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ORIGINAL ARTICLE

# Effect of three peptidase inhibitors on antinociceptive potential and toxicity with intracerebroventricular administration of dynorphin A (1–17) or (1–13) in the rat

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**Abstract**
*Purpose* The N- and C-terminal regions of dynorphin (Dyn) A (1–17) activate opioid and N-methyl-D-aspartate receptors, respectively. Earlier studies demonstrated that Dyn-converting enzyme cleaved Dyn A (1–17) mainly at the $Arg^6$–$Arg^7$ bond, resulting in the production of N- and C-terminal region peptide fragments, and that this enzyme was not inhibited by a mixture of the three peptidase inhibitors (PIs) amastatin (A), captopril (C), and phosphoramidon (P). The purpose of the present study was to evaluate antinociceptive potential and toxicity with intracerebroventricular administration of Dyn A (1–17) or (1–13) under pretreatment with a mixture of A, C, and P and/or Dyn-converting enzyme inhibitor (*p*-hydroxymercuribenzoate).
*Methods* Peptide fragments from Dyn A (1–17) following incubation with membrane preparation under pretreatment with a mixture of the three PIs was identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI–TOF–MS). Infusion of drugs and peptides into the third ventricle in rats was performed via indwelling cannulae. Induction of antinociception and toxicity by Dyn A (1–17), Dyn A (1–13), Dyn A (1–6), or Dyn A (7–17) were determined by the tail-flick test and induction of barrel rotation, respectively. The effects of the PIs on antinociception and toxicity were evaluated by a dose−response study and a comparison of differences among various combinations of Dyn A (1–17) or Dyn A (1–13) and the three PIs and *p*-hydroxymercuribenzoate.
*Results* MALDI–TOF–MS analysis identified Dyn A (1–6) and Dyn A (1–10) fragments as products following incubation of Dyn A (1–17) with membrane preparation of rat midbrain under pretreatment with a mixture of the three PIs. Pretreatment with a mixture of the three PIs produced an approximately 30-fold augmentation in antinociception induced by low-dose intracerebroventricular administration of Dyn A (1–17) or (1–13) in a μ-, δ- and κ-opioid receptor antagonist-reversible manner, but without signs of toxicity such as barrel rotation in the rat. Dyn A (1–17)-induced antinociception and toxicity was greater than that of Dyn A (1–6), Dyn A (1–13), or Dyn A (7–17) at the same dose. Dyn A (1–17)-induced antinociception and toxicity under pretreatment with various combinations of the three PIs and *p*-hydroxymercuribenzoate was greater than that with a mixture of the three PIs alone.
*Conclusion* These findings suggest that administration of a mixture of the three PIs increases Dyn A (1–17)- or (1–13)-induced antinociception under physiological conditions without toxicity.

**Keywords** Dynorphin A · Peptidase · Dynorphin-converting enzyme · Antinociception · Toxicity

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## Introduction

Dynorphin (Dyn) A (1–17) activates both opioid and non-opioid receptors, with its N- and C-terminal regions activating opioid and N-methyl-D-aspartate (NMDA) receptors, respectively. At physiological concentrations, Dyn A (1–17) or Dyn A (1–13) activates opioid receptors. At supra-physiological levels, however, Dyn A (1–17), Dyn A (1–13), or their C-terminal peptide fragments may act via NMDA receptors, exerting an excitotoxic effect on neurons and oligodendroglia and potentially destabilizing astroglia [1].

Dyn-converting enzyme (DCE) has been suggested to cleave Dyn A (1–17) mainly at the $Arg^6$–$Arg^7$ bond, resulting in the production of N- and C-terminal region peptide fragments [2]. An earlier matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI–TOF–MS) analysis identified Dyn A (1–6) as one of the most prominent products resulting from incubation of Dyn A (1–17) with extract of rat caudate putamen under pretreatment with a mixture of the three peptidase inhibitors (PIs)—amastatin (A, an aminopeptidase inhibitor), captopril (C, a dipeptidyl carboxypeptidase inhibitor), and phosphoramidon (P, an endopeptidase-24.11 inhibitor)—for 40 min at 37 °C [3]. The results of this earlier in vitro study suggested that the activity of DCE was not completely inhibited under pretreatment with a mixture of these three PIs [2].

Previous in vitro studies showed that N-terminal region peptides of Dyn A such as [$Leu^5$]-enkephalin (LE) [4] or Dyn A (1–8) [5] incubated with ileal or striatal membrane preparation for 60 min at 37 °C remained intact in the presence of a mixture of the three PIs, but were completely hydrolyzed in their absence. These results support previous in vivo studies showing that intracerebroventricular (i.c.v.) administration of a mixture of the three PIs increased LE-induced antinociception by >500-fold [6] and that by Dyn A (1–8) by >100-fold [7], which was mediated mainly by μ-opioid receptors.

A competition radioligand binding assay demonstrated that Dyn A (1–17) and Dyn A (1–13) bind to κ-opioid receptors with somewhat higher affinity than μ-opioid or δ-opioid receptors, indicating that they are endogenous ligands for κ-opioid receptors [8, 9]. In contrast, previous in vivo studies demonstrated that N-terminal region peptide fragments of Dyn A (1–17) such as LE [6] and Dyn A (1–8) [7] act mainly on μ-opioid receptors under pretreatment with the three PIs. These results predict that DCE will produce N-terminal region peptides such as Dyn A (1–6) from Dyn A (1–17) or Dyn A (1–13) in brain under pretreatment with the three PIs, resulting in antinociception mediated mainly by μ-opioid rather than κ-opioid receptors.

Dyn A (1–17) or (1–13) induces more toxicity than their C-terminal region peptide fragments such as Dyn A (13–17) [10]. Hence, high doses of Dyn A (1–17) or (1–13) induce toxicity as well as antinociception [11]. We previously demonstrated that a mixture of the three PIs increased the antinociception of Dyn A N-terminal region peptides such as LE [6, 12] and Dyn A (1–8) [7]. These earlier studies suggest that low doses of Dyn A (1–17) or (1–13) under pretreatment with a mixture of the three PIs results in antinociception without toxicity because DCE produces N- and C-terminal region fragments.

The purpose of the present study was to evaluate the effect of A-, C-, and P-sensitive peptidases and DCE on the antinociceptive effects and toxicity of Dyn A (1–17), Dyn A (1–13), or their peptide fragments in rat brain under physiological conditions. The effects of opioid receptor antagonists on antinociception under pretreatment with PIs were also investigated.

## Materials and methods

The present animal experiments were performed in strict accordance with the guidelines of Tokai University (http://www.u-tokai.ac.jp/about/concept/guidance.html) and were approved by the Animal Investigation Committee of Tokai University.

### Chemicals

Dyn A (1–17), Dyn A (1–13), A, and P were purchased from Peptide Institute Inc. (Minoh, Japan). Dyn A (1–6) was purchased from Phoenix Pharmaceuticals, Inc. (Mannheim, Germany). Dyn A (7–17) was purchased from Abgent (San Diego, USA). Captopril, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ (CTOP, a μ-opioid receptor antagonist) nor-binaltorphimine dihydrochloride (nor-BNI, a κ-opioid receptor antagonist), and naltrindole hydrochloride (NTI, a δ-opioid receptor antagonist) were purchased from Sigma Japan (Tokyo, Japan). Naloxone hydrochloride (NOX, a non-selective opioid receptor antagonist) was purchased from Daiichi-Sankyo Company, Limited (Tokyo, Japan). *p*-hydroxymercuribenzoate (PHMB; a DCE inhibitor) was purchased from Merck Japan (Tokyo, Japan). All chemicals apart from nor-BNI, NTI, and PHMB were dissolved in saline. Nor-BNI and NTI were dissolved in water. *p*-hydroxymercuribenzoate was dissolved in saline with 0.1 N-NaOH up to pH 9.0. The solution for all drugs used was prepared to the desired concentration just before use. Each drug was injected at a volume of 10 μL. The PIs were administered 10 min before administration of the opioid receptor agonist or saline as a control.

### Preparation for total homogenates and membrane fractions from rat midbrain

Male Wistar rats (180–220 g each; Nihon Clea, Tokyo, Japan) were housed in an air-conditioned room at a control temperature of 22–24 °C and a humidity of 50–60 % with a 12-h light/dark cycle (light on: 7:00) and food and water freely available. The rats were allowed to adapt to the novel laboratory environment for 1 week.

The midbrain periaqueductal gray (PAG) matter is continuous with the periventricular gray matter surrounding the third cerebral ventricle in brain [13]. The PAG is a major component of a descending pain inhibitory system. A-, C-, or P-sensitive peptidase is bound to cellular membrane [14]. Thus, midbrain membrane preparation was selected for MALDI–TOF–MS analysis of peptide fragments from Dyn A (1–17) under pre-treatment with a mixture of the three PIs. The tissues of rat midbrain were homogenized in 15 volumes of 50 mM Tris–HCl (pH 7.4) buffer with a Teflon-glass homogenizer using a similar method as described previously [14]. The homogenate was centrifuged at $800\times g$ for 15 min. The supernatant was re-centrifuged at $10{,}000\times g$ for 20 min. The new supernatants were collected and adjusted with the buffer to a concentration of 2 mg/ml protein, yielding the membrane fraction for the following experiment. Protein concentrations were determined according to the Lowry method, using bovine serum albumin as standard [15]. The PI solution containing A, C and P (20 μM each) was pre-incubated with the membrane fraction samples at 37 °C for 20 min before 0.1 nmol Dyn A (1–17) was added to them using a similar method as described previously [3]. Incubations were terminated after 40 min by adding 2 volumes of methanol to withdrawn incubation samples. The samples were evaporated in a SpeedVac centrifuge and subsequently desalted on a ZipTip $C_{18}$ column (Millipore, Bedford, MA, USA).

### MALDI–TOF–MS for peptide identification

MS spectra were recorded on an AXIMA–QIT–TOF–MS (Shimadzu, Kyoto, Japan) using 2,5-dihydroxybenzoic acid (SDHB; Bruker Daltonics Japan, Tokyo) spots as matrix. Peptide calibration standard II (Part-No. 222570, Bruker Daltonics Japan) was used for external near neighbor calibration.

### Intracerebroventricular 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) were mounted on a stereotaxic frame and implanted with stainless-steel guide cannulae (internal diameter of 0.35 mm) under inhalation anesthesia with nitrous oxide, oxygen, and isoflurane (2 %) 5–7 days prior to the day of the experiment. The lower end of the injection cannula (30-G needle, Terumo Co., Tokyo, Japan) was aimed at the third cerebral ventricle (3.0 mm posterior to the bregma and 7.5 mm ventral to the surface of the skull) according to the atlas of Paxinos and Watson [16]. The injection cannula was attached to a motor-driven, 50-μl microsyringe by polyethylene tubing (PE-20; Clay Adams, Parsippany, NJ, USA). Distribution of the drug solution in the cerebroventricular system was verified by infusion of 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 Dyn A (1–17), Dyn A (1–13), or Dyn A (1–6) was measured by the tail immersion assay, with 55 °C as the nociceptive stimulus [17, 18]. The latency to flick the tail with 55 °C water was measured before and at 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min after administration. The latency to flick the tail before administration was approximately 1 s. A cut-off 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: % MPE = [(test latency − baseline latency)/(5 − baseline latency)] × 100. The area under the curve (AUC) value for the antinociceptive action of the drug on each rat was calculated for some experiments.

### Animal experimental protocol

#### *Dose–response study*

Ten minutes following i.c.v. administration of a mixture of the three PIs (10 nmol each) or saline, Dyn A (1–17), Dyn A (1–13), or Dyn A (1–6) was administered intracerebroventricularly. To determine whether the antinociceptive effect was increased by administration of the PIs, the rats were tested in the following groups: Group 1, Dyn A (1–17) (0.1–3.0 nmol) alone or with a mixture of the three PIs; Group 2, Dyn A (1–13) (0.1–3.0 nmol) alone or with a mixture of the three PIs; Group 3, Dyn A (1–6) (1–3 nmol) alone or with a mixture of the three PIs. To determine whether Dyn A (1–17)-, Dyn A (1–13)-, or Dyn A (7–17)-induced toxicity (as evidenced by the rats exhibiting barrel rotation, i.e.,, spinning repeatedly around their longitudinal axis, or death) was exacerbated by increase in dose, the following groups were tested—Group 1, Dyn A (1–17) (0.1–30 nmol) alone or with a mixture of the three PIs; Group 2, Dyn A (1–13) (0.1–30 nmol) alone or with a

mixture of the three PIs; Group 3, ten or 30 nmol Dyn A (7–17) alone or with a mixture of the three PIs.

*Combination of Dyn A (1–17) and PHMB together with a mixture of the three PIs*

Ten minutes following i.c.v. administration of PHMB together with a mixture of the three PIs (10 nmol each), Dyn A (1–17) (1 nmol) was administered intracerebroventricularly. To test whether the antinociceptive effect of Dyn A (1–17) was increased by joint administration of PHMB and the PIs, the rats were tested in the following groups—Group 1, Dyn A (1–17) alone; Group 2, Dyn A (1–17) with a mixture of the three PIs; Group 3, Dyn A (1–17) with PHMB; Group 4, Dyn A (1–17) in combination PHMB and a mixture of the three PIs.

*Effect of each PI alone, in paired combinations, and all together on Dyn A (1–17)-induced antinociception*

To investigate the effects of each PI alone, paired combinations of the PIs, and all the PIs together on Dyn A (1–17) (1 nmol)-induced antinociception, 10 nmol of each PI, paired combinations of the PIs (10 nmol each of AC, CP, or AP), or all three PIs together (10 nmol each) were administered intrathecally.

*Selective or non-selective opioid receptor antagonists*

To investigate the effect of opioid receptor antagonists on Dyn A (1–17) (1 nmol), Dyn A (1–13) (3 nmol) or Dyn A (1–6) (10 nmol)-induced antinociception with pretreatment with a mixture of the three PIs, NOX (1 mg/kg, subcutaneously), CTOP (3 nmol, i.c.v.) [19], nor-BNI (20 mg/kg, subcutaneously) [20], and NTI (132 or 66 nmol, i.c.v.) [21] were injected at 20, 15, 30 min, and 24 h, respectively, before i.c.v. administration of Dyn A (1–17), Dyn A (1–13) or Dyn A (1–6).

Statistical analyses

The results are given as the mean and standard error of the mean (SEM) of the data. The 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 after drug administration was obtained in a two-way (drugs and time) repeated measures analysis of variance (ANOVA), the 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, the 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, the Dunn's comparison test was applied to determine significance.

## Results

Identification of peptide fragments from Dyn A (1–17)

MALDI–TOF–MS analysis identified Dyn A (1–6) (theoretical monoisotopic mass 712.377 $[M + H^+]$), Dyn A (1–10) (theoretical monoisotopic mass 1234.716 $[M + H^+]$) and Dyn A (1–17) (theoretical monoisotopic mass 2147.198 $[M + H^+]$) as *m/z* 712.433, 1234.973 and 2147.601, respectively, following incubation of Dyn A (1–17) with membrane preparation of rat midbrain under pretreatment with a mixture of the three PIs (Fig. 1). No other peptide fragments from Dyn A (1–17) were identified.

Toxicity induced by i.c.v. administration of Dyn A (1–17), Dyn A (1–13), or Dyn A (1–6)

At 0.1, 0.3, 1 or 3 nmol Dyn A (1–17) in the absence of PIs (10 nmol), none of the rats ($n = 5$–8, each group) died or exhibited barrel rotation. Four out of 5 rats died within 5 min of administration of 30 nmol Dyn A (1–17) in the absence of PIs. These rats exhibited convulsion but no barrel rotation. The remaining 1 out of 5 rats exhibited barrel rotation within 15 min after administration. This behaviour lasted for 2 min. At 10 nmol Dyn A (1–17) in the absence of PIs, 1 out of 6 rats was dead within 15 min and 2 rats exhibited barrel rotation within 10 min. At 0.1, 0.3, 1 or 3 nmol Dyn A (1–17) under pretreatment with PIs, none of the rats ($n = 5$–7, each group) died or exhibited barrel rotation. At 10 nmol Dyn A (1–17) under pretreatment with PIs, 4 out of 5 rats died within 5 min, with the remaining 1 rat exhibiting barrel rotation within 5 min.

At 0.1, 0.3, 1, 3 or 10 nmol Dyn A (1–13) in the absence of PIs, none of the rats ($n = 5$–10, each group) died or exhibited barrel rotation. At 30 nmol Dyn A (1–13) in the absence of PIs, 1 out of 6 rats died within 5 min and 1 rat exhibited barrel rotation within 15 min. At 0.1, 0.3, 1, 3 or 10 nmol Dyn A (1–13) under pretreatment with PIs, none of the rats ($n = 5$–10, each group) died or exhibited barrel rotation. At 30 nmol Dyn A (1–13) under pretreatment with PIs, 3 out of 7 rats died within 5 min and 3 rats exhibited barrel rotation within 5 min. At 10 nmol Dyn A (1–13) in the absence of PIs or under pretreatment with PIs, none of the rats died or exhibited barrel rotation.

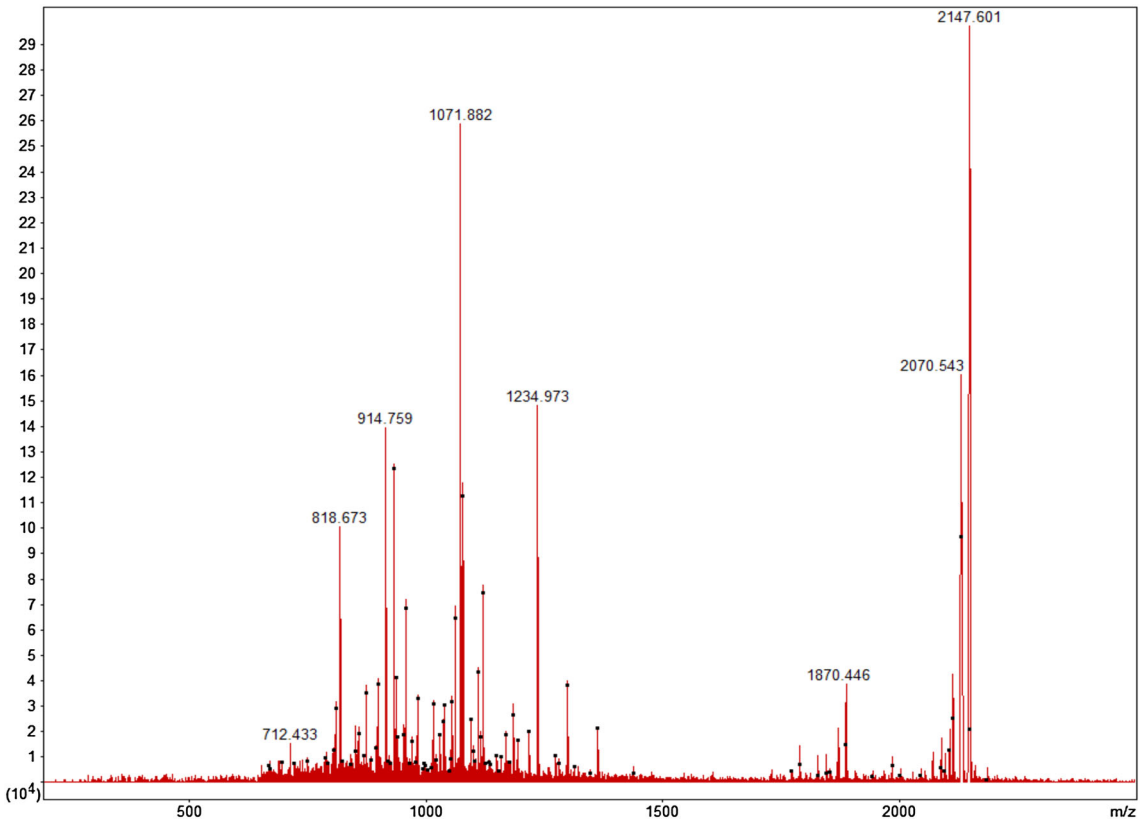


**Fig. 1** MADLI-TOF–MS spectrum of Dyn A (1–17) (*m/z* 2147.601 [M + $H^+$]) following incubation with membrane preparation of rat midbrain under pretreatment with a mixture of the three PIs for 40 min. The fragments Dyn A (1–6) and Dyn A (1–10) were identified as *m/z* 712.433 [M + $H^+$] and 1234.973 [M + $H^+$], respectively

At any dose (1–30 nmol) of Dyn A (1–6) in the absence of PIs or under pretreatment with PIs, none of the rats died or exhibited barrel rotation ($n$ = 5–7, each group).

## Toxicity induced by i.c.v. administration of Dyn A (7–17)

At 30 nmol Dyn A (7–17) in the absence of PIs or under pretreatment with PIs, none of 5 rats died or exhibited barrel rotation.

## Effects of PIs on Dyn A (1–17)-induced antinociception

Figures 2a and b show change over time in Dyn A (1–17)-induced antinociception from 10 min following i.c.v. administration of saline or a mixture of the three PIs (10 nmol each), respectively. The results showed that Dyn A (1–17) exerted a dose-dependent and prolonged antinociceptive effect on the tail-flick response. Sharp symbols on the $AUC_{0–120\ min}$ value of Dyn A (1–17) at doses of 0.3, 1, or 3 nmol under pretreatment with a mixture of the three PIs indicate significant differences compared to that of Dyn A (1–17) under pretreatment with saline (Fig. 2c). Asterisks placed over $AUC_{0–120\ min}$ values for Dyn A (1–17) at doses of 1 or 3 nmol under pretreatment with a mixture of the three PIs indicate significant differences compared with saline under pretreatment with the three PIs. Furthermore, asterisks placed over the $AUC_{0–120\ min}$ values for Dyn A (1–17) under pretreatment with saline indicate significant differences compared with under saline pretreatment with saline (Fig. 2c). The antinociceptive effect of i.c.v. administration of 0.3 nmol Dyn A (1–17) with a mixture of the three PIs (10 nmol each) had the same onset, offset, and duration of action as that with 10 nmol Dyn A (1–17) alone (Fig. 4a). The $AUC_{0–120\ min}$ value for % MPE of 0.3 nmol Dyn A (1–17) with a mixture of the three PIs (10 nmol each) was approximately equal to that for 10 nmol Dyn A (1–17) alone (Fig. 4b). Thus, i.c.v. administration of Dyn A (1–17) under i.c.v. pretreatment with the three PIs (10 nmol each) induced a 30-fold increase in the antinociceptive effect on the tail-flick response. At 10 nmol Dyn A (1–17) in the absence of PIs, 1 out of 6 rats was dead and 2 rats exhibited barrel rotation. These results indicate that the antinociceptive potency of Dyn A (1–17) may increase by >30-fold under pretreatment with a mixture of the three PIs without toxicity.

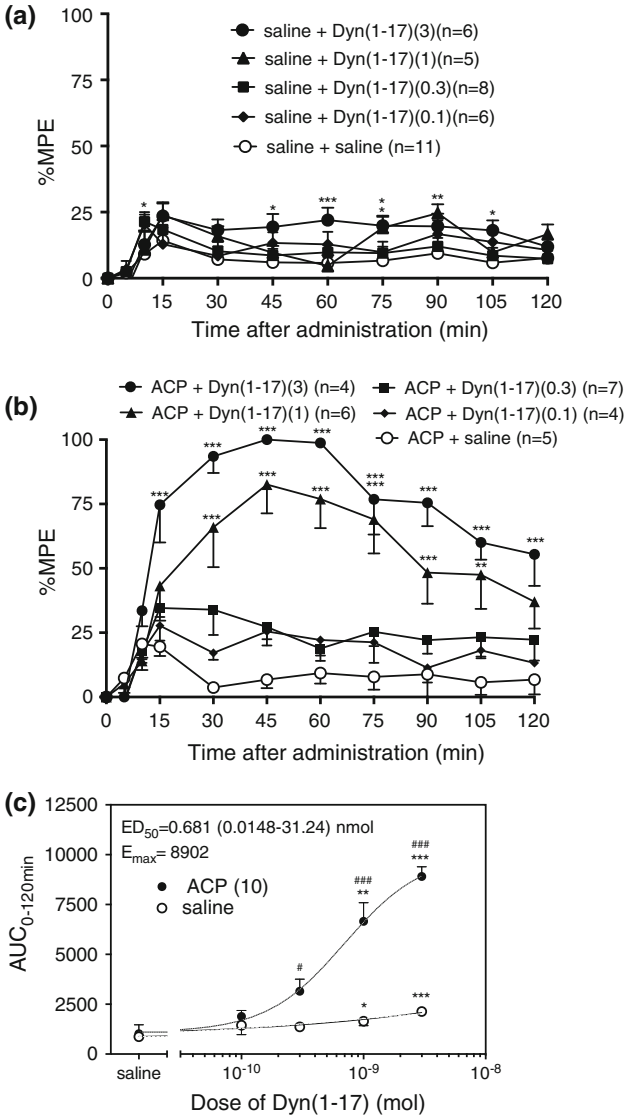


**Fig. 2** Dose-dependent antinociception by i.c.v. administration of Dyn A (1–17) under pretreatment with saline or a mixture of PIs (ACP). *Upper* (**a**) and *middle panels* (**b**) indicate time course of % MPE of Dyn A (1–17) (0.1–3 nmol) under pretreatment with saline and ACP, respectively. Significantly different from saline–saline or ACP−saline-treated control by Dunn's post hoc test following two-way repeated measures ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. *Lower panel* (**c**) shows $AUC_{0–120\ min}$ for value of % MPE indicated in *upper* (**a**) and *middle panels* (**b**). Significantly different from saline–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 under pretreatment with saline according to Dunn's post hoc test following two-way repeated measures ANOVA, $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$

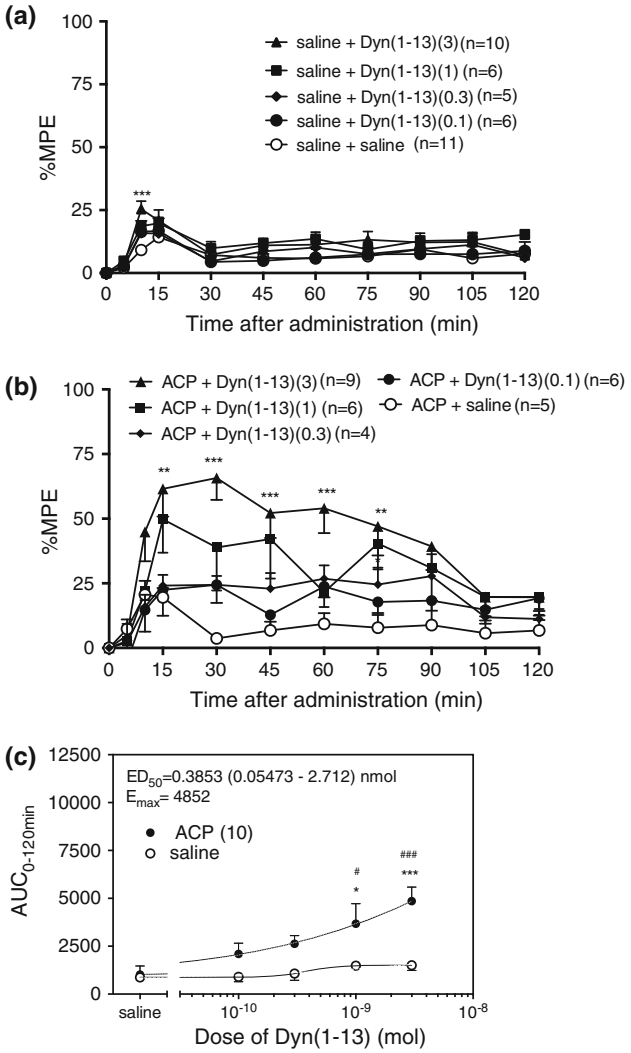


**Fig. 3** Dose-dependent antinociception by i.c.v. administration of Dyn A (1–13) under pretreatment with saline or a mixture of PIs (ACP). *Upper* (**a**) and *middle panels* (**b**) indicate time course of % MPE of Dyn A (1–13) (0.1–3 nmol) under pretreatment with saline and ACP, respectively. Significantly different from saline–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* (**c**) shows $AUC_{0–120\ min}$ for value of % MPE indicated in *upper* (**a**) and *middle panels* (**b**). Significantly different from saline–saline 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 under pretreatment with saline according to Dunn's post hoc test following two-way repeated measures ANOVA, $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$

## Effects of PIs on Dyn A (1–13)-induced antinociception

Figures 3a and b show change over time in Dyn A (1–13)-induced antinociception from 10 min following i.c.v. administration of saline or a mixture of the three PIs (10 nmol each), respectively. These results showed a dose-dependent and prolonged antinociceptive effect of Dyn A (1–13) on the tail-flick response. The $AUC_{0–120\ min}$ value demonstrated that induction of antinociception by Dyn A (1–13) at doses of 1 or 3 nmol under pretreatment with a mixture of the three PIs was significantly greater than that with the PIs alone (Fig. 3c). Sharp symbols on the

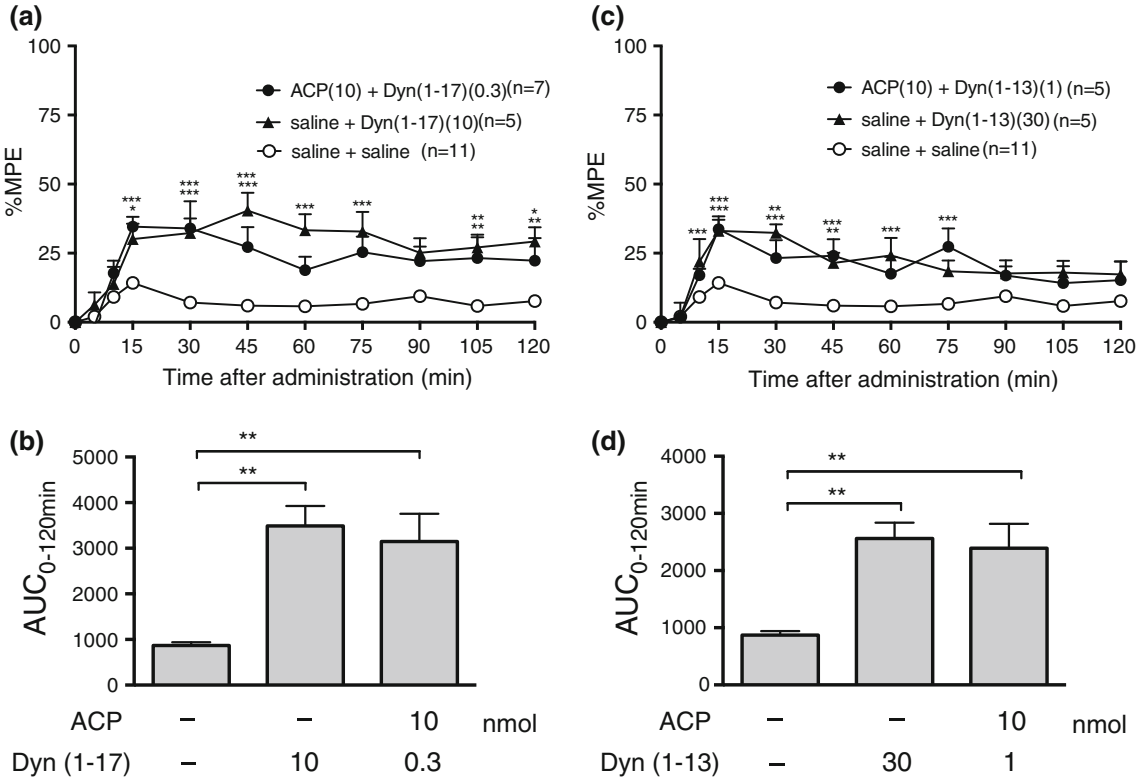


**Fig. 4** Potentiating effect of PIs on antinociception induced by i.c.v. administration of Dyn A (1–17) or Dyn A (1–13). *Upper panel* (**a**) indicates time course of % MPE of Dyn A (1–17) (0.3 nmol) under pretreatment with ACP (10 nmol each) and Dyn A (1–17) (10 nmol) under pretreatment with saline. *Upper panel* (**c**) indicates time course of % MPE of Dyn A (1–13) (1 nmol) under pretreatment with ACP (10 nmol each) and Dyn A (1–13) (30 nmol) under pretreatment with saline. Significantly different from saline–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* (**b**) and (**d**) shows $AUC_{0-120\ min}$ for value of % MPE indicated in *upper panel* (**a**) and (**c**), respectively. Significantly different from saline–saline-treated control according to Dunn's post hoc test following Kruskal–Wallis test, $^{**}P < 0.01$

$AUC_{0-120\ min}$ value of Dyn A (1–13) at doses of 1 or 3 nmol under pretreatment with a mixture of the three PIs indicate significant differences compared to that of Dyn A (1–13) under pretreatment with saline (Fig. 3c). Asterisks placed over $AUC_{0-120\ min}$ values for Dyn A (1–13) at doses of 1 or 3 nmol under pretreatment with a mixture of the three PIs indicate significant differences compared with saline under pretreatment with a mixture of the three PIs (Fig. 3c). The antinociceptive effect of i.c.v. administration of 1 nmol Dyn A (1–13) with a mixture of the three PIs (10 nmol each) had the same onset, offset, and duration of action as that with 30 nmol Dyn A (1–13) alone (Fig. 4c). The $AUC_{0-120\ min}$ value for % MPE of 1 nmol Dyn A (1–13) with a mixture of the three PIs (10 nmol each) was approximately equal to that for 30 nmol Dyn A (1–13) alone (Fig. 4d). Thus, i.c.v. administration of Dyn A (1–13) under i.c.v. pretreatment with the three PIs (10 nmol each) induced a 30-fold increase in the antinociceptive effect on the tail-flick response. At 30 nmol Dyn A (1–13) in the absence of PIs, 1 out of 6 rats died and 1 rat exhibited barrel rotation. These results indicate that the antinociceptive potency of Dyn A (1–17) may increase by >30-fold under pretreatment with a mixture of the three PIs without toxicity.

## Effects of PHMB on Dyn A (1–17)-induced toxicity and antinociception

At 1 nmol Dyn A (1–17) with PHMB and the three PIs, 1 out of 6 rats exhibited barrel rotation within 5 min. At 1 nmol Dyn A (1–17) with PHMB, none of 6 rats died or exhibited barrel rotation. The antinociceptive potency of 1 nmol Dyn A (1–17) under pretreatment with PHMB and PIs was significantly higher than that of 1 nmol Dyn A (1–17) with PIs (Fig. 5). Furthermore, the antinociceptive potency of 1 nmol Dyn A (1–17) under pretreatment with PIs was significantly higher than that of 1 nmol Dyn A (1–17) with PHMB (Fig. 5).

## Effects of PIs on Dyn A (1–6)-induced antinociception

Figures 6a and b show change over time in Dyn A (1–6)-induced antinociception from 10 min following i.c.v. administration of saline or a mixture of the three PIs

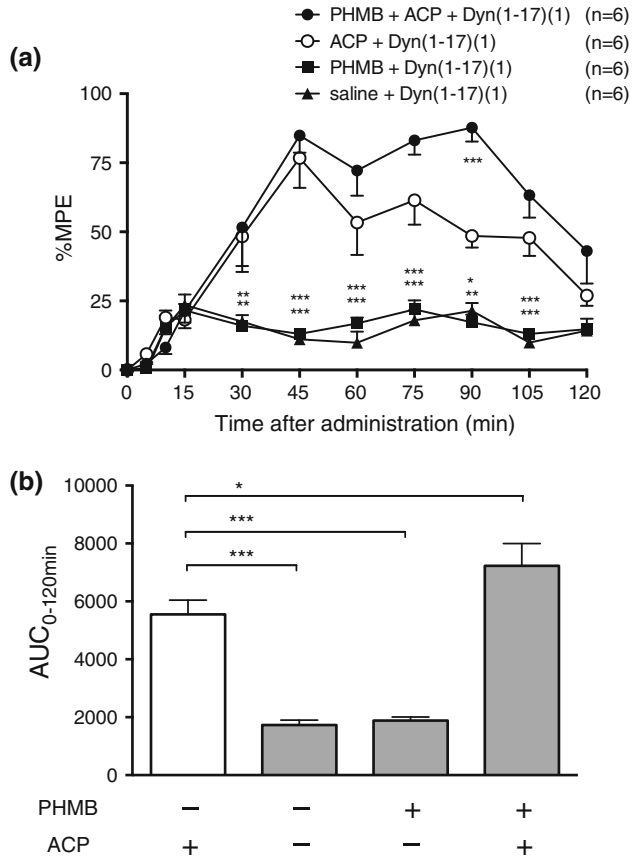


**Fig. 5** Dose-dependent antinociception by i.c.v. administration of Dyn A (1–17) under pretreatment with saline or a mixture of three PIs (ACP) along with/without PHMB. *Upper panel* (**a**) indicates time course of % MPE of Dyn A (1–17) (1 nmol) under pretreatment with saline and ACP with/without PHMB, respectively. Significantly different from saline−ACP-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* (**b**) shows $AUC_{0–120\ min}$ for value of % MPE indicated in *upper panel* (**a**). Significantly different from saline−ACP-treated control according to Dunn's post hoc test following Kruskal–Wallis test, $^{*}P < 0.05$ and $^{***}P < 0.001$

(10 nmol each), respectively. The results showed a dose-dependent and prolonged antinociceptive effect of Dyn A (1–6) on the tail-flick response. The $AUC_{0–60\ min}$ value demonstrated that induction of antinociception by Dyn A (1–6) at a dose of 10 or 30 nmol under pretreatment with a mixture of the three PIs was significantly greater than that with the PIs alone (Fig. 6c). Sharp symbols on the $AUC_{0–60\ min}$ value of Dyn A (1–6) at doses of 3, 10, or 30 nmol under pretreatment with a mixture of the three PIs indicate significant differences compared to that of Dyn A (1–6) under pretreatment with saline (Fig. 6c). Asterisks placed over the $AUC_{0–60\ min}$ values for Dyn A (1–6) at doses of 10 or 30 nmol under pretreatment with a mixture of the three PIs indicate significant differences compared with saline under pretreatment with a mixture of the three PIs (Fig. 6c).

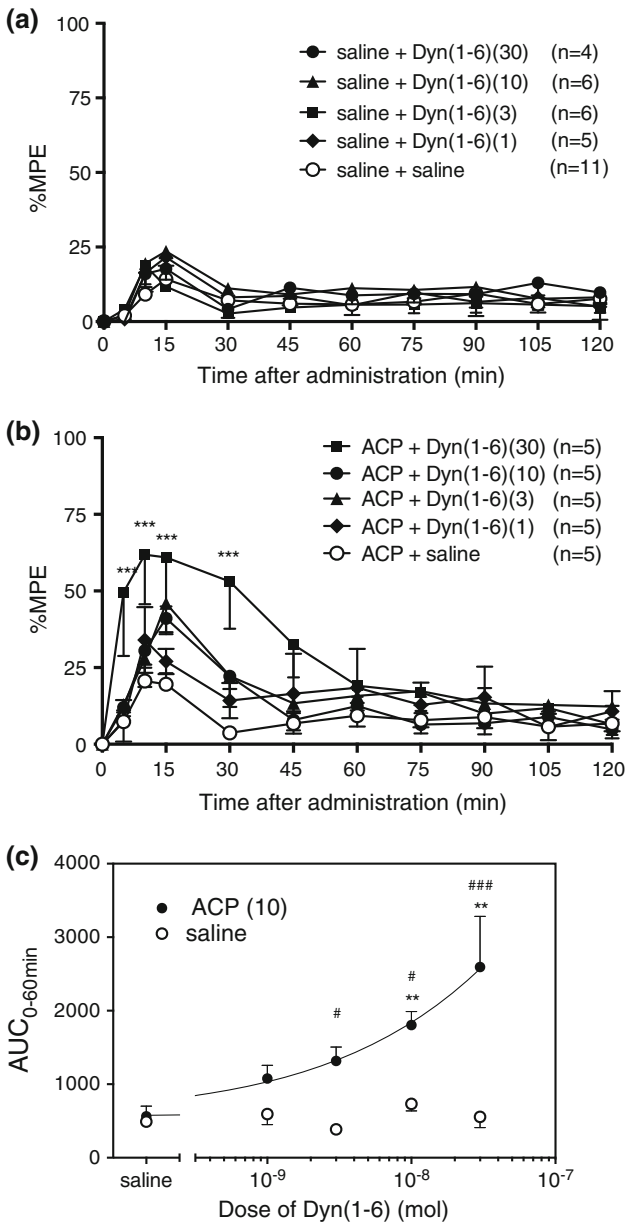


**Fig. 6** Dose-dependent antinociception by i.c.v. administration of Dyn A (1–6) under pretreatment with saline or mixture of the three PIs (ACP). *Upper* (**a**) and *middle panels* (**b**) indicate time course of % MPE of Dyn A (1–6) (1–30 nmol) under pretreatment with saline and ACP, respectively. Significantly different from saline–saline-treated control in Dunn's post hoc test following two-way repeated measures ANOVA; $^{***}P < 0.001$. *Lower panel* (**c**) shows $AUC_{0–60\ min}$ for value of % MPE indicated in *upper* (**a**) and *middle panels* (**b**). Significantly different from saline–saline or ACP−saline-treated control according to Dunn's post hoc test following Kruskal–Wallis test; $^{**}P < 0.01$. Significantly different from under pretreatment with saline according to Dunn's post hoc test following two-way repeated measures ANOVA, $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$

## Effect of each PI alone, in paired combinations, and all together on Dyn A (1–17)-induced antinociception

The administration of A, C or P alone did not potentiate the magnitude of 1 nmol Dyn A (1–17)-induced

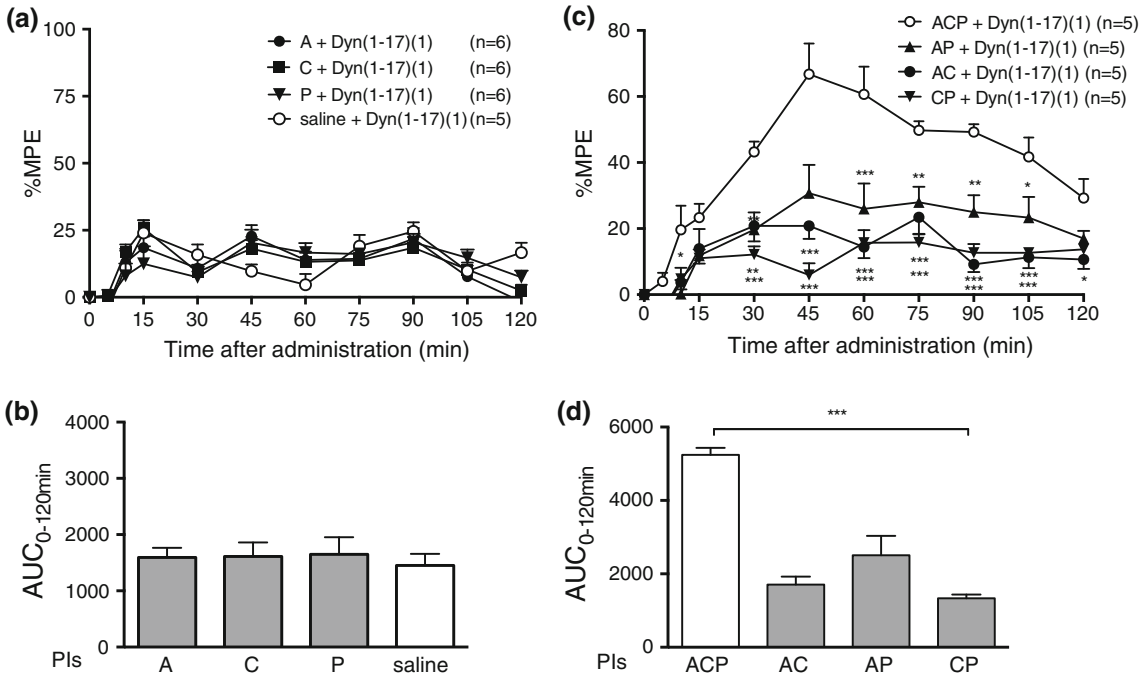


**Fig. 7** Comparison of effect of single PI (*A* amastatin, *C* captopril, *P* phosphoramidon, 10 nmol each) or combination of two PIs (*AC* amastatin and captopril, *CP* captopril and phosphoramidon, *AP* amastatin and phosphoramidon, 10 nmol each) and mixture of three PIs (*ACP* amastatin, captopril and phosphoramidon, 10 nmol each) on antinociception induced by i.c.v. administration of Dyn A (1–17). *Upper panel* (**a**) and (**c**) indicates time course of % MPE of Dyn A (1–17) (1 nmol) and pretreatment with single PI and combination of two PIs, respectively. Significantly different from saline-treated control or a mixture of three PIs-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* (**b**) and (**d**) shows $AUC_{0–120\ min}$ for value of % MPE indicated in *upper panel* (**a**) and (**c**), respectively. Significantly different from a mixture of three PIs-treated control according to Dunn's post hoc test following Kruskal–Wallis test, $***P < 0.001$

antinociception (Fig. 7). The antinociceptive potency of Dyn A (1–17) pretreatment with a mixture of the three PIs was significantly higher than that with the combination of CP; it was also higher than that with AC or AP, but not significantly so (Fig. 7). These results indicate that any residual single peptidase inactivates substantial amounts of Dyn A (1–17) in the rat.

### Effects of antagonists on Dyn A (1–17)-, Dyn A (1–13)-, or Dyn A (1–6)-induced antinociception under pretreatment with a mixture of the three PIs

The antinociceptive potency of Dyn A (1–17) or Dyn A (1–13) under pretreatment with PIs was significantly attenuated by NOX, CTOP, NTI or nor-BNI (Figs. 8, 9). The antinociceptive potency of Dyn A (1–6) under pretreatment with PIs was significantly attenuated by CTOP; it was also attenuated by NTI, but not significantly (Fig. 10).

## Discussion

The present study showed that the antinociceptive potential and toxicity of Dyn A (1–17), Dyn A (1–13), Dyn A (1–6), or Dyn A (7–17) in the absence of PIs or under pretreatment with a mixture of the three PIs depended on the dose and length of the peptide. This is in good agreement with previous studies showing that Dyn A (1–17) had a greater analgesic effect than Dyn A (1–13) in the absence of PIs [22], and that Dyn A (1–17) or its fragments caused dose-dependent toxicity, with toxic potential ranked in the order of Dyn A (1–17) > Dyn A (1–13) > Dyn A (13–17) in the absence of PIs [10].

The results of the present in vivo study showed that the antinociceptive potency of Dyn A (1–17) under pretreatment with a mixture of the three PIs together with PHMB was significantly higher than that with a mixture of the three PIs alone. This is in good agreement with MALDI–TOF–MS analysis in the present and earlier study [3], which identified Dyn A (1–6) fragments as one of the products following incubation of Dyn A (1–17) with extract of rat midbrain and caudate putamen under pretreatment with a mixture of the three PIs, respectively. In addition to Dyn A (1–6), MALDI–TOF–MS analysis in the present and earlier study [3] identified Dyn A (1–10) fragment as one of the products following incubation of Dyn A (1–17) with the extract. This peptide fragment should be involved in antinociception induced by Dyn A

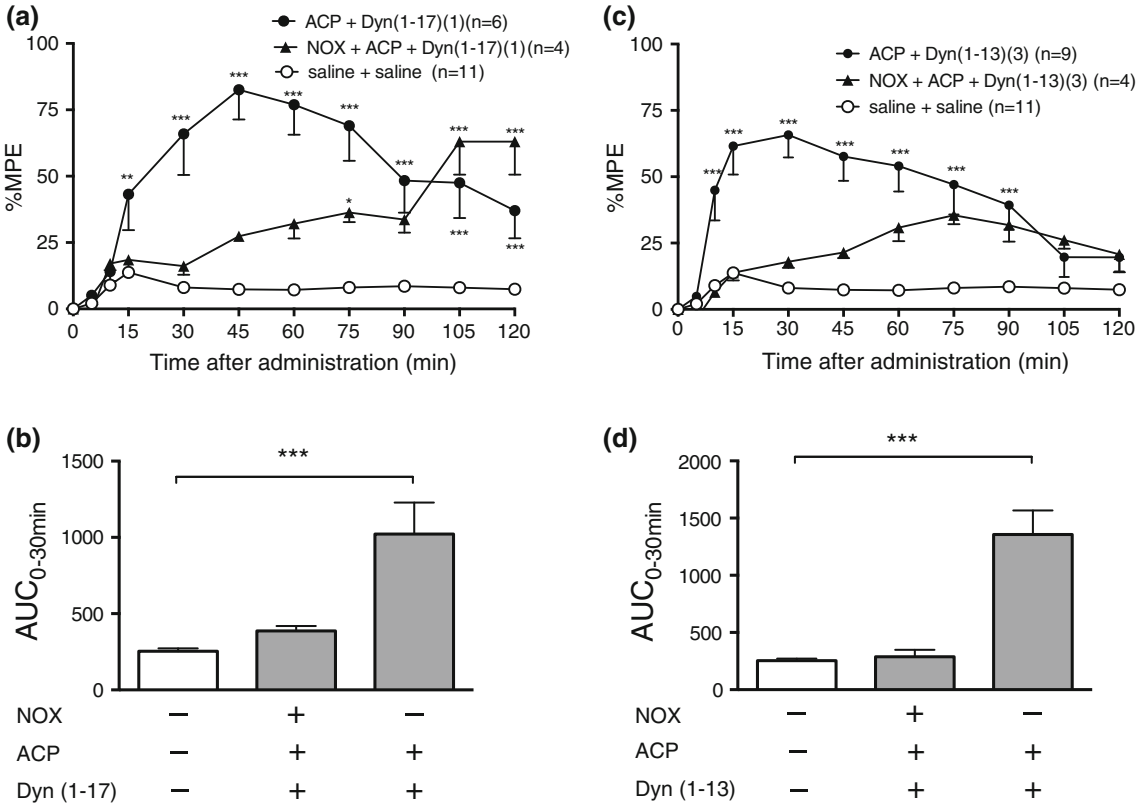


**Fig. 8** Effect of NOX, a non-selective opioid receptor antagonist, on the antinociceptive potency of Dyn A (1–17) or Dyn A (1–13) under pretreatment with a mixture of the three PIs. *Upper panel* (**a**) and (**c**) indicate time course of % MPE of Dyn A (1–17) (1 nmol) and Dyn A (1–13) (3 nmol)-induced antinociception under pretreatment with PIs following administration of NOX (1 mg/kg), respectively. Significantly different from saline-administered 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* (**b**) and (**d**) shows $AUC_{0-60\ min}$ for value of % MPE indicated in *upper panel* (**a**) and (**c**), respectively. Significantly different from saline-administered group according to Dunn's post hoc test following Kruskal–Wallis test, $***P < 0.001$

(1–17) under pretreatment with a mixture of the three PIs, although the enzyme cleaving at $Pro^{10}$–$Lys^{11}$ bond remains to be determined. Further study needs to evaluate its antinociceptive effects and selectivity for opioid peptide receptor types in vivo and to elucidate the enzyme.

MALDI–TOF–MS analysis in the present and earlier study [3] identified intact (e.g., non-degraded) Dyn A (1–17) following incubation of Dyn A (1–17) with extract of rat midbrain and caudate putamen under pretreatment with a mixture of the three PIs, respectively. These suggest that pretreatment with a mixture of the three PIs results in substantial amounts of residual intact Dyn A (1–17) or Dyn A (1–13) and production of peptide fragments from their N- and C-terminal regions in an in vivo animal model. This may explain why a mixture of the three PIs produces an approximately 30-fold increase in the analgesic potency of Dyn A (1–17) or Dyn A (1–13) with no signs of toxicity. Another possible explanation is that intact Dyn A (1–17) or Dyn A (1–13) has a higher binding affinity to opioid receptors than to NMDA receptors, as demonstrated in previous studies, in which Dyn A (1–13) showed high affinity ($IC_{50}$ $7 \times 10^{-10}$ M) to μ-opioid receptors in guinea pig ileum [23] and Dyn A (1–17) showed low affinity ($IC_{50}$ $5 \times 10^{-7}$ M) to NMDA receptors in rat striatum [24].

Interestingly, the MALDI–TOF–MS analysis in the present and earlier study [3] identified the N-terminal peptide fragment Dyn A (1–6) of Dyn A (1–17), but not the corresponding C-terminal peptide fragment Dyn A (7–17). In addition, we confirmed that MALDI used in this study could ionize Dyn A (7–17) (data not shown). The absence of Dyn A (7–17) is supported by another study which evaluated the metabolism of Dyn A (1–17) in rat striatum by using microdialysis [25]. These results suggested that C-terminal peptide fragments such as Dyn A (7–17) were catabolized to generate shorter products that are extremely weak, with no signs of toxicity, albeit under pretreatment with a mixture of the three PIs. This indicates that a mixture of the three PIs produces an approximately 30-fold increase in the analgesic potency of Dyn A without signs of toxicity.

MALDI–TOF–MS analysis in the present study identified Dyn A (1–6) and Dyn A (1–10), but not other

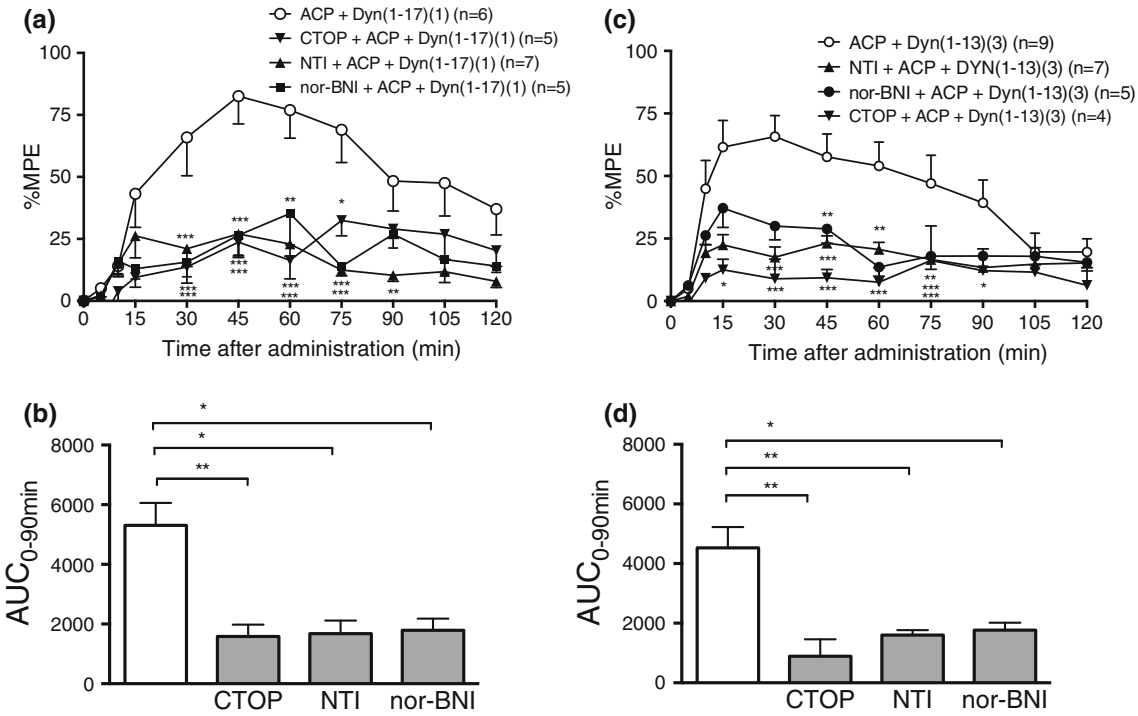


**Fig. 9** Effects of opioid receptor selective antagonists on the antinociceptive potency of Dyn A (1–17) (1 nmol) or Dyn A (1–13) (3 nmol) under pretreatment with PIs. *Upper panel* (**a**) and (**c**) indicate time course of % MPE of Dyn A (1–17) and Dyn A (1–13)-induced antinociception under pretreatment with PIs following administration of the three opioid receptor antagonists CTOP (3 nmol), nor-BNI (20 mg/kg), or NTI (132 nmol), respectively. Significantly different from Dyn A (1–17) or Dyn A (1–13) under pretreatment with PIs 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* (**b**) and (**d**) shows $AUC_{0-90\ min}$ for value of % MPE indicated in *upper panel* (**a**) and (**c**), respectively. Significantly different from Dyn A (1–17) or Dyn A (1–13) under pretreatment with PIs according to Dunn's post hoc test following Kruskal–Wallis test, $*P < 0.05$ and $**P < 0.01$

N-terminal peptide fragments of Dyn A (1–17) in rat midbrain. This is not in agreement with the results of the earlier MALDI–TOF–MS analysis study showing detection of Dyn A (1–7), Dyn A (1–11), Dyn A (1–12) and Dyn A (1–13) in rat caudate putamen [3]. This discrepancy may be related to differences in enzymatic activity, such as A-, C- or P-sensitive peptidases and DCE, between the regions [3]. Further study are needed to elucidate the reason.

To our knowledge, the present study is the first to comprehensively assess the antinociceptive potential of Dyn A (1–17) under pretreatment with single PIs or in combinations of two or three with or without a DCE inhibitor. The antinociceptive potency of Dyn A (1–17) under pretreatment with a mixture of the three PIs was significantly higher than that of Dyn A (1–17) with PHMB. No single PI significantly increased the antinociceptive potency of Dyn A (1–17). Moreover, the antinociceptive potency of Dyn A (1–17) under pretreatment with a mixture of the three PIs was higher than that with any pair of the three PIs. These results demonstrate that a mixture of the three PIs is required to inhibit degradation of intact Dyn A (1–17) in rat brain, and that any residual single peptidase inactivates substantial amounts of Dyn A (1–17).

The present results showed that administration of PHMB or P alone did not potentiate antinociception after i.c.v. administration of Dyn A (1–17). This is not in agreement with the results of an earlier study using the mouse formalin test [26]. This discrepancy may be related to differences in the nociceptive stimulus employed. Several lines of evidence support this possibility. First, low-level stimulation of nociception such as that involved in the second phase of the formalin test may evoke capsaicin-sensitive C-fiber-mediated responses, whereas the acute pain arising from higher-level stimulation such as that involved in skin heating may result in the recruitment of capsaicin-insensitive Aδ-fibers [27]. Second, Aδ or C fiber nociceptors fall under a different descending control path from the nucleus raphe magnus [28]. Third, the potency of morphine has been shown to be dependent on the nociceptive test used, even when all other factors such as species, strain, age, sex, and cut-off value are held constant [29].

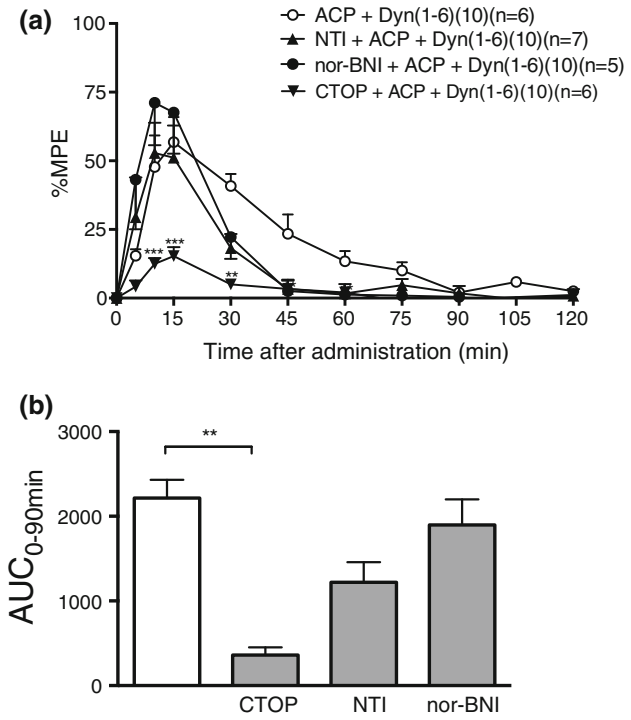


**Fig. 10** Effects of opioid receptor selective antagonists on the antinociceptive potency of Dyn (1–6) under pretreatment with PIs. *Upper panel* (**a**) indicates time course of % MPE of Dyn (1–6) (10 nmol)-induced antinociception under pretreatment with PIs following administration of the three opioid receptor antagonists CTOP (3 nmol), nor-BNI (20 mg/kg), or NTI (66 nmol). Significantly different from Dyn A (1–6) under pretreatment with PIs according to Dunn's post hoc test following two-way repeated measures ANOVA, $***P < 0.001$. *Lower panel* (**b**) shows $AUC_{0–90\ min}$ for value of % MPE indicated in *upper panel* (**a**). Significantly different from Dyn A (1–6) under pretreatment with PIs according to Dunn's post hoc test following Kruskal–Wallis test, $**P < 0.01$

A competition radioligand binding assay showed that Dyn A bound to κ-opioid receptors with somewhat higher affinity than to μ-opioid or δ-opioid receptors, indicating that it is an endogenous ligand for κ-opioid receptors [8]. The N-terminal region peptide fragments of Dyn A such as LE [6] and Dyn A (1–8) [7] act mainly on μ-opioid receptors under pretreatment with the three PIs. As observed in the present study, Dyn A (1–6) also acts mainly on μ-opioid receptors under pretreatment with the three PIs, whereas Dyn A (1–17) or Dyn A (1–13) acts on μ-opioid, δ-opioid and κ-opioid receptors. These results are in good agreement with previous studies showing that the N-terminal region of Dyn A acts on μ-opioid and δ-opioid receptor activity, whereas the C-terminal and central region of Dyn A acts on κ-opioid receptor activity [11, 30, 31]. Thus, it appears that the selectivity of Dyn A (1–17) or Dyn A (1–13) for opioid receptors shifts from κ-opioid to μ-opioid receptors following cleavage at its $Arg^6$–$Arg^7$ bonds by DCE. This conversion is interesting given that the effects of μ-opioid receptors differ from those of κ-opioid receptors. In fact, there is a growing body of evidence to suggest that activation of κ-opioid receptors opposes a variety of μ-opioid receptor-mediated actions throughout the brain and spinal cord [32]. The results of the present study may contribute to clinical and experimental knowledge demonstrating that functional interaction among μ-opioid, δ-opioid and κ-opioid receptor types involves in opioid dependence, tolerance, etc. 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 low-dose i.c.v. administration of Dyn A (1–17) or Dyn A (1–13) under pretreatment with a mixture of PIs increased antinociception 30-fold, but without signs of toxicity in rat. This antinociception was mediated by μ-, δ- and κ-opioid receptors. The antinociceptive potency and toxicity of Dyn A (1–17), Dyn A (1–13), or their peptide fragments depended on their dose and length. The results of the present study indicate that inactivation of A-, C-, or P-sensitive enzymes leads to an increase in low-dose Dyn A (1–17)- or Dyn A (1–13)-induced antinociception without toxicity.

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