Sialorphin Potentiates Effects of [Met\(^5\)]Enkephalin without Toxicity by Action other than Peptidase Inhibition

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ABSTRACT

This dose-response study investigated the effects of sialorphin on [Met\(^5\)]enkephalin (ME)-induced inhibition of contractions in mouse vas deferens and antinociception in male rats. Differences were compared among combinations of three chemical peptidase inhibitors: amastatin, captopril, and phosphoramidon. The ratio of potencies of ME in mouse vas deferens pretreated with both sialorphin (100 \(\mu\)M) and a mixture of the three peptidase inhibitors (1 \(\mu\)M each) was higher than that with the mixture of peptidase inhibitors alone at any dose. Intrathecal administration of sialorphin (100–400 nmol) significantly and dose dependently increased ME (3 nmol)-induced antinociception with the mixture of three peptidase inhibitors (10 nmol each). The degree of antinociception with a combination of any two of the peptidase inhibitors (10 nmol each) in the absence of sialorphin was less than that in the presence of sialorphin (200 nmol). Pretreatment with both sialorphin (200 nmol) and the mixture of three peptidase inhibitors (10 nmol each) produced an approximately 100-fold augmentation in ME (10 nmol)-induced antinociception, but without signs of toxicity such as motor dysfunction in rats. Radioligand receptor binding assay revealed that sialorphin did not affect either binding affinity or maximal binding capacity of \([\alpha\text{-Ala}^2,N\text{-MePhe}^4,Gly-ol^5]\)enkephalin. These results indicate that sialorphin potentiates the effects of ME without toxicity by a mechanism other than peptidase inhibition and with no effect on its affinity to \(\mu\)-opioid receptors.

SIGNIFICANCE STATEMENT

Sialorphin is regarded as an endogenous peptidase inhibitor that interacts with enkephalin-degrading enzymes. The results of these in vitro and in vivo studies confirm that sialorphin potentiates the effects of [Met\(^5\)]enkephalin without toxicity by an action other than peptidase inhibition. This suggests that sialorphin offers the advantage of reducing or negating the side effects of opioid drugs and endogenous opioid peptides.

Introduction

Earlier studies have demonstrated rapid degradation of opioid peptides by any of five types of peptidase: 1) aminopeptidase N (EC 3.4.11.2), which cleaves the Tyr\(^1\)-Gly\(^2\) amide bond; 2) dipeptidyl peptidase III (EC 3.4.14.4), which hydrolyzes the Gly\(^2\)-Gly\(^3\) bond; 3) dipeptidyl carboxypeptidase (EC 3.4.15.1, also known as the angiotensin I–converting enzyme); 4) neutral endopeptidase (EC 3.4.24.11, also known as enkephalinase), which cleaves the Gly\(^3\)-Phe\(^4\) bond; and 5) carboxypeptidase A (EC 3.4.17.1) (Khakht et al., 2012; Morales-Mulia et al., 2012). The membrane-bound three enzymes, aminopeptidase N (APN), dipeptidyl carboxypeptidase, and neutral endopeptidase (NEP), play an essential role in the degradation of [Met\(^5\)]enkephalin (ME) in three different types of isolated preparation: guinea pig ileum (Aoki et al., 1984), mouse vas deferens (MVD) (Aoki et al., 1986), and rat vas deferens (Cui et al., 1986). A mixture of the following three peptidase inhibitors (PIs) significantly increased the antinociceptive effects of ME: amastatin, an aminopeptidase inhibitor; captopril, a dipeptidyl carboxypeptidase inhibitor; and phosphoramidon, an endopeptidase-24.11 inhibitor (Murata et al., 2014). This finding was in good agreement with those of earlier studies employing high-performance liquid chromatography that showed that a mixture of these PIs almost completely inhibited the degradation of ME (Hiranuma and Oka, 1986). Widely distributed throughout the human body, opioid receptors are activated by endogenous peptides and exogenous ligands (Stein, 1995). High-dose administration of opioids can lead to lethal toxicity in multiple organ systems (Boyer, 2012).

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104
Earlier studies by the present group revealed that administration of a mixture of PIs enhanced antinociception induced by low-dose administration of dynorphin without toxicity (Ajimi et al., 2015; Matsuda et al., 2017). These results demonstrated that the use of PIs synergizes and potentiates intrinsic signaling pathways, which would allow a reduction in the doses required and subsequent avoidance of toxicity.

Two endogenous peptidase inhibitors have recently been isolated from rat and human saliva: sialorphin (Gln-His-Asn-Pro-Arg) from the former and opiorphin (Gln-Arg-Phe-Ser-Arg) from the latter (Kamysz et al., 2013). Administration of either was reported to induce an antinociceptive effect through activation of opioid receptors (Rougeot et al., 2003; Wisner et al., 2006). Rougeot et al. (2003) suggested that opiorphin protects enkephalins from degradation by two peptidases (NEP and APN), thus improving the affinity of enkephalins without directly interacting with opioid receptors themselves (Tóth et al., 2012; Benyhe et al., 2014; Sitbon et al., 2016).

Increasingly, compounds are being discovered that directly modulate receptors via distinct allosteric, rather than orthosteric, sites. Allosteric modulators usually demonstrate higher selectivity for individual receptor subtypes, which means they are safer than orthosteric-site ligands (Conn et al., 2009). Allosteric modulators can be divided into three basic classes: the first class only affects the binding affinity of orthosteric ligands, whereas the second modulates the efficacy of orthosteric ligands, either in addition to or without affecting the affinity of orthosteric ligands. It is assumed that modulators in both of these classes (the so-called pure allosteric modulators) exert no detectable effect in the absence of orthosteric ligands. In contrast, the third class are effective independently of their allosteric effects (Langmead and Christopoulos, 2006).

Cannabidiol (CB1 cannabinoid receptor agonist) and salvinarin-A (a κ-opioid receptor agonist) were reported to be negative allosteric modulators of opioid receptors (Kathmann et al., 2006; Rothman et al., 2007). One earlier study using high-throughput screening also identified two compounds (BMS-986121 and BMS-986122) as positive allosteric modulators (PAMs) of μ-opioid receptors (Burford et al., 2013). BMS-986122 enhances the recruitment of β-arrestin to μ-opioid receptors by endomorphin-1 and potentiates G protein–mediated decrease in cAMP accumulation produced by endomorphin-1. Two analogs (BMS-986123 and BMS-986124) of BMS-986122 were identified as silent allosteric modulators (SAMs) of this allosteric site. Although these SAMs exert no PAM activity, they have been shown to competitively antagonize the effects of BMS-986122 (Burford et al., 2013). In this dose-response study, the effects of sialorphin on Mβ-induced inhibition of contractions in MVD and antinociception in male rats were investigated to determine whether they were mediated as an inhibitor of neutral endopeptidase. Differences were compared among combinations of three PIs. A radioligand receptor binding assay was used to establish the effect of sialorphin on Bmax and binding affinity (Kd) of [α-Ala2,N-MePhe4,Gly-ol5]enkephalin (DAMGO).

Materials and Methods

All animal experiments were performed strictly in accordance with the guidelines of this institution (Tokai University, http://www.u-tokai.ac.jp/about/concept/guidance.html). Approval for the study protocol was obtained from the Animal Investigation Committee of Tokai University (Approval No: 191029 and 191031).

Animals

Male Wistar rats (7 to 8 weeks old, 180–220 g each, n = 276; NIH Clea, Tokyo, Japan) and male ICR JCL mice (9 to 10 weeks old, weighing 30–40 g each, n = 40; NIH Clea) were housed in an air-conditioned room at a control temperature of 24–26°C and 50%–60% humidity, with a 12-hour light/dark cycle (lights on: 07:00 hours) and food and water freely available. The animals were allowed 1 week to adapt to the novel laboratory environment. Opioid-induced antinociception is strongly affected by sex. Endopeptidase-24.11 inhibitor SCH 34826 induced significantly greater antinociceptive effects and stress-induced opioid analgesia in male than in female deer mice (Kavaliers and Innes, 1993). Remarkably, synthesis of sialorphin shows significant sexual dimorphism. The expression of gene and peptide levels in adult male rats are 1000-fold and 100- to 500-fold higher than those in adult females, respectively (Rosinski-Chupin et al., 1988, 1993, 2001; Messaoudi et al., 2004). Sialorphin is released into the bloodstream from the submandibular gland and can adapt to acute stress, depending on the degree of adrenergic receptor activity. In view of this, all experimental procedures in the present study were performed exclusively on male mice and rats.

Chemicals

The following were obtained from the sources indicated: sialorphin (PH Japan, Hiroshima, Japan); and [Met5]enkephalin (ME), amastatin, and phosphoramidon (The Peptide Institute, Inc., Minoh, Japan). The following were all purchased from SIGMA Japan (Tokyo, Japan): captopril, β-Phe-Cys-Tyr-β-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP, a μ-opioid receptor antagonist), nor-binaltorphimine dihydrochloride (nor-BNI, a κ-opioid receptor antagonist), naltrindole hydrochloride (NTI, a δ-opioid receptor antagonist), and BMS-986124 (a silent allosteric modulator of μ-opioid receptor). The nonselective opioid receptor antagonist naloxone hydrochloride (NOX) was purchased from Daiichi-Sankyo Company, Limited (Tokyo, Japan). All of the above chemicals were dissolved in saline, except for nor-BNI and NTI, which were dissolved in water, and BMS-986124, which was dissolved in 50% DMSO and 50% saline. The desired concentration of each solution was prepared at the time it was to be used, and each was administered intrathecally at a volume of 10 μl. Administration of the PIs was performed at 10 minutes prior to that of the opioid receptor agonist or saline as a control.

Radioligand Receptor Binding Assay

A membrane fraction was prepared from rat whole brain according to the method of Benyhe et al. (1997) with some modifications. The animals were decapitated. The brains were then rapidly removed and critically hypotonic in 0.32 M sucrose. Aliquots of the tissue were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose. Aliquots of the membrane fraction were then washed in liquid nitrogen and then stored at −80°C. Immediately before use in the binding assays, the membranes were thawed and resuspended in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged (40,000g at 4°C for 20 minutes) to remove sucrose. After incubation in 50 mM Tris-HCl buffer (pH 7.4) containing ACP (final concentration 1 μM each) with or without sialorphin (final concentration 100 μM) for 15 minutes, the membrane suspensions (protein concentration of 0.5 mg/ml) were incubated in glass tubes for 60 minutes at 25°C with the radioligand [3H]DAMGO in a final volume of 1 ml. Assays for Scatchard analysis were carried out at various concentrations of [3H]DAMGO. Nonspecific binding was measured in the presence of unlabeled 10 μM DAMGO. Incubation was terminated by rapid filtration through Whatman GF/C glass fiber filters. After washing three times in 5 ml ice-cold buffer (50 mM
Tris-HCl, pH 7.4), radioactivity was measured in a scintillation cocktail (Pico-Fluor Plus; Perkin Elmer Japan, Tokyo) using a scintillation counter (2810; Perkin Elmer Japan). Experiments were carried out in duplicate and repeated two times. Ligand binding data were evaluated using computer software (GraphPad Prism, version 6.0c; GraphPad Software, San Diego, CA).

In Vitro Isolated Preparations

Mouse vas deferens were stimulated with a 5-minute interval between each experimental condition, unless specifically mentioned. Mouse vas deferens were stimulated with a supramaximal rectangular pulse of 1.0 millisecond in duration and at a frequency of 0.1 Hz using an electronic stimulator (SEN-3201; Nihon Koden, Tokyo, Japan). To determine IC_{50}, the degree (%) to which each opioid depressed muscle twitch was plotted against its log concentration. The effect of each PI on ME-induced depression of contractions was determined by administering each inhibitor at least 3 minutes before the enkephalin. The percent difference was calculated as follows: percent difference = [(IC_{50} before each treatment − IC_{50} after each treatment)/IC_{50} before each treatment] × 100. These differences are shown in the tables.

Intrathecal Administration

Based on a method described in an earlier study (Murata et al., 2014), intrathecal catheters were implanted in male Wistar rats (7 to 8 weeks old) under inhalation anesthesia with nitrous oxide, oxygen, and isoflurane (2%). An 8.5-cm polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ) was inserted caudally to the thoracolumbar level of the skull and was plugged with a 30-gauge steel wire. Only rats with normal motor function and behavior were used for the study 7 days later. Drugs were injected at a volume of 10 µl, followed by 10 µl saline over 1 minute.

Tail-Flick Test

To eliminate bias, each investigator was blind to the drug administered. In accordance with the method in earlier studies by the present group (Kitamura et al., 2000; Takahashi et al., 2007; Akahori et al., 2008; Murata et al., 2014; Ajimi et al., 2015; Matsuda et al., 2017), noxious stimulation was achieved by immersing the tail of each rat in hot water (55°C) for a maximum of 5 seconds. This time limit was set to prevent injury to the animal in accordance with the result from earlier studies by the present group showing that persistent pain occurs when the tail is placed in hot water for more than 5 seconds (data not shown). The average baseline latency was approximately 0.8–1.6 seconds. After determining baseline latencies three times at 15-minute intervals, each drug was administered, and tail-flick latencies were determined at 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 minutes after. The following formula was used to calculate the percent of maximal possible effect (MPE) for each animal at each time: %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.

Assessment of Motor Function

Motor function was assessed using a method previously described, with some modification (Drummond and Moore, 1989; Kakinohana et al., 2006; Shirasawa et al., 2009). A motor deficit index (MDI) graded as follows was used: 0, normal; 1, toes flat under the body when walking, but axia present; 2, knuckle walking; 3, movement of legs but unable to walk; or 4, no movement, drugs legs. Each drug was administered, and MDI was assessed at 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 minutes after. The AUC value for MDI of each drug was also calculated.

Statistical Analyses

The results are shown as means and S.E.M. The statistical analysis software package (GraphPad Prism, version 6.0c; GraphPad Software) was used to compare across experimental conditions. The Friedman test and Dunn’s post hoc test were used to evaluate differences among groups. Dunn’s multiple comparison test was used to determine significance at each time point when a significant difference among the %MPE data after drug administration was obtained by means of a two-way (drugs and time) repeated measures ANOVA. Dunn’s multiple comparison test was also used to determine significance at each dose when a significant difference among groups in the AUC data were obtained with a two-way (drugs and dose) ANOVA. When a significant difference within groups was obtained in the Kruskal-Wallis test, Dunn’s comparison test was applied to determine significance. The Mann-Whitney test was used for intergroup comparisons. A P value less than 0.05 was considered to indicate a statistical significance.

In Vitro Isolated Preparations Experimental Protocol

Ratio of Potencies of ME in MVD Pretreated with ACP with or without Sialorphin. Four MVDs were tested (Fig. 1). After administration of ACP alone, ME was added and washed out with Krebs’ solution after their maximal effects had been noted. Subsequent to this experiment, the evaluation of ACP with sialorphin was carried out.

Enhanced Effect of Sialorphin on the Inhibitory Potency of ME. Four MVD were tested (Table 1). ME was added and washed out after their maximal effects had been noted among each dose of sialorphin from low to high dose.

Enhanced Effect of Combination of ACP on the Inhibitory Potency of ME in the Presence or Absence of Sialorphin. Sixteen MVDs were divided into four groups: AC, CP, AP, ACP (n = 4 each) (Table 2).
TABLE 1
Enhanced effect of sialorphin on the inhibitory potency of ME in MVD

<table>
<thead>
<tr>
<th>Sia (M)</th>
<th>IC\textsubscript{50} (× 10\textsuperscript{-5} M)</th>
<th>Ratio of Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>17.11 ± 1.69</td>
<td>1</td>
</tr>
<tr>
<td>10\textsuperscript{-6}</td>
<td>11.94 ± 0.41</td>
<td>1.45 ± 0.19</td>
</tr>
<tr>
<td>10\textsuperscript{-5}</td>
<td>5.83 ± 0.59</td>
<td>3.07 ± 0.56</td>
</tr>
<tr>
<td>10\textsuperscript{-4}</td>
<td>3.00 ± 0.30</td>
<td>5.70 ± 0.09**</td>
</tr>
<tr>
<td>2 × 10\textsuperscript{-4}</td>
<td>3.55 ± 0.24</td>
<td>4.90 ± 0.65*</td>
</tr>
</tbody>
</table>

Sia, sialorphin. Dunn’s post hoc test compared with ME alone, *P < 0.05, **P < 0.01, n = 4.

**Enhanced Effect of Sialorphin on the Inhibitory Potency of ME in the Presence of ACP.** Four MVDs were tested. ME was added and washed out after their maximal effects had been noted among each dose of ACP and sialorphin from low to high dose (Table 3).

**Inhibitory Effect of Sialorphin and ACP on Electrically Evoked Contractions in MVD before and after Administration of ME.** Sixteen MVDs were divided two groups (n = 4) (Fig. 2). Each drug was sequentially added to the MVD.

**Animal Experimental Protocol**

Combination of ME and Sialorphin Together with ACP. A previous study demonstrated that pretreatment with intrathecal administration of a combination of 10 nmol each of amastatin, captopril, and phosphoramidon completely inhibited peptidase-induced degradation of ME (Murata et al., 2014). In the present study, intrathecal administration of ME (1 nmol) was performed at 10 minutes after intrathecal administration of sialorphin alone or in combination with ACP (amastatin, captopril, and phosphoramidon; 10 nmol each). The rats were put into the following groups to determine whether joint administration of sialorphin and ACP increased the antinociceptive effect of ME: group 1, ME alone; group 2, ME with sialorphin; group 3, ME with ACP; or group 4, ME with the combination of sialorphin and ACP.

Dose-Response Study. Intrathecal administration of ME was performed at 10 minutes after intrathecal administration of ACP (10 nmol each) or saline. The rats were placed in the following groups to determine whether administration of ACP increased the antinociceptive effect: group 1, ME (1–10 nmol) with ACP (10 nmol each) alone or together with sialorphin (200 nmol); group 2, ME (3 nmol) with sialorphin (100–300 nmol) alone or together with ACP (10 nmol each); or group 3, ME (3 nmol) with sialorphin (200 nmol) alone or together with ACP (5–50 nmol each).

Effect of Sialorphin with Paired Combinations of PIs on ME-Induced Antinociception. To investigate the effects of sialorphin together with paired combinations of PIs on ME-induced antinociception, ME (3 nmol) was administrated intrathecally under pretreatment with each of the following combinations: AC (10 nmol each), CP (10 nmol each), or AP (10 nmol each).

Sialorphin-Induced Antinociception. Sialorphin alone, ACP (10 nmol each) alone, or both in combination were administrated intrathecally to determine their antinociceptive effect.

**Results**

Enhancement of Potency of ME by Sialorphin in MVD. The results showed that electrically evoked contractions in MVD were significantly inhibited by administration of ME and that this inhibitory potency was dose dependently augmented under pretreatment with sialorphin (Table 1, Friedman test, P < 0.0001) (mean rank difference: 10\textsuperscript{-6} M, 4.0; 10\textsuperscript{-5} M, 8.0; 10\textsuperscript{-4} M, 15.0; 2 × 10\textsuperscript{-4} M, 13.0). Figure 1 shows ME-induced inhibition after administration of ME (1, 3, or 10 nmol) alone or with sialorphin (200 nmol). The results revealed that sialorphin augmented ME-induced inhibition at any dose of ACP (F\textsubscript{5.38} = 5.618, P = 0.0006). The sharp symbol above the ratio of potency values with ACP at doses of 20 nmol each under pretreatment with sialorphin indicates significant differences compared with that at the same dose without sialorphin. The enhancing effect of ACP on ME-induced inhibition was also dose-dependent, reaching a maximum at a dose of 1 × 10\textsuperscript{-5} M (Fig. 1, Kruskal-Wallis test followed by Dunn’s post hoc test, *P < 0.05 and **P < 0.01, n = 4). The inhibitory potency of ME on electrically evoked contractions in MVD was significantly higher under pretreatment with AP and sialorphin than with AP alone; that with AC, CP, or ACP with sialorphin was also higher than that without sialorphin, but not significantly so (Table 2, Friedman test, P = 0.0003). Sialorphin itself had no intrinsic efficacy (A-2 in Fig. 2, Mann-Whitney test, P = 0.1143, n = 4). In contrast, sialorphin enhanced the efficacy of ME with ACP (B-6 in Fig. 2, Mann-Whitney test, P = 0.0286, n = 4) in a dose-dependent manner (Table 3, Friedman test, P < 0.0001) (mean rank difference: sialorphin 0 M, 4.00; 10\textsuperscript{-4} M, 9.75; 2 × 10\textsuperscript{-4} M, 10.25).

**TABLE 2**
Enhanced effect of combination of three PIs on the inhibitory potency of ME in MVD in the presence or absence of sialorphin

<table>
<thead>
<tr>
<th>PIs (× 10\textsuperscript{-5} M each)</th>
<th>IC\textsubscript{50} (× 10\textsuperscript{-5} M)</th>
<th>Ratio of Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sia (None)</td>
<td>Sia (1 × 10\textsuperscript{-5} M)</td>
</tr>
<tr>
<td>AC</td>
<td>5.75 ± 0.75</td>
<td>4.18 ± 0.32</td>
</tr>
<tr>
<td>CP</td>
<td>1.98 ± 0.22</td>
<td>1.49 ± 0.12</td>
</tr>
<tr>
<td>AP</td>
<td>2.46 ± 0.27</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>ACP</td>
<td>1.34 ± 0.21</td>
<td>0.83 ± 0.11</td>
</tr>
</tbody>
</table>

Sia, sialorphin. Dunn’s post hoc test compared with without sialorphin, *P < 0.05, n = 4.
Each drug was sequentially added to the MVD (Fig. 2). The ratio of potencies of ME in MVD under pretreatment with sialorphin \(2 \times 10^{-4} \text{M}\) and ACP was higher than those with ACP alone at any dose (Fig. 1, \(F_{5, 38} = 5.618, P = 0.0006\)).

### TABLE 3
Enhanced effect of sialorphin on the inhibitory potency of ME in MVD in the presence of ACP

<table>
<thead>
<tr>
<th>ACP (M each)</th>
<th>Sia (M)</th>
<th>IC(_{50}) (x10(^{-10}) M)</th>
<th>Ratio of Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>177.56 ± 25.07</td>
<td></td>
</tr>
<tr>
<td>5 x 10(^{-6})</td>
<td>None</td>
<td>6.60 ± 0.31</td>
<td>27.36 ± 4.39</td>
</tr>
<tr>
<td>5 x 10(^{-6})</td>
<td>10(^{-4})</td>
<td>4.09 ± 0.46</td>
<td>45.20 ± 8.87*</td>
</tr>
<tr>
<td>5 x 10(^{-6})</td>
<td>2 x 10(^{-4})</td>
<td>4.12 ± 0.55</td>
<td>44.88 ± 8.28**</td>
</tr>
</tbody>
</table>

Sia, sialorphin.

Dunn's post hoc test compared with ME alone, \(*P < 0.05; **P < 0.01, n = 4\).

### Effect of Sialorphin on ME-Induced Antinociception.
The antinociceptive effect observed with intrathecal administration of 1 nmol ME with 400 nmol sialorphin was similar to that with ACP (10 nmol each) in terms of onset, offset, and duration of action (Fig. 3A, \(F_{30, 210} = 4.074, P < 0.0001\)). The AUC\(_{0–45}\) min value for %MPE of 1 nmol ME with 400 nmol sialorphin was approximately equal to that with a mixture of the three PIs (10 nmol each) (Fig. 3B, Kruskal-Wallis test, \(P = 0.0007\)). Figure 4A shows ME-induced antinociception from 10 minutes after intrathecal administration of sialorphin (100, 200, or 400 nmol) (\(n = 5\) each sialorphin dose) alone or with ACP (10 nmol each) (\(n = 5\) each sialorphin dose). The results revealed that sialorphin augmented ME-induced antinociception under pretreatment with ACP in a dose-dependent manner (\(F_{3, 32} = 3.675, P = 0.0221\)). Sharp symbols above the AUC\(_{0–45}\) min values for sialorphin at all doses with ACP on ME indicate significant differences compared with that for sialorphin with saline on ME. Asterisks placed below the AUC\(_{0–45}\) min values for 200 nmol sialorphin with ACP on ME indicate significant differences compared with saline with ACP on ME (Kruskal-Wallis test, \(P = 0.0093\)). The AUC\(_{0–45}\) min value for sialorphin and ACP without ME (Kruskal-Walllis test, \(P = 0.0009\)). Asterisks placed above the AUC\(_{0–45}\) min values for ACP (10 nmol each) with or without sialorphin on ME indicate significant differences compared with that with or without sialorphin on saline (Kruskal-Wallis test, \(P = 0.0381\)). Figure 4B shows ME (3 nmol)-induced antinociception from 10 minutes after intrathecal administration of sialorphin (100, 200, or 400 nmol) (\(n = 5\) each sialorphin dose) alone or with ACP (10 nmol each) (\(n = 5\) each sialorphin dose). The results revealed that sialorphin augmented ME-induced antinociception under pretreatment with or without sialorphin in a dose-dependent manner (\(F_{3, 32} = 4.531, P = 0.0093\)). The AUC\(_{0–45}\) min value for sialorphin and ACP without ME (Kruskal-Walllis test, \(P = 0.0009\)). Asterisks placed above the AUC\(_{0–45}\) min values for sialorphin and ACP without ME (Kruskal-Walllis test, \(P = 0.0009\)). Asterisks placed above the AUC\(_{0–45}\) min values for ACP (10 nmol each) with or without sialorphin on ME indicate significant differences compared with that with or without sialorphin on saline (Kruskal-Wallis test, \(P = 0.0381\)). Figure 4B shows ME (3 nmol)-induced antinociception from 10 minutes after intrathecal administration of sialorphin (100, 200, or 400 nmol) (\(n = 5\) each sialorphin dose) alone or with ACP (10 nmol each) (\(n = 5\) each sialorphin dose). The results revealed that sialorphin augmented ME-induced antinociception under pretreatment with ACP in a dose-dependent manner (\(F_{3, 32} = 3.675, P = 0.0221\)). Sharp symbols above the AUC\(_{0–45}\) min values for sialorphin at all doses with ACP on ME indicate significant differences compared with that for sialorphin with saline on ME. Asterisks placed below the AUC\(_{0–45}\) min values for 200 nmol sialorphin with ACP on ME indicate significant differences compared with saline with ACP on ME (Kruskal-Wallis test, \(P = 0.0090\)). Figure 4C shows ME (3 nmol)-induced antinociception from 10 minutes after intrathecal administration of 200 nmol sialorphin (\(n = 5\) each ACP dose) or saline (\(n = 5\) each ACP dose) with ACP at doses of 0, 3, 10, or 30 nmol each. The results showed that ACP augmented ME-induced antinociception under pretreatment with or without sialorphin in a dose-dependent manner (\(F_{3, 32} = 4.531, P = 0.0093\)). The AUC\(_{0–45}\) min value for sialorphin and ACP without ME (Kruskal-Walllis test, \(P = 0.0009\)). Asterisks placed above the AUC\(_{0–45}\) min values for ACP (10 nmol each) with or without sialorphin on ME indicate significant differences compared with that with or without sialorphin on saline (Kruskal-Wallis test, \(P = 0.0381\)).
demonstrated that induction of ME-induced antinocicep-
tion by 200 nmol sialorphin and ACP (3, 10, or 30 nmol each) was
significantly greater than that with ACP alone. Sharp symbols above the AUC0–45 min values for ACP (10 nmol
each) together with 200 nmol sialorphin on ME indicate
significant differences compared with that for ACP alone on
ME. Asterisks placed above the AUC0–45 min values for ACP (10 nmol each) with 200 nmol sialorphin on ME indicate
significant differences compared with that for saline with
sialorphin on ME (Kruskal-Wallis test, P = 0.0045). Asterisks
placed above the AUC0–45 min values for ACP (10 nmol each)
on ME indicate significant differences compared with that for saline on ME (Kruskal-Wallis test, P = 0.0209).

Effect of Sialorphin with Combinations of PI (AC,
CP, AP) on ME-Induced Antinociception. A significant
increase was observed in the antinociceptive potency of
ME (3 nmol) under pretreatment with ACP (10 nmol each),
CP (10 nmol each), or AP (10 nmol each) in the presence of
200 nmol sialorphin in comparison with AC, CP, or AP alone
(Fig. 4D) (AC, AP, CP each; P = 0.0286).

Antinociceptive Effects of Sialorphin or ACP Alone. A
significant increase in antinociception was observed after in-
trathecal administration of ACP (10 nmol each) alone or in
combination with sialorphin (200 nmol each); sialorphin alone,
however, yielded no increase in antinociception (Fig. 5A)
(Kruskal-Wallis test, P = 0.0003) (mean rank difference:
sialorphin + saline, −5.667; ACP + saline, −10.40; sialorphin +
ACP, −16.33).

Effect of Antagonists on ME-Induced Antinocicep-
tion under Pretreatment with Sialorphin and ACP. The
results showed that NOX significantly attenuated the
antinociceptive effect of ME under pretreatment with ACP
(Fig. 5B). The antinociceptive potency of ME under pretreat-
ment with ACP after administration of NOX (1 mg/kg) was
approximately equal to that under pretreatment with saline
(data not shown). The antinociceptive effect of ME was atten-
uated dose dependently by NOX under pretreatment with
sialorphin (200 nmol) and ACP (10 nmol each) (Kruskal-Wallis
test, P = 0.0028). The antinociceptive potency of ME (3 nmol)
under pretreatment with sialorphin and ACP after administra-
tion of NOX (1 or 2 mg/kg) was approximately equal to that of
saline alone (mean rank difference: NOX 0.5 mg/kg, 3.75;
1 mg/kg, 10.63; 2 mg/kg, 8.50; saline, 15.30) (Fig. 5B). The
antinociceptive potency of ME under pretreatment with
sialorphin (200 nmol) and ACP (10 nmol each) was signif-
icantly attenuated by CTOP (3 nmol) or NTI (132 nmol); it
was not attenuated by nor-BNI (20 mg/kg) (Fig. 5C) (Krus-
kal-Wallis test, P = 0.0032) (mean rank difference: CTOP,
10.95; NTI, 9.60; nor-BNI, 1.40).

Effect of Silent Allosteric Modulator of μ-Opioid
Receptor on ME-Induced Antinociception under Pre-
treatment with Sialorphin and ACP. Assuming that allo-
steric modulators have higher selectivity for receptor subtypes
and that PAMs have an ability to augment the efficacy of
orthosteric ligands in addition to affecting the affinity of
orthosteric ligands, the effect of BMS-986124 was assessed
in the presence of a high dose of sialorphin and a low dose of
ME. The results showed that BMS-986124 significantly
dose dependently attenuated the enhancing effects of
sialorphin (400 nmol) on ME (1 nmol)-induced antinocicep-
tion under pretreatment with ACP (Fig. 5D) (Kruskal-Wallis test,
P = 0.0019). The antinociceptive potency of ME under
pretreatment with sialorphin and ACP after administra-
tion of BMS-986124 (5 nmol) was approximately equal to
that under pretreatment with ACP alone (mean rank difference:
5 nmol, −2.00; 1 nmol, −9.00; 0 nmol, −12.60).

Effect of Sialorphin and PI on Motor Dysfunction
Induced by Intrathecal Administration of ME. No
significant differences were observed in the antinociceptive
effects between 1000 nmol ME alone and 10 nmol ME under
pretreatment with sialorphin (200 nmol) and ACP (Fig. 6,
A and B) (Kruskal-Wallis test, P = 0.0092) (mean rank
difference: saline + ME, 3.4; ACP + ME, 8.0). Intrathecal
administration of 1000 nmol ME induced significant motor
dysfunction (Fig. 6, C and D) (Kruskal-Wallis test, P =
0.0010). In contrast, no motor dysfunction was observed
with 10 nmol ME under pretreatment with sialorphin and
ACP (mean rank difference: saline + ME, −7.5; ACP +
ME, 0.0).

Effect of Sialorphin on Kd and B max of DAMGO in
Presence of ACP. Sialorphin showed no effect on the
binding affinity of [3H]DAMGO: Kd in the absence of
sialorphin was 1.149 nM (95% CI: 0.7834–1.515); Kd in
the presence of 100 μM sialorphin was 1.272 nM (95% CI:
0.9092–1.635) (Fig. 7). Sialorphin also showed no effect
on maximal binding capacity of [3H]DAMGO: B max in the
absence of sialorphin was 9.11 fmol/mg tissue (95% CI: 8.232–9.99),
whereas $B_{\text{max}}$ in the presence of 100 $\mu$M sialorphin was 9.228 fmol/mg (95% Cl: 8.41–10.048) (Fig. 7).

**Discussion**

The results of the present study showed that pretreatment with both sialorphin and a mixture of three PIs produced an at least 100-fold augmentation in antinociception by dose. Significantly different from saline pretreated with ACP with (closed circle; $n = 6$ each dose) or without (open circle; $n = 6$ each dose) sialorphin according to Dunn’s post hoc test after Kruskal-Wallis test; *$P < 0.05$; **$P < 0.01$. Significantly different from under pretreatment with ACP alone in control at the same dose of ME according to Dunn’s post hoc test after two-way repeated measures ANOVA; *$P < 0.05$; **$P < 0.01$. B) Sialorphin-dependent antinociception with intrathecal administration of ME under pretreatment with (closed circle; $n = 5$ each dose) or without (open circle; $n = 5$ each dose) a mixture of PIs (ACP) by dose. Significantly different from sialorphin-treated control at the same dose according to Dunn’s post hoc test after Kruskal-Wallis test; *$P < 0.01$; **$P < 0.001$. Significantly different from ME (3 nmol) under pretreatment with ACP alone in control according to Dunn’s post hoc test after two-way repeated measures ANOVA; *$P < 0.05$. C) ACP-dependent antinociception with intrathecal administration of ME (3 nmol) under pretreatment with (closed circle; $n = 5$ each dose) or without sialorphin (open circle; $n = 5$ each dose) by dose. Significantly different from under pretreatment with or without sialorphin according to Dunn’s post hoc test after Kruskal-Wallis test; *$P < 0.01$; **$P < 0.001$. Significantly different from under pretreatment with ACP without sialorphin at the same dose of ACP according to Dunn’s post hoc test after two-way repeated measures ANOVA; ***$P < 0.001$. (D) Comparison of effect of paired combinations of PIs (AC, CP, and AP; 10 nmol each) on antinociception induced by intrathecal administration of ME (3 nmol) with (closed circle; $n = 4$ each combination of PIs) or without sialorphin (200 nmol) (open circle; $n = 5$ each combination of PIs). A value of zero indicates intrathecal administration of saline. Significantly different from in control treated with pairs of PIs alone according to Mann-Whitney test; *$P < 0.05$.

**Fig. 4.** (A) ME-dependent antinociception with intrathecal administration of a mixture of PIs (ACP) with or without sialorphin by dose. Significantly different from saline pretreated with ACP with (closed circle; $n = 6$ each dose) or without (open circle; $n = 6$ each dose) sialorphin according to Dunn’s post hoc test after Kruskal-Wallis test; *$P < 0.05$; **$P < 0.01$. Significantly different from under pretreatment with ACP alone in control at the same dose of ME according to Dunn’s post hoc test after two-way repeated measures ANOVA; *$P < 0.05$; **$P < 0.01$. (B) Sialorphin-dependent antinociception with intrathecal administration of ME under pretreatment with closed circle; $n = 5$ each dose) or without (open circle; $n = 5$ each dose) a mixture of PIs (ACP) by dose. Significantly different from sialorphin-treated control at the same dose according to Dunn’s post hoc test after Kruskal-Wallis test; *$P < 0.01$; **$P < 0.001$. Significantly different from ME (3 nmol) under pretreatment with ACP alone in control according to Dunn’s post hoc test after two-way repeated measures ANOVA; *$P < 0.05$. (C) ACP-dependent antinociception with intrathecal administration of ME (3 nmol) under pretreatment with (closed circle; $n = 5$ each dose) or without sialorphin (open circle; $n = 5$ each dose) by dose. Significantly different from under pretreatment with or without sialorphin according to Dunn’s post hoc test after Kruskal-Wallis test; *$P < 0.01$; **$P < 0.001$. Significantly different from under pretreatment with ACP without sialorphin at the same dose of ACP according to Dunn’s post hoc test after two-way repeated measures ANOVA; ***$P < 0.001$. (D) Comparison of effect of paired combinations of PIs (AC, CP, and AP; 10 nmol each) on antinociception induced by intrathecal administration of ME (3 nmol) with (closed circle; $n = 4$ each combination of PIs) or without sialorphin (200 nmol) (open circle; $n = 5$ each combination of PIs). A value of zero indicates intrathecal administration of saline. Significantly different from in control treated with pairs of PIs alone according to Mann-Whitney test; *$P < 0.05$. 

potentiation of orthosteric ligands as allosteric modulators rather than by inhibition of acetylcholine degradation. Either PAM or PIs can potentiate the analgesic effects of opioid drugs or endogenous opioids, without potentiation of the side effects. Thus, administration of opioid compounds containing sialorphin may offer the advantage of reducing or negating the side effects of the former.

Sialorphin did not affect the $B_{\text{max}}$ of DAMGO in the presence of ACP. This suggests that sialorphin only inhibits amastatin-sensitive aminopeptidase, captopril-sensitive dipeptidyl carboxypeptidase I, and phosphoramidon-sensitive endopeptidase-24.11. This is in good agreement with the results of an earlier high-performance liquid chromatography analysis, which revealed that ME was almost solely catabolized by these three peptidases in ileal and striatal membrane preparations (Hiranuma and Oka, 1986).

The present study demonstrated that BMS-986124 significantly attenuated the enhancing effects of sialorphin on ME-induced antinociception and that sialorphin affected neither the binding affinity nor the maximal binding capacities of [3H]DAMGO. These results are not, however, in agreement with the results of an earlier study showing that...
BMS-986124 acted as a silent allosteric modulator at the site where BMS-986121 or 986122 binds (Burford et al., 2013). This discrepancy may be related to “activity switching” of allosteric modulators. Similar to orthosteric opioid ligands (Morgan et al., 1999), allosteric modulators appear to act as agonists or antagonists, or to act as a positive or negative allosteric modulators, depending on the pain test and drugs used (Burford et al., 2015). For example, positive
allosteric modulator BMS-986122 increased the affinity of full μ-opioid receptor agonists (high intrinsic efficacy) with no change in maximal response, whereas it increased maximal response with minimal effects on agonist affinity for partial (low intrinsic efficacy) agonists (Livingston and Traynor, 2014). Although BMS-986124 did not significantly increase the potency of morphine at a high dose, it increased the maximal response of morphine at a high dose, but with only a slight decrease at low dose (Burford et al., 2013). Further investigation using PAMs such as BMS-9861242 to assess the impact of allosteric modulators on the antinociceptive effects of ME compared with sialorphin is required.

Rougeot et al. (2003) suggested that opiorphin interacts indirectly with opioid receptors (Toth et al., 2012; Benyhe et al., 2014; Sitbon et al., 2016). This was proposed based on two findings (Toth et al., 2012): 1) that opiorphin increased the maximal binding capacities ($B_{max}$) of $[^{3}H]$Met-enkephalin-Arg^6-Phe ([$^{3}H$]MRF), but not $[^{3}H]$endoendorphin-1, at both 0°C and 24°C in a saturation binding assay and 2) that opiorphin increased the affinity of MRF in competition studies with $[^{3}H]$MRF binding more than a mixture of PIs (bestatin, captopril, thiorphan, bacitracin, phenylmethylsulfonyl fluoride, benzamidine, soybean trypsin inhibitor, EDTA, and EGTA) not only at 24°C (MVF IC$_{50}$ in the presence of opiorphin was 5.8 nM; IC$_{50}$ in the presence of a mixture of the PIs was 26.4 nM). The finding of the present study that opiorphin increased the affinity of ME in competition with $[^{3}H]$DAMGO in rat spinal cord (Waksman et al., 1986; Noble et al., 2001). This dose can be taken to correspond to approximately 200 μM based on the finding that the amount of cerebrospinal fluid was approximately 100 μl in rat (200–300 body weight) (Consiglio and Lucion, 2000).

Although sialorphin alone induced no increase in antinociception in the tail-flick test in the present study, intravenous administration of sialorphin alone did induce an antinociceptive effect in the pin-pain and formalin tests in another study (Rougeot et al., 2003). Such discrepancies may be explained by differences in the nociceptive stimulus applied between any two studies (Mélik Parsadaniantz et al., 2015), such as that seen between the present study and that of Rougeot et al. (2003).

In conclusion, the results of the present study indicate that sialorphin increases the antinociceptive effects of ME to μ-opioid receptors without toxicity by an action other than peptidase inhibition.

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**Participated in research design:** Kan, Yoshikawa, Miura.

**Conducted experiments:** Kan, Yoshikawa, Watanabe, Iwao.
Sialorphin Potentiates Effects of [Met]Enkephalin 113


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