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Effect of Peptidase Inhibitors on Dynorphin A (1-17) or (1-13)-Induced Antinociception and Toxicity at Spinal Level

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Abstract

Our group has earlier demonstrated that three enzymes sensitive to peptidase inhibitors (PIs), amastatin (A)-, captopril (C)-, and phosphoramidon (P), played an important role in inactivation of enkephalins at the spinal level. Dynorphin-converting enzyme (DCE) hydrolyzes dynorphin (Dyn) A (1-17) or Dyn A (1-13) mainly at the Arg^6-Arg^7 bond. Dynorphin A and its derived peptides interact with opioid and glutamate receptors at their N- and C-terminals, respectively. The purpose of the present study was to evaluate the antinociceptive potency and toxicity of intrathecal administered Dyn A (1-17), Dyn A (1-13), or Dyn A (1-6) under pretreatment with ACP and/or the DCE inhibitor p-hydroxymercuribenzoate (PHMB). The effect of these PIs on Dyn A (1-17)-induced inhibition of electrically-evoked contractions in mouse vas deferens was also investigated. The inhibitory potency of Dyn A (1-17) on electrically-evoked contractions in mouse vas deferens under pretreatment with ACP was higher than that with AC, AP, or CP. Pretreatment with ACP augmented Dyn A (1-17) or (1-13)-induced antinociception by approximately 50- or 30-fold with no sign of allodynia when administered intrathecally at low doses. Pretreatment with ACP and PHMB induced neuropathy. These findings showed that intrathecal administration of low-dose Dyn A (1-17) or DynA (1-13) increased antinociception under pretreatment with ACP, but without signs of allodynia in rat.

Keywords

Dynorphin A, Peptidase, Dynorphin-Converting Enzyme, Antinociception, Allodynia

1. Introduction

Dynorphin (Dyn) A (1-17) and (1-13) interacts with both opioid and N-methyl-D-aspartate (NMDA) receptors: their N-terminals activate the former with high affinity, while its C-terminals activate the latter with low affinity [1] [2] [3] [4]. In fact, intrathecal (i.t.) low dose (0.5 - 2 nmol) administration of Dyn A induced an antinociceptive effect [5] [6], whereas that at a high dose induced long-lasting mechanical allodynia and motor dysfunction [7] [8] [9] [10].

Three peptidases, an aminopeptidase N (APN), a dipeptidylcarboxypeptidase, and neutral endopeptidase-24.11 (NEP), play an important role in degradation of opioid peptides. High-performance liquid chromatography revealed that [Leu^5]-enkephalin (LE) [11] or Dyn A (1-8) [12] remained intact in the presence of a mixture of peptidase inhibitors (PIs) when incubated with membrane preparation, but was completely hydrolyzed after incubation in their absence.

Preparations isolated *in vitro* allow a drug to be quantified and its efficacy, potency, and affinity compared more accurately than can be done with *in vivo* methods [13]. Earlier research on the inhibitory potency of LE or Dyn A (1-8) against electrically-evoked contractions in mouse vas deferens (MVD) demonstrated that it was enhanced by exposure to various combinations of amastatin (A), captopril (C), and phosphoramidon (P) [14] [15]. These results correspond with the results of the previous *in vivo* studies showing that intracerebroventricular (i.c.v.) administration of ACP increased LE-, Dyn A (1-8)-, and Dyn A (1-17)-induced antinociception by more than 500- [14], 100- [16], and 30-fold [17], respectively.

Dynorphin-converting enzyme (DCE) hydrolyzes Dyn A mainly at the Arg^6-Arg^7 bond, resulting in the production of N- and C-terminal region peptide fragments, and this enzyme is not inhibited by ACP [18]. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) identified N-terminal peptide fragment Dyn A (1-6) from Dyn A (1-17), but not the corresponding C-terminal peptide fragment, Dyn A (7-17), after incubation of Dyn A (1-17) with membrane fraction from rat midbrain or caudate putamen under pretreatment with ACP [17] [19]. These results suggest that C-terminal peptide fragments such as DynA (7-17) are catabolized, generating shorter, extremely weak, non-toxic products, particularly under pretreatment with ACP [17].

The purpose of the present study was to evaluate antinociceptive potency and toxicity with i.t. administration of Dyn A (1-17), Dyn A (1-13), or Dyn A (1-6) under pretreatment with ACP and/or DCE inhibitor p-hydroxymercuribenzoate (PHMB) in rat spinal cord under physiological conditions. In an attempt to further characterize the pharmacological action of PIs, the effect of ACP on Dyn A (1-17)-induced inhibition of electrically-evoked contractions in MVD was also evaluated.

2. Materials and Methods

The present animal experiments were performed in strict accordance with the guidelines (http://www.u-tokai.ac.jp/about/concept/guidance.html) of Tokai Uni-

versity and with the approval of the Animal Investigation Committee of this institute.

2.1. Chemicals

Dynorphin 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). Captopril, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP, a μ-opioid receptor antagonist), nor-binaltorphiminedihydrochloride (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 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. The PHMB was dissolved in saline with 0.1 N-NaOH up to a pH of 9.0. The solution for all drugs used was prepared to the desired concentration just before use. In accordance with the method of earlier studies, CTOP (3 nmol, i.t.), nor-BNI (20 mg/kg, subcutaneously), and NTI (66 nmol, i.t.) were injected 15 min, 30 min, and 24 h, respectively, before i.t. administration of the PIs [20] [21] [22] [23].

2.2. *In Vitro* Isolated Preparations

Male ICR JCL mice (30 - 40 g each; Nihon Clea, Tokyo, Japan) were used. Mouse vas deferens was harvested and prepared for electrical stimulation as described previously [24]. The percent (%) inhibition of stimulated muscle twitch produced by each opioid was plotted against its log concentration to determine the IC_{50} (concentration required to produce 50% inhibition of the twitch). When investigating the effect of PIs on Dyn A (1-17)-induced inhibition of contractions, a period of 3 min was given before administration of Dyn A (1-17). The % difference shown in the tables was calculated as follows: % difference = [(IC_{50} before each treatment − IC_{50} after each treatment)/IC_{50} before each treatment] × 100 [24].

2.3. Intrathecal Administration

Intrathecal catheters were implanted in Male Wistar rats (180 - 220 g each; Nihon Clea, Tokyo, Japan) under inhalation anesthesia with nitrous oxide, oxygen, and isoflurane (2%) as described previously [17] [25]. 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 experiments 7 days later. Drugs were injected at a volume of 10 μl followed by 10 μl saline over 1 min.

2.4. 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 [26] [27]. The latency to flick the tail was measured as described previously [17]. A cut-off time of 5 sec was used to prevent any injury to the tail. The % 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 each drug was also calculated in some of the experiments.

2.5. Von Frey Test

The threshold for tactile allodynia was measured with a series of von Frey filaments (von Frey Filaments; Bioseb, Vitrolles, France), ranging from 2.44 to 5.88 (0.03 - 60.0 g), according to the methods of Park *et al.* [28] and Zhu *et al.* [29]. The rats were placed in individual transparent plastic boxes with a wire mesh floor at least 15 min before testing began to allow acclimatization to the environment. The filaments were pushed against the plantar surface of the right hind paw. Tactile thresholds were measured at intervals before and after administration of drugs. Results were reported as the mean value of 4 readings from the right hind paw in each rat.

2.6. Animal Experimental Protocol

2.6.1. Dyn A-Dependent Antinociception with or without ACP by Dose

Ten minutes following i.t. administration of ACP or saline, Dyn A (1-17), Dyn A (1-13), Dyn A (1-6), or saline was administered by the same route. To determine whether the antinociceptive effect increased with i.t. administration of ACP (10 nmol each), the rats were tested in the following groups: Group 1, Dyn A (1-17) (0.03 - 1 nmol) alone or with ACP; Group 2, Dyn A (1-13) (0.1 - 3 nmol) alone or with ACP; and Group 3, Dyn A (1-6) (0.3 - 3 nmol) alone or with ACP.

2.6.2. Combination of Dyn A (1-17) and PHMB Together with ACP

Ten minutes following i.t. administration of PHMB and ACP, Dyn A (1-17) (0.3 nmol) was administered intrathecally. To determine whether the antinociceptive effect of Dyn A (1-17) was increased by joint administration of PHMB and ACP, the rats were tested in the following groups: Group 1, Dyn A (1-17) alone; Group 2, Dyn A (1-17) with ACP; Group 3, Dyn A (1-17) with PHMB; and Group 4, Dyn A (1-17) in combination PHMB and ACP.

2.6.3. Selective or Non-Selective Opioid Receptor Antagonists

To investigate the effect of opioid receptor antagonists on Dyn A (1-17) (0.3 nmol) or DynA (1-13) (0.3 nmol)-induced antinociception under pretreatment with ACP, NOX (1 mg/kg, subcutaneously), CTOP (3 nmol, i.t.) [22], NTI (66 nmol, i.t.) [21], and nor-BNI (20 mg/kg, subcutaneously) [23] were injected at 20 min, 15 min, 30 min, and 24 hr, respectively, before i.t. administration of Dyn A (1-17) or Dyn A (1-13).

2.7. Statistical Analyses

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 6.0 c, Graph Pad Software, San Diego, CA, USA) for a comparison across 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), Dunn's multiple comparison test was applied to determine the significance at each time point. When a significant difference was observed in the AUC data among the groups in a two-way (drugs and dose) repeated 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.

3. Results

3.1. Enhanced Effect of PIs in Paired Combinations or all Together on Dyn A (1-17)-Induced Inhibition in Isolated Preparation

The results showed that Dyn A (1-17) significantly inhibited electrically-evoked contractions in MVD. The inhibitory potency of Dyn A (1-17) was dose-dependently augmented by ACP (Table 1). Administration of paired combinations (2 μM each of AP, AC, or CP) or all three PIs together (2 μM ACP) revealed that any two combination or ACP increased Dyn A (1-17)-induced inhibition of electrically-evoked contractions in MVD. This effect was significantly stronger with ACP than with CP; it was also stronger than with administration of AP or AC, but not significantly so (Table 2).

Table 1. IC_{50} values and ratio of potency of Dyn A (1-17) under pretreatment of ACP.

ACP (μM each)	IC_{50} (nM)	Ratio of potency
0	11.37 ± 3.97	
1	4.53 ± 2.11	2.77 ± 1.54
2	3.18 ± 0.97	3.51 ± 1.27*
5	3.15 ± 1.42	3.81 ± 1.74*

Each value represents mean ± S.E.M. of data obtained from four MVD. The ratio of potency was significantly increased in 2 or 5 μM ACP as compared to these in 0 μM ACP; *$P < 0.05$ by Dunn's post-hoc test following Kruskal-Wallis test.

Table 2. IC_{50} values and ratio of potency of Dyn A (1-17) under pretreatment of combination of PIs.

PIs (2 μM each)	IC_{50} (nM)	Ratio of potency
None	11.37 ± 3.97	
ACP	3.18 ± 0.97	3.51 ± 1.27
AP	3.90 ± 1.53	2.88 ± 0.86
AC	4.64 ± 1.14	2.30 ± 0.53
CP	6.10 ± 1.85	1.80 ± 0.52*

Each value represents mean ± S.E.M. of data obtained from four MVD. The ratio of potency was significantly deceased in CP as compared to ACP; *$P < 0.05$ by Dunn's post-hoc test following Kruskal-Wallis test.

3.2. Effect of ACP on Dyn A (1-17)- or Dyn A (1-13)-Induced Antinociception

Change over time in Dyn A (1-17)-induced antinociception with i.t. administration of saline and ACP is shown in Figure 1(a) and Figure 1(b), respectively.

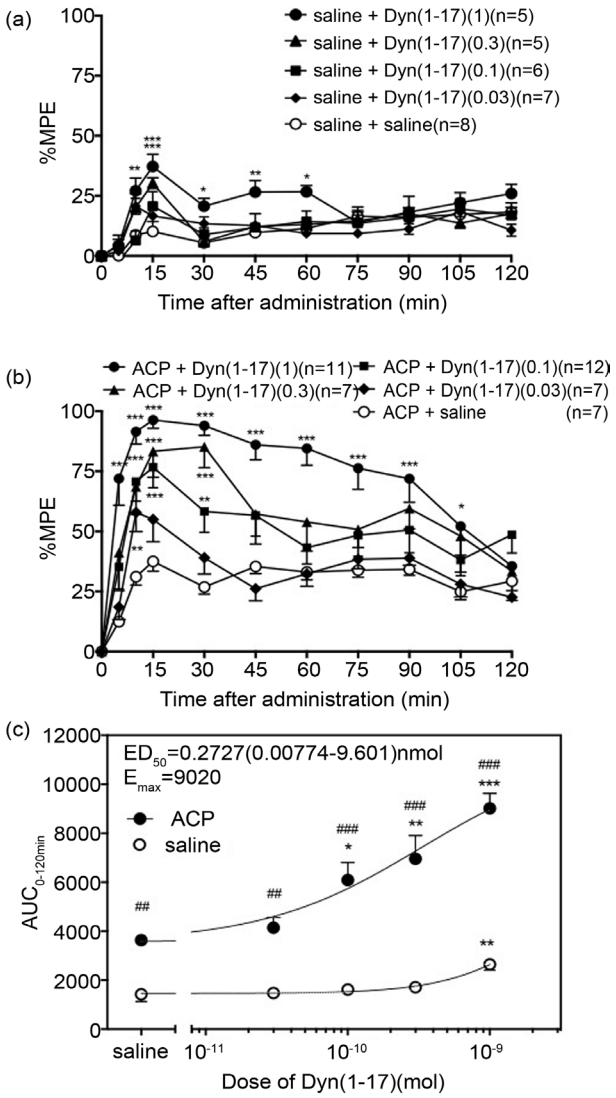

Figure 1. Dose-dependent antinociception by i.t. administration of Dyn A (1-17) under pretreatment with saline or ACP. Upper (a) and middle panels (b) indicate time course of %MPE of Dyn A (1-17) (0.03 - 1 nmol) under pretreatment with saline and ACP, respectively. Significantly different from saline-saline 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 $AUC_{0\text{-}120min}$ for value of %MPE indicated in upper (a) and middle panels (b). Where asterisks have been placed above $AUC_{0\text{-}120min}$ values for Dyn A (1-17) under pretreatment with ACP, this indicates significant differences in comparison with for saline alone or saline under pretreatment with ACP according to Dunn's post-hoc test following the Kruskal-Wallis test; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Where sharp symbols have been placed above $AUC_{0\text{-}120min}$ values for Dyn A (1-17) under pretreatment with ACP, this indicates significant differences in comparison with those for Dyn A (1-17) under pretreatment with saline according to Dunn's post-hoc test following two-way repeated measures ANOVA; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

Change over time in Dyn A (1-13)-induced antinociception after i.t. administration of saline and ACP is shown in Figure 2(a) and Figure 2(b), respectively. A prolonged and dose-dependent antinociceptive effect was observed on the tail-flick response by administration of Dyn A (1-17) and Dyn A (1-13).

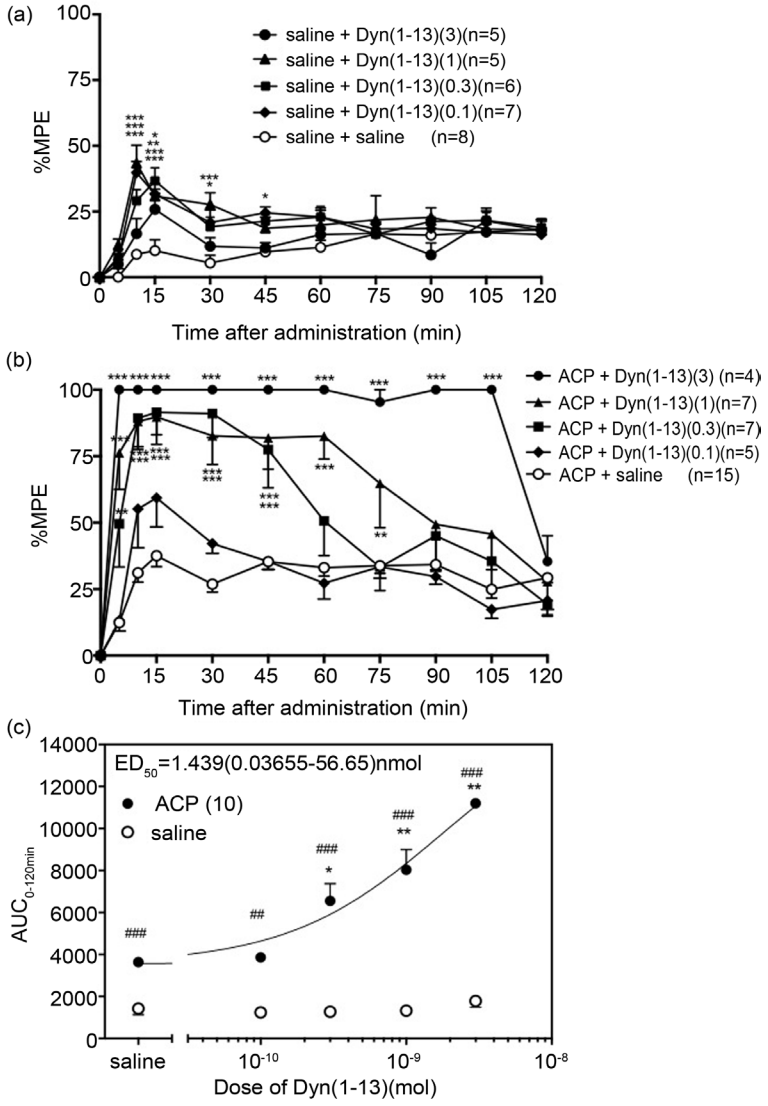

Figure 2. Dose-dependent antinociception by i.t. administration of Dyn A (1-13) under pretreatment with saline or ACP. Upper (a) and middle panels (b) indicate time course of %MPE of Dyn A (1-13) (0.1 - 3 nmol) under pretreatment with saline and ACP, respectively. Significantly different from saline-saline 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 $AUC_{0\text{-}120min}$ for value of %MPE indicated in upper (a) and middle panels (b). Where asterisks have been placed above $AUC_{0\text{-}120min}$ values for Dyn A (1-13) under pretreatment with ACP, this indicates significant differences in comparison with for saline alone or saline under pretreatment with ACP according to Dunn's post-hoc test following the Kruskal-Wallis test; $^{*}P < 0.05$ and $^{**}P < 0.01$. Where sharp symbols have been placed above $AUC_{0\text{-}120min}$ values for Dyn A (1-13) under pretreatment with ACP, this indicates significant differences in comparison with those for Dyn A (1-13) under pretreatment with saline according to Dunn's post-hoc test following two-way repeated measures ANOVA; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

The onset, offset, and duration of the antinociceptive effect induced by i.t. administration of 0.1 nmol Dyn A (1-17) under pretreatment with ACP was the same as that with 5 nmol Dyn A (1-17) alone (Figure 3(a)). Administration of 0.1 nmol Dyn A (1-17) under pretreatment with ACP yielded an $AUC_{0\text{-}120min}$ value for %MPE similar to that with 5 nmol Dyn A (1-17) alone (Figure 3(b)). The onset, offset, and duration of the antinociceptive effect induced by i.t. administration of 0.3 nmol Dyn A (1-13) under pretreatment with ACP was similar to that with 10 nmol Dyn A (1-13) alone (Figure 3(c)). Administration of 0.3 nmol Dyn A (1-13) under pretreatment with ACP yielded an $AUC_{0\text{-}120min}$ value for %MPE similar to that with 10 nmol Dyn A (1-13) alone (Figure 3(d)).

Intrathecal administration of Dyn A (1-17) under pretreatment with ACP induced a 50-fold increase in the antinociceptive effect on the tail-flick response, while that of Dyn A (1-13) increased it 30-fold.

3.3. Effect of ACP on Dyn A (1-6)-Induced Antinociception

Figure 4(a) shows change over time in Dyn A (1-6)-induced antinociception after i.t. administration of saline, while Figure 4(b) shows that with ACP. A

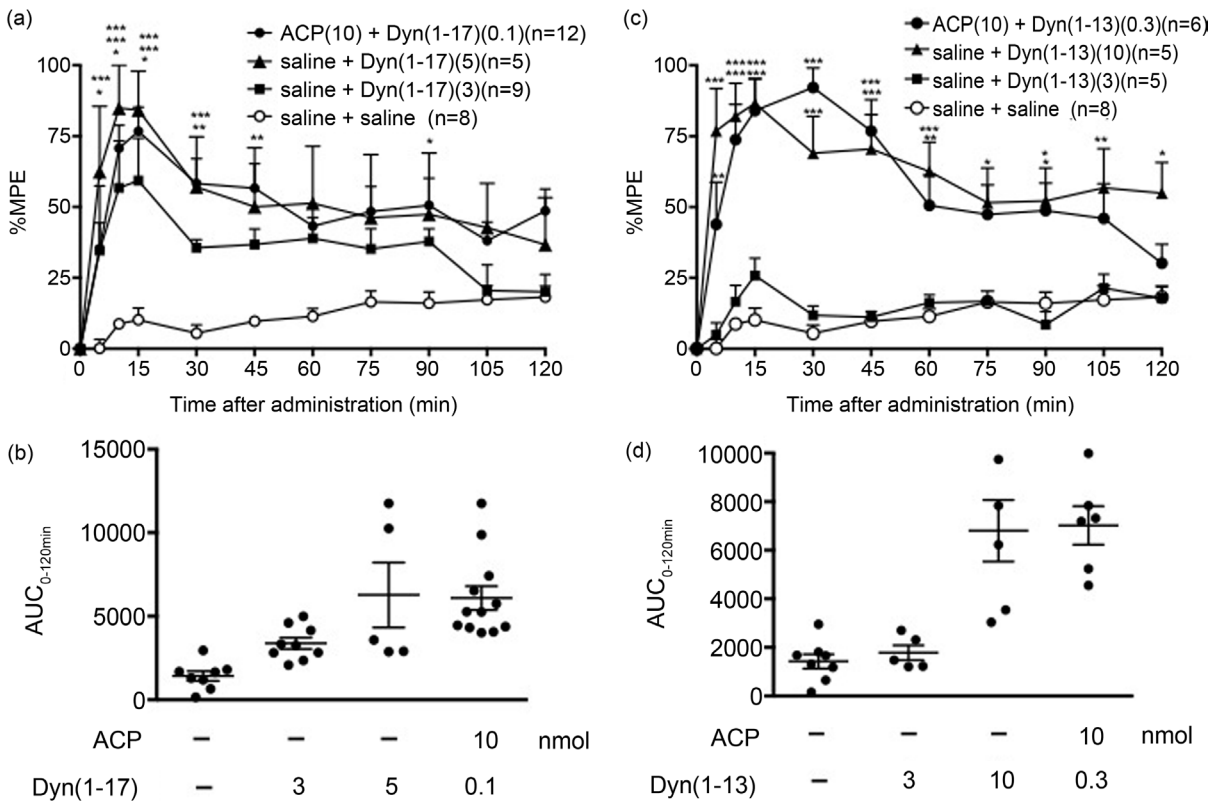

Figure 3. Potentiating effect of ACP on antinociception induced by i.t. administration of Dyn A (1-17) or Dyn A (1-13). Upper panel (a) indicates time course of %MPE of Dyn A (1-17) (0.1 nmol) under pretreatment with ACP (10 nmol each) and Dyn A (1-17) (3 or 5 nmol) under pretreatment with saline. Upper panel (c) indicates time course of % MPE of Dyn A (1-13) (0.3 nmol) under pretreatment with ACP (10 nmol each) and Dyn A (1-13) (3 or 10 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 $AUC_{0\text{-}120min}$ for value of % MPE indicated in upper panel (a) and (c), respectively.

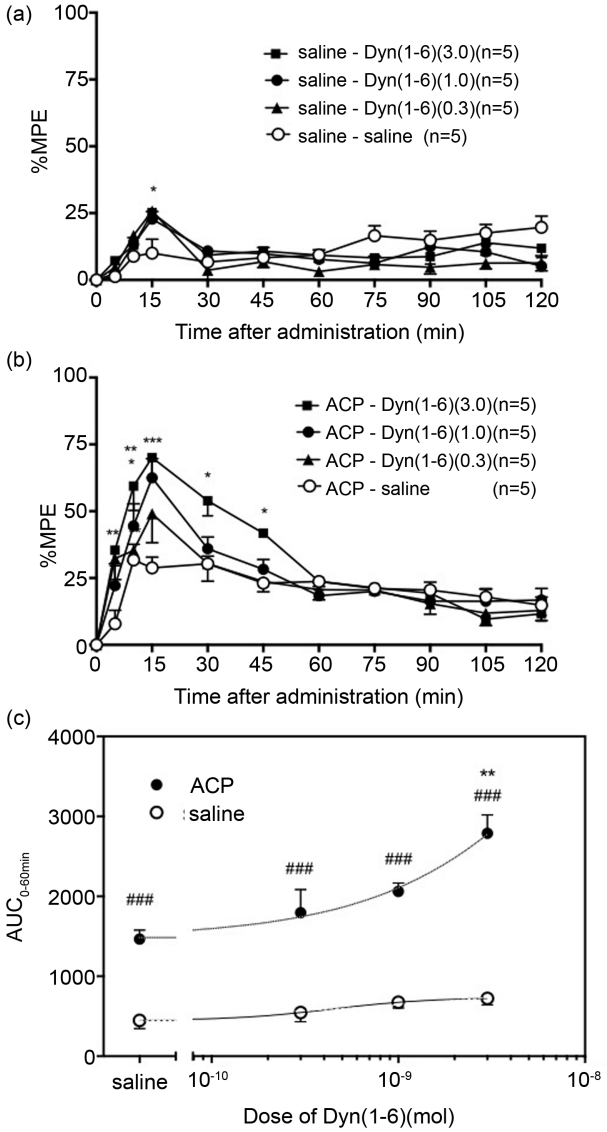

Figure 4. Dose-dependent antinociception by i.t. administration of Dyn A (1-6) under pretreatment with saline or ACP. Upper (a) and middle panels (b) indicate time course of %MPE of Dyn A (1-6) (0.3 - 3 nmol) under pretreatment with saline and ACP, respectively. Significantly different from saline-saline 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 $AUC_{0\text{-}60min}$ for value of %MPE indicated in upper (a) and middle panels (b). Where asterisks have been place above $AUC_{0\text{-}60min}$ values for Dyn A (1-6) under pretreatment with ACP, this indicates significant differences in comparison with saline under pretreatment with ACP according to Dunn's post-hoc test following the Kruskal-Wallis test; $^{**}P < 0.01$. Where sharp symbols have been place above $AUC_{0\text{-}60min}$ values for Dyn A (1-6) under pretreatment with ACP, this indicates significant differences in comparison with that for Dyn A (1-6) under pretreatment with saline according to Dunn's post-hoc test after two-way repeated measures ANOVA; $^{###}P < 0.001$.

prolonged and dose-dependent antinociceptive effect was observed on the tail-flick response with administration of Dyn A (1-6). The $AUC_{0\text{-}60min}$ value demonstrated significantly greater induction of antinociception with 3 nmol Dyn A (1-6) under pretreatment with ACP than with ACP alone (Figure 4(c)).

3.4. Effect of Antagonists on Dyn A (1-17)- or Dyn A (1-13)-Induced Antinociception under Pretreatment with ACP

The antinociceptive potency of Dyn A (1-17) or Dyn A (1-13) under pretreatment with ACP was significantly attenuated by NOX, CTOP, or nor-BNI; it was also was attenuated by NTI, but not significantly so (Figure 5 and Figure 6).

3.5. Effect of PHMB on Dyn A (1-17)-Induced Antinociception

The antinociceptive potency of 0.3 nmol Dyn A (1-17) under pretreatment with ACP was significantly higher than that of 0.3 nmol Dyn A (1-17) alone or with PHMB (10 nmol) (Figure 7). The antinociceptive potency of 0.3 nmol Dyn A (1-17) under pretreatment with PHMB and ACP was approximately equal to that of 0.3 nmol Dyn A (1-17) under pretreatment with ACP alone (Figure 7). Ten minutes following i.t. administration of PHMB and ACP, 0.3 nmol Dyn A (1-17) was administered in 6 rats. Two of the rats showed no abnormal signs as a result. Meanwhile, subtle and transient paralysis occurred in two more, and these animals showed the lowest $AUC_{0\text{-}120min}$ values in the tail flick test. The results

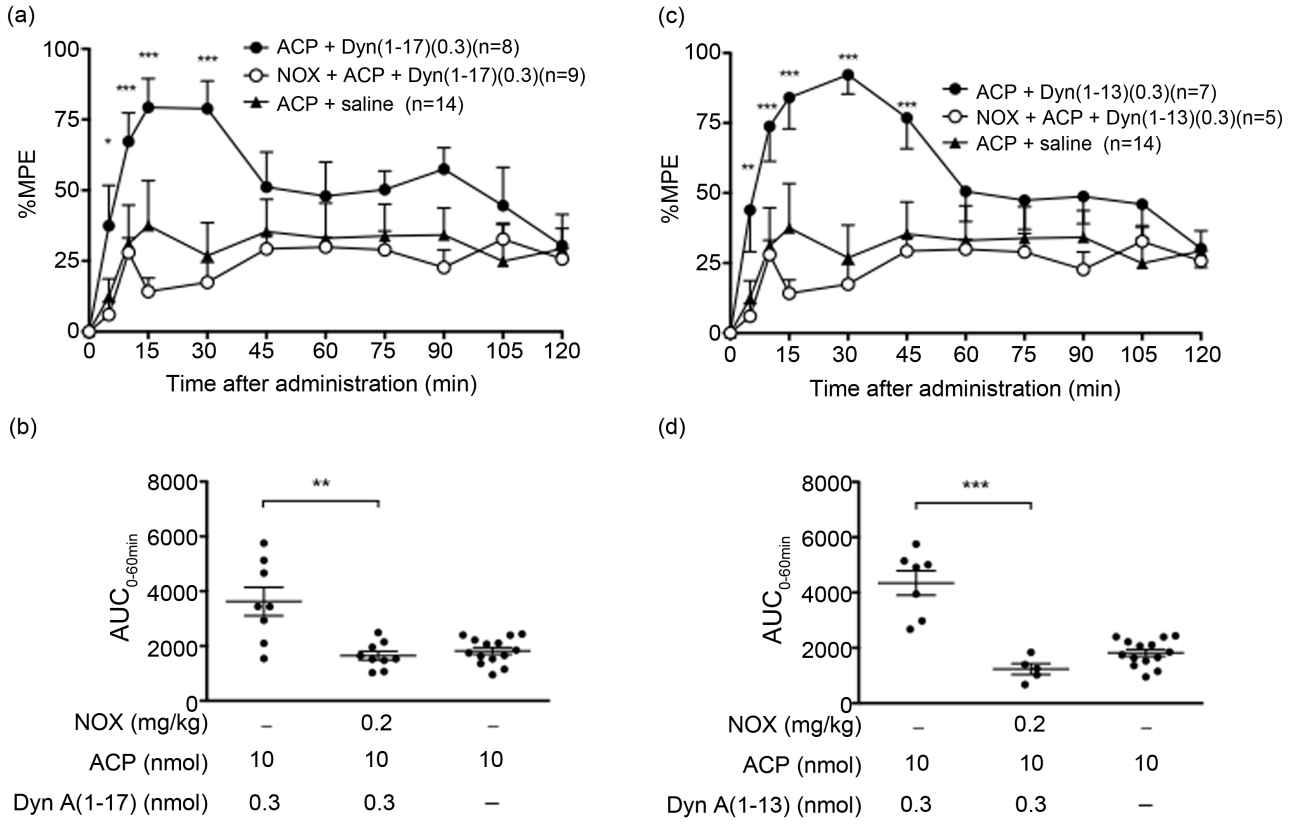

Figure 5. Effect of non-selective opioid receptor antagonist NOX on antinociceptive potency of Dyn A (1-17) or Dyn A (1-13) under pretreatment with ACP. Upper panels (a) and (c) indicate time course of %MPE of Dyn A (1-17) (0.3 nmol) and DynA (1-13) (0.3 nmol)-induced antinociception under pretreatment with ACP following administration of NOX (0.2 mg/kg), respectively. Significantly different from NOX-administrated 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 panels (b) and (d) show $AUC_{0\text{-}60min}$ for value of %MPE indicated in upper panels (a) and (c), respectively. Significantly different from NOX-administrated group according to Dunn's post-hoc test following Kruskal-Wallis test; $^{**}P < 0.01$ and $^{***}P < 0.001$.

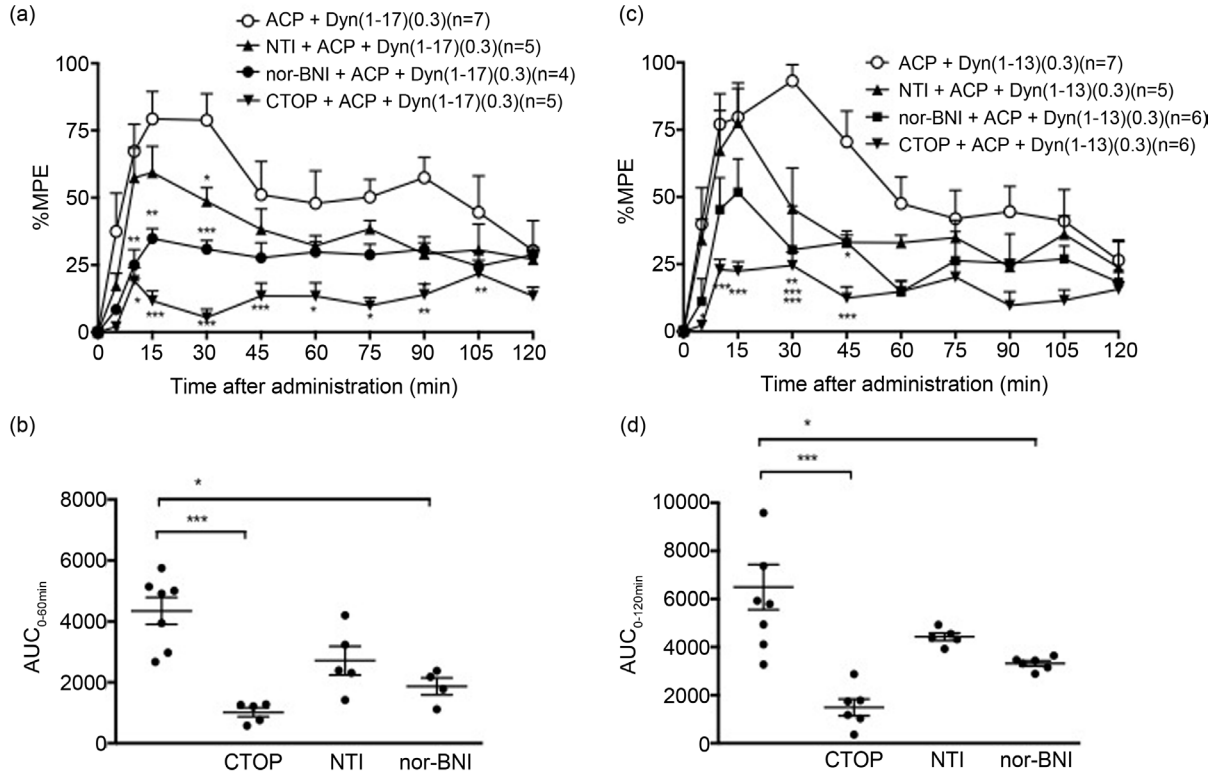

Figure 6. Effect of opioid receptor selective antagonists on antinociceptive potency of Dyn A (1-17) or Dyn A (1-13) under pretreatment with ACP. Upper panels (a) and (c) indicate time course of %MPE of Dyn A (1-17) (0.3 nmol) and DynA (1-13) (0.3 nmol)-induced antinociception under pretreatment with ACP following administration of three opioid receptor antagonists CTOP (3 nmol), nor-BNI (20 mg/kg), or NTI (66 nmol), respectively. Significantly different from Dyn A (1-17) or Dyn A (1-13) under pretreatment with ACP according to Dunn's post-hoc test following two-way repeated measures ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Lower panels (b) and (d) show $AUC_{0\text{-}60min}$ and $AUC_{0\text{-}120min}$ for value of %MPE indicated in upper panels (a) and (c), respectively. Significantly different from Dyn A (1-17) under pretreatment with ACP according to Dunn's post-hoc test following Kruskal-Wallis test; $^{*}P < 0.05$ and $^{***}P < 0.001$.

of the tail flick and von Frey tests in all of four of these animals are reported here. The two remaining rats, however, showed prolonged and severe paralysis, making both the tail flick and von Frey test unviable. Thus, 4 out of 6 rats showed some signs of neuropathy following i.t. administration of 0.3 nmol Dyn A (1-17) under pretreatment with both PHMB and ACP.

3.6. ACP Attenuates Allodynia Induced by i.t. Administration of Dyn A (1-17)

No significant differences were observed in the baseline threshold stimulus between each group before administration of Dyn A (1-17) (3 or 5 nmol) alone or Dyn A (1-17) (0.1, 0.3 nmol) with ACP and/or PHMB (10 nmol). The threshold stimulus intensity required to evoke withdrawal was in the order of 4.93 to 5.88 (8.0 - 60.0 g) in normal rat (Figure 8). Intrathecal administration of Dyn A (1-17) (3, 5 nmol) produced significant mechanical allodynia, with tactile thresholds falling from approximately 3.61 to 4.08 (0.4 - 1.0 g) (Figure 8(a)). In contrast, no allodynia was observed with Dyn A (1-17) (0.1, 0.3 nmol) with ACP or PHMB (Figure 8).

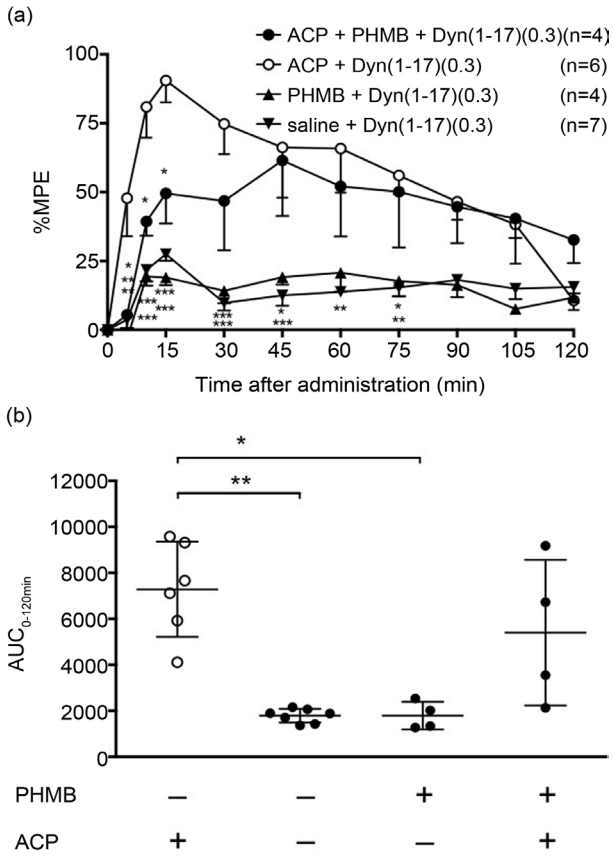

Figure 7. Antinociception by i.t. administration of Dyn A (1-17) under pretreatment with saline or ACP together with/without PHMB. Upper panel (a) indicates time course of %MPE of Dyn A (1-17) (0.3 nmol) under pretreatment with saline and ACP with and without PHMB, respectively. Significantly different 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 $AUC_{0\text{-}120min}$ 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 $^{**}P < 0.01$.

4. Discussion

The present results revealed that the antinociceptive potency of Dyn A (1-17), Dyn A (1-13), or Dyn A (1-6) depended on the dose and length of the peptide when administered i.t. in the absence of, or under pretreatment with, ACP. This is in good agreement with the results of earlier studies showing that Dyn A (1-17)-induced antinociception at the supraspinal level was greater than that of Dyn A (1-6) or Dyn A (1-13) at the same dose [17], and that Dyn A (1-17) had a greater analgesic effect than Dyn A (1-13) in the absence of ACP [30]. Pretreatment with ACP increased antinociception with low-dose Dyn A (1-17), with neither hyperalgesia nor paralysis. The present findings then provide further support for the view that C-terminal peptide fragments of Dyn A (1-17), such as Dyn A (7-17), are catabolized into shorter products that are extremely weak toxicity under pretreatment with ACP [17], thus reducing neuropathic symptoms.

Intrathecal administration of Dyn A (1-17) and LE [31] under pretreatment with ACP augmented antinociception by at least 50- and 100-fold, respectively.

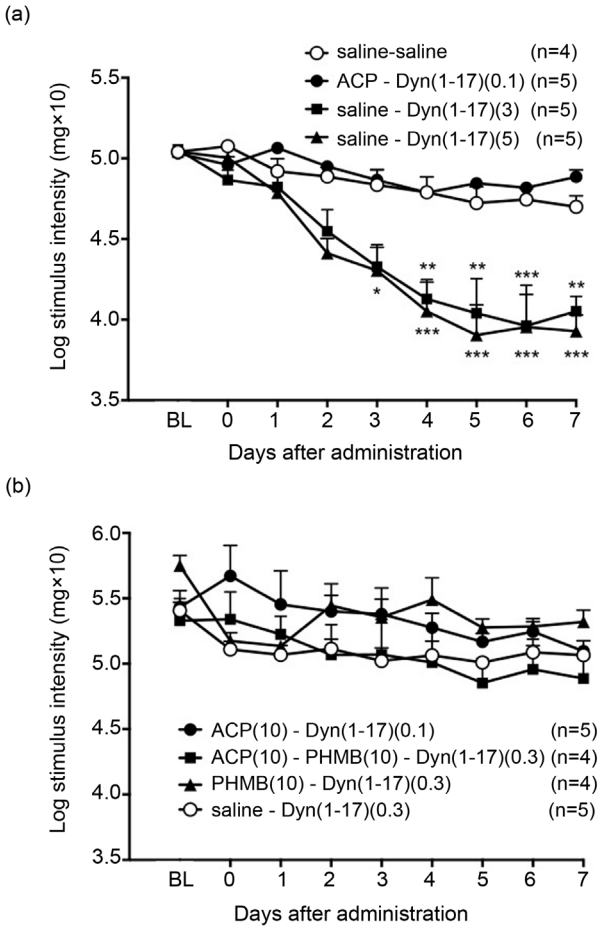

Figure 8. Duration of tactile threshold stimulus intensity required to evoke withdrawal with Dyn A (1-17) (0.3, 3 or 5 nmol) alone or Dyn A (1-17) (0.1, 0.3 nmol) with ACP and/or PHMB administration. Upper and lower panels (a) and (b) indicate tactile thresholds measured at intervals by von Frey filaments. Significantly different from Dyn A (1-17) under pretreatment with ACP and/or PHMB according to Dunn's post-hoc test following two-way repeated measures ANOVA; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

These findings coincide well with the results of earlier histological studies showing that the distribution of NEP and APN was highly concentrated in the substantia gelatinosa of the spinal cord, a region closely associated with μ-opioid receptors and enkephalins [32] [33] [34] [35]. Taken together, this suggests that this co-localization of peptidases and opioid peptides plays a critical role in nociception through the latter's inactivation in the spinal cord.

Pretreatment of paired combinations (2 μM each of AP, AC, or CP) or all three PIs together (2 μM ACP) revealed that AP or ACP significantly increased Dyn A (1-17)-induced inhibition of electrically-evoked contractions in MVD. This effect was significantly stronger with ACP than with AC or CP; it was also stronger than with administration of AP, but not significantly so. These results demonstrate that ACP is required to inhibit degradation of intact Dyn A (1-17), and that any residual pair of peptidases inactivates substantial amounts of Dyn A (1-17). This is in good agreement with the results of an earlier study by this group demonstrating that the antinociceptive potency of Dyn A (1-17) was

higher under pretreatment with ACP than with any paired combination of these PIs at the supraspinal level [17].

Both the present and earlier studies demonstrated that LE (IC_{50}: 1.74 nM) [36] showed greater potency than Dyn A (1-17) (IC_{50}: 4.53 nM) under pretreatment with ACP in isolated MVD preparation. On the other hand, Dyn A (1-17) (ED_{50}: 0.272 nmol) showed greater potency than LE (ED_{50}: 4.5 nmol) [31] under pretreatment with ACP in the tail flick test. This discrepancy might be explained by differences in the opioid receptor subtype mainly responsible for each effect between isolated MVD preparation and spinal cord. The inhibitory potency of the δ-opioid receptor agonist in isolated MVD preparation is greater than that of the others [37]. [Leu^5]-enkephalin [38] and Dyn A [1] [39] mainly bind to δ- and κ-opioid receptors, respectively, so the inhibitory potency of LE in isolated MVD preparation is greater than that of Dyn A (1-17). In contrast, the present and earlier [31] studies using opioid receptor selective antagonists showed that i.t. administration of LE or Dyn A (1-17) under pretreatment with ACP induced antinociception by μ-opioid receptors, in particular. These results correspond well with those showing that regional distribution of NEP and APN overlaps that of μ-opioid, but not δ-opioid receptors [32] [34], and that the N-terminal region of Dyn A (1-17) interacts with both μ-opioid and δ-opioid receptors [40] [41] [42].

One study demonstrated that DCE was an important enzyme in Dyn A-induced antinociception and toxicity at the spinal cord level [6]. This indicates that pretreatment with PHMB and ACP would safeguard the integrity of i.t. administered Dyn A (1-17). Another study reported that toxicity ranked in the order of Dyn A (1-17) > Dyn A (1-13) > Dyn A (13-17), whereas LE caused no neuronal toxicity [2]. The present results showed that the antinociceptive potency of 0.3 nmol Dyn A (1-17) under pretreatment with PHMB and ACP was not significantly higher than that with ACP alone. The 2 rats showing the lowest values for antinociceptive potency exhibited subtle lower limb paralysis following administration of 0.3 nmol Dyn A (1-17) under pretreatment with PHMB and ACP, but recovered completely after 24 hr. Potency in these two animals was lower than the lowest observed with ACP in the absence of PHMB. This reduction in potency may have resulted from hyperalgesia, as suggested by an earlier study reporting that i.t. administration of high-dose Dyn A (1-17) induced hyperalgesia [43].

Neuropathy was observed in a total of 4 out of 6 rats after i.t. administration of 0.3 nmol Dyn A (1-17) under pretreatment with PHMB and ACP. Both the tail flick and von Frey test could not be used to evaluate allodynia in 2 out of 4 rats which developed neuropathy due to severe and long-lasting paralysis of the tails and lower limbs after administration of 0.3 nmol Dyn A (1-17) under pretreatment with PHMB and ACP. Meanwhile, subtle and transient paralysis occurred in the two remaining rats, and these animals showed the lowest $AUC_{0\text{-}120min}$ values and stimulus intensity in the tail flick and von Frey test, respectively. Two other rats showed no abnormal signs. These may lead to no difference in Dyn A

(1-17)-induced antinociception and allodynia under pretreatment with ACP between with and without concomitant administration of PHMB. The character and frequency of symptoms of neuropathic pain may depend on the dose of Dyn A (1-17), which would correspond to the concentration of Dyn A (1-17) in the intrathecal space. Indeed, earlier studies have reported persistent allodynia with i.t. administration of high-dose (15 - 50 nmol) Dyn A [7] [10]. Further support for this hypothesis comes from evidence that high doses of Dyn A enhance intracellular levels of Ca^{2+} via simultaneous activation of NMDA and κ-opioid receptors, whereas low doses activate only κ-opioid receptors [7].

5. Conclusion

In conclusion, the present results showed that inactivation of A-, C-, or P-sensitive enzymes leads to an increase in low-dose Dyn A (1-17)-induced antinociception without signs of allodynia at the spinal level. The antinociceptive potency and induction of allodynia by Dyn A (1-17), Dyn A (1-13), or their peptide fragments depended on their dose and length. 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.

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Disclosures

There is no conflict of interest in this study.

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Abbreviations

A: amastatin,
ANOVA: analysis of variance,
APN: aminopeptidase N,
AUC: area under the curve,
C: captopril,
CTOP: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2,
DCE: dynorphiin-converting enzyme,
Dyn: dynorphin,
ED_{50}: median effective dose,
IC_{50}: concentration required to produce 50% inhibition,
i.c.v.: intracerebroventricular,
i.t.: intrathecal,
LE: [Leu5]-enkephalin,
MPE: maximal possible effect,
MVD: mouse vas deferens,
NEP: neutral endopeptidase-24.11,
NMDA: N-methyl-D-aspartate,
nor-BNI: nor-binaltorphimine dihydrochloride,
NOX: naloxone hydrochloride,
NTI: naltrindole hydrochloride,
P: phosphoramidon,
PHMB: p-hydroxymercuribenzoate,
PIs: peptidase inhibitors,
S.E.M.: standard error of the mean.