was completely inhibited by phosphoramidon (40). Therefore, when EA stimulation promotes the release of supraspinal opioid peptides (Met-enk and/or β-end), which stimulate the release of anti-opiate peptides, the degradation of both opioid peptides and anti-opiate peptides may be reduced similarly by i.c.v.-PIs. However, when the distribution of opioid peptides released by EA is different from that of anti-opiate peptides released by the opioid peptides, different effects of i.c.v.-PIs on opioid peptides and anti-opiate peptides may be induced. Particularly, if the anti-opiate peptides are released more closely to the lateral ventricle than the opioid peptides induced by EA stimulation, the inhibition of degradation of anti-opiate peptides may be more prominent than that of opioid peptides after i.c.v.-PIs, and EA antinociception could be reduced by i.c.v.-PIs. In fact, the immunoreactive fibers containing FMRF amide were identified around the lateral ventricle in the forebrain of frog brain (41). However, the mechanism of the activation of the anti-opiate peptide system induced by endogenous and exogenous opioid peptides and the role of anti-opiate peptides in exogenous opioid peptides and EA-antinociception are not yet clear and remain to be elucidated.

In conclusion, the present data indicate that Met-enk hydrolyzing enzymes may be involved in the degradation of not only Met-enk but also β-end and Dyn in rat CNS and that the supraspinal opioid peptides, Met-enk and/or β-end, may be involved in EA antinociception induced by stimulation of low frequency. This suggests the possible existence of an anti-opiate peptide system in the CNS that may be activated by EA stimulation and is susceptible to Met-enk hydrolyzing enzymes.

REFERENCES
1 Basbaum AI and Fields HL: Endogenous pain control system: Brainstem spinal pathway and endorphin circuitry. Annu Rev Neurosci 7, 309–338 (1984)
2 Kishioka S, Morita N, Kitabata Y, Yamanishi T, Miyamoto Y, Ozaki M and Yamamoto H: Dynorphin-(1–13): Antinociceptive action and its effects on morphine analgesia and acute tolerance. Jpn J Pharmacol 60, 197–207 (1992)
3 Larson AA, Vaught JL and Takemori AE: The potentiation of spinal analgesia by leucine enkephalin. Eur J Pharmacol 61, 381–383 (1989)
4 Malick JB and Goldstein JM: Analgesic activity of enkephalins following intracerebral administration in the rat. Life Sci 20, 827–832 (1977)
5 Kuraishi Y, Sato M, Harada Y, Akaia A, Shibata T and Takagi H: Analgesic action of intrathecal and intracerebral β-endorphin in rats: Comparison with morphine. Eur J Pharmacol 67, 143–146 (1980)
6 Piercey MF, Varner K and Schroeder LA: Analgesic activity of intraspinally administered dynorphin and ethylketocyclazocine. Eur J Pharmacol 80, 283–284 (1982)
7 Herman BH, Leslie F and Goldstein A: Behavioral effects and in vivo degradation of intraventricularly administered dynorphin-(1–13) and d-Ala2-dynorphin-(1–11) in rats. Life Sci 27, 883–892 (1980)
8 Walker JM, Katz RJ and Akil H: Behavioral effects of dynorphin 1–13 in the mouse and rat: Initial observations. Peptides 1, 341–345 (1980)
9 Chen X-H and Han J-S: All three types of opioid receptors in the spinal cord are important for 2/15 Hz electroacupuncture analgesia. Eur J Pharmacol 211, 203–210 (1992)
10 Han J-S, Xie G-X and Zhou Z-F: Acupuncture mechanisms in
rabbids studied with microinjection of antibodies against \( \beta \)-endorphin, enkephalin and substance P. Neuropharmacology 23, 1–5 (1984)

11 Han JS and Xie GX: Dynorphin: Important mediator for electroacupuncture analgesia in the spinal cord of the rabbit. Pain 19, 367–376 (1984)

12 Hambrock JM, Morgan BA, Rance MJ and Smith CFC: Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates. Nature 262, 782–783 (1976)

13 Erdos EG, Johnson AR and Boyden NT: Hydrolysis of enkephalin by cultured human endothelial cells and by purified pep tidyl dipeptidase. Biochem Pharmacol 27, 843–848 (1978)

14 Malfroy B, Swerts JP, Guyon A, Roques BP and Schwartz JC: High-affinity enkephalin-degrading peptidase in brain is increased after morphine. Nature 276, 523–526 (1978)

15 Hirayama T and Oka T: Effects of peptidase inhibitors on the [Met]-enkephalin hydrolysis in ileal and striatal preparations of guinea pig: almost complete protection of degradation by the combination of amastatin, captopril and thiorphan. Jpn J Pharmacol 41, 437–446 (1986)

16 Oka T, Liu X-F, Kajita T, Ohgiya N, Ghoda K, Taniguchi T, Arai Y and Matsunomiya T: Effects of the subcutaneous administration of enkephalins on tail-flick response and righting reflex of developing rats. Dev Brain Res 69, 271–276 (1992)

17 Kuno Y, Aoki K, Kajiwara M, Ishii K and Oka T: The relative potency of enkephalins and \( \beta \)-endorphin in guinea pig ileum, mouse vas deferens and rat vas deferens after the administration of peptidase inhibitors. Jpn J Pharmacol 41, 273–281 (1986)

18 Aoki K, Kajiwara M and Oka T: The role of bestatin-sensitive aminopeptidase, angiotensin converting enzyme and thiorphan-sensitive "enkephalinase" in the potency of enkephalins in the guinea pig ileum. Jpn J Pharmacol 36, 59–65 (1984)

19 Aoki K, Kajiwara M and Oka T: The inactivation of [Met]-enkephalin by bestatin-sensitive aminopeptidase, captopril-sensitive pep tidyl dipeptidase A and thiorphan-sensitive endopeptidase-24.11 in mouse vas deferens. Jpn J Pharmacol 40, 297–302 (1986)

20 Cui S, Kajiwara M, Ishii K, Aoki K, Sakamoto J, Matsunomiya T and Oka T: The enhancing effects of amastatin, phosphoramidon and captopril on the potency of [Met]-enkephalin in rat vas deferens. Jpn J Pharmacol 42, 43–49 (1986)

21 Paxinos G and Watson C: The Rat Brain in Stereotaxic Coordinates, 2nd edition. Academic Press, San Diego (1986)

22 Tallarida RJ and Murray RB: Manual of Pharmacologic Calculations with Computer Programs, 2nd edition. Springer-Verlag, New York (1987)

23 Chaillet P, Marciau-Collado H, Costentin J, Yi C-C, De La Baume S and Schwarz J-C: Inhibition of enkephalin metabolism by, and antinociceptive activity of, bestatin, an aminopeptidase inhibitor. Eur J Pharmacol 86, 329–336 (1983)

24 McKnigh AT, Corbett AD and Kosterlitz HW: Increase in potencies of opioid peptides after peptidase inhibition. Eur J Pharmacol 86, 393–402 (1983)

25 Nakazawa T, Ikeda M, Kaneko T, Yamatsu K, Kitagawa K and Kiso Y: Bestatin potentiates the antinociception but not the motor dysfunction induced by intracerebrally administered dynorphin-B in mice. Neuropeptides 13, 277–283 (1989)

26 Molineaux CJ and Ayala M: An inhibitor of endopeptidase-24.15 blocks the degradation of intraventricularly administered dynorphins. J Neurochem 55, 611–618 (1990)

27 Tseng LF, Towell JF and Fujimoto JM: Spinal release of immunoreactive Met-enkephalin by intraventricular \( \beta \)-endorphin and its analogs in anesthetized rats. J Pharmacol Exp Ther 237, 65–74 (1986)

28 Miyamoto Y, Morita N, Kitabata Y, Yamanishi T, Kishioka S, Ozaki M and Yamamoto H: Antinoceptive synergy between supraspinal and spinal sites after subcutaneous morphine evidenced by CNS morphine content. Brain Res 552, 136–140 (1991)

29 Han JS: The role of endogenous opioid peptides (EPO) in acupuncture-induced analgesia (AA]. In Proceedings of China-Japan Medical Conference, Beijing, Vol 4, p 628 (1992)

30 Han JS, Fei H and Zhou ZF: Met-enkephalin-[Arg]2-Phe]-like immunoreactive substances mediated electroacupuncture analgesia in the periaqueductal gray of the rabbit. Brain Res 322, 289–296 (1984)

31 Jin W-Q, Zhou Z-F and Han J-S: Electroacupuncture and morphine analgesia potentiated by bestatin and thiorphan administered to the nucleus accumbens of the rabbit. Brain Res 380, 317–324 (1986)

32 Galina ZH and Kastin AJ: Existence of antipeptide systems as illustrated by MIF-1/Tyr-MIF-1. Life Sci 39, 2153–2159 (1986)

33 Tseng LF and Huang FY: Release of immunoreactive [Met]enkephalin and cholecystokinin from the spinal cord by \( \beta \)-endorphin administered intraventricularly in the pentobarbital-anesthetized rat. Eur J Pharmacol 215, 309–312 (1992)

34 Tang J, Yang H-YT and Costa E: Inhibition of spontaneous and opiate-modified nociception by an endogenous neuropeptide with Phe-Met-Arg-Phe-NH2-like immunoreactivity. Proc Natl Acad Sci USA 81, 5002–5005 (1984)

35 Cawley JN: Comparative distribution of cholecystokinin and other neuropeptides; Why is this peptide different from all other peptides? Ann NY Acad Sci 448, 1–8 (1985)

36 Webster E, Evans CJ, Samuelsson SJ and Barchas JD: Novel peptidic neuronal system in rat brain and pituitary. Science 214, 1248–1251 (1981)

37 Deschoot-Lanckman M: Enzymatic degradation of cholecystokinin in the central nervous system. Ann NY Acad Sci 448, 87–98 (1985)

38 Deschoot-Lanckman M, Bui ND, Noyer M and Christophe J: Degradation of cholecystokinin-like peptides by a crude rat brain synaptosomal fraction: A study by high pressure liquid chromatography. Regul Pept 2, 15–30 (1981)

39 Saito A, Goldfine ID and Williams JA: Characterization of receptors for cholecystokinin and related peptides in mouse cerebral cortex. J Neurochem 37, 483–490 (1981)

40 Turner AJ, Matsas R and Kenny AJ: Metabolism of cholecystokinin by endopeptidase-24.11. Ann NY Acad Sci 448, 666–668 (1985)

41 Dockray GJ, Vaillant C and Williams RG: New vertebrate-gut peptide related to a molluscan neuropeptide and an opioid peptide. Nature 293, 656–657 (1981)