

Increase in Antinociceptive Effect of [Leu⁵]Enkephalin after Intrathecal Administration of Mixture of Three Peptidase Inhibitors

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Objective: Previous *in vitro* studies have shown that the degradation of [Leu⁵]enkephalin during incubation with cerebral membrane preparations is almost completely prevented by a mixture of three peptidase inhibitors such as amastatin, captopril, and phosphoramidon. The present *in vivo* study was performed to examine the antinociceptive effect of [Leu⁵]enkephalin administered intrathecally under pretreatment with these three peptidase inhibitors.

Methods: A tail-flick test was used to determine the nociceptive threshold after administration of [Leu⁵]enkephalin. The time-course profiles of the latency to flick the tail and the area under the time response curve were evaluated for the antinociceptive action of the drug.

Results: The antinociceptive effect of [Leu⁵]enkephalin administered intrathecally on the tail-flick test was increased more than 100-fold under i.t. pretreatment with three peptidase inhibitors. The antinociceptive effect produced by [Leu⁵]enkephalin in rats pretreated with any single peptidase inhibitor increased antinociception compared to that with saline. The antinociceptive potency of [Leu⁵]enkephalin under pretreatment with any combination of two peptidase inhibitors was smaller than that in rats pretreated with three peptidase inhibitors together. These results indicate that any residual single peptidase inactivates significant amounts of [Leu⁵]enkephalin.

Conclusion: The present data, together with those of earlier studies, clearly demonstrate that amastatin-, captopril-, and phosphoramidon-sensitive enzymes play an important role in the inactivation of [Leu⁵]enkephalin administered intrathecally in rat.

Key words: [Leu⁵]enkephalin; antinociception; amastatin; captopril; phosphoramidon

INTRODUCTION

Endogenous opioid peptides play a vital role in the regulation of many physiological functions, including antinociception [1]. Enkephalins undergo rapid enzymatic degradation by 5 types of peptidase [2, 3]: 1) aminopeptidase N (EC 3.4.11.2, APN, also known as CD13), which cleaves the Tyr¹–Gly² amide bond; 2) membrane bound-dipeptidyl peptidase III (EC 3.4.14.4, DPP), which hydrolyzes the Gly²–Gly³ bond; 3) dipeptidyl carboxypeptidase (EC 3.4.15.1, also known as the angiotensin-converting enzyme known as ACE); 4) neutral endopeptidase (EC 3.4.24.11, NEP, also known as neprilysin, enkephalinase, or CD10), which cleaves the Gly³–Phe⁴ bond; and 5) carboxypeptidase A (EC 3.4.17.1)(Fig.1).

[Leu⁵]enkephalin (LE) incubated with ileal or striatal membrane fraction for 60 min at 37°C remains intact in the presence of only three peptidase inhibitors (PIs) of five ones: amastatin (an aminopeptidase inhibitor), captopril (a dipeptidyl carboxypeptidase inhibitor), or phosphoramidon (an endopeptidase-24.11 inhibitor). It is, however, completely hydrolyzed after incubation in the absence of these PIs [4]. This suggests that these 3 membrane-bound peptidases play a

role in the degradation of LE. The relative importance of three enzymes in the inactivation of LE has been also examined in three *in vitro* isolated preparations: guinea-pig ileum, mouse vas deferens, and rat vas deferens. The results showed that APN played the greatest role in both guinea-pig ileum and rat vas deferens, while it played a similar role to either NEP or ACE in mouse vas deferens [5]. In addition to LE, a mixture of three PIs largely prevented the hydrolysis of endogenous opioid peptides [Met⁵]enkephalin (ME), [Met⁵]enkephalin-Arg⁶-Phe⁷ (ME-RF), [Met⁵]enkephalin-Arg⁶-Gly⁷-Leu⁸ (ME-RGL), and dynorphin A (1–8)(dyn(1–8)) in cerebral membrane preparation [4, 6–8]. Additionally, the close proximity of these enzymes to opioid receptors in isolated preparations such as guinea pig ileum [9], mouse vas deferens [10], and rat vas deferens [11], suggests that they act to terminate the physiological action of these endogenous opioid peptides.

It has been suggested that hydrolysis of LE by amastatin-, phosphoramidon-, or captopril-sensitive enzymes produces fragments such as free Tyr, [Tyr-Gly-Gly], [des-Tyr], and [des-Tyr-Gly-Gly] which display very low, if any, agonist activity at opioid receptors [12]. Therefore, hydrolysis of LE by these three peptidases

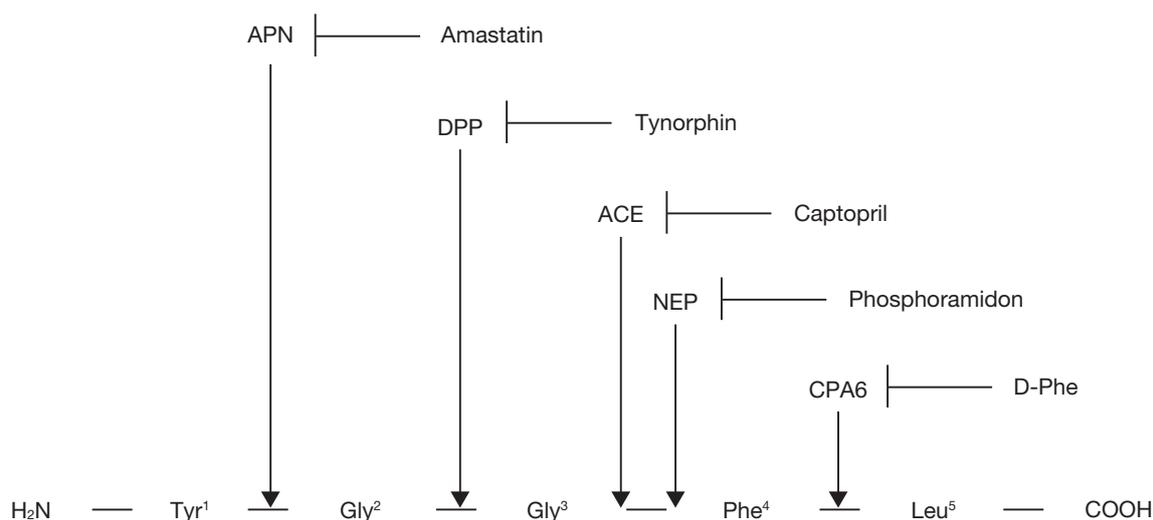


Fig. 1 Cleavage sites of peptidases on LE and those inhibitors. The sequence of LE is shown with sharp arrowheads pointing to the bonds cleaved by the peptidases. Lines ending with blunt arrowheads show inhibition. APN, aminopeptidase N; DPP, membrane bound-dipeptidyl peptidase III; ACE, dipeptidyl carboxypeptidase; NEP, neutral endopeptidase; CPA6, carboxypeptidase A; D-Phe, D-phenylalanine

should yield a decrease in its potency. In fact, the potency of LE in *in vitro* isolated preparations was found to show a significant increase by exposure to amastatin, phosphoramidon, or captopril [4, 5].

Several reports have shown that a single PI or two PIs augmented enkephalin-induced antinociception. 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. In fact, antinociception induced by intracerebroventricular (i.c.v.) administration of dyn (1-8), ME-RF, or ME-RGL was increased more than 100-fold by i.c.v. pretreatment with three PIs [13-15].

One of the most frequently used tests of nociception has been tail flick. Tail flick response is known to be a spinal reflex, but is likely modulated by descending influences from the brainstem [16]. Previous our study demonstrated that i.c.v. administration of LE was increased more than 500-fold by i.c.v. pretreatment with three PIs using tail flick test. We made hypothesis antinociceptive effects induced by intrathecal (i.t.) administration of LE pretreated with PIs may be more potent than those by i.c.v. administration. The effect of pretreatment with PIs on antinociception induced by i.t. administration of LE were investigated in this study to compare them with i.c.v. administration of LE or those of other opioid peptides and evaluate the real analgesic potency of spinal levels of LE.

MATERIALS AND METHODS

Chemicals

LE, amastatin (A), and phosphoramidon (P) were purchased from Peptide Institute Inc. (Minoh, Japan). Captopril (C), CTOP (D-Phe - Cys - Tyr - D-Trp - Orn - Thr - Pen - Thr - NH₂; μ receptor antagonist), naltrindole hydrochloride (δ receptor antagonist), and norbinaltorphimine dihydrochloride (κ receptor antagonist) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). All chemicals except naltrindole

hydrochloride and norbinaltorphimine dihydrochloride were dissolved in saline immediately before use. Naltrindole hydrochloride and norbinaltorphimine dihydrochloride were dissolved in water immediately before use.

Intrathecal administration

The present animal experiments were performed in strict accordance with the guidelines of Tokai University, and were approved by the Institutional Animal Care and Use Committee at 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 at the thoracolumbar level of the spinal cord in the intrathecal space through an incision in the atlanto-occipital membrane [17]. 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- μ l microsyringe by polyethylene tubing (PE-20; Clay Adams, Parsippany, NJ, USA). Drugs were injected at a volume of 10 μ l followed by 10 μ l saline over 1 min. LE was injected into intrathecal space of rats at 10 min following administration of single, two combination of or three mixture of peptidase inhibitors — amastatin, captopril and phosphoramidon — via the same route. After tail flick test, the distribution of the drug solution in the spinal system was verified by infusion of 0.3% Evans blue dissolved in saline after the experiment (Fig.2).

Tail-flick test

Induction of antinociception by LE was measured by the tail immersion assay, with 55 °C as the nocicep-

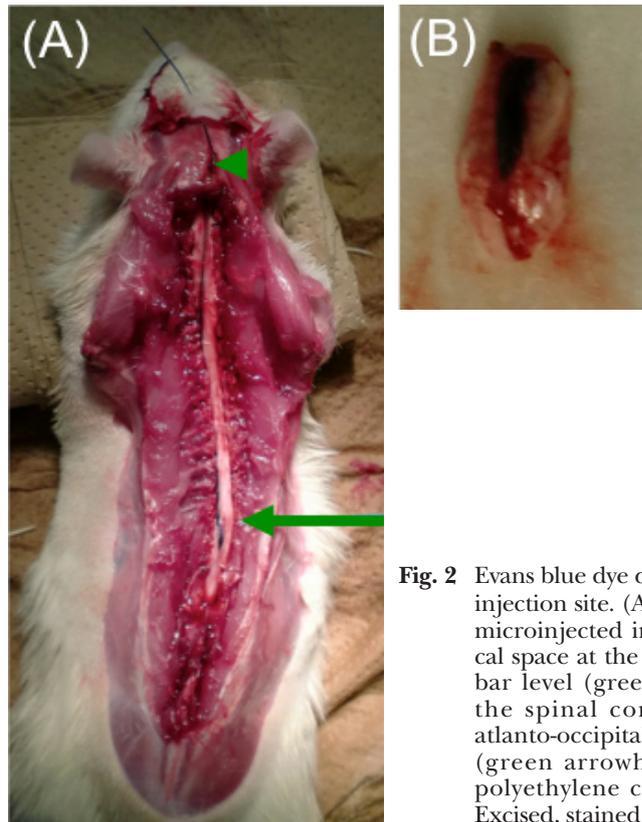


Fig. 2 Evans blue dye depicts spinal injection site. (A) Drug were microinjected into intrathecal space at the thoracolumbar level (green arrow) of the spinal cord through atlanto-occipital membrane (green arrowhead) using polyethylene catheter. (B) Excised, stained spinal cord.

tive stimulus [18]. 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 sec. A cut-off time of 5 sec 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: $\%MPE = [(test\ latency - baseline\ latency) / (5 - baseline\ latency)] \times 100$. The area under the curve (AUC) value for the antinociceptive action of the drug on each rat was calculated for some of the experiments.

Statistical analysis

The results are given as the mean and standard error of the mean (S.E.M.) of the data. The statistical analysis was conducted using computer software (Prism, version 5.0c, GraphPad Software, San Diego, CA) 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.

RESULTS

Effects of PIs on LE-induced inhibition of tail-flick response

Figs. 3-I and 3-II show change over time in LE-induced antinociception from 10 min following i.t. administration of saline or a mixture of the three PIs (10 nmol each). The results showed a dose-dependent and prolonged antinociceptive effect on the tail-flick response. The $AUC_{0-45min}$ value demonstrated that induction of antinociception by LE at doses of 500, 200, 100, 50, or 10 nmol under pretreatment with a mixture of the three PIs was significantly greater than that with the PIs alone (Fig. 3-III). The $AUC_{0-60min}$ value also showed that the antinociceptive effect of LE at doses of 500, 200, or 100 nmol under pretreatment with a mixture of the three PIs was significantly greater than that with the PIs alone (Fig. 3-IV). A significant change was also observed in antinociception following i.t. administration of a mixture of the PIs (10 nmol each) alone (Fig. 3-III, 3-IV). The antinociceptive effect of i.t. administration of 10 nmol LE with a mixture of the three PIs (10 nmol each) had the same onset, offset, and duration of action as that with 1000 nmol LE alone (Fig. 4-I). The $AUC_{0-45min}$ value for %MPE of 10 nmol LE with a mixture of the three PIs (10 nmol each) was approximately equal to that for 1000 nmol LE alone (Fig. 4-II). Thus, i.t. administration of LE under i.t. pretreatment with the three PIs (10 nmol each) induced a 100-fold increase in the antinociceptive effect on the tail-flick response. However, it is possible that the potency of LE is increased more than 100-fold by the three PIs, as the potency of LE at doses of more than 1000 nmol in rats not treated with PIs could not be estimated due to the unavailability of high concen-

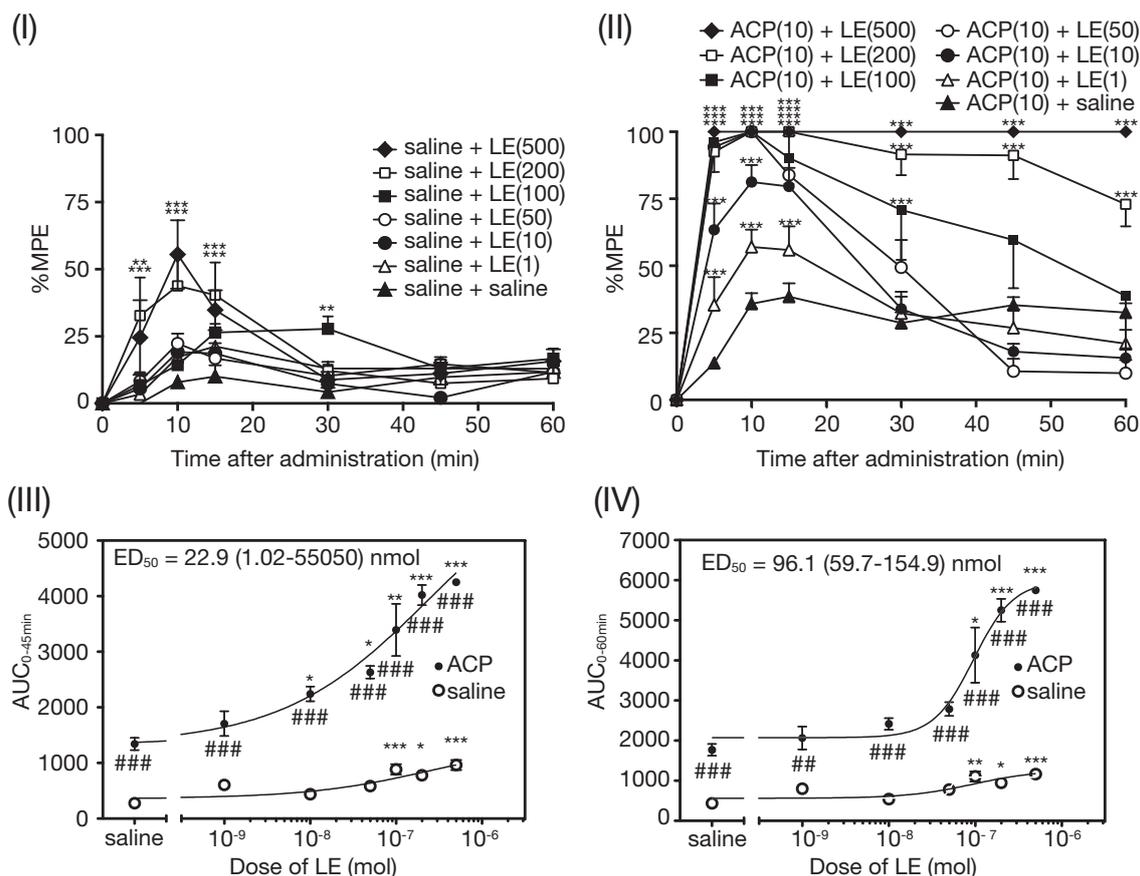


Fig. 3 Dose-dependent antinociception by i.t. administration of LE and pretreatment with saline or a mixture of PIs (ACP). Upper panel (I) and (II) indicates time course of %MPE of LE (1nmol, $n = 6$; 10 nmol, $n = 9$; 50 nmol, $n = 14$; 100 nmol, $n = 7$; 200 nmol, $n = 6$; 500 nmol, $n = 7$) pretreatment with saline and LE (1 nmol, $n = 12$; 10 nmol, $n = 18$; 50 nmol, $n = 8$; 100 nmol, $n = 6$; 200 nmol, $n = 8$; 500 nmol, $n = 4$) pretreatment with 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 ANOVA; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Lower panel (III) and (IV) shows $AUC_{0-45min}$ and $AUC_{0-60min}$, respectively, for value of %MPE indicated in both of panel (I) and (II). Significantly different from saline-saline treated control or ACP-saline treated control according to Dunn's post-hoc test following Kruskal-Wallis test; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Significantly different from pretreatment with saline according to Dunn's post-hoc test following two-way repeated measures ANOVA; ### $P < 0.001$.

trations of LE owing to its low solubility.

Pretreatment with a mixture of the three PIs (1, 3, 10, or 30 nmol) dose-dependently increased the antinociceptive effects induced by LE (10 nmol) (Fig. 5). The antinociceptive potency of LE (10 nmol) under pretreatment with the three PIs at doses of 3, 10, or 30 nmol was significantly greater than that of LE (10 nmol) alone (Fig. 5-II).

Effects of single PI or two peptidase inhibitor combinations of PIs (AC, AP, CP) on LE-induced antinociceptive effects

To examine the effect of a single PI (A, C, P) on LE (10 nmol)-induced antinociception, amastatin (A), captopril (C), or phosphoramidon (P) was administered i.t. at a dose of 10 nmol. Each PI significantly increased the magnitude of LE-induced antinociception (Fig. 6-I, 6-III). To examine the effect of two PIs on LE (10 nmol)-induced antinociception, combinations of PIs (AC, AP, or CP) were administered i.t. The LE-induced antinociceptive effect induced by any combination of PIs was significantly lower than that of the three PIs

together (ACP) (Fig. 6-II, 6-IV). These results indicate that any residual single peptidase inactivates substantial amounts of LE in rat at the spinal level.

Effects of antagonists on combination of PIs and LE-induced inhibition of tail-flick response

To investigate the effect of opioid receptor antagonists on LE-induced antinociception in the presence of a mixture of PIs, 10nmol i.t. CTOP, 66 nmol i.t. naltrindole hydrochloride (NTI), or 10 mg/kg subcutaneous norbinaltorphimine dihydrochloride (nor-BNI) was administered. Five or 20 min following administration of CTOP or NTI, a mixture of the three PIs was administered. Twenty-four hours following administration of nor-BNI, PIs were administered i.t. The antinociceptive potency of LE under pretreatment with PIs was significantly attenuated by CTOP or NTI; it was also attenuated by nor-BNI, but not significantly so (Fig. 7).

DISCUSSION

The $AUC_{0-60min}$ value for antinociception with i.t. and i.c.v. administration of 10 nmol LE pretreatment

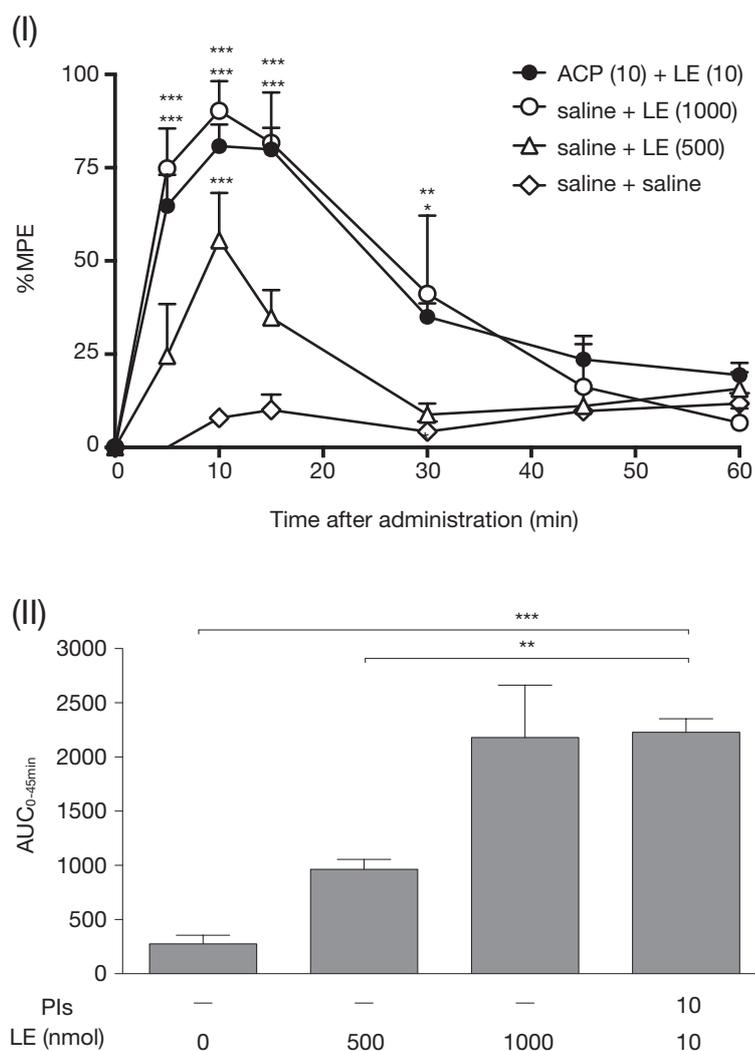


Fig. 4 Potentiating effect of PIs on antinociception induced by i.t. administration of LE. Upper panel (I) indicates time course of %MPE of LE (0 nmol, n = 8; 10 nmol, n = 18; 500 nmol, n = 7; and 1000 nmol, n = 5) and pretreatment with saline and ACP (10 nmol each), respectively. Significantly different 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_{0-45min} for value of %MPE indicated in upper panel (I). Significantly different from LE (10 nmol) and pretreatment with ACP according to Dunn's post-hoc test following Kruskal-Wallis test; *P < 0.05, **P < 0.01 and ***P < 0.001.

with PIs was approximately 2500 (present study) and 1800 (our earlier study [19]), respectively. These results indicate that antinociception induced by i.t. administration of LE pretreated with PIs is more potent than those by i.c.v. administration. The results of the present study showed that pretreatment with a mixture of three PIs produced an at least 100-fold augmentation in antinociception induced by i.t. administration of LE in rat. An earlier study demonstrated that pretreatment with a mixture of three PIs produced an approximately 500-fold augmentation of this effect by i.c.v. administration of LE [19]. On the other hand, in another study, the inhibitory potency of LE in isolated guinea pig ileum and mouse vas deferens was increased by approximately 6.3- and 7.6-fold, respectively [20]. Whereas LE did not induce analgesia when injected alone, even at high doses, it did so strongly at low doses when co-injected with peptidase inhibitors [21]. Taken together with the fact that the potency of LE should be decreased by its hydrolysis by these three PIs [8], these results provide further support for the view that amastatin-, captopril-, and phosphoramidon-sensitive enzymes play an important role in inactivation of LE at both the spinal and supraspinal level [19].

A significant dose-dependent change was observed in antinociception following i.t. administration of a mixture of the three PIs alone. This is in good agree-

ment with the results of studies 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 an antinociceptive response in the hot plate and writhing tests in mouse and the tail flick test in rat [22].

The AUC_{0-60min} value for antinociception with i.t. administration of 10 nmol ME and LE was approximately 4000 (Murata *et al.*, in submission) and 2500 (present study), respectively. The AUC_{0-60min} value for antinociception with i.c.v. administration of 1 nmol ME and LE was approximately 2100 (Murata *et al.*, in submission) and 1000 [19], respectively. This indicates that ME is approximately twice as potent as LE in its antinociceptive effect. This is consistent with the results of other investigations indicating that the affinity of ME was approximately twice as potent as that of LE [23]. Taken together, these results suggest that co-administration of a mixture of three PIs allows for evaluation of the real analgesic potency of opioid peptides at the spinal or supraspinal level.

In the present study, administration of a single PI or any combination of two PIs revealed that any single residual peptidase inactivates substantial amounts of LE. On the other hand, several studies have shown that a single PI or two PIs augmented enkephalin-induced antinociception [24-27]. However, the cur-

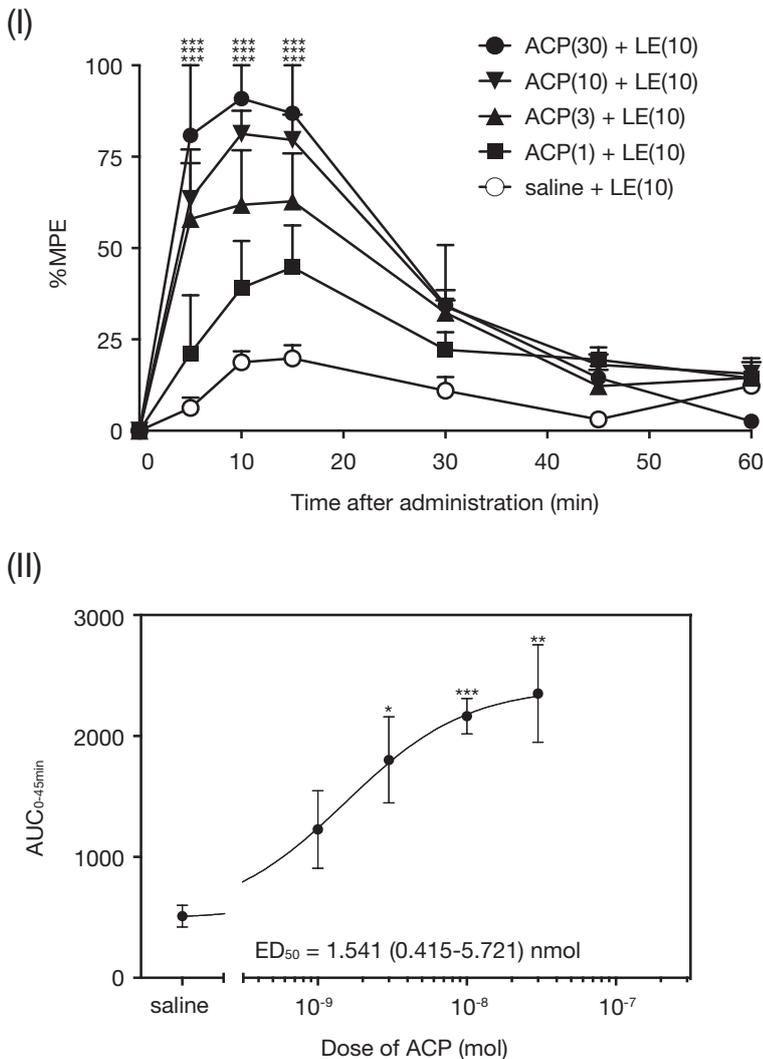


Fig. 5 Dose-dependent antinociception by i.t. administration of LE pretreatment with saline or a mixture of PIs (ACP). Upper panel (I) indicate time course of %MPE of LE (10 nmol) and pretreatment with saline or ACP (1 nmol, $n = 6$; 3 nmol, $n = 6$; 10 nmol, $n = 18$; 30 nmol, $n = 5$). Significantly different from saline-LE (10 nmol) group 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) shows $AUC_{0-45min}$ for value of %MPE indicated in upper (I). Significantly different from saline-LE (10 nmol) group according to Dunn's post-hoc test following Kruskal-Wallis test; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

rent results strongly suggest that those studies may have only estimated the partial analgesic potency of enkephalins, including LE. Several lines of evidence support this. First, the antinociceptive effect induced by i.c.v. administration of dyn(1-8), ME, ME-RF, or ME-RGL is inactivated by any residual single peptidase [4, 6-8]. Second, NEP was localized to discrete regions of the gray matter at all spinal cord levels [28]. The substantia gelatinosa displayed rich NEP staining which overlapped the inner and outer zones of lamina II. The regional distribution of NEP overlapped that of enkephalin and substance-P rich regions of the spinal cord. These findings indicate a role for NEP in the metabolic regulation of opioid peptides in spinal cord [29]. Third, APN has been found to be involved in the degradation of enkephalins in association with NEP [23]. A high concentration of APN was observed in regions of the gray matter at dorsal and ventral horn [30]. These findings indicate that the co-localization of APN, NEP and enkephalins plays a key role in control of nociception in spinal cord. Fourth, 85-90% of the metabolism of LE in rat plasma is due to the combined action of bestatin-sensitive aminopeptidase M and captopril-sensitive ACE [31]. Approximately 80%, 10%, 6%, and 3% of the metabolism of LE in human plasma is due to aminopeptidases, dipeptidylaminopeptidases, dipeptidylcarboxypeptidases, and carboxypeptidases,

respectively [32]. In the presence of human saliva, LE was partially hydrolyzed by aminopeptidases, dipeptidylaminopeptidases, and dipeptidylcarboxypeptidases [33].

The present results showed the involvement of μ and δ opioid receptors in antinociception induced by i.t. administration of LE, as suggested by the fact that CTOP and NTI significantly attenuated antinociception. This is consistent with the results of other investigations. First, comparative study of pA_2 values has shown that μ -opioid receptors were involved in antinociception induced by i.c.v. administration of LE under i.c.v. pretreatment with the three PIs [19]. Second, the K_i values of LE are 3.4 and 4.0 nM for μ and δ opioid receptor, respectively, indicating that LE has almost the same affinity to μ opioid and δ opioid receptors [34]. Third, the μ , δ , and κ opioid receptors were found to be distributed at approximately 70%, 20%, and 10%, respectively, in rat spinal cord [35, 36]. Fourth, activation of μ or δ opioid receptors inhibited the amplitude of the A δ fiber- or C fiber-evoked excitatory postsynaptic current in rat spinal cord [37-39] 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)

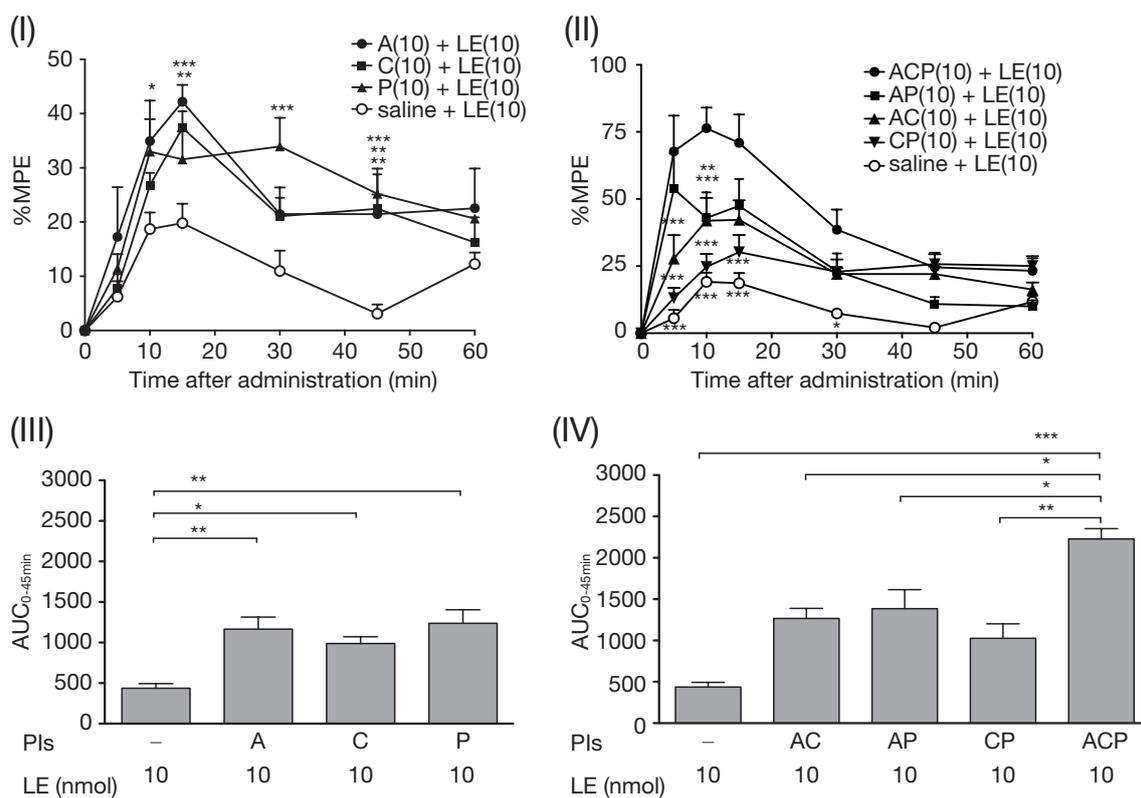


Fig. 6 Comparison of effect of combination of single PI (A, C, P), two PIs (AC, CP, AP) or three PIs (ACP) on antinociception induced by i.t. administration of LE. Upper panel (I) indicates time course of %MPE of LE (10 nmol) and pretreatment with saline or single PI (A, $n = 4$; P, $n = 4$; C, $n = 7$; saline, $n = 9$; 10 nmol each PI), respectively. Upper panel (II) indicates time course of %MPE of LE (10 nmol) and pretreatment with saline or two PIs (AC, $n = 10$; AP, $n = 9$; CP, $n = 10$; ACP, $n = 18$; saline, $n = 9$; 10 nmol each PI) 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; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Lower panel (III) and panel (IV) shows AUC_{0-45min} for value of %MPE indicated in upper panel (I) and (II), respectively. Significantly different from LE and pretreatment with saline (III) or ACP (IV) according to Dunn's post-hoc test following Kruskal-Wallis test; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

[40]. Fifth, 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 [28]. The co-distribution of peptidases, opioid-binding sites and enkephalins, along with the physiological effects of PI, strongly supports the view that peptidases are mainly involved in terminating enkephalinergic signals [28, 41]. Sixth, the presence of μ opioid receptors was found to be essential in the antinociceptive action of δ -selective opioid agonists in μ opioid receptor knockout animals [42].

In conclusion, the present results showed that LE-induced antinociception was increased by more than 100-fold under pretreatment with three peptidase inhibitors in a μ and δ opioid receptor antagonist-reversible manner at the spinal level in rat. The antinociceptive effect produced by LE under pretreatment with a single PI increased antinociception compared to that with saline. The antinociceptive potency of LE under pretreatment with any combination of two peptidase inhibitors was smaller than that with three peptidase inhibitors. These findings indicate that amastatin-, captopril-, and phosphoramidon-sensitive enzymes play an important role in the inactivation of

LE at the spinal level.

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REFERENCES

- 1) König M, Zimmer A M, Steiner H, Holmes P V, Crawley J N, Brownstein M J and Zimmer A. Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Nature* 1996; 383(6600): 535-8.
- 2) Khaket T P, Singh J, Attri P and Dhanda S. Enkephalin degrading enzymes: metalloproteases with high potential for drug development. *Curr Pharm Des* 2012; 18(2): 220-30.
- 3) Morales-Mulia M, de Gortari P, Amaya M I and Mendez M. Activity and expression of enkephalinase and aminopeptidase N in regions of the mesocorticolimbic system are selectively modified by acute ethanol administration. *J Mol Neurosci* 2012; 46(1): 58-67.
- 4) Hiranuma T, Kitamura K, Taniguchi T, Kobayashi T, Tamaki R, Kanai M, Akahori K, Iwao K and Oka T. Effects of three pepti-

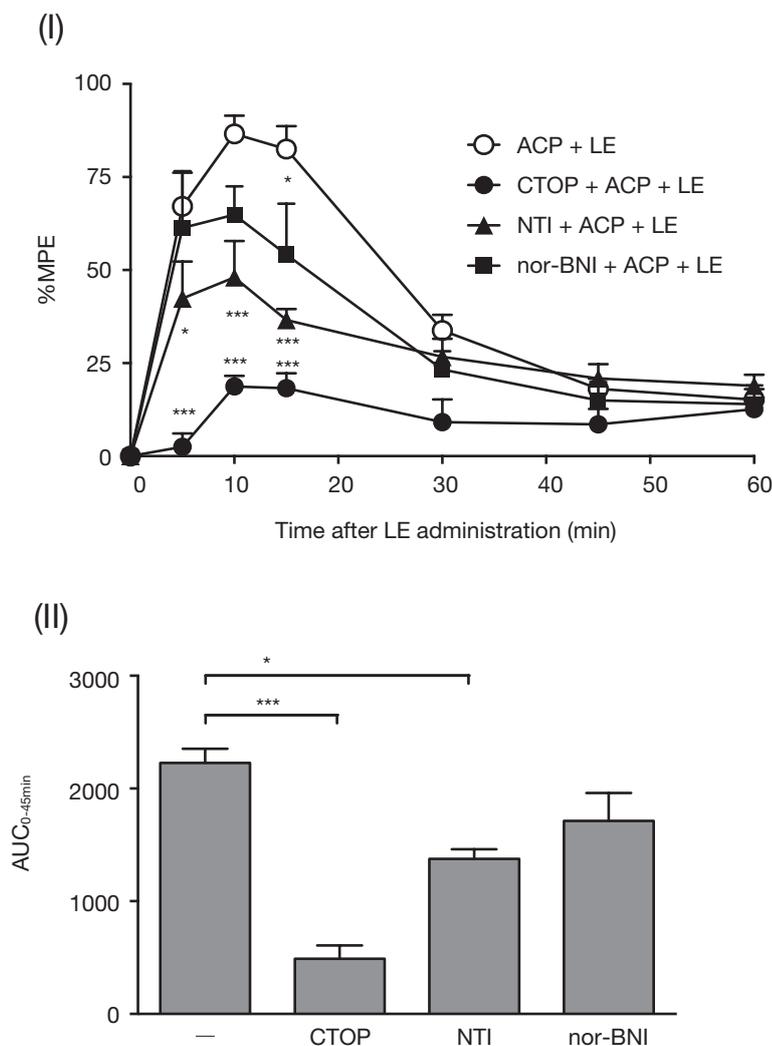


Fig. 7 Effect of opioid receptor antagonists on LE-induced antinociception under pretreatment with PIs. Upper panel (I) indicate time course of %MPE of PIs by LE (10 nmol)-induced antinociception under pretreatment with PIs following administration of CTOP (n = 5), naltrindole hydrochloride (NTI; n = 7), or norbinaltorphimine dihydrochloride (nor-BNI; n = 5), respectively. Significantly different from LE pretreated with ACP-LE; n = 11) according to Dunn's post-hoc test following two-way repeated measures ANOVA; *P < 0.05 and ***P < 0.001. Lower panel (II) show AUC_{0-45min} for value of %MPE indicated in upper panel (I). Significantly different from LE pretreatment with antagonists according to Dunn's post-hoc test following Kruskal-Wallis test; **P < 0.01.

dase inhibitors, amastatin, captopril and phosphoramidon, on the hydrolysis of [Met⁵]-enkephalin-Arg⁶-Phe⁷ and other opioid peptides. *Naunyn Schmiedebergs Arch Pharmacol* 1998; 357(3): 276-82.

- 5) Oka T, Aoki K, Kajiwara M, Ishii K, Kuno Y, Hiranuma T and Matsumiya T. Inactivation of [Leu⁵]-enkephalin in three isolated preparations: relative importance of aminopeptidase, endopeptidase-24.11 and peptidyl dipeptidase A. *NIDA Res Monogr* 1986; 75(259-62).
- 6) Hiranuma T, Iwao K, Kitamura K, Matsumiya T and Oka T. Almost complete protection from [Met⁵]-enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-enk-RGL) hydrolysis in membrane preparations by the combination of amastatin, captopril and phosphoramidon. *J Pharmacol Exp Ther* 1997; 281(2): 769-74.
- 7) Hiranuma T, Kitamura K, Taniguchi T, Kanai M, Arai Y, Iwao K and Oka T. Protection against dynorphin-(1-8) hydrolysis in membrane preparations by the combination of amastatin, captopril and phosphoramidon. *J Pharmacol Exp Ther* 1998; 286(2): 863-9.
- 8) Hiranuma T and Oka T. Effects of peptidase inhibitors on the [Met⁵]-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* 1986; 41(4): 437-46.
- 9) 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* 1984; 36(1): 59-65.
- 10) Aoki K, Kajiwara M and Oka T. The inactivation of [Met⁵]-enkephalin by bestatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A and thiorphan-sensitive endopeptidase-24.11 in mouse vas deferens. *Jpn J Pharmacol* 1986; 40(2): 297-302.
- 11) Cui S Y, Kajiwara M, Ishii K, Aoki K, Sakamoto J, Matsumiya T and Oka T. The enhancing effects of amastatin, phosphoramidon and captopril on the potency of [Met⁵]-enkephalin in rat vas deferens. *Jpn J Pharmacol* 1986; 42(1): 43-9.
- 12) Morley J S. Structure-activity relationships of enkephalin-like peptides. *Annu Rev Pharmacol Toxicol* 1980; 20(81-110).
- 13) Kanai M, Takahashi S, Kosaka K, Iwao K, Kobayashi H and Oka T. [Met⁵]-enkephalin-Arg-Gly-Leu-induced antinociception is greatly increased by peptidase inhibitors. *Eur J Pharmacol* 2002; 453(1): 53-8.
- 14) Kitamura K, Akahori K, Yano H, Iwao K and Oka T. Effects of peptidase inhibitors on anti-nociceptive action of dynorphin-(1-8) in rats. *Naunyn-Schmiedeberg's archives of pharmacology* 2000; 361(3): 273-8.
- 15) Takahashi S, Jin X L, Kosaka K, Yoshikawa M, Kobayashi H and Oka T. The enhancing effects of peptidase inhibitors on the antinociceptive action of [Met⁵]-enkephalin-Arg⁶-Phe⁷ in rats. *J Pharmacol Sci* 2007; 105(1): 117-21.
- 16) Le Bars D, Gozariu M and Cadden S W. Animal models of nociception. *Pharmacol Rev* 2001; 53(4): 597-652.
- 17) Yaksh T L and Rudy T A. Chronic catheterization of the spinal subarachnoid space. *Physiology & behavior* 1976; 17(6): 1031-6.
- 18) Janssen P A, Niemegeers C J and Dony J G. The inhibitory effect of fentanyl and other morphine-like analgesics on the warm water induced tail withdrawal reflex in rats. *Arzneimittelforschung* 1963; 13(502-7).
- 19) Akahori K, Kosaka K, Jin X L, Arai Y, Yoshikawa M, Kobayashi H and Oka T. Great increase in antinociceptive potency of [Leu⁵] enkephalin after peptidase inhibition. *Journal of pharmacological sciences* 2008; 106(2): 295-300.
- 20) 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* 1986; 41(3): 273-81.
- 21) Chen W, Song B, Lao L, Perez O A, Kim W and Marvizon J C. Comparing analgesia and mu-opioid receptor internalization produced by intrathecal enkephalin: requirement for peptidase inhibition. *Neuropharmacology* 2007; 53(5): 664-76.
 - 22) Noble F, Soleilhac J M, Soroca-Lucas E, Turcaud S, Fournie-Zaluski M C and Roques B P. Inhibition of the enkephalin-metabolizing enzymes by the first systemically active mixed inhibitor prodrug RB 101 induces potent analgesic responses in mice and rats. *J Pharmacol Exp Ther* 1992; 261(1): 181-90.
 - 23) Waksman G, Bouboutou R, Devin J, Bourgoin S, Cesselin F, Hamon M, Fournie-Zaluski M C and Roques B. In vitro and in vivo effects of ketorphan on enkephalin metabolism in rodent brain. *European journal of pharmacology* 1985; 117(2): 233-43.
 - 24) Costentin J, Vlaiculescu A, Chaillet P, Ben Natan L, Aveaux D and Schwartz J C. Dissociated effects of inhibitors of enkephalin-metabolising peptidases or naloxone on various nociceptive responses. *Eur J Pharmacol* 1986; 123(1): 37-44.
 - 25) Norman J A and Chang J Y. Proteolytic conversion of [Met] enkephalin-Arg6-Gly7-Leu8 by brain synaptic membranes. Characterization of formed peptides and mechanism of proteolysis. *J Biol Chem* 1985; 260(5): 2653-6.
 - 26) Stine S M, Yang H Y and Costa E. Inhibition of in situ metabolism of [3H](met5)-enkephalin and potentiation of (met5)-enkephalin analgesia by captopril. *Brain research* 1980; 188(1): 295-9.
 - 27) Van Amsterdam J G and Llorens-Cortes C. Inhibition of enkephalin degradation by polorphan: effects on striatal [Met5] enkephalin levels and jump latency in mouse hot plate test. *European journal of pharmacology* 1988; 154(3): 319-24.
 - 28) Waksman G, Hamel E, Fournie-Zaluski M C and Roques B P. Autoradiographic comparison of the distribution of the neutral endopeptidase "enkephalinase" and of mu and delta opioid receptors in rat brain. *Proc Natl Acad Sci U S A* 1986; 83(5): 1523-7.
 - 29) Back S A and Gorenstein C. Fluorescent histochemical localization of neutral endopeptidase-24.11 (enkephalinase) in the rat spinal cord. *J Comp Neurol* 1989; 280(3): 436-50.
 - 30) Noble F, Banisadr G, Jardinaud F, Popovici T, Lai-Kuen R, Chen H, Bischoff L, Parsadaniantz S M, Fournie-Zaluski M C and Roques B P. First discrete autoradiographic distribution of aminopeptidase N in various structures of rat brain and spinal cord using the selective iodinated inhibitor [125I]RB 129. *Neuroscience* 2001; 105(2): 479-88.
 - 31) Weinberger S B and Martinez J L, Jr. Characterization of hydrolysis of [leu]enkephalin and D-ala2-[L-leu]enkephalin in rat plasma. *J Pharmacol Exp Ther* 1988; 247(1): 129-35.
 - 32) Zolotarev Iu A, Sokolov O, Kost N V, Vas'kovskii B V, Miasoedov N F and Zozulia A A. [Leu-enkephalin homogeneously labeled with tritium in studying the Selank inhibiting effect on the enkephalin-degrading enzymes of human plasma]. *Bioorganicheskaia khimiia* 2004; 30(3): 234-40.
 - 33) Marini M and Roda L G. Enkephalin-degrading enzymes and their inhibitors in human saliva. *Peptides* 2000; 21(1): 125-35.
 - 34) Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell G I and Reisine T. Pharmacological characterization of the cloned kappa-, delta-, and mu-opioid receptors. *Mol Pharmacol* 1994; 45(2): 330-4.
 - 35) Besse D, Lombard M C, Zajac J M, Roques B P and Besson J M. Pre- and postsynaptic distribution of mu, delta and kappa opioid receptors in the superficial layers of the cervical dorsal horn of the rat spinal cord. *Brain Res* 1990; 521(1-2): 15-22.
 - 36) Stevens C W, Lacey C B, Miller K E, Elde R P and Seybold V S. Biochemical characterization and regional quantification of mu, delta and kappa opioid binding sites in rat spinal cord. *Brain Res* 1991; 550(1): 77-85.
 - 37) Danzebrink R M, Green S A and Gebhart G F. Spinal mu and delta, but not kappa, opioid-receptor agonists attenuate responses to noxious colorectal distension in the rat. *Pain* 1995; 63(1): 39-47.
 - 38) Dickenson A H, Sullivan A F, Knox R, Zajac J M and Roques B P. Opioid receptor subtypes in the rat spinal cord: electrophysiological studies with mu- and delta-opioid receptor agonists in the control of nociception. *Brain Res* 1987; 413(1): 36-44.
 - 39) Ikoma M, Kohno T and Baba H. Differential presynaptic effects of opioid agonists on Adelta- and C-afferent glutamatergic transmission to the spinal dorsal horn. *Anesthesiology* 2007; 107(5): 807-12.
 - 40) Jutkiewicz E M. RB101-mediated protection of endogenous opioids: potential therapeutic utility? *CNS Drug Rev* 2007; 13(2): 192-205.
 - 41) Noble F and Roques B P. Protection of endogenous enkephalin catabolism as natural approach to novel analgesic and antidepressant drugs. *Expert Opin Ther Targets* 2007; 11(2): 145-59.
 - 42) Sora I, Funada M and Uhl G R. The mu-opioid receptor is necessary for [D-Pen2,D-Pen5]enkephalin-induced analgesia. *Eur J Pharmacol* 1997; 324(2-3): R1-2.