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# Phenylmethanesulfonyl fluoride, a serine protease inhibitor, suppresses naloxone-precipitated withdrawal jumping in morphine-dependent mice

Wataru Nemoto [a], Tasuku Sato [a], Osamu Nakagawasai [a], Fukie Yaoita [a], Jerzy Silberring [b], Takeshi Tadano [c], Koichi Tan-No [a,*]

[a] Department of Pharmacology, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan
[b] Department of Biochemistry and Neurobiology, Faculty of Materials Science and Ceramics, AGH University of Science and Technology, Krakow, Poland
[c] Laboratory of Environmental and Health Sciences, College of Medical Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa 920-1192, Japan

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ABSTRACT

We have previously shown that intracerebroventricular (i.c.v.) administration of cysteine protease inhibitors suppresses naloxone-precipitated withdrawal jumping in morphine-dependent mice, presumably through the inhibition of dynorphin degradation (see (Tan-No, K., Sato, T., Shimoda, M., Nakagawasai, O., Niijima, F., Kawamura, S., Furuta, S., Sato, T., Satoh, S., Silberring, J., Terenius, L., Tadano, T., 2010. Suppressive effects by cysteine protease inhibitors on naloxone-precipitated withdrawal jumping in morphine-dependent mice. Neuropeptides 44, 279–283)). In the present study, we examined the effect of phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor, on naloxone-precipitated withdrawal jumping in morphine-dependent mice. The doses of morphine (mg/kg per injection) were subcutaneously given twice daily for 2 days [day 1 (30) and day 2 (60)]. On day 3, naloxone (8 mg/kg) was intraperitoneally administered 3 h after the final injection of morphine (60 mg/kg), and the number of jumps was immediately recorded for 20 min. Naloxone-precipitated withdrawal jumping was significantly suppressed by i.c.v. administration of PMSF (4 nmol), given 5 min before each morphine treatment during the induction phase, with none given on the test day. The expression of tissue plasminogen activator (tPA), a serine protease that converts plasminogen to plasmin, in the prefrontal cortex was significantly increased in morphine-dependent and -withdrawal mice, as compared with saline-treated mice. Moreover, *trans*-4-(aminomethyl)-cyclohexanecarboxylic acid (300 pmol), an antiplasmin agent, and ($Tyr^1$)-thrombin receptor activating peptide 7 (0.45 and 2 nmol), an antagonist of protease activated receptor-1 (PAR-1), significantly suppressed naloxone-precipitated withdrawal jumping. The present results suggest that PMSF suppresses naloxone-precipitated withdrawal jumping in morphine-dependent mice, presumably through the inhibition of activities of tPA and plasmin belonging to the serine proteases family, which subsequently activates PAR-1.



## 1. Introduction

It is well known that bioactive peptides are converted to shorter, bioactive fragments and/or degraded by several proteases, following a loss of activity. We have examined the effect of various protease inhibitors on the functions in the central nervous system (CNS). The findings regarding cysteine protease inhibitors that have been found so far are described as follows: (1) *p*-hydroxymercuribenzoate (PHMB), a general cysteine protease inhibitor, and Boc-Tyr-Gly-NHO-Bz (BYG-Bz), a representative of a novel class of cysteine protease inhibitors, when co-administered with dynorphin A or dynorphin B, significantly prolong antinociception induced by intrathecal (i.t.) and intracerebroventricular (i.c.v.) injection of these dynorphins in the mouse formalin and capsaicin tests (Tan-No et al., 1996, 2001, 2005a); (2) i.t.-administered PHMB produces antinociceptive effect in capsaicin test through the inhibition of endogenous dynorphin degradation (Tan-No et al., 1998); (3) i.t.-administered *N*-ethylmaleimide, another cysteine protease inhibitor, produces nociceptive behavior through the inhibition of endogenous dynorphin degradation in uninjured mice (Tan-No et al., 2005b); (4) *N*-ethylmaleimide and BYG-Bz suppress the development of antinociceptive tolerance to morphine, presumably through the inhibition of dynorphin degradation when administered i.t. 5 min before each morphine pretreatment during the induction (Tan-No et al., 2008). These observations indicate that cysteine protease inhibitors modulate CNS pathways engaged in

*Abbreviations:* Boc-, tert-butyloxycarbonyl; -Bz, benzoyl.

* Corresponding author. Tel.: +81 22 727 0122; fax: +81 22 727 0123.
*E-mail address:* koichi@tohoku-pharm.ac.jp (K. Tan-No).

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pain transmission and morphine tolerance, through the inhibition of endogenous dynorphin degradation. Moreover, we have recently reported that i.c.v. administration of cysteine protease inhibitors suppresses naloxone-precipitated withdrawal jumping in morphine-dependent mice, presumably through the inhibition of dynorphin degradation, whereas the inhibitors for aminopeptidase, endopeptidase 24.11, and angiotensin-converting enzyme cause no changes (Tan-No et al., 2010). On the other hand, it has been suggested that tissue plasminogen activator (tPA)–plasmin system, which possesses serine protease activity, is involved in the rewarding effect of morphine (Nagai et al., 2004). However, it has not been clarified whether serine protease inhibitors suppress naloxone-precipitated withdrawal jumping in morphine-dependent mice. Therefore, in the present study, we examined the effect of i.c.v.-administered phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor, on naloxone-precipitated withdrawal jumping in morphine-dependent mice; as a result, PMSF suppressed the jumping. We also examined the possible mechanisms involved in these phenomena.

## 2. Materials and methods

### 2.1. Animals

Male ddY-strain mice (5 weeks old, weighing 22–25 g, Japan SLC, Japan) were used for all experiments. Animals were housed in plastic cages with free access to standard food and tap water under conditions of constant temperature (23 ± 1 °C) and relative humidity (55 ± 5%), on a 12 h light–dark cycle (light 8:00 a.m.–8:00 p.m.). Groups of 9–18 mice for behavioral experiments and four mice for Western blotting experiments were used only once. All experiments were performed following the approval of the Ethics Committee for Animal Experiments at Tohoku Pharmaceutical University, and in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

### 2.2. Drugs

The following drugs and chemicals were used: naloxone, PMSF (Sigma–Aldrich, USA); *trans*-4-(aminomethyl)-cyclohexanecarboxylic acid (*t*-AMCHA) (Nacalai Tesque, Japan); ($Tyr^1$)-thrombin receptor activating peptide 7 (($Tyr^1$)-TRAP-7) (Bachem, Switzerland); morphine hydrochloride (Sankyo, Japan). For i.c.v. injections, test compounds, except PMSF, were dissolved in Ringer's solution. PMSF was dissolved in Ringer's solution containing 2% ethanol. Morphine and naloxone were dissolved in physiological saline.

### 2.3. Intracerebroventricular injections

I.c.v. injections were made directly into the lateral ventricle of unanesthetized mice as described by a previous report (Tan-No et al., 1995, 2001, 2010). Namely, the compounds and those vehicles were administered in a volume of 5 μl by free hand at a rate of 10–15 s into the right-side lateral cerebroventricle of an unanesthetized mouse by using a 50 μl Hamilton microsyringe.

### 2.4. Induction and assessment of physical dependence on morphine

The experimental protocol for induction and assessment of physical dependence on morphine were performed as described in our previous report (Tan-No et al., 2010). Briefly, the doses of morphine (mg/kg per injection) were subcutaneously (s.c.) administered twice daily, at 9:00 a.m. and 7:00 p.m., for 2 days [day 1 (30) and day 2 (60)]. On day 3, naloxone-induced withdrawal jumping was measured to assess physical dependence on morphine. Namely, naloxone (8 mg/kg) was intraperitoneally administered 3 h after the final injection of morphine at a dose of 60 mg/kg. Immediately after naloxone injection, mice were placed in a cylinder (10 cm diameter × 50 cm height) and the number of jumps was measured during a 20 min period.

### 2.5. Treatment with test drugs

I.c.v. administration of various drugs was performed 5 min prior to each morphine injection during the induction phase, to determine the effects of these compounds on the development of a physical dependence on morphine. No treatment was given on the test day.

### 2.6. Western blotting

Twelve mice were randomly divided into three equal groups. In a first group, the doses of morphine (mg/kg per injection) were s.c. administered twice daily for 2 days [day 1 (30) and day 2 (60)]. A second group of mice, serving as control, was given physiological saline alone. The animals in both the first and second groups were sacrificed by decapitation 14 h after the last morphine or physiological saline injection. In a third group, the doses of morphine (mg/kg per injection) were s.c. administered twice daily for 2 days [day 1 (30) and day 2 (60)]. On day 3, naloxone (8 mg/kg) was intraperitoneally administered 3 h after the final injection of morphine at a dose of 60 mg/kg, and the mice were sacrificed by decapitation 10 min later. After sacrifice, the brain was immediately removed and the prefrontal cortex was dissected on an ice-cold glass plate. Individual tissue samples were homogenized in 150 μl of CelLytic™ MT Cell Lysis Reagent (Sigma–Aldrich) and centrifuged at 15,000 × *g* for 15 min at 4 °C. Supernatants were collected and protein concentration was determined using Advanced Protein Assay Reagent (Cytoskeleton Inc., USA). Supernatants were then dissolved in 4× Laemmli sample buffer (300 mM Tris–HCl (pH 6.8), 8% SDS, 60% glycerol, 12% 2-mercaptoethanol), and boiled at 95 °C for 5 min. All dissections and sample preparation were performed on the same day. Aliquots of the obtained extracts (30 μg of protein/well for each extract) were loaded onto a 10% SDS–polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane, which was then incubated with blocking solution (10 mM Tris–HCl (pH 7.4), 100 mM NaCl and 5% skim milk) for 1 h and probed with rabbit polyclonal anti tPA antibody (Santa Cruz Biotechnology, USA) or rabbit polyclonal anti β-actin antibody (Cell Signaling Technology, USA) overnight at 4 °C. The membrane was then washed with blocking solution without milk, incubated with horseradish peroxidase-linked secondary antibody (Cell Signaling Technology) for 2 h and analyzed with an ECL plus Western blotting detection system (Amersham Life Sciences, USA).

### 2.7. Statistics

Results are presented as means ± S.E.M. Significant differences between groups were determined by Fisher's PLSD post hoc test for multiple comparisons, following analysis of variance (ANOVA) using the StatView-J5.0 software (SAS Institute, Inc., USA). In all comparisons, $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Effect of PMSF on naloxone-precipitated withdrawal jumping

Naloxone-precipitated withdrawal mice show significantly aggravated morphine-withdrawal symptoms including jumping