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ORIGINAL ARTICLE

Potentiation of [Met^5]enkephalin-induced antinociception by mixture of three peptidase inhibitors in rat

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Abstract
Purpose Previous in vitro studies have shown that degradation of opioid peptides during incubation with cerebral membrane preparations is almost completely prevented by a mixture of three peptidase inhibitors (PIs), namely, amastatin, captopril, and phosphoramidon. In the present in vivo study, we evaluate the effects of intrathecal administration of these PIs on antinociception by [Met^5]-enkephalin (ME) or PIs themselves.
Methods Drugs were administered into the thoracolumbar level of the spinal cord in the intrathecal space in rat. Induction of antinociception was measured by the tail immersion assay, with 55 °C as the nociceptive stimulus. Effects of PIs on antinociception were evaluated by dose–response study (ME, 1–20 nmol; PIs, 1–20 nmol each), by comparison of differences among two combinations of PIs (amastatin and captopril; captopril and phosphoramidon; amastatin and phosphoramidon) and three PIs (amastatin, captopril, and phosphoramidon), and by using opioid receptor selective antagonists.
Results Intrathecal administration of ME with these three PIs or PIs alone significantly and dose dependently increased antinociception in a μ- and δ-opioid receptor antagonist-reversible manner; moreover, the degree of antinociception with a combination of any two of these was less than that with all three, indicating that any residual single peptidase could inactivate significant amounts of ME.
Conclusion The present data, together with those of earlier studies, clearly demonstrate that amastatin-, captopril-, and phosphoramidon-sensitive enzymes play an important role in inactivation of opioid peptides at the spinal level.

Keywords Antinociception · Peptidase-inhibitors · Methionine-enkephalin · Tail-flick response

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Introduction

Endogenous opioid peptide levels depend on the activity of opioid peptide-degrading enzymes, which terminate their action at the synaptic cleft. Enkephalins are hydrolyzed by five types of peptidase [1, 2]: (1) aminopeptidase N (EC 3.4.11.2, APN, also called CD13), which cleaves the Tyr^1–Gly^2 amide bond; (2) membrane-bound-dipeptidyl peptidase III (EC 3.4.14.4, DPP), which hydrolyzes the Gly^2–Gly^3 bond; (3) peptidyl-dipeptidase A (EC 3.4.15.1, also called the angiotensin-converting enzyme, ACE), (4) neutral endopeptidase (EC 3.4.24.11, NEP, also called neprilysin, enkephalinase, or CD10), which cleaves the Gly^3–Phe^4 bond, and (5) carboxypeptidase A (EC 3.4.17.1, CPA). [Met^5]enkephalin (ME) incubated with ileal or striatal membrane fraction for 60 min at 37 °C remains intact in the presence of the three peptidase inhibitors (PIs) amastatin (APN inhibitor), captopril (ACE inhibitor), and thiorphan (NEP inhibitor), whereas it is completely hydrolyzed after incubation in their absence [3]. This

finding suggests that the three membrane-bound peptidases, such as amastatin-sensitive APN, captopril-sensitive ACE, and thiorphan or phosphoramidon-sensitive NEP, have a role in the degradation of ME. In fact, these three peptidases are located in very close proximity to the opioid receptors in isolated membrane preparation of guinea pig ileum [4], mouse vas deferens [5], and rat vas deferens [6]. Because the hydrolysis products of ME by either amastatin-, captopril-, or phosphoramidon-sensitive enzymes such as free Tyr and [Tyr-Gly-Gly]-, [des-Tyr]-, and [des-Tyr-Gly-Gly]-fragments are suggested to have very low, if any, agonist activity at opioid receptors [3], the potency of ME should be decreased by its hydrolysis with these three peptidases. In addition to ME, a mixture of three PIs largely prevented the hydrolysis of endogenous opioid peptides [Leu^5]enkephalin (LE), [Met^5]enkephalin-Arg^6-Phe^7, [Met^5]enkephalin-Arg^6-Gly^7-Leu^8, or dynorphin A (1-8) in cerebral membrane preparations [3, 7–9].

Several reports have shown that a single PI or two PIs augment enkephalin-induced antinociception [10–13]. However, the partial analgesic potency of enkephalin may have only been estimated in these studies, as in vivo studies have demonstrated that significant amounts of enkephalins are still hydrolyzed by any combination of two peptidase inhibitors [3, 14, 15]. In fact, antinociception induced by intracerebroventricular (i.c.v.) administration of [Leu^5]enkephalin and dynorphin (1–8) increased more than 100 fold by i.c.v. pretreatment with three PIs [16, 17]. The effects of pretreatment with PIs on antinociception induced by intrathecal (i.t.) administration of ME were investigated in this study to evaluate the real analgesic potency of spinal levels of ME and compare them with those of other opioid peptides.

Materials and methods

Chemicals

Amastatin (A), phosphoramidon (P), and ME were purchased from Peptide Institute (Minoh, Japan). Captopril (C), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP, a μ-opioid receptor antagonist), norbinaltorphimine dihydrochloride (nor-BNI, a κ-opioid receptor antagonist), and naltrindole hydrochloride (NTI, a δ-opioid receptor antagonist) were purchased from Sigma Japan (Tokyo, Japan). All chemicals except nor-BNI and NTI were dissolved in saline; nor-BNI and NTI were dissolved in water. The solution for all peptides used was prepared to the desired concentration just before use. According to previous studies, CTOP (3 nmol, i.t.), nor-BNI (10 mg/kg, subcutaneously), and NTI (66 nmol, i.t.) was injected 15 min, 30 min, and 24 h, respectively, before i.t. administration of PIs [18–21].

Intrathecal administration

The present animal experiments were performed in strict accordance with the guidelines of Tokai University, and were approved by the Animal Investigation Committee of Tokai University. Male Wistar rats (180–220 g each; Nihon Clea, Tokyo, Japan) were implanted with intrathecal catheters under inhalation anesthesia with nitrous oxide, oxygen, and isoflurane (2 %). An 8.5-cm polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, USA) was inserted caudally to the thoracolumbar level of the spinal cord in the intrathecal space through an incision in the atlanto-occipital membrane [22]. The external part of the catheter was tunneled subcutaneously to exit from the top of the skull and was plugged with a 30-gauge steel wire. After surgery, all rats were housed individually in a temperature- and light-controlled environment with free access to food and water. Only rats with normal motor function and behavior were used for the study 7 days later. The polyethylene catheter was attached to a motor-driven, 50-μl microsyringe by polyethylene tubing (PE-20; Clay Adams). Drugs were injected at a volume of 10 μl followed by 10 μl saline over 1 min. The distribution of the drug solution in the spinal system was verified by infusion of 10 μl 0.3 % Evans blue dissolved in saline after the experiment.

Tail-flick test

The investigators were blind to all drug treatments carried out in these experiments. Induction of antinociception by ME was measured by the tail immersion assay, with 55 °C as the nociceptive stimulus [23]. The latency to flick the tail from the 55 °C water was measured before and at 5, 10, 15, 30, 45, and 60 min after administration. The latency to flick the tail before administration was approximately 1 s. A cutoff time of 5 s was used to prevent any injury to the tail. The percent of maximal possible effect (MPE) for each animal at each time was calculated using the following formula: $\%\text{MPE} = [(\text{test latency} - \text{baseline latency})/(5 - \text{baseline latency})] \times 100$. The AUC (area under the curve) value for the antinociceptive action of the drug on each rat was calculated for some experiments.

Experimental protocol

Dose–response study

Ten minutes following i.t. administration of a mixture of the three PIs (10 nmol each) or saline, ME or saline was administered intrathecally. To test whether the antinociceptive effect was produced by PIs, rats were tested in the following groups: group 1, ME (1–20 nmol) alone; group

2, ME (1–20 nmol) with a mixture of three PIs (10 nmol each inhibitor); group 3, a mixture of three PIs (1–20 nmol each inhibitor) alone; and group 4, ME (10 nmol) with a mixture of three PIs (1–20 nmol each inhibitor).

Combinations of PIs

To examine the effect of two PIs on ME-induced antinociception, combinations of PIs (AC, amastatin, and captopril; CP, captopril and phosphoramidon; AP, amastatin and phosphoramidon) and three PIs (ACP, amastatin, captopril, and phosphoramidon) were administered intrathecally.

Opioid receptor selective antagonist

To investigate the effect of opioid receptor antagonists on antinociception induced by a mixture of PIs alone or ME with pretreatment with three PIs, CTOP (3 nmol, i.t.), nor-BNI (10 mg/kg, subcutaneously), and NTI (66 nmol, i.t.) was injected 15 min, 30 min, and 24 h, respectively, before i.t. administration of ME [18–21].

Statistical analysis

The results are given as the mean and standard error of the mean (SEM) of the data. A statistical analysis was conducted using computer software (Prism, version 6.0c; GraphPad Software, San Diego, CA, USA) for a comparison across the experimental conditions. When a significant difference among the %MPE data during the experiment after drug administration was obtained in a two-way (drugs and time) repeated-measures analysis of variance (ANOVA), Dunn's multiple comparison test was applied to determine the significance at each time point. When a significant difference among the groups of AUC data was obtained in a two-way (drugs and dose) ANOVA, Dunn's multiple comparison test was applied to determine the significance at each dose. When a significant difference within groups was obtained in the Kruskal–Wallis test, Dunn's comparison test was applied to determine significance. The power of statistical comparison was assessed using the StatMate 2 program (GraphPad Software, La Jolla, CA, USA).

Results

Effects of PIs on ME-induced antinociception

Pretreatment with the PIs by i.t. administration increased and prolonged ME-induced antinociception (Fig. 1). Intrathecal administration of ME with a mixture of the three PIs significantly increased antinociception compared

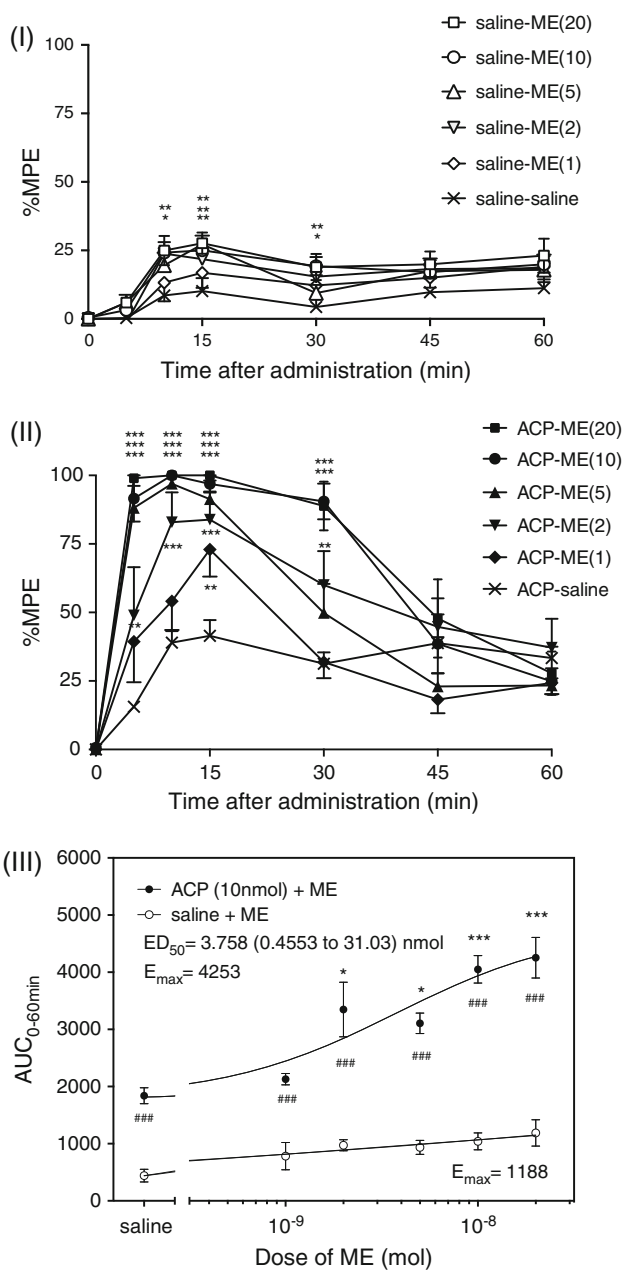

Fig. 1 Dose-dependent antinociception by i.t. administration of [Met^5]enkephalin (ME) under pretreatment with saline or a mixture of peptidase inhibitors (PIs) (amastatin, captopril, and phosphoramidon: ACP). Results represent mean with SEM of data from five to seven rats in each group. *Upper* (I) and *middle panels* (II) indicate time course of %MPE of ME (1–20 nmol) and pretreatment with saline and ACP, respectively. Significantly different from saline–saline-treated control or ACP–saline-treated control in Dunn's post hoc test following two-way repeated-measures analysis of variance (ANOVA); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. *Lower panel* (III) shows $AUC_{0-60min}$ for value of %MPE indicated in *upper* (I) and *middle panels* (II). Significantly different from saline–saline-treated control or ACP–saline-treated control according to Dunn's post hoc test following Kruskal–Wallis test; $^{*}P < 0.05$ and $^{***}P < 0.001$. Significantly different from pretreatment with saline according to Dunn's post hoc test following two-way repeated-measures ANOVA; $^{\#\#\#}P < 0.001$

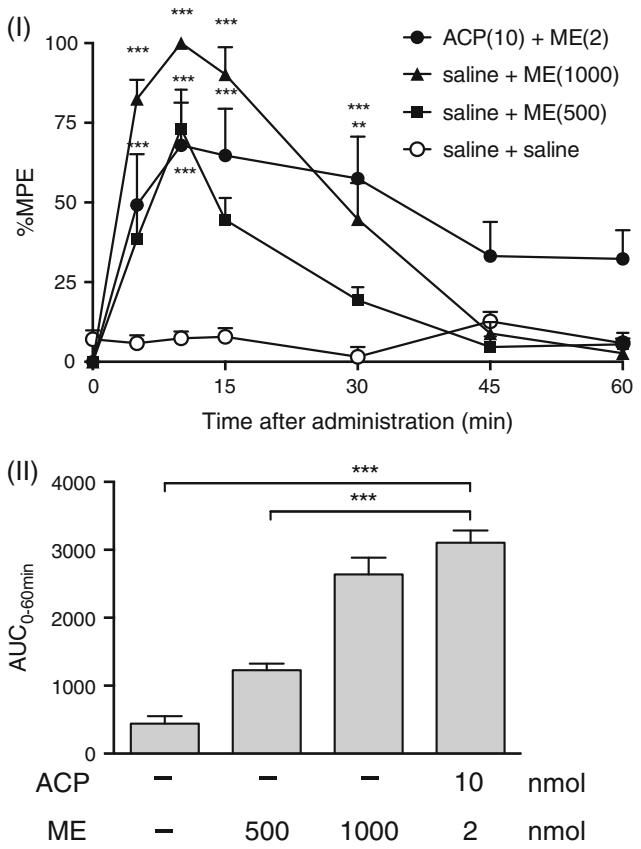

Fig. 2 Potentiating effect of PIs on antinociception induced by intrathecal (i.t.) administration of ME. Results represent mean with SEM of data from five to seven rats in each group. *Upper panel* (I) indicates time course of %MPE of ME (2, 500, and 1,000 nmol) and pretreatment with saline and ACP (10 nmol each), respectively. Significantly different from saline–saline-treated control according to Dunn's post hoc test following two-way repeated-measures ANOVA; $^{**}P < 0.01$ and $^{***}P < 0.001$. *Lower panel* (II) shows $AUC_{0-60min}$ for value of %MPE indicated in *upper panel* (I). Significantly different from ME (2 nmol) and pretreatment with ACP according to Dunn's post hoc test following Kruskal–Wallis test; $^{***}P < 0.001$

to that with saline at all doses of ME tested. The $AUC_{0-60min}$ value for %MPE with 10 nmol ME under pretreatment of a mixture of the three PIs (10 nmol each) was approximately 4,000. The $AUC_{0-60min}$ value for %MPE with 2 nmol ME under pretreatment of a mixture of the three PIs (10 nmol each) was approximately equal to that of 1,000 nmol ME alone (Fig. 2). Antinociception with i.t. administration of 2 nmol ME under pretreatment of a mixture of the three PIs (10 nmol each) was similar in terms of onset, offset, and duration of action to that with 1,000 nmol ME alone (Fig. 2). Thus, antinociception with i.t. administration of ME increased more than 500 fold by i.t. administration of a mixture of the three PIs in the tail-flick test (10 nmol each). However, it is possible that the potency of ME was increased by more than 500 fold by the three PIs, as the potency of ME at a dose of more than 1,000 nmol in rats not treated with PIs could not be estimated because high concentrations of ME are not available as it has low solubility. A mixture of three PIs dose dependently increased the antinociceptive effect of ME (10 nmol) (Fig. 3).

Antinociceptive effects of PIs by themselves

Significant increase of antinociception was observed in rats following i.t. administration of a mixture of the PIs alone (Fig. 3).

Effect of combinations of PIs (AC, AP, CP) on ME-induced antinociception

The magnitude of ME-induced antinociception under pretreatment with the combination of AC (amastatin and captopril) or CP (captopril and phosphoramidon) was significantly lower than that of the three ACP (amastatin, captopril, and phosphoramidon). That of AP (amastatin and phosphoramidon) was also lower, but not significantly, than that of the three ACPs (Fig. 4), indicating that any residual single peptidase could inactivate substantial amounts of ME.

Attenuation of antinociception with combination with ME and PIs or with PIs alone by opioid peptide antagonists

CTOP or NTI significantly attenuated the antinociceptive potency of the PIs alone; it was also attenuated by Nor-BNI, but not significantly so (Fig. 5). The antinociceptive potency of ME under pretreatment with a mixture of the three PIs was significantly attenuated by CTOP or NTI; it was also attenuated by nor-BNI, but not significantly so (Fig. 5).

Discussion

The results of the present study showed that pretreatment with a mixture of three PIs produced at least 500-fold augmentation in antinociception induced by i.t. administration of ME in rats. An earlier study demonstrated that pretreatment with a mixture of three PIs increased antinociception induced by i.c.v. administration of ME [24, 25]. Taken together with the fact that the potency of ME should be decreased by its hydrolysis by these three PIs [3], these results provide further support for the view that amastatin-, captopril-, and phosphoramidon-sensitive enzymes have an important role in inactivation of ME at both the spinal and supraspinal level.

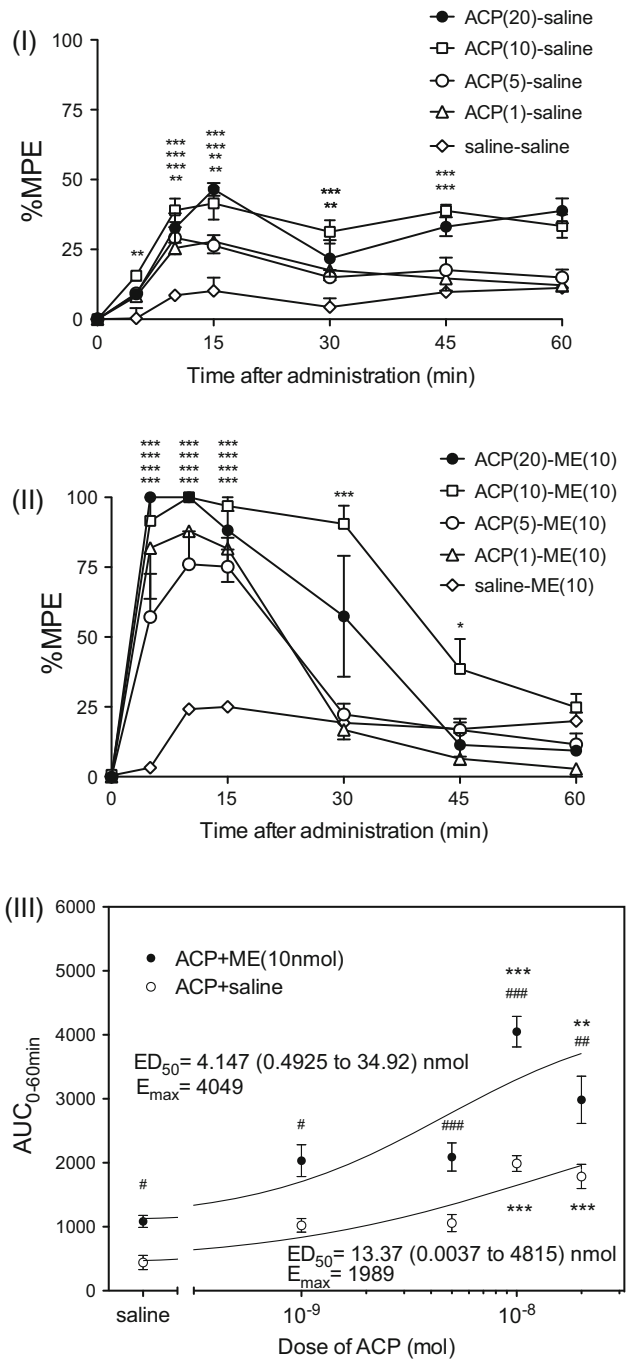

Fig. 3 Dose-dependent antinociception by i.t. administration of ME and pretreatment with saline or a mixture of PIs (ACP). *Upper* (I) and *middle panels* (II) indicate time course of %MPE of ME (10 nmol) and pretreatment with saline and ACP (1–20 nmol), respectively. Significantly different from saline–saline-treated control or ACP–saline-treated control according to Dunn's post hoc test following two-way repeated-measures ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. *Lower panel* (III) shows $AUC_{0-60min}$ for value of %MPE indicated in *upper* (I) and *middle panels* (II). Significantly different from saline–saline-treated control or ACP–saline-treated control according to Dunn's post hoc test following Kruskal–Wallis test; $^{**}P < 0.01$ and $^{***}P < 0.001$. Significantly different from saline pretreatment with ACP according to Dunn's post hoc test following two-way repeated-measures ANOVA; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$

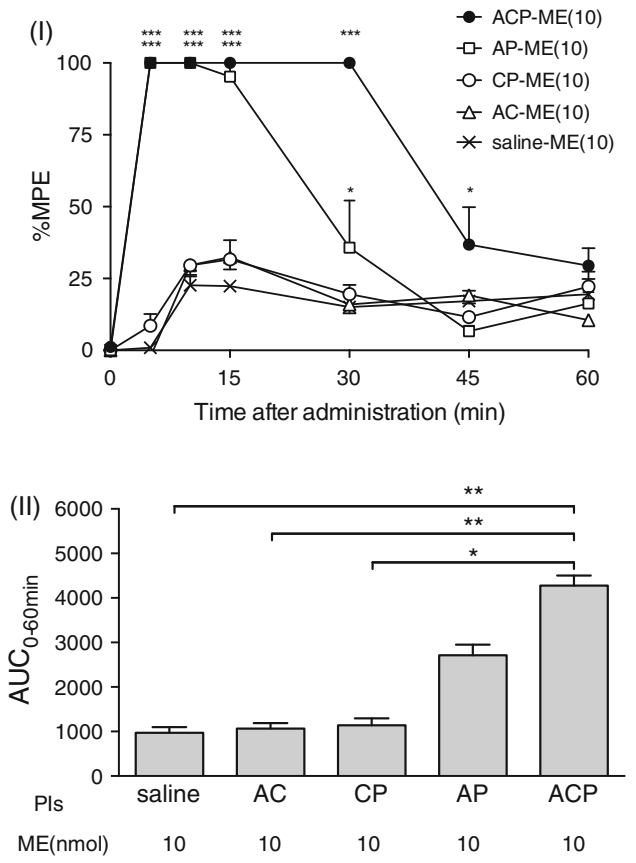

Fig. 4 Comparison of effect of combination of two PIs (AC: amastatin and captopril; CP: captopril and phosphoramidon; AP: amastatin and phosphoramidon) and three PIs (ACP: amastatin, captopril, and phosphoramidon) on antinociception induced by i.t. administration of ME. Results represent mean with SEM of data from five rats in each group. *Upper panel* (I) indicates time course of %MPE of ME (10 nmol) under pretreatment with saline, two PIs (AC, CP, AP; 10 nmol each) and three PIs (ACP, 10 nmol each), respectively. Significantly different from saline-treated control according to Dunn's post hoc test following two-way repeated-measures ANOVA; $^{*}P < 0.05$ and $^{***}P < 0.001$. *Lower panel* (II) shows $AUC_{0-60min}$ for value of %MPE indicated in *upper panel* (I). Significantly different from ME and pretreatment with ACP according to Dunn's post hoc test following Kruskal–Wallis test; $^{*}P < 0.05$ and $^{**}P < 0.01$

This is the first study to demonstrate that a significant dose-dependent change was observed in antinociception following i.t. administration of a mixture of the three PIs alone. This result is in good agreement with the results of a study using RB-101, a compound that combines one APN inhibitor and one NEP inhibitor linked by their mercapto groups. Intravenous or intraperitoneal administration of RB-101 induced antinociceptive response in the hot plate and the writhing test in mice and the tail flick tests in rats [26]. On the other hand, no significant change was observed in antinociception following i.t. administration of a mixture of the three PIs alone in previous study by using a paw pressure test [27]. This discrepancy

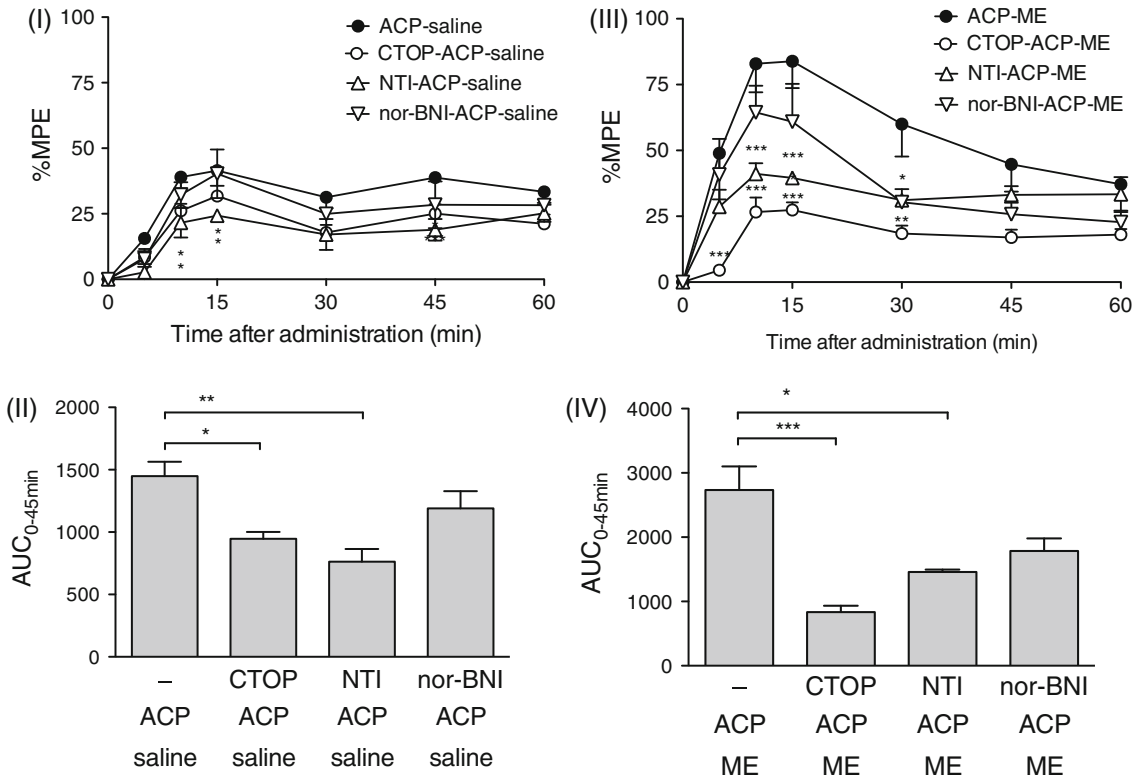

Fig. 5 Effect of opioid receptor antagonists on PIs by themselves (I, II) or ME-induced (III, IV) antinociception under pretreatment with PIs. *Upper panel* (I) and (III) indicate time course of %MPE of PIs by themselves and ME (10 nmol)-induced antinociception under pretreatment with PIs following administration of opioid receptor antagonists, respectively. Significantly different from PIs alone (ACP–saline) or ME and pretreatment with PIs (ACP–ME) according to Dunn's post hoc test following two-way repeated-measures ANOVA; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. *Lower panel* (II) and (IV) show $AUC_{0–45min}$ for value of %MPE indicated in *upper panel* (I) and *panel* (III), respectively. Significantly different from PIs alone (ACP–saline) or ME and pretreatment with PIs (ACP–ME) according to Dunn's post hoc test following Kruskal–Wallis test; $*P < 0.05$ and $**P < 0.01$

may be related to the difference in temperature as the nociceptive stimulus. Several lines of evidence also support this possibility. First, low rates of skin heating, for example, may evoke capsaicin-sensitive, C fiber-mediated responses, whereas higher rates may recruit the involvement of capsaicin-insensitive Aδ nociceptors [28]. Second, Aδ or C fiber nociceptors are under different descending control from the nucleus raphe magnus [29]. Third, i.t. administration of endomorphin had lesser antinociceptive effect by using the paw pressure test than the tail-flick test [30]. Fourth, NEP and APN are distributed in high concentrations in the dorsal horn of the spinal cord, and in moderate concentrations in the central periaqueductal gray matter and thalamus, where enkephalins are colocalized [31, 32]. These structures have a key role in control of nociceptive messaging [33]. Fifth, i.c.v. administration of hydrocinnamic acid and intraperitoneal administration of D-phenylalanine, known NEP inhibitors, also results in different levels of ME in brain tissue in C57BL/6J mice [34]. Taken together, these results suggest that regional differences in opioid metabolism indicate that peptidases evoke tissue-specific patterns of enkephalin regulation.

Induction of antinociception by i.t. administration of ME as evidenced by the tail-flick response was increased more than 500 fold by pretreatment of a mixture of the three PIs. This finding is in good agreement with the results of earlier studies showing that the antinociceptive effect of i.c.v. administration of ME and i.t. administration of LE was increased 1,000- and 100 fold, respectively, by pretreatment with a mixture of three PIs [35, 36], whereas the inhibitory potency of ME in isolated guinea pig ileum and mouse vas deferens was increased by approximately 6.8- and 21 fold, respectively [14].

The $AUC_{0–60min}$ value for antinociception with i.t. administration of 10 nmol ME and LE under pretreatment with a mixture of the three PIs was approximately 4,000 (present study) and 2,500 [36], respectively. The antinociception of ME was about twice as potent as that of LE; this is inconsistent with the results of other investigations indicating that the affinity of ME was approximately twice as potent as that of LE [37]. Taken together, these results

suggest that coadministration of a mixture of three PIs allows for evaluation of the real analgesic potency of opioid peptides at the spinal or supraspinal level.

The magnitude of antinociception of ME with AC or CP was significantly lower than that with all three PIs (ACP); it was also lower with the combination of AP than with ACP, although not significantly so. These results demonstrate that any residual single peptidase can inactivate substantial amounts of ME. One reason the potentiation of antinociception differs among PIs may be related to the selectivity of these peptidases on ME. This proposition is supported by evidence that the affinity of NEP for ME is 1,000 times higher than that of ACE [38]. Several reports have shown that a single PI or two PIs augment enkephalin-induced antinociception [10–13]. However, the results of the present and previous studies indicate that these studies may have only estimated the partial analgesic potency of enkephalin [3, 14–17, 25]. Moreover, the activities of NEP and APN show a similar distribution among μ- and δ-opioid receptors in several regions of the brain, suggesting that they play a more essential role than ACE in the regulation of ME levels in the spinal cord [31, 32].

The present results showed the involvement of μ- and δ-opioid receptors in antinociception induced by i.t. administration of ME under pretreatment with a mixture of the three PIs, as suggested by the fact that CTOP and NTI significantly decreased antinociception. In addition, CTOP and NTI significantly decreased antinociception induced by i.t. administration of the three PIs alone, which is consistent with the results of other investigations. First, antinociception induced by i.c.v. administration of ME and the three PIs was significantly prevented in the presence of naloxone [25]. Second, ME has high affinity for δ-opioid receptors [39]. Third, μ-opioid receptors alone were proposed to be preferentially involved in supraspinal antinociception (hot plate and writhing tests), but both μ- and δ-opioid receptors were implicated in spinal antinociception (tail-flick and motor response to electrical stimulation) [40]. Fourth, high levels of NEP and μ-opioid receptor-binding sites were detected at the level of periaqueductal gray and in the substantia gelatinosa of the spinal cord, where only sparse δ-opioid receptors could be detected [32]. The codistribution of peptidase- and opioid-binding sites, along with the physiological effects of PI, strongly supports the view that peptidases are mainly involved in terminating enkephalinergic signals [32]. Fifth, Nox and NTI blocked antinociception induced by RB101 [26]. Sixth, the distribution of NEP in rat brain did not correlate selectively with that of a particular opioid receptor subtype but overlapped the localizations of both μ- and δ-opioid receptors [32]. The close similarity observed in the distribution of peptidases and that of μ- and δ-opioids could account for most antinociception elicited by PIs.

Peptidase inhibitors alone induce antinociception through some type of opioid receptor activity, which is similar to enkephalins; they are not selective endogenous ligands [40]. The antinociceptive effect of PIs alone is produced through activation of both μ- and δ-opioid receptors. Inhibitors of opioid peptide-degrading enzymes possess the advantage that they lack opioid side effects such as respiratory depression, tolerance, and physical dependence [41]. Although little is known about the effects of PIs on other physiologically and behaviorally relevant peptides, the present findings suggest that PIs and other inhibitors of opioid peptide-degrading enzymes may have potential as novel therapeutic compounds for treatment of pain.

In conclusion, the present results showed that ME-induced antinociception in rat was increased more than 500 fold by pretreatment with three peptidase inhibitors in a μ- and δ-opioid receptor antagonist-reversible manner. PIs alone also induces antinociception in a μ- and δ-opioid receptor antagonist-reversible manner. These findings indicate that amastatin-, captopril-, and phosphoramidon-sensitive enzymes have an important role in the inactivation of ME at the spinal level.

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