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Sialorphin potentiates effects of [Met⁵]enkephalin without toxicity by action other than
peptidase inhibition

Takugi Kan, Masanobu Yoshikawa, Mariko Watanabe, Masaaki Miura, Kenji Ito,
Mitsumasa Matsuda, Kayoko Iwao, Hiroyuki Kobayashi, Takeshi Suzuki and
Toshiyasu Suzuki

TK, MW, MM, KI, MM, TS, and TS: Department of Anesthesiology, Tokai University
School of Medicine

MY and HK: Department of Clinical Pharmacology, Tokai University School of
Medicine

KI: Education and Research Support Center, Tokai University School of Medicine

JPET # 266080

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Corresponding author: Masanobu Yoshikawa

Address: Department of Clinical Pharmacology, Tokai University
School of Medicine, Isehara, Kanagawa 259-1193, Japan

Tel: +81-463-93-1121

Fax: +81-463-93-2896

E-mail: yoshikaw@is.icc.u-tokai.ac.jp

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JPET # 266080

ABBREVIATIONS:

A, amastatin; ACP, amastatin and captopril and phosphoramidon; ANOVA, analysis of variance; APN, aminopeptidase N; C, captopril; AUC, the area under the curve; Bmax, maximal binding capacities; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂; DAMGO, (D-Ala(2)-N-MePhe(4)-Gly-ol(5)-enkephalin; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; IC₅₀, concentration required to produce 50% inhibition of contraction; i.t., intrathecal; Kd, binding affinity; MDI, motor deficit index; ME, [Met⁵]enkephalin; MERF, [Met⁵]enkephalin-Arg⁶-Phe⁷; MPE, maximal possible effect; MVD, mouse vas deferens; AUC, area under the curve; NEP, neutral endopeptidase; nor-BNI, nor-binaltorphimine dihydrochloride; NOX, naloxone hydrochloride; NTI, naltrindole hydrochloride; P, phosphoramidon; PIs, peptidase inhibitors; PROL1, proline-rich lacrimal 1; SMR1, submandibular rat1; Tris-HCl, Tris(hydroxymethyl)aminomethane hydrochloride; Vcsa1, variable coding sequence A1

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JPET # 266080

Abstract

This dose-response study investigated the effects of sialorphin on [Met⁵]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 μM) and a mixture of the three peptidase inhibitors (1 μM each) was higher than that with the mixture of peptidase inhibitors alone at any dose. Intrathecal administration of sialorphin (100 to 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 DAMGO (D-Ala(2)-N-MePhe(4)-Gly-ol(5)-enkephalin). These results indicate that sialorphin potentiates the effects of ME without toxicity by a mechanism other than peptidase

JPET # 266080

inhibition and with no effect on its affinity to μ -opioid receptors.

Significance Statement

Sialorphin is regarded as an endogenous peptidase inhibitor which interacts with enkephalin-degrading enzymes. The results of these *in vitro* and *in vivo* studies confirm that sialorphin potentiates the effects of ME 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.

JPET # 266080

Introduction

Earlier studies have demonstrated rapid degradation of opioid peptides by any of 5 types of peptidase: 1) aminopeptidase N (EC 3.4.11.2), which cleaves the Tyr¹–Gly² amide bond; 2) dipeptidyl peptidase III (EC 3.4.14.4), which hydrolyzes the Gly²–Gly³ 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 Gly³–Phe⁴ bond; and 5) carboxypeptidase A (EC 3.4.17.1) (Khaket 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 [Met⁵]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 (A), an aminopeptidase inhibitor; captopril (C), a dipeptidyl carboxypeptidase inhibitor; and phosphoramidon (P), 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 (HPLC) that showed that a mixture of these PIs almost completely inhibited the degradation of ME (Hiranuma and Oka, 1986).

JPET # 266080

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). 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 and colleagues 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 itself (Toth 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

JPET # 266080

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, while 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 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 salvinorin-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 (PAM) 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 analogues (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).

JPET # 266080

In this dose-response study, the effects of sialorphin on ME-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 DAMGO (D-Ala(2)-N-MePhe(4)-Gly-ol(5)-enkephalin).

JPET # 266080

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 (7-8 weeks-old, 180–220 g each, n=276; Nihon Clea, Tokyo, Japan) and male ICR JCL mice (9-10 weeks-old, weighing 30-40 g each, n=40; Nihon Clea, Tokyo Japan) were housed in an air-conditioned room at a control temperature of 24°C–26°C and 50%–60% humidity, with a 12-hour light/dark cycle (lights on: 07:00 hrs) 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; Rosinski-Chupin et al., 1993; Rosinski-Chupin et al., 2001; Messaoudi et al., 2004). Sialorphin is released into the

JPET # 266080

bloodstream from the submandibular gland and prostate in response 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 (A), and phosphoramidon (P) (The Peptide Institute Inc., Minoh, Japan). The following were all purchased from SIGMA Japan (Tokyo, Japan): captopril (C), D-Phe-Cys-Tyr-D-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 non-selective opioid receptor antagonist naloxone hydrochloride (NOX) was purchased from Daiichi-Sankyo Company, Limited (Tokyo, Japan). All of the above were dissolved in saline, except for nor-BNI and NTI, which were dissolved in water, and BMS-986124, which was dissolved in 50% dimethyl sulfoxide (DMSO) and 50% saline. The desired concentration of each solution was prepared at the time it was to be used, and each administered i.t. at a volume of 10 μ l. Administration of the PIs was performed at 10

JPET # 266080

min 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 (Benyhe et al., 1997) with some modifications. The animals were decapitated. The brains were then rapidly removed and washed several times with ice-cold 50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.4). Membrane fraction was resuspended in 5 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose. Aliquots of the membrane fraction were frozen 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 (40,000 × g, at 4°C, for 20 min) to remove sucrose. Following 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 min, the membrane suspensions (protein concentration of 0.5 mg/ml) were incubated in glass tubes for 60 min at 25°C with the radioligand [³H] DAMGO (D-Ala(2)-N-MePhe(4)-Gly-ol(5)-enkephalin) in a final volume of 1 ml. Assays for Scatchard analysis were carried out at various concentrations of [³H] DAMGO. Non-specific binding was measured in the presence of unlabeled 10 mM DAMGO. Incubation was terminated by rapid filtration through Whatman GF/C glass fiber filters. After washing 3 times in 5 ml ice-cold buffer (50 mM

JPET # 266080

Tris-HCl, pH 7.4) radioactivity was measured in a scintillation cocktail (Pico-Fluor Plus, Perkin Elmer Japan) using a scintillation counter (2810, Perkin Elmer Japan, Tokyo). Experiments were carried out in duplicate and repeated 2 times. Ligand binding data were evaluated using computer software (GraphPad Prism, Version 6.0c, GraphPad Software, San Diego, CA, USA).

In vitro isolated preparations

Mouse vas deferens obtained from male ICR JCL mice (9-10 weeks-old) was subjected to electrical stimulation according to a method previously reported (Hughes et al., 1975; Oka et al., 1982). Four MVD samples were suspended separately in organ baths containing 4 mL Krebs solution each (millimolar concentration: NaCl, 118; KCl, 4.75; CaCl₂, 2.54; KH₂PO₄, 1.19; NaHCO₃, 3.25; glucose, 11); the temperature in the bath was maintained at 36°C and air bubbled through. Each drug was added in 40-μL amounts, after which it was washed out with 4 mL Krebs solution; the temperature in the bath was maintained at 36°C throughout this process. This was repeated 4 times, with a 5-minute interval between each experimental condition, unless specifically mentioned. Mouse vas deferens was stimulated with a supramaximal rectangular pulse of 1.0 msec in duration and at a frequency of 0.1 Hz using an electronic stimulator SEN-3201 (Nihon Koden, Tokyo, Japan). To determine the concentration required to produce 50% inhibition of contraction (IC₅₀), the degree

JPET # 266080

(%) 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 min before the enkephalin. The % difference was calculated as follows: % difference = $[(IC_{50} \text{ before each treatment} - IC_{50} \text{ after each treatment}) / IC_{50} \text{ before each treatment}] \times 100$. These differences are shown in the tables.

Intrathecal administration

Based on a method described in an earlier study (Murata et al., 2014), intrathecal (i.t.) catheters were implanted in male Wistar rats (7-8 weeks-old) under inhalation anesthesia with nitrous oxide, oxygen, and isoflurane (2%). An 8.5-cm polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, USA) was inserted caudally to the thoracolumbar level of the spinal cord in the intrathecal space through an incision in the atlanto-occipital membrane (Yaksh and Rudy, 1976). The external part of the catheter was tunneled subcutaneously to exit from the top of the skull and was plugged with a 30-gauge steel wire. Only rats with normal motor function and behavior were used for the study 7 days later. Drugs were injected at a volume of 10 μ l followed by 10 μ l saline over 1 min.

Tail-flick test

To eliminate bias, each investigator was blind to the drug administered. In

JPET # 266080

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), nociceptive stimulation was achieved by immersing the tail of each rat in hot water (55°C) for a maximum of 5 sec. 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 to 1.6 sec. After determining baseline latencies 3 times at 15-minute intervals, each drug was administered and tail flick latencies determined at 5, 10, 15, 30, 45, 60, 75, 90, 105 and 120 min after. The following formula was used to calculate the percent of maximal possible effect (MPE) for each animal at each time: $\%MPE = [(test\ latency - baseline\ latency)/(5 - baseline\ latency)] \times 100$. 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 ataxia present; 2, knuckle walking; 3, movement of

JPET # 266080

legs but unable to walk; or 4, no movement, drags legs. Each drug was administered and MDI assessed at 5, 10, 15, 30, 45, 60, 75, 90, 105 and 120 min after. The area under the curve (AUC) value for MDI of each drug was also calculated.

Statistical analyses

The results are shown as the mean and standard error of the mean. The statistical analysis software package (GraphPad Prism, Version 6.0c, GraphPad Software, San Diego, CA, USA) was used to compare across experimental conditions. The Friedman test followed by 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 analysis of variance (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 was 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 inter-group comparisons. A *P*-value less than 0.05 was considered to indicate a statistical significance.

In vitro isolated preparations experimental protocol

JPET # 266080

1. Ratio of potencies of ME in MVD pretreated with ACP with or without sialorphin

(Figure 1)

Four MVD were tested. Following 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.

2. Enhanced effect of sialorphin on the inhibitory potency of ME (Table 1)

Four MVD were tested. ME was added and washed out after their maximal effects had been noted among each dose of sialorphin from low to high dose.

3. Enhanced effect of combination of ACP on the inhibitory potency of ME in the presence or absence of sialorphin (Table 2)

Sixteen MVDs were divided 4 groups; AC, CP, AP, ACP (n=4 each).

4. Enhanced effect of sialorphin on the inhibitory potency of ME in the presence of ACP (Table 3)

Four MVD 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.

5. Inhibitory effect of sialorphin and ACP on electrically-evoked contractions in MVD at before and after administration of ME (Figure 2)

Sixteen MVDs were divided 2 groups (n=4). Each drug was sequentially added to the

JPET # 266080

MVD.

Animal experimental protocol

1. Combination of ME and sialorphin together with ACP

A previous study demonstrated that pretreatment with i.t. administration of a combination of 10 nmol each of amastatin (A), captopril (C) and phosphoramidon (P) completely inhibited peptidase-induced degradation of ME (Murata et al., 2014). In the present study, i.t. administration of ME (1 nmol) was performed at 10 min after i.t. 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.

2. Dose-response study

Intrathecal administration of ME was performed at 10 min following i.t. 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)

JPET # 266080

alone or together with ACP (3-30 nmol each).

3. *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 administered i.t. under pretreatment with each of the following combinations: AC (amastatin, captopril; 10 nmol each), CP (captopril, phosphoramidon; 10 nmol each), or AP (amastatin, phosphoramidon; 10 nmol each).

4. *Sialorphin-induced antinociception*

Sialorphin alone, ACP (10 nmol each) alone, or both in combination were administered i.t. to determine their antinociceptive effect.

5. *Selective or non-selective opioid receptor antagonists*

The effect of opioid receptor antagonists on ME (3 nmol, i.t.)-induced antinociception under pretreatment with saline (i.t.), sialorphin (i.t.) and ACP (10 nmol each, i.t.) was investigated by injection of NOX (0.5-2.0 mg/kg, subcutaneously), CTOP (3 nmol, i.t.) (Walker, 2006), NTI (132 nmol, i.t.; final concentration of DMSO, 0.5%) (Malmberg and Yaksh, 1992), or nor-BNI (20 mg/kg, subcutaneously; final concentration of DMSO, 8%) (Xie et al., 2008) at 20 min, 15 min, 30 min, and 24 hr, respectively, before i.t. administration of ME. These antagonists caused no observable toxicity after administration.

JPET # 266080

6. Silent allosteric modulator of μ -opioid receptors

The effect of a silent allosteric modulator of μ -opioid receptors on ME (1 nmol)-induced antinociception under pretreatment with sialorphin (400 nmol) and ACP (10 nmol each) was investigated by i.t. injection of BMS-986124 (1 or 5 nmol; final concentration of DMSO, 0.75%) at 15 min before i.t. administration of sialorphin and ACP. This SAM caused no observable toxicity after administration.

Results

Enhancement of potency of ME by sialorphin in MVD

The results showed 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^{-6} M, 4.0; 10^{-5} M, 8.0; 10^{-4} M, 15.0; 2×10^{-4} M, 13.0). Figure 1 shows ME-induced inhibition following 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 ($F_{5, 38} = 5.618$, $P = 0.0006$). 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 to 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^{-5} M

JPET # 266080

(Figure 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 (amastatin, phosphoramidon) and sialorphin than with AP alone; that with AC (amastatin, captopril), CP (captopril, phosphoramidon), or ACP (amastatin, captopril, phosphoramidon) 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 Figure 2, Mann-Whitney test, $P = 0.1143$, $n = 4$). In contrast, sialorphin enhanced the efficacy of ME with ACP (B-6 in Figure 2, Mann-Whitney test, $P = 0.0286$, $n = 4$) and in a dose-dependent manner (Table 3, Friedman test, $P < 0.0001$)(mean rank difference: sialorphin 0 M, 4.00; 10^{-4} M, 9.75; 2×10^{-4} M, 10.25). Each drug was sequentially added to the MVD (Figure 2). The ratio of potencies of ME in MVD under pretreatment with sialorphin (2×10^{-4} M) and ACP were higher than those with ACP alone at any dose (Figure 1, $F_{5, 38} = 5.618$, $P = 0.0006$).

Effect of sialorphin on ME-induced antinociception

The antinociceptive effect observed with i.t. 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 (Figure 3a, $F_{30, 210} = 4.074$, $P < 0.0001$). The $AUC_{0-45\text{min}}$ value for %MPE of 1 nmol ME with 400 nmol sialorphin was approximately equal to that with

JPET # 266080

a mixture of the three PIs (10 nmol each) (Figure 3b, Kruskal-Wallis test, $P = 0.0007$). Figure 4a shows ME-induced antinociception from 10 min following i.t. administration of the combination of 200 nmol sialorphin and ACP (10 nmol each)($n=6$ each dose) or ACP alone ($n=6$ each dose). A dose-dependent and prolonged antinociceptive effect on the tail-flick response was observed with administration of ME (1, 3, or 10 nmol)($F_{3,40} = 4.350$, $P = 0.0096$). The sharp symbols above the $AUC_{0-45\text{min}}$ values with 3 nmol ME under pretreatment with sialorphin and ACP indicate significant differences compared to that with ME under pretreatment with ACP. Asterisks placed above the $AUC_{0-45\text{min}}$ values for ME at doses of 3 or 10 nmol under pretreatment with sialorphin and ACP indicate significant differences compared to that for sialorphin and ACP without ME (Kruskal-Wallis test, $P = 0.0009$). Asterisks placed above the $AUC_{0-45\text{min}}$ values for ACP (10 nmol each) with or without sialorphin on ME indicate significant differences compared to that with or without sialorphin on saline (Kruskal-Wallis test, $P = 0.0381$). Figure 4b shows ME (3 nmol)-induced antinociception from 10 min following i.t. 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 $AUC_{0-45\text{min}}$ values for sialorphin at all doses with ACP on ME indicate

JPET # 266080

significant differences compared to that for sialorphin with saline on ME. Asterisks placed below the $AUC_{0-45\text{min}}$ values for 200 nmol sialorphin with ACP on ME indicate significant differences compared to saline with ACP on ME (Kruskal-Wallis test, $P = 0.0090$). Figure 4c shows ME-induced antinociception from 10 min following i.t. 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 $AUC_{0-45\text{min}}$ value demonstrated that induction of ME-induced antinociception by 200 nmol sialorphin and ACP (3, 10, or 30 nmol each) was significantly greater than that with ACP alone. Sharp symbols above the $AUC_{0-45\text{min}}$ values for ACP (10 nmol each) together with 200 nmol sialorphin on ME indicate significant differences compared to that for ACP alone on ME. Asterisks placed above the $AUC_{0-45\text{min}}$ values for ACP (10 nmol each) with 200 nmol sialorphin on ME indicate significant differences compared to that for saline with sialorphin on ME (Kruskal-Wallis test, $P = 0.0045$). Asterisks placed above the $AUC_{0-45\text{min}}$ values for ACP (10 nmol each) alone on ME indicate significant differences compared to that for saline on ME (Kruskal-Wallis test, $P = 0.0209$).

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

JPET # 266080

A significant increase was observed in the antinociceptive potency of ME (3 nmol) under pretreatment with AC (amastatin, captopril; 10 nmol each), CP (captopril, phosphoramidon; 10 nmol each) or AP (amastatin, phosphoramidon; 10 nmol each) in the presence of 200 nmol sialorphin in comparison with AC, CP or AP alone (Figure 4d)(AC, AP, CP each; $P = 0.0286$).

Antinociceptive effects of sialorphin or ACP alone

A significant increase in antinociception was observed following i.t. administration of ACP (10 nmol each) alone or in combination with sialorphin (200 nmol each); sialorphin alone, however, yielded no increase in antinociception (Figure 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 antinociception under pretreatment with sialorphin and ACP

The results showed that NOX significantly attenuated the antinociceptive effect of ME under pretreatment with ACP (Figure 5b). The antinociceptive potency of ME under pretreatment with ACP following administration of NOX (1 mg/kg) was approximately equal to that under pretreatment with saline (data not shown). The antinociceptive effect of ME was attenuated dose-dependently by NOX under pretreatment with sialorphin (200 nmol) and ACP (10 nmol each) (Kruskal-Wallis test,

JPET # 266080

$P = 0.0028$). The antinociceptive potency of ME (3 nmol) under pretreatment with sialorphin and ACP following administration 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) (Figure 5b). The antinociceptive potency of ME under pretreatment with sialorphin (200 nmol) and ACP (10 nmol each) was significantly attenuated by CTOP (3 nmol) or NTI (132 nmol); it was not attenuated by nor-BNI (20 mg/kg) (Figure 5c)(Kruskal-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 pretreatment with sialorphin and ACP

Assuming that allosteric modulators have higher selectivity for receptor subtypes and that PAM 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 high dose of sialorphin and low dose of ME. The results showed that BMS-986124 significantly and dose-dependently attenuated the enhancing effects of sialorphin (400 nmol) on ME (1 nmol)-induced antinociception in the presence of ACP (Figure 5d)(Kruskal-Wallis test, $P = 0.0019$). The antinociceptive potency of ME under pretreatment with sialorphin and ACP following administration of BMS-986124 (5 nmol) was approximately equal to that under pretreatment with ACP

JPET # 266080

alone (mean rank difference: 5 nmol, -2.00; 1 nmol, -9.00; 0 nmol, -12.60).

Effect of sialorphin and PIs on motor dysfunction induced by i.t. 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 (Figure 6a and 6b) (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 (Figure 6c and 6d)(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 Bmax of DAMGO in presence of ACP

Sialorphin showed no effect on the binding affinity of [3 H] DAMGO: Kd in the absence of sialorphin was 1.149 nM (95% CI: 0.7834 - 1.515); Kd in the presence of 100 μ M sialorphin was 1.272 nM (95% CI: 0.9092 - 1.635)(Figure 7). Sialorphin also showed no effect on maximal binding capacity of [3 H] DAMGO: Bmax in the absence of sialorphin was 9.11 fmol/mg tissue (95% CI: 8.232 - 9.99), while Bmax in the presence of 100 μ M sialorphin was 9.228 fmol/mg (95% CI: 8.41 - 10.048)(Figure 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

JPET # 266080

antinociception induced by i.t. administration of ME without signs of toxicity such as motor dysfunction in rat. This supports the hypothesis that augmenting the affinity and efficacy of low doses of opioid drugs or endogenous opioids would increase antinociception, but with no concomitant increase in toxicity (Thompson et al., 2015). The present results do not preclude the notion that sialorphin may enhance inhibition of peptidases; rather, they suggest that another mechanism is involved in sialorphin-induced increase in the efficacy of ME. This is in good agreement with the results an earlier study showing that acetylcholinesterase inhibitor galantamine increased the frequency of ion-channel opening of nicotinic acetylcholine receptors as PAM (Samochocki et al., 2003). This earlier result suggests that galantamine-enhanced activity in nicotinic acetylcholine receptors is mainly due to 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_{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

JPET # 266080

endopeptidase-24.11. This is in good agreement with the results of an earlier HPLC analysis, which revealed that ME was almost solely catabolized by these 3 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). While BMS-986124 did not significantly increase the potency of morphine at high dose, it increased the maximal response of morphine at high dose, but with only a slight decrease at low dose (Burford et al.,

JPET # 266080

2013). Further investigation using PAMs such as BMS-9861242 to assess the impact of allosteric modulators on the antinociceptive effects of ME compared to sialorphin is required.

Rougeot *et al.* 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): one, that opiorphin increased the maximal binding capacities (B_{max}) of [³H][Met⁵]enkephalin-Arg⁶-Phe⁷ ([³H]MERF), but not [³H]endomorphin-1, at both 0 °C and 24°C, in a saturation binding assay; and that opiorphin increased the affinity of MERF in competition studies with [³H]MERF 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 (MERF IC₅₀ in the presence of opiorphin was 5.8 nM; IC₅₀ in the presence of a mixture of the PIs was 59 nM), but also at 0°C (IC₅₀ in the presence of opiorphin was 15.2 nM; IC₅₀ in the presence of a mixture of the PIs was 26.4 nM). The finding of the present study that sialorphin did not affect the binding affinity of [³H]DAMGO is in agreement with this earlier observation. Two parallel signaling cascades have been reported in opioid receptors (Georgoussi *et al.*, 2012). They functionally interact with G protein, inhibiting the ‘adenylate cyclase-cAMP-protein kinase A’ signaling pathway and subsequently inducing opioid analgesia. β-arrestin-mediated

JPET # 266080

signaling is critical for receptor desensitization and trafficking, including phosphorylation and internalization of opioid receptors (Shukla et al., 2011; Allouche et al., 2014). Further investigation into the molecular features of such signal cascades in opioid receptors would help elucidate functions of sialorphin other than that involved in peptidase inhibition.

Sialorphin was identified as the amino terminal region of submandibular rat1 (SMR1), a 146-amino acid encoded by the *Vcsa1* (variable coding sequence A1) gene. The VCS genes are divided into two subgroups: VCSA, which includes *Vcsa1*, and VCSB (Chupin and Rougeon, 2009). Human VCSB includes *PROL1* (proline-rich lacrimal 1), which encodes opiorphin, which is functionally equal to sialorphin as an endogenous PI (Morris et al., 2007) (Wisner et al., 2006). Expression of *Vcsa1* and *PROL1* is hormonally regulated by androgens (Messaoudi et al., 2004) (Rosinski-Chupin et al., 2001) (Tong et al., 2008). Interestingly, androgen or testosterone reduces pain-induced response (English et al., 2000; Kaergaard et al., 2000; Aloisi et al., 2004). Independently of hormonal regulation, intraperitoneal administration of beta-adrenergic agonists increased SMR1 levels in rat submandibular gland, resulting in secretion into plasma and saliva (Rougeot et al., 1994; Rougeot et al., 2000). A large number of experimental and clinical studies demonstrated that pain is associated with increased activity of the sympathoadrenal

JPET # 266080

system, leading to elevation of the plasma concentrations of epinephrine, an endogenous beta-adrenergic receptor agonist (Cryer, 1980) (DeTurck and Vogel, 1980) (Taylor et al., 1989). Taken together, these findings suggest that pain induces expression and secretion of sialorphin by hormonal and adrenergic regulation, resulting in relief of pain.

In the present study, sialorphin was observed to enhance ME-induced inhibition of electrically-evoked contractions in MVD in the absence of ACP; no such effect was seen in the tail-flick test, however. This discrepancy may be explained by differences in peptidase activity in MVD and rat spinal cord. Indeed, high amounts of NEP and APN were detected in rat spinal cord (Waksman et al., 1986; Noble et al., 2001). In the present study, the IC_{50} values of ME were approximately 20 nM in the *in vitro* experiments using isolated MVD, which was in good agreement with the results of earlier studies. However, one earlier study found that i.t. administration of ME at a dose of 20 nmol had no antinociceptive effect (Murata et al., 2014). This dose can be taken to correspond to approximately 200 μ M based on the finding that the amount of cerebrospinal fluid was approximately 100 μ l 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

JPET # 266080

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 (Melik Parsadaniantz et al., 2015), such as that seen between the present study and that of Rougeot et al. (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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Authorship Contributions

Participated in research design: Kan, Yoshikawa and Miura.

Conducted experiments: Kan, Yoshikawa, Watanabe and Iwao

Contributed new reagents or analytic tools: Ito and Matsuda.

Performed data analysis: Kan and Yoshikawa.

Wrote or contributed to the writing of the manuscript: Kan, Yoshikawa, Kobayashi, Takeshi Suzuki and Toshiyasu Suzuki.

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JPET # 266080

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JPET # 266080

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JPET # 266080

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JPET # 266080

Legends for Figures

Figure 1: Ratio of potencies of ME in MVD pretreated with ACP with or without sialorphin (200 μ M). Significantly different from ME alone (open circle) or sialorphin-ME (closed circle) 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 ACP at same dose of ACP according to Dunn's post-hoc test following two-way repeated measures ANOVA; ### $P < 0.001$.

Figure 2: Inhibitory effect of sialorphin and ACP on electrically-evoked contractions in MVD at before and after administration of ME. Compounds were added sequentially (dots shown in upper figures). In the lower table, data are reported as the means of results from four samples in independent experiments (A and B). Significantly different from in ACP (1×10^{-5} M) pre-treated control according to Mann-Whitney test.

Figure 3: Potentiating effect of PIs on antinociception induced by i.t. administration of ME with or without sialorphin. Upper panel (a) indicates time course of %MPE of ME (1 nmol) under pretreatment with saline and ACP with or without sialorphin. Significantly different from ME alone-treated control (open circle) according to Dunn's post-hoc test following two-way repeated measures ANOVA; * $P < 0.05$, ** $P < 0.01$,

JPET # 266080

and $***P < 0.001$. Lower panel (b) shows $AUC_{0-45min}$ for value of %MPE indicated in upper panel (a). A value of zero indicates i.t. administration of saline. Significantly different from ME alone-treated control according to Dunn's post-hoc test following Kruskal-Wallis test; $***P < 0.001$.

Figure 4: (a) ME-dependent antinociception with i.t. administration of 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 following Kruskal-Wallis test; $*P < 0.05$ and $**P < 0.01$. Significantly different from under pretreatment with ACP alone in control at same dose of ME according to Dunn's post-hoc test following two-way repeated measures ANOVA; $\#P < 0.05$ and $##P < 0.01$. (b) Sialorphin-dependent antinociception with i.t. administration of ME under pretreatment with (closed circle; n=5 each dose) or without (open circle; n=5 each dose) mixture of PIs (ACP) by dose. Significantly different from sialorphin-treated control at same dose according to Dunn's post-hoc test following Kruskal-Wallis test; $##P < 0.01$ and $###P < 0.001$. Significantly different from ME (3 nmol) under pretreatment with ACP alone in control according to Dunn's post-hoc test following two-way repeated measures ANOVA; $*P < 0.05$. (c) ACP-dependent antinociception with i.t. administration of ME (3 nmol)

JPET # 266080

under pretreatment with (closed circle; n=5 each dose) or without sialorphin (open circle; n=5 each dose) by dose. Significantly different from ME under pretreatment with or without sialorphin according to Dunn's post-hoc test following Kruskal-Wallis test; $**P < 0.01$ and $***P < 0.001$. Significantly different from under pretreatment with ACP without sialorphin at same dose of ACP according to Dunn's post-hoc test following 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 i.t. 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 i.t. administration of saline. Significantly different from in control treated with pairs of PIs alone according to Mann-Whitney test; $*P < 0.05$.

Figure 5: (a) Antinociceptive effect of saline alone (n=5), sialorphin alone (n=6), ACP alone (n=5), or sialorphin with ACP (n=6). A value of zero indicates i.t. administration of saline. Significantly different from saline-treated control (open circle) according to Dunn's post-hoc test following Kruskal-Wallis test; $*P < 0.05$ and $***P < 0.001$. (b) The effect of naloxone (0.5-2.0 mg/kg, subcutaneously, n=4 each) on ME (3 nmol, i.t.)-induced antinociception under pretreatment with saline (i.t.), sialorphin (200 nmol, i.t.) and ACP (10 nmol each, i.t.). To evaluate the effects of naloxone, saline was

JPET # 266080

administered subcutaneously and intrathecally (n=5). A value of zero indicates administration of saline. Significantly different from in saline-administered group (open circle, subcutaneously and i.t. administration, n=5) according to Dunn's post hoc test following Kruskal–Wallis test, $***P < 0.001$. (c) ME (1 nmol) -induced antinociception under pretreatment with sialorphin and PIs following administration of three opioid receptor selective antagonists CTOP (3 nmol; n=4), NTI (132 nmol; n=5), or nor-BNI (20 mg/kg; n=5). A value of zero indicates i.t. administration of saline. Significantly different from ME under pretreatment with saline (i.t.)(open circle, n=5), sialorphin and PIs according to Dunn's post hoc test following Kruskal–Wallis test, $*P < 0.05$. (d) Effect of BMS-986124 (0, 1 and 5 nmol; n=5 each), a silent allosteric modulator of μ -opioid receptor, on antinociception by i.t. administration of ME under pretreatment with sialorphin (400 nmol) and mixture of PIs (ACP). Significantly different from ME under pretreatment with a mixture of PIs (ACP, 10 nmol each) alone following administration of DMSO (final concentration, 0.14%)(open circle; n=5) according to Dunn's post hoc test following Kruskal–Wallis test, $*P < 0.05$ and $**P < 0.01$.

Figure 6: Effects of sialorphin or ACP on ME-induced antinociception without motor dysfunction. (a) Time course of %MPE of ME (1000 nmol) under pretreatment with saline, and ME (10 nmol) under pretreatment with ACP with or without sialorphin. (b)

JPET # 266080

Effects of sialorphin or ACP on ME-induced antinociception. $AUC_{0-45min}$ for value of %MPE indicated in left panel (a). Significantly different from 10 nmol ME with both sialorphin and ACP -treated group according to Dunn's post-hoc test following Kruskal-Wallis test; $**P < 0.01$. (c) Time course of value of motor deficit index (MDI) of ME (1000 nmol) under pretreatment with saline, and ME (10 nmol) under pretreatment with ACP with or without sialorphin. (d) Effects of sialorphin or ACP on ME-induced motor dysfunction. $AUC_{0-45min}$ for value of MDI indicated in left panel (c). Significantly different from ME alone-treated control according to Dunn's post-hoc test following Kruskal-Wallis test; $**P < 0.01$.

Figure 7: Saturation binding experiments using [3H] DAMGO (a μ -opioid receptor agonist) were carried out in the absence or presence of sialorphin (100 μM) under treatment with ACP (1 μM each). The graph presents the specific binding signal of [3H] DAMGO. Scatchard analysis subsequently was performed (inset graph).

JPET # 266080

Table 1. Enhanced effect of sialorphin on the inhibitory potency of ME in MVD

Sia (M)	IC ₅₀ ($\times 10^{-9}$ M)	Ratio of Potency
None	17.11 \pm 1.69	1
10 ⁻⁶	11.94 \pm 0.41	1.45 \pm 0.19
10 ⁻⁵	5.83 \pm 0.59	3.07 \pm 0.56
10 ⁻⁴	3.00 \pm 0.30	5.70 \pm 0.09 **
2 \times 10 ⁻⁴	3.55 \pm 0.24	4.90 \pm 0.65 *

Dunn's post hoc test compared to ME alone, * $P < 0.05$ and ** $P < 0.01$, n=4

JPET # 266080

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

PIs (10 ⁻⁶ M each)	IC ₅₀ (×10 ⁻⁹ M)		Ratio of Potency	
	Sia (None)	Sia (1 × 10 ⁻⁴ M)	Sia (None)	Sia (1 × 10 ⁻⁴ M)
AC	5.75 ± 0.75	4.18 ± 0.32	3.70 ± 0.59	5.10 ± 0.90
CP	1.98 ± 0.22	1.49 ± 0.12	10.57 ± 1.36	13.92 ± 1.78
AP	2.46 ± 0.27	0.79 ± 0.07	8.67 ± 1.48	26.68 ± 4.34 *
ACP	1.34 ± 0.21	0.83 ± 0.11	16.34 ± 3.24	26.58 ± 5.50

Dunn's post hoc test compared to without sialorphin, **P* < 0.05, n=4

JPET # 266080

Table 3. Enhanced effect of sialorphin on the inhibitory potency of ME
 in MVD in the presence of ACP

ACP (M each)	Sia (M)	IC ₅₀ (×10 ⁻¹⁰ M)	Ratio of Potency
None	None	177.56 ± 25.07	1
5×10 ⁻⁶	None	6.60 ± 0.31	27.27 ± 4.39
5×10 ⁻⁶	10 ⁻⁴	4.09 ± 0.46	45.20 ± 8.87 *
5×10 ⁻⁶	2×10 ⁻⁴	4.12 ± 0.55	44.88 ± 8.28 **

Dunn's post hoc test compared to ME alone, *P < 0.05 and **P < 0.01, n=4

Figure 1

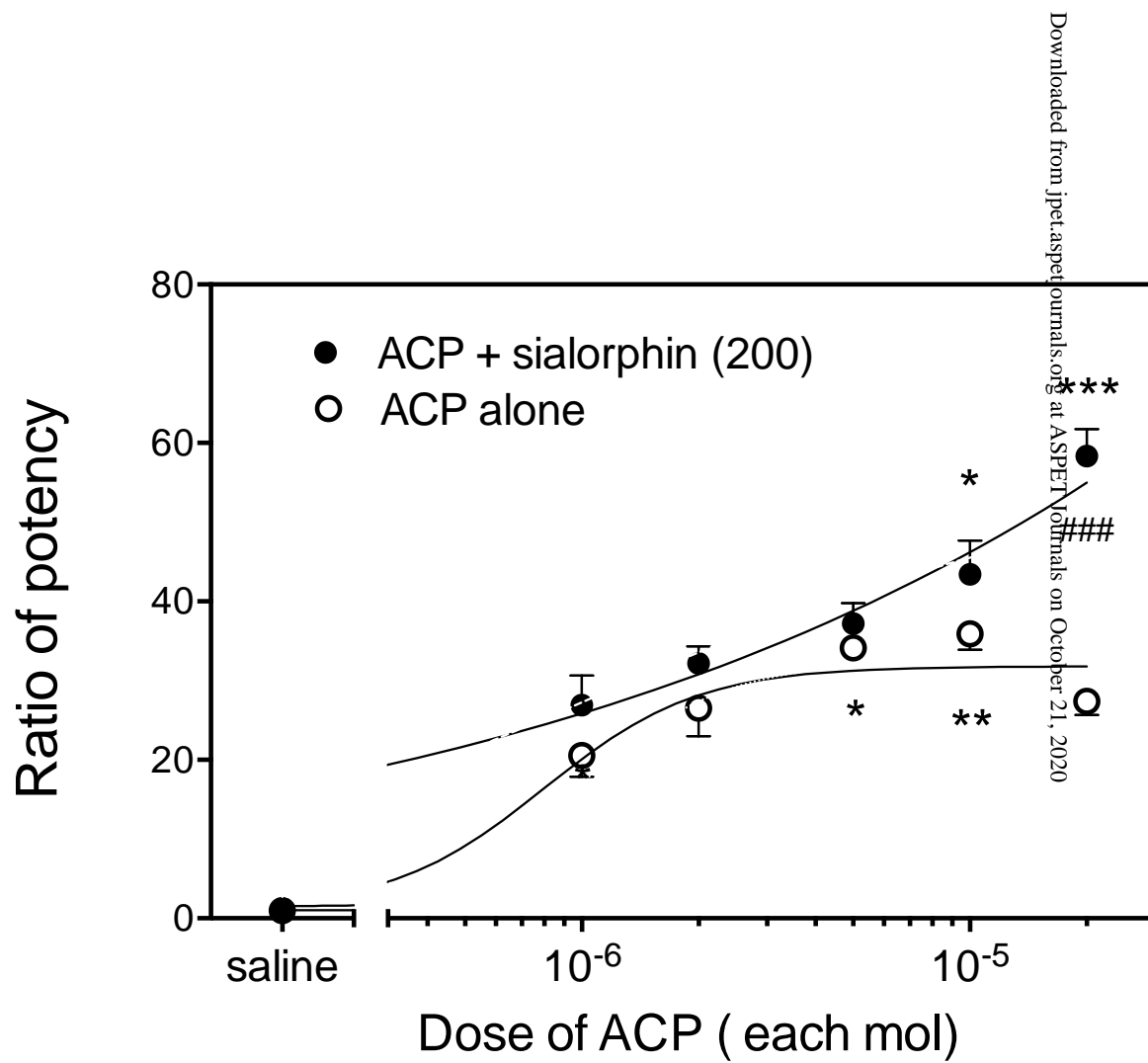
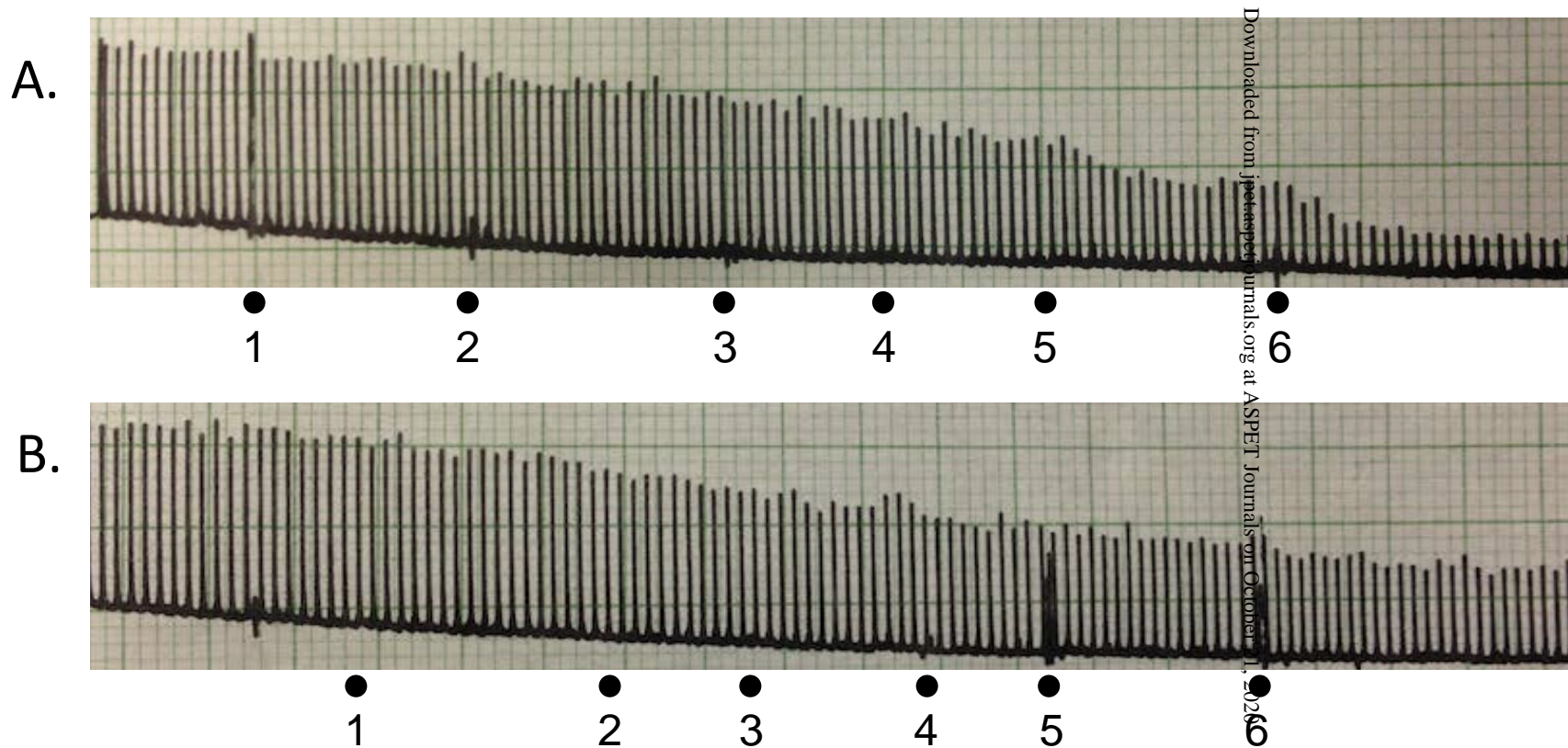


Figure 2

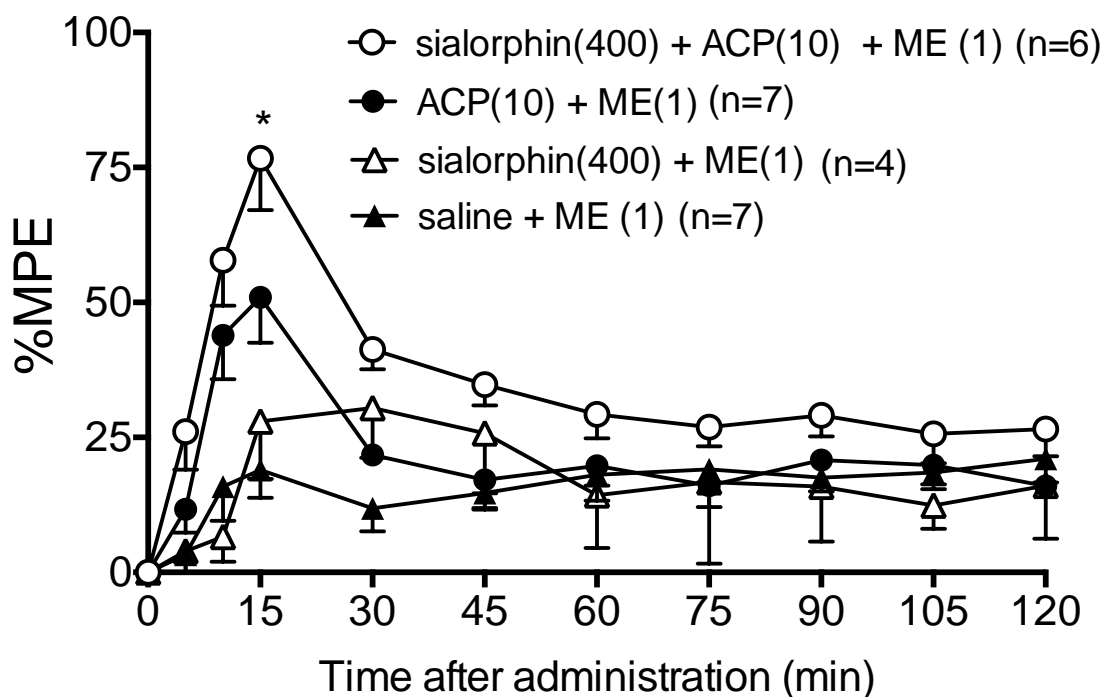


A	Drug (M)	inhibition rate (%)	B	Drug (M)	inhibition rate (%)
1	ACP (1×10^{-5})	0.46 ± 0.32	1	ACP (5×10^{-6})	1.39 ± 0.43
2	Sia (2×10^{-4})	2.84 ± 1.28	2	ME (1×10^{-10})	12.40 ± 1.90
3	ME (1×10^{-10})	16.21 ± 2.87	3	ME (2×10^{-10})	25.03 ± 0.43
4	ME (2×10^{-10})	29.44 ± 3.25	4	ME (3×10^{-10})	35.55 ± 1.85
5	ME (5×10^{-10})	63.48 ± 2.75	5	ACP (1×10^{-5})	37.69 ± 2.68
6	ME (1×10^{-9})	86.71 ± 1.81	6	Sia (2×10^{-4})	54.37 ± 2.46

$p=0.1143$ (for comparison between A and B points 1-3)
 $p=0.0286$ (for comparison between A and B points 5-6)

Figure 3

(a)



(b)

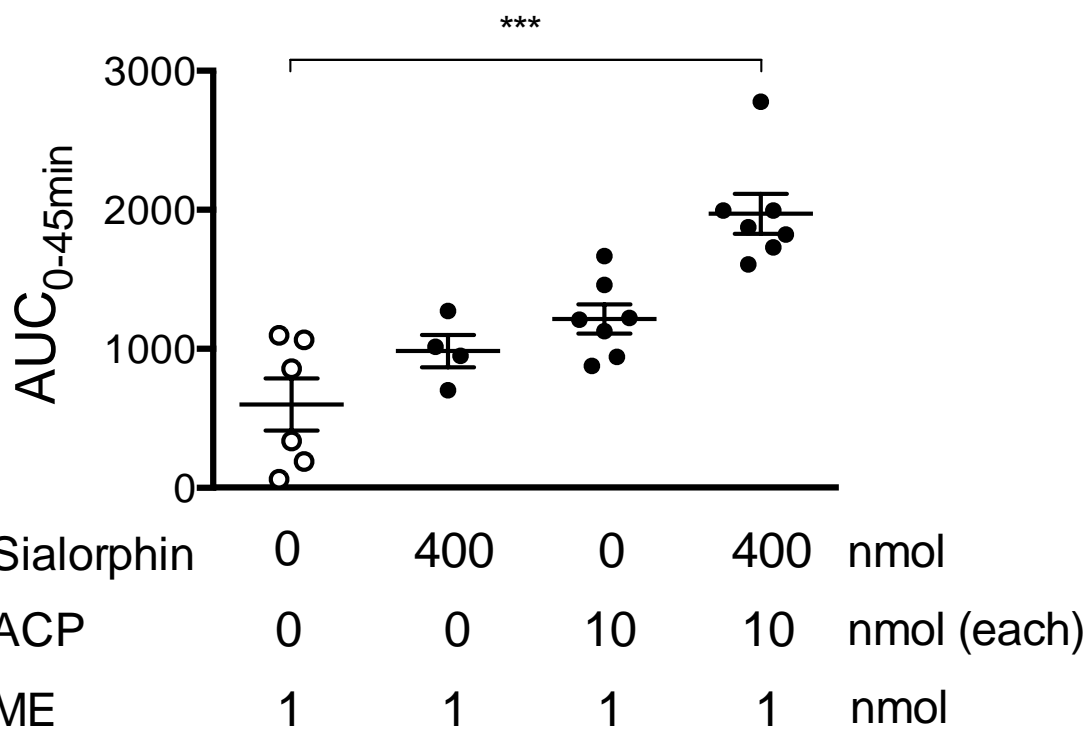
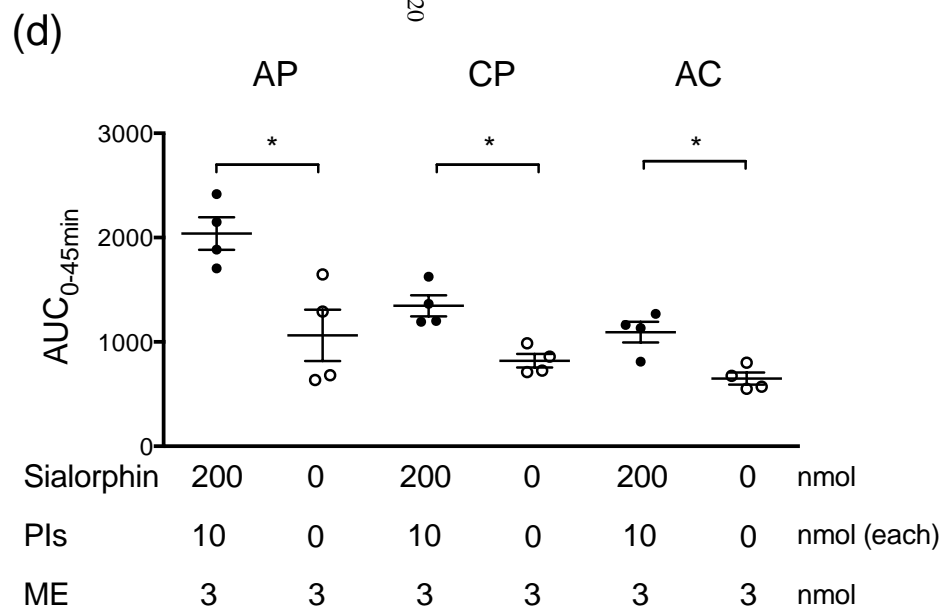
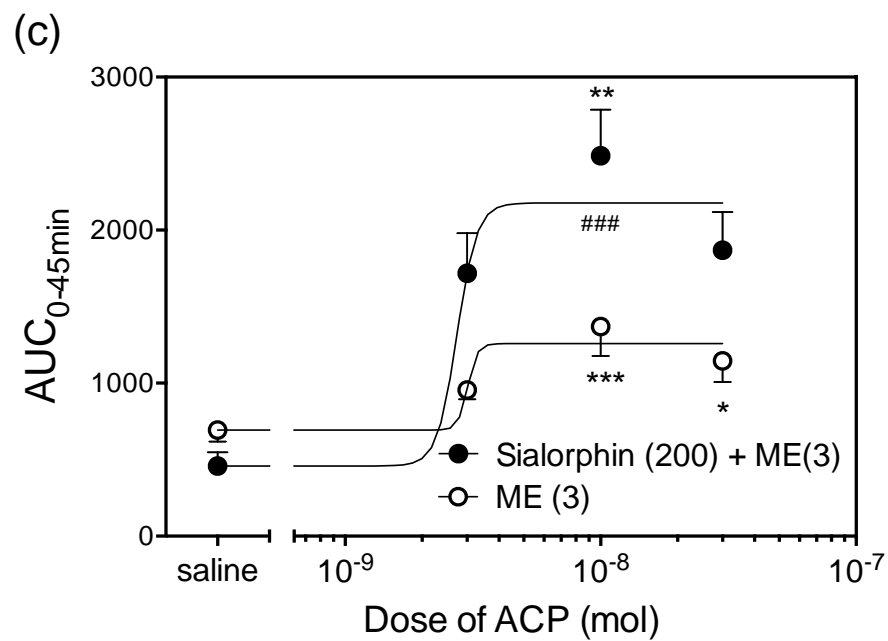
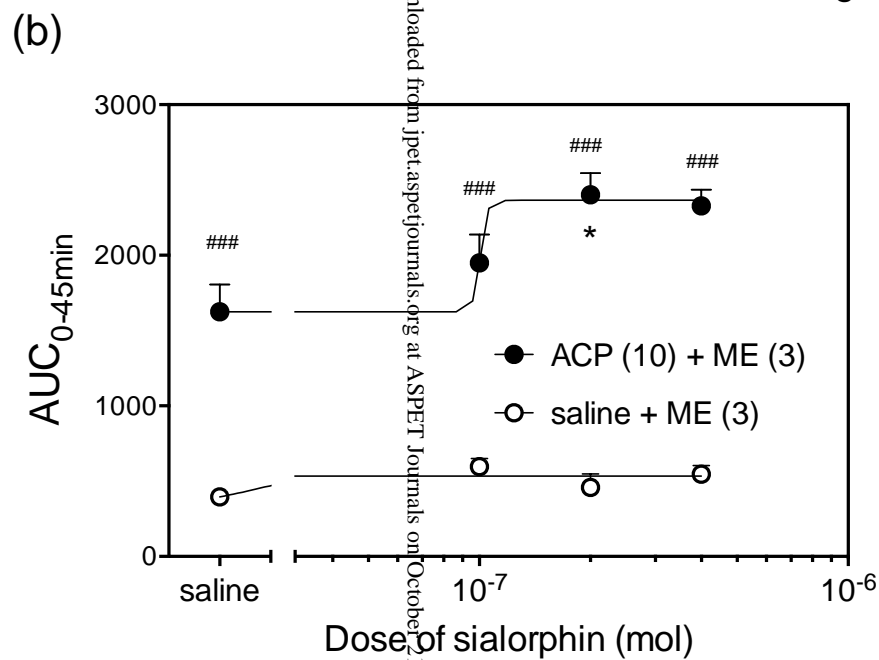
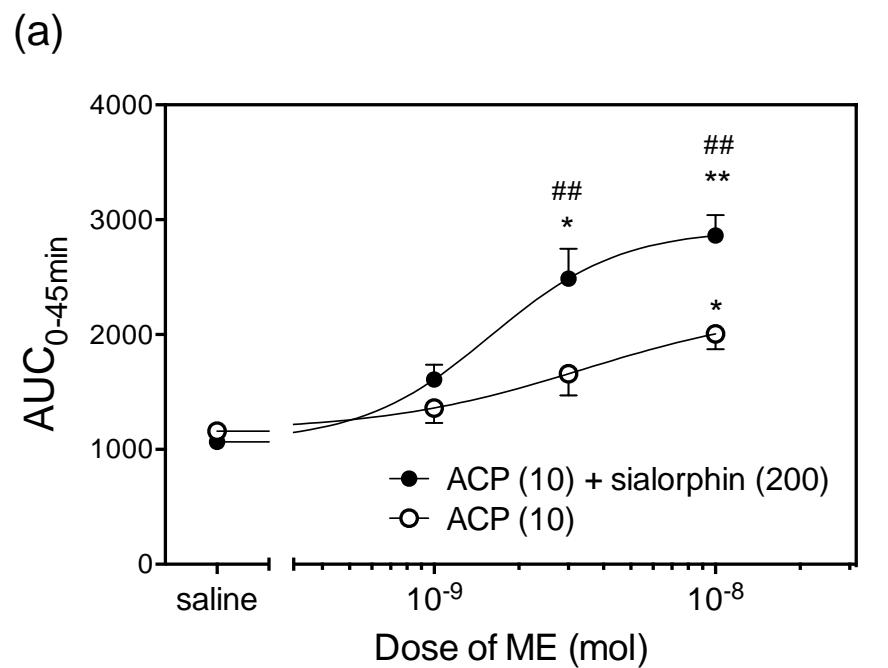
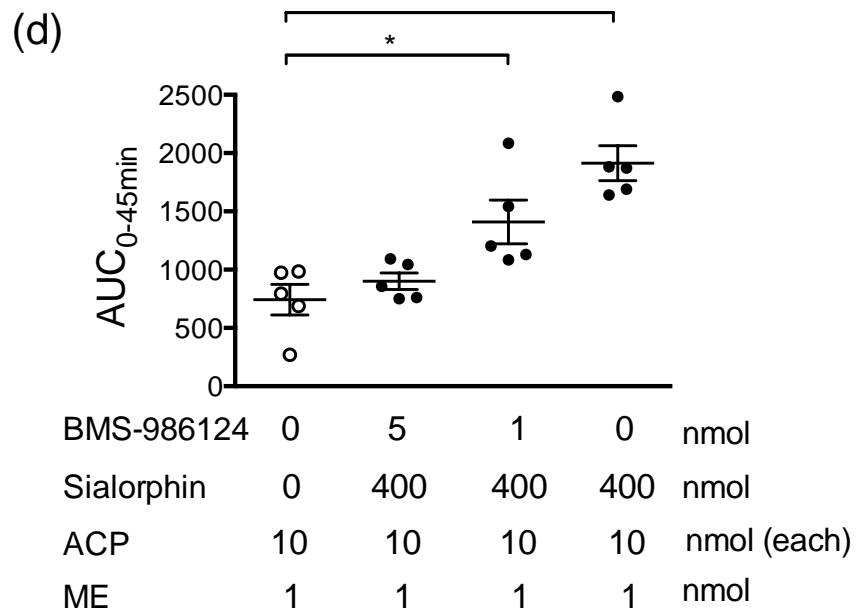
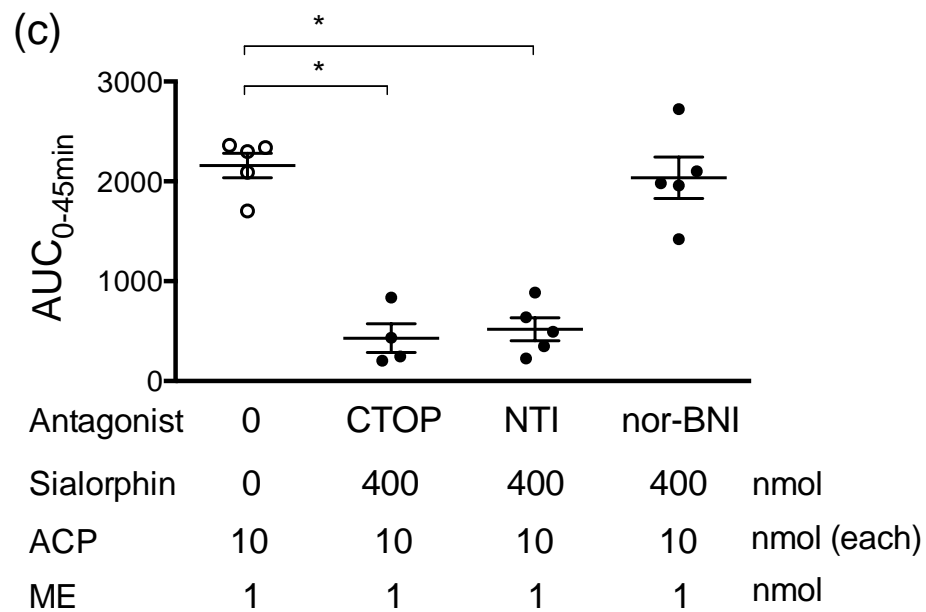
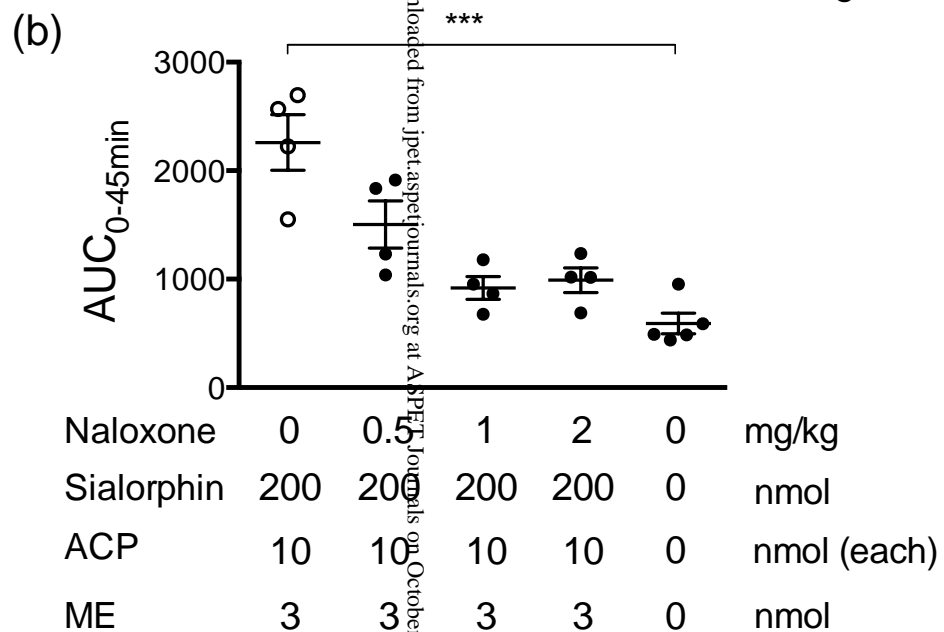
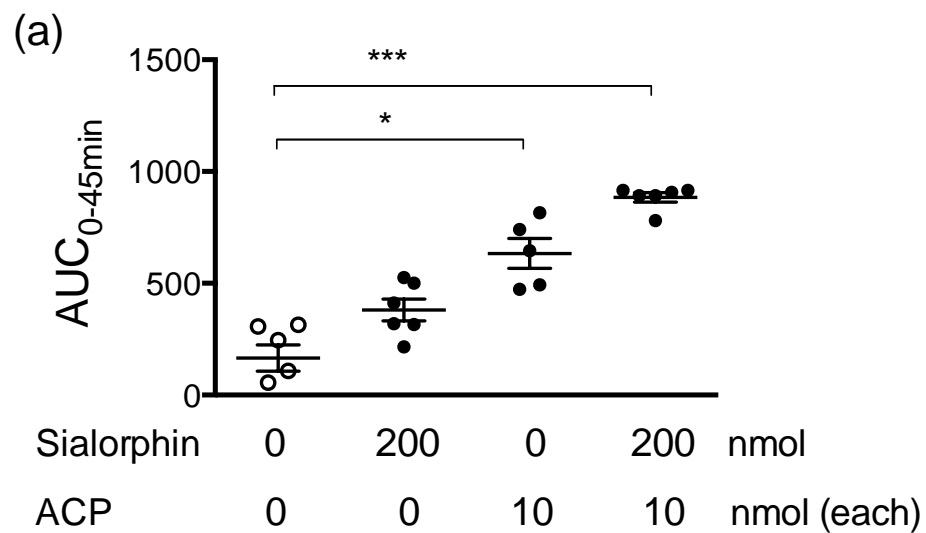


Figure 4



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Figure 5



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Figure 6

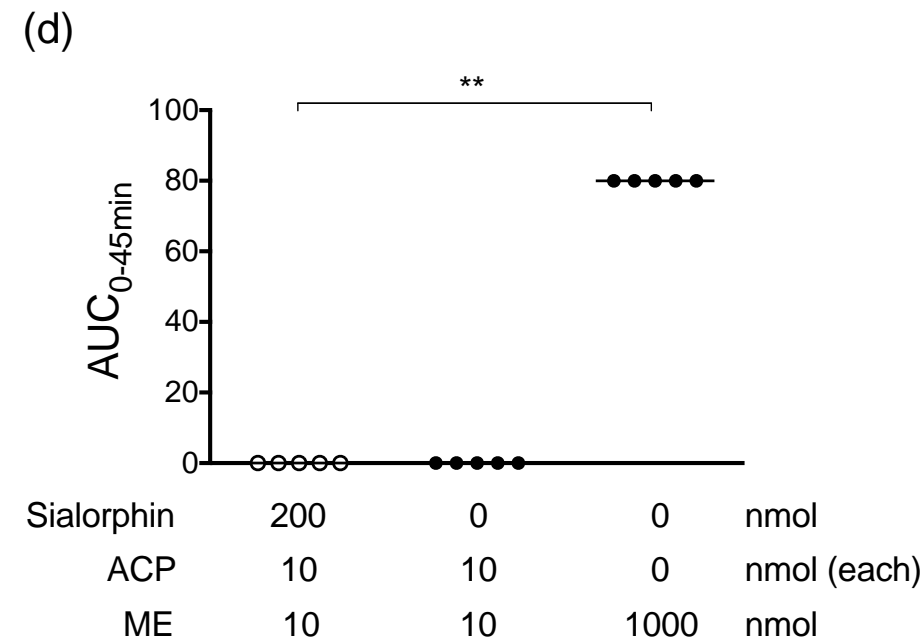
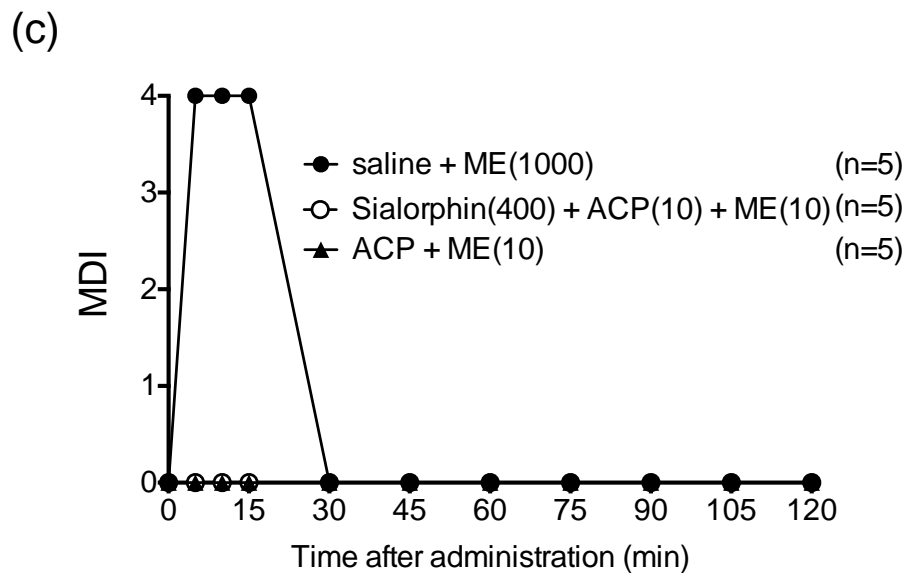
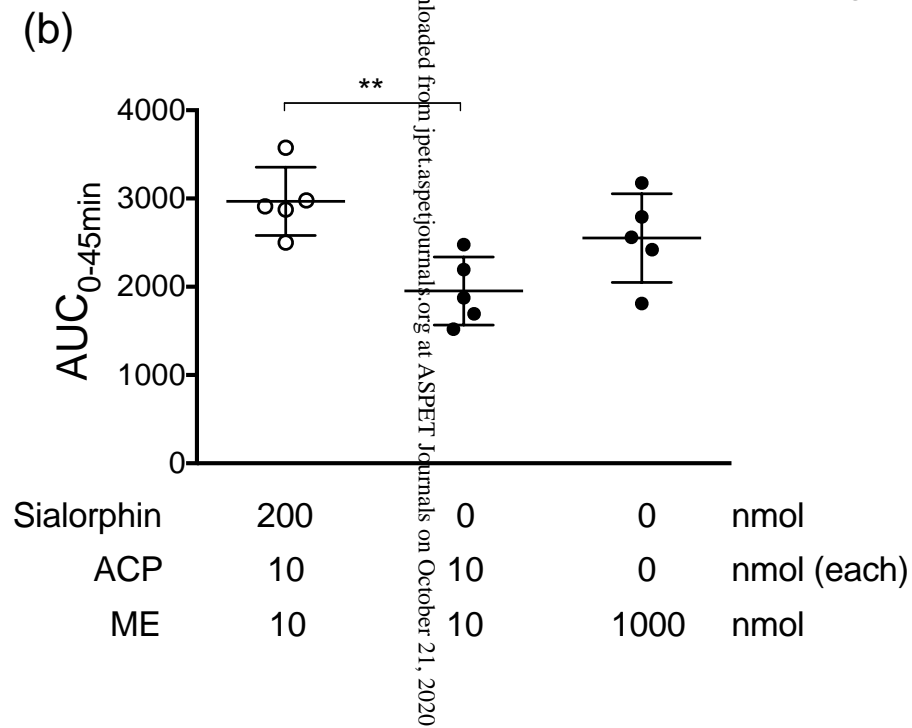
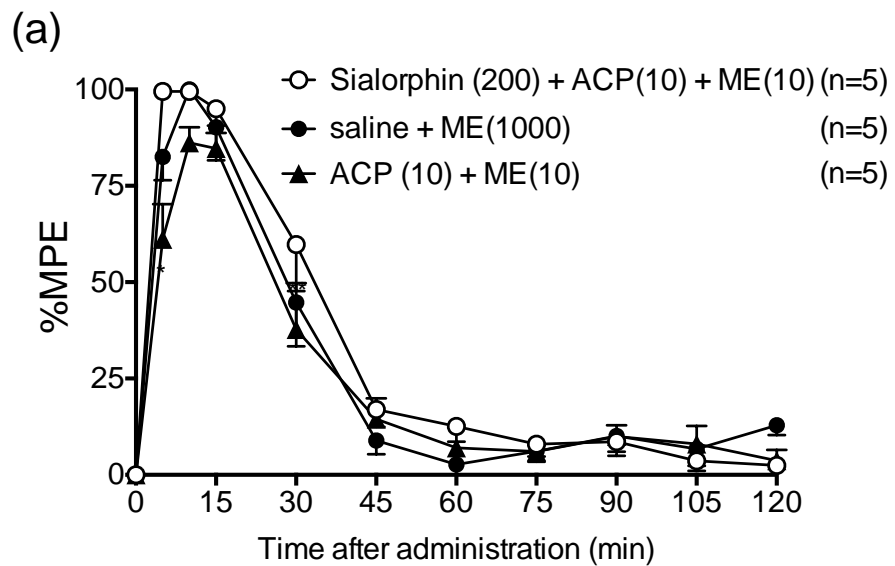


Figure 7

