JPET Fast Forward. Published on November 23, 2004 as DOI: 10.1124/jpet.104.075697

JPET #75697

Title

A Tyr-W-MIF-1 Analogue Containing D-Pro^2 Acts as a Selective μ_2-Opioid Receptor Antagonist in the Mouse

Authors

Hiroyuki Watanabe, Daisuke Nakayama, Kanenori Ito, Chizuko Watanabe, Hirokazu Mizoguchi, Tsutomu Fujimura, Kimie Murayama, Shunsuke Kawamura, Takumi Sato, Chikai Sakurada, Tsukasa Sakurada, and Shinobu Sakurada

Laboratory of Origin

Department of Physiology and Anatomy, Tohoku Pharmaceutical University, Sendai, Japan (H.W., D.N., K.I., C.W., H.M., S.S.); Division of Biochemical Analysis, Central Laboratory of Medical Sciences, Juntendo University School of Medicine, Tokyo, Japan (T.F., K.M.); Educational Center for Pharmaceutical Sciences, Tohoku Pharmaceutical University, Sendai, Japan (S.K.); Department of Pharmacology, Nihon Pharmaceutical University, Saitama, Japan (T. Sato); and Department of Biochemistry, Daiichi College of Pharmaceutical Sciences, Fukuoka, Japan (C.S., T. Sakurada)

Running Title Page

a) Running title: μ_2-Opioid receptor antagonist

b) Corresponding author:

Shinobu Sakurada, Ph.D.

Department of Physiology and Anatomy

Tohoku Pharmaceutical University

4-4-1 Komatsushima, Aoba-ku,

Sendai 981-8558, Japan

Tel: +81-22-234-4181

Fax: +81-22-275-2013

E-mail: s-sakura@tohoku-pharm.ac.jp

c) The number of text pages: 33

The number of figures: 3

The number of tables: 3

The number of references: 24

The number of words in the Abstract: 208

The number of word in the Introduction: 498

The number of words in the Discussion: 1333

d) Abbreviations:

ACSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2
DAMGO	[D-Ala^2,$NMePhe^4$,$Gly(ol)^5$]enkephalin
D-Pro^2-endomorphin-1	Tyr-D-Pro-Trp-Phe-NH_2
D-Pro^2-endomorphin-2	Tyr-D-Pro-Phe-Phe-NH_2
D-Pro^2-Tyr-W-MIF-1	Tyr-D-Pro-Trp-Gly-NH_2
Endomorphin-1	Tyr-Pro-Trp-Phe-NH_2
Endomorphin-2	Tyr-Pro-Phe-Phe-NH_2
i.t.	intrathecal/intrathecally
MIF-1	melanocyte-stimulating hormone-release inhibiting factor-1
95% CI	95% confidence interval
% MPE	percent of maximum possible effect
Tyr-W-MIF-1	Tyr-Pro-Trp-Gly-NH_2

e) Section: Neuropharmacology

Abstract

The antagonistic properties of Tyr-D-Pro-Trp-Gly-NH_2 (D-Pro^2-Tyr-W-MIF-1), a Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1) analogue, on the antinociception induced by the μ-opioid receptor agonists Tyr-W-MIF-1, [D-Ala^2,$NMePhe^4$,$Gly(ol)^5$]enkephalin (DAMGO), Tyr-Pro-Trp-Phe-NH_2 (endomorphin-1), and Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2) was studied in the mouse paw-withdrawal test. D-Pro^2-Tyr-W-MIF-1 injected intrathecally (i.t.) had no apparent effect on the thermal nociceptive threshold. D-Pro^2-Tyr-W-MIF-1 (0.1-0.4 nmol) co-administered i.t. showed a dose-dependent attenuation of the antinociception induced by Tyr-W-MIF-1 without affecting endomorphin- or DAMGO-induced antinociception. However, higher doses of D-Pro^2-Tyr-W-MIF-1 (0.8-1.2 nmol) significantly attenuated endomorphin-1- or DAMGO-induced antinociception, whereas the antinociception induced by endomorphin-2 was still not affected by D-Pro^2-Tyr-W-MIF-1. Pretreatment i.t. with various doses of naloxonazine, a μ_1-opioid receptor antagonist, attenuated the antinociception induced by Tyr-W-MIF-1, endomorphin-1, endomorphin-2, or DAMGO. Judging from the ID_{50} values for naloxonazine against the antinociception induced by the μ-opioid receptor agonists, the antinociceptive effect of Tyr-W-MIF-1 is extremely less sensitive to naloxonazine than those of endomorphin-1 or DAMGO. In contrast, endomorphin-2-induced antinociception is extremely sensitive to naloxonazine. The present results clearly suggest that D-Pro^2-Tyr-W-MIF-1 is the selective antagonist to be identified for the μ_2-opioid receptor in the mouse spinal cord. D-Pro^2-Tyr-W-MIF-1 may also discriminate between Tyr-W-MIF-1-induced antinociception and the antinociception induced by endomorphin-1 or DAMGO, all of which show a preference for the μ_2-opioid receptor in the spinal cord.

Introduction

Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1) has been isolated from human the cerebral cortex (Erchegyi et al., 1992) and bovine hypothalamus (Hackler et al., 1993), and named for its structural similarity to the melanocyte-stimulating hormone-release inhibiting factor-1 (MIF-1) family of brain peptides (Reed et al., 1994). Tyr-W-MIF-1 has a high affinity for μ-opioid receptors (Erchegyi et al., 1992; Hackler et al., 1993; Erchegyi et al., 1993) and its own specific non-opioid receptors in the brain (Zadina et al., 1990), without any appreciable affinities for δ- and κ-opioid receptors (Zadina et al., 1994a,b). Tyr-W-MIF-1 has been reported to show a prolonged and naloxone-reversible antinociception after both intracerebroventricular (i.c.v.) and intrathecal (i.t.) administration (Gergen et al., 1996a,b). Tyr-W-MIF-1 also showed a potent inhibition of the electrically-elicited contraction of the guinea pig ileum, a property of an agonistic for μ- or κ-opioid receptors (Erchegyi et al., 1992, 1993). The Tyr-W-MIF-1-induced inhibition of the contractions was eliminated by the selective μ-opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), but not by the selective κ-opioid receptor antagonist nor-binaltorphimine (Erchegyi et al., 1992). This evidence clearly suggests that Tyr-W-MIF-1 is a potent agonist for μ-opioid receptors.

The μ-opioid receptor has been divided into μ_1- and μ_2-opioid receptors based on their sensitivity to the μ-opioid receptor antagonist naloxonazine, which irreversibly binds to μ_1-opioid receptors (Hahn et al., 1982; Ling et al., 1986). In fact, the antinociception mediated by the spinal or supraspinal μ-opioid receptors can be divided into naloxonazine (35 mg/kg, s.c.)-sensitive (μ_1-opioid receptor-mediated) antinociception and naloxonazine-insensitive (μ_2-opioid

receptor-mediated) antinociception (Sakurada et al., 1999; Sato et al., 1999). The antinociception induced by Tyr-W-MIF-1 was significantly attenuated by pretreatment with β-funaltrexamine, but not by naloxonazine (Zadina et al., 1993; Gergen et al., 1996a,b), indicating that Tyr-W-MIF-1-induced antinociception may be mediated through the spinal or supraspinal μ_2-opioid receptors. However, the extensive characterization of μ_2-opioid receptor-mediated antinociception has been limited because a selective antagonist for the μ_2-opioid receptor was not available.

The antinociception induced by the endogenous μ-opioid receptor agonists Tyr-Pro-Trp-Phe-NH_2 (endomorphin-1) and Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2) is considered to be mediated by the spinal μ_2- and μ_1-opioid receptors, respectively. This contention is supported by the evidence that the antinociception induced by i.t. administration of endomorphin-2, but not endomorphin-1, is suppressed by the pretreatment with the μ_1-opioid receptor antagonist naloxonazine (Sakurada et al., 1999, 2000a). We recently found that Tyr-D-Pro-Trp-Phe-NH_2 (D-Pro^2-endomorphin-1) and Tyr-D-Pro-Phe-Phe-NH_2 (D-Pro^2-endomorphin-2), in which the L-Pro^2 of endomorphin-1 and endomorphin-2 has been replaced with D-Pro^2, selectively attenuated the antinociception induced by endomorphin-1 and endomorphin-2, respectively (Sakurada et al., 2002). This evidence suggests the possibility that the synthetic peptides, which have replaced the L-Pro^2 of their parent peptide with D-Pro^2, are antagonists against their parent peptide. Based on the above hypothesis, in the present study, we newly synthesized D-Pro^2-Tyr-W-MIF-1 as a possible and primary antagonist for the Tyr-W-MIF-1 binding site, probably the μ_2-opioid receptor.

The purpose of the present study is now to characterize the antagonistic properties of D-

Pro^2-Tyr-W-MIF-1 against the spinal antinociception induced by four distinct μ-opioid receptor agonists, Tyr-W-MIF-1, [D-Ala^2,NMePhe^4,Gly(ol)^5]enkephalin (DAMGO), endomorphin-1, and endomorphin-2.

Methods

All experiments were approved by and conformed to the guidelines of the Committee of Animal Experiments at Tohoku Pharmaceutical University. Every effort was made to minimize the number of animals and any suffering to the animal used in the following experiments.

Animals. Male ddY mice weighing 22-25 g (SLC, Hamamatsu, Japan) were housed in a light- and temperature-controlled room (light on at 09:00 and off at 21:00; 23°C). Food and water were available *ad libitum*. Animals were used only once.

Assessment of Antinociceptive Response. The antinociceptive response was assessed with the thermal paw-withdrawal test, using an automated tail-flick unit (BM kiki, Tokyo, Japan). Mice were adapted to the testing environment for at least 1 h before any stimulation. Each animal was restrained with a soft cloth to reduce visual stimuli, and the light beam as a noxious radiant heat stimulation was applied from underneath the glass floor toward the hind paw. The light beam focused on the plantar surface of the hind paw, and the latency for the paw-withdrawal response against the noxious radiant heat stimulation was measured. The intensity of the noxious radiant heat stimulation was adjusted so that the pre-drug latency for the paw-withdrawal response was 2.5-3.5 s. The antinociceptive effect was expressed as percent of the maximum possible effect (% MPE), which was calculated with the following equation: $[(T_1-T_0)/(10-T_0)]x100$, where T_0 and T_1 are the pre-drug and post-drug latencies for the paw-withdrawal response, respectively. To prevent tissue damage in paw, the noxious radiant heat stimulation was terminated automatically if the mouse did not lift the paw within 10 s. The measurement of the paw-withdrawal latency was performed by only one individual who was uninformed for drug treatment for each mouse.

Intrathecal Administration. The i.t. administration was performed according to the procedure described by Hylden and Wilcox (1980) using a 10-μl Hamilton microsyringe with a 29-gauge needle. The injection volume was 2 μl.

Drugs. Drugs used were Tyr-W-MIF-1 (Bachem, San Carlos, CA); DAMGO (Sigma, St. Louis, MO); endomorphin-1 (Tocris Cookson, Bristol, UK); endomorphin-2 (Tocris Cookson); deltorphin II (Bachem); (-)-U-50,488 hydrochloride (Tocris Cookson); D-Pro^2-Tyr-W-MIF-1 (synthesized in our laboratory); β-funaltrexamine hydrochloride (Tocris Cookson); naloxonazine dihydrochloride (RBI, Natick, MA); naltrindole hydrochloride (Tocris Cookson); and nor-binaltorphimine dihydrochloride (Tocris Cookson). All drugs were dissolved in sterile artificial cerebrospinal fluid (ACSF) containing 7.4 g of NaCl, 0.19 g of KCl, 0.19 g of $MgCl_2$, and 0.14 g of $CaCl_2$ 1000 ml^{-1}.

Statistical Analysis. The data are expressed as the mean ± S.E.M. The statistical significance of the differences between groups was assessed with a one-way analysis of variance (ANOVA) followed by either Dunnett's test or Newman-Keuls's test, or a two-way ANOVA followed by Bonferroni's test. The ED_{50}, ID_{50}, and hill slope values with their 95% confidence intervals were calculated with a computer-associated curve-fitting program (GraphPad Prism, GraphPad Software, Inc., San Diego, CA). For the statistical significance of differences between groups, the entire curves were compared using the F-test, according to the instruction provided with GraphPad Prism.

Results

The Antinociception Induced by Tyr-W-MIF-1. Groups of mice were treated with ACSF or various i.t. doses of Tyr-W-MIF-1 (2.0-16 nmol), and the antinociception was measured 5, 10, 15, 20, 30, and 45 min after the treatment. As shown in Fig. 1a and 1b, Tyr-W-MIF-1 given i.t. produced a marked dose-dependent antinociception. The antinociception induced by Tyr-W-MIF-1 developed rapidly, reached its peak at 10 min, and then gradually disappeared by 30 min after the treatment (Fig. 1a). The ED_{50} value of Tyr-W-MIF-1 for antinociception at the peak time was 5.89 (95% CI: 4.42-7.85) nmol (Fig. 1b). At the peak time, 16 nmol of Tyr-W-MIF-1 produced approximately an 80% MPE.

Effects of β-Funaltrexamine, nor-Binaltorphimine and Naltrindole on the Antinociception Induced by Tyr-W-MIF-1. Groups of mice were pretreated i.t. with the μ-opioid receptor antagonist β-funaltrexamine (4.0 nmol), the κ-opioid receptor antagonist nor-binaltorphimine (4.0 nmol) or ACSF 24 h before, or with the δ-opioid receptor antagonist naltrindole (0.033 nmol) or ACSF 5 min before the i.t. administration of Tyr-W-MIF-1 (16 nmol), and the antinociception induced by Tyr-W-MIF-1 was measured 10 min after the treatment. The antinociception induced by i.t. administration of Tyr-W-MIF-1 was almost eliminated by i.t. pretreatment with β-funaltrexamine, while i.t. pretreatment with nor-binaltorphimine or naltrindole failed to affect the Tyr-W-MIF-1-induced antinociception (Table 1). The same pretreatment with either nor-binaltorphimine or naltrindole completely attenuated the antinociception induced by i.t. administration of either the κ-opioid receptor agonist U-

50,488H or the δ-opioid receptor agonist deltorphin II, respectively (data not shown).

Effect of Naloxonazine on the Antinociception Induced by μ-Opioid Receptor Agonists. Groups of mice were pretreated i.t. with various doses of the μ_1-opioid receptor antagonist naloxonazine (1.4-44.2 nmol) or ACSF 24 h before the i.t. administration of equipotent doses of Tyr-W-MIF-1 (16 nmol), DAMGO (20 pmol), endomorphin-1 (5.0 nmol), or endomorphin-2 (5.0 nmol). The antinociception induced by i.t. administration of Tyr-W-MIF-1, DAMGO, endomorphin-1, and endomorphin-2 were measured 10 min, 5 min, 5 min, and 5 min after the treatment, respectively, as peak effects. The pretreatment with naloxonazine attenuated the antinociception induced by these four distinct μ-opioid receptor agonists in a dose-dependent manner (Fig. 2a). However, naloxonazine at a dose of 5.5 nmol only significantly antagonized the antinociceptive effect induced by endomorphin-2, without affecting the antinociception induced by endomorphin-1, DAMGO and Tyr-W-MIF-1. Higher doses (11.1 or 22.1 nmol) of naloxonazine significantly attenuated endomorphin-1- and DAMGO-induced antinociception, but still had no effect against the antinociception induced by Tyr-W-MIF-1. The Tyr-W-MIF-1-induced antinociception was significantly, but not completely, attenuated by the pretreatment with a much higher dose of naloxonazine (44.2 nmol), which completely eliminated the antinociception induced by endomorphin-1 and DAMGO. The ID_{50} values for naloxonazine against the antinociception induced by endomorphin-2, DAMGO, endomorphin-1, and Tyr-W-MIF-1 were 4.58 (95% CI: 3.82-5.49), 13.66 (95% CI: 10.35-18.04), 15.79 (95% CI: 14.99-16.63), and 35.34 (95% CI: 33.69-37.07) nmol, respectively (Fig. 2b, Table 2). The dose-response curves for inhibition by naloxonazine against DAMGO- and endomorphin-1-induced antinociception were statistically distinct from those against endomorphin-2- and Tyr-W-MIF-1-

induced antinociception.

Effect of D-Pro^2-Tyr-W-MIF-1 on the Antinociception Induced by μ-Opioid Receptor Agonists. Groups of mice were co-administered various i.t. doses of D-Pro^2-Tyr-W-MIF-1 (0.025-1.2 nmol) with equipotent doses of Tyr-W-MIF-1 (16 nmol), DAMGO (20 pmol), endomorphin-1, (5.0 nmol) or endomorphin-2 (5.0 nmol), and the antinociception induced by Tyr-W-MIF-1, DAMGO, endomorphin-1, and endomorphin-2 were measured 10 min, 5 min, 5 min, and 5 min after the treatment, respectively, as peak effects. D-Pro^2-Tyr-W-MIF-1 at any of the doses used did not show any antinociceptive or hyperalgesic effect by itself at 5 or 10 min after the treatment. Co-administered D-Pro^2-Tyr-W-MIF-1 dose-dependently attenuated the antinociception induced by Tyr-W-MIF-1 (Fig. 3a). The ID_{50} value for D-Pro^2-Tyr-W-MIF-1 against Tyr-W-MIF-1-induced antinociception was 0.21 (95% CI: 0.15-0.28) nmol (Fig. 3b, Table 2). In contrast, co-administered D-Pro^2-Tyr-W-MIF-1 at a dose of 0.4 nmol, which almost completely attenuated Tyr-W-MIF-1-induced antinociception, did not affect the antinociception induced by DAMGO and endomorphin-1, whereas higher doses of D-Pro^2-Tyr-W-MIF-1 (0.8-1.2 nmol) significantly attenuated the antinociception induced by either DAMGO or endomorphin-1. The ID_{50} values for D-Pro^2-Tyr-W-MIF-1 against DAMGO- and endomorphin-1-induced antinociception were 0.98 (95% CI: 0.82-1.18) and 0.75 (95% CI: 0.63-0.89) nmol, respectively (Fig. 3b, Table 2). The dose-response curves for inhibition by D-Pro^2-Tyr-W-MIF-1 against DAMGO- and endomorphin-1-induced antinociception were statistically distinct from that against Tyr-W-MIF-1-induced antinociception. On the other hand, the antinociception induced by endomorphin-2 was not affected by co-administration of D-Pro^2-Tyr-W-MIF-1 at any of the doses used (Fig. 3a and 3b, Table 2).

Effect of D-Pro^2-Tyr-W-MIF-1 on the Antinociception Induced by U-50,488H and Deltorphin II. Groups of mice were co-administered various i.t. doses of D-Pro^2-Tyr-W-MIF-1 (0.025-1.2 nmol) with equipotent doses of the κ-opioid receptor agonist U-50,488H (30 nmol) or δ-opioid receptor agonist deltorphin II (4 nmol), and the antinociception induced by U-50,488H and deltorphin II were measured 10 min and 5 min after the treatment, respectively. As shown in Table 3, co-administered D-Pro^2-Tyr-W-MIF-1 at any of the doses used failed to affect the antinociception induced by either U-50,488H or deltorphin II.

Discussion

Tyr-W-MIF-1 induced a dose-dependent antinociception in the paw-withdrawal test after spinal administration. The effects of Tyr-W-MIF-1-induced antinociception occurred at 5-10 min after the i.t. injection and disappeared at 30 min after the injection, whereas those of endomorphin-1, which is structurally similar to Tyr-W-MIF-1 with a Phe in position 4, occurred within 1 min after i.t. injection and were reduced at 15 min. The time-course of antinociception with Tyr-W-MIF-1 is similar to that with DAMGO (Sakurada et al., 2001).

We used a variety of i.t. doses of naloxonazine to determine the sensitivity to antagonists of the μ-opioid receptor subclasses involved in the antinociceptive responses to Tyr-W-MIF-1. There is biochemical and pharmacological evidence supporting the existence of μ-opioid receptor subclasses which are localized in the spinal and supraspinal structures involved in the modulation of nociception (Wolozin and Pasternak, 1981; Moskowitz and Goodman, 1985). At least two μ-opioid receptor subclasses have been proposed: μ_1- and μ_2-opioid receptors. β-Funaltrexamine irreversibly antagonizes both μ_1- and μ_2-opioid receptors and inhibits both supraspinal and spinal antinociception, whereas naloxonazine selectively antagonizes the μ_1-opioid receptors and inhibits supraspinal antinociception. Recent behavioral pharmacological studies suggest the presence of μ_1-opioid receptors sensitive to naloxonazine in spinal sites as assayed with the formalin, hot-plate, tail-pressure, and tail-flick tests (Sakurada et al., 1999; Sato et al., 1999; Sakurada et al., 2000b). Autoradiographic studies show that μ_1- and μ_2-opioid receptor subclasses are localized in the spinal and supraspinal structures involved in the modulation of nociception (Moskowitz et al., 1985). The difference in μ_1 and μ_2 binding could

be due to induced differences in the receptor conformation.

It is noteworthy that both the s.c. 35 mg/kg dose and the i.t. 5.5 nmol/mouse dose of naloxonazine are reasonable doses to selectively block μ_1-opioid receptors in mice (Ling et al., 1986; Sakurada et al., 2000a). Recent studies have shown that the antinociceptive response to DAMGO is not blocked by pretreatment with naloxonazine at a dose of 35 mg/kg s.c. or 5.5 nmol/mouse i.t., whereas higher doses of naloxonazine (52.5, 65.6 or 78.8 mg/kg s.c. or 11.1 nmol/mouse i.t.) significantly attenuated DAMGO-induced antinociception (Sakurada et al., 2000a), indicating that naloxonazine at high doses loses much of its selectivity for μ_1-opioid receptors (Sakurada et al., 2000a). The antinociception induced by i.t. administration of Tyr-W-MIF-1 was significantly attenuated by pretreatment with β-funaltrexamine, whereas the antinociceptive activity was not antagonized by pretreatment with a reasonable i.t. dose of naloxonazine, i.e., 5.5 nmol/mouse. The present results with naloxonazine on Tyr-W-MIF-1-induced antinociception are in agreement with those of Gergen et al. (1996b). This result suggests that the antinociception with Tyr-W-MIF-1 is mediated through μ_2-opioid receptors, since higher doses of i.t. naloxonazine attenuated the antinociception with Tyr-W-MIF-1 (Fig. 2a and 2b). Furthermore, i.t. pretreatment with the κ-opioid receptor antagonist nor-binaltorphimine or the δ-opioid receptor antagonist naltrindole did not attenuate the antinociception induced by i.t. administration of Tyr-W-MIF-1 (Table 1). These results strongly support the previous reports that the antinociception induced by i.t. administration of Tyr-W-MIF-1 was mediated by stimulation of the μ_2-opioid receptor at the spinal cord level (Gergen et al., 1996b). Unexpectedly, higher doses of naloxonazine (11.1 or 22.1 nmol/mouse i.t.), which suppressed the antinociception with DAMGO, did not significantly inhibit the Tyr-W-MIF-1-induced

antinociception. Even the highest dose of naloxonazine (44.2 nmol/mouse, i.t.) did not completely antagonize the antinociception with Tyr-W-MIF-1.

Two new endogenous opioid peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) have been found to be highly selective for μ-opioid receptors (Zadina et al., 1997). Both endomorphin-1 and endomorphin-2 significantly increase nociceptive thresholds after both i.t. and i.c.v. administration, and these effects are antagonized by the μ-opioid receptor selective antagonists naloxone and β-funaltrexamine. However, more recent results indicate that different subclasses of μ-opioid receptors may be involved in the antinociceptive effects induced by endomorphin-1 and endomorphin-2. The antinociception induced by endomorphin-1 is blocked by the μ_1- and μ_2-opioid receptor antagonist β-funaltrexamine, but not by the selective μ_1-opioid receptor antagonist naloxonazine, whereas the antinociception induced by endomorphin-2 is blocked by both β-funaltrexamine and naloxonazine (Tseng et al., 2000; Sakurada et al., 2002).

A μ_1-opioid receptor antagonist, naloxonazine, was more effective in blocking the antinociceptive effects in mice induced by endomorphin-2 than by endomorphin-1 (Sakurada et al., 1999). A reasonable dose of naloxonazine, 35 mg/kg (s.c.) or 5.5 nmol/mouse (i.t.), to obtain a relative μ_1-opioid receptor selectivity (Ling et al., 1986), did not attenuate the antinociceptive effects induced by i.t. administration of endomorphin-1 (Sakurada et al., 2000a) or Tyr-W-MIF-1 (Gergen et al., 1996b) but did attenuate the antinociception due to endomorphin-2, suggesting that endomorphin-1 acts as a μ_2-opioid receptor agonist and endomorphin-2 acts as a μ_1-opioid receptor agonist at the spinal site. Thus, based on antagonism by naloxonazine, endomorphin-1, but not endomorphin-2, has behavioral and pharmacological similarities to Tyr-W-MIF-1.

We have demonstrated that D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2, analogs of the endomorphins (Sakurada et al., 2002; Hung et al., 2002), are opioid receptor antagonists which selectively block the antinociception induced by endomorphin-1 and endomorphin-2, respectively, in the spinal cord. Furthermore, D-Pro^2-endomorphin-2 attenuated the antinociception induced by i.t. administration of endomorphin-2 or the selective μ_1-opioid receptor agonist Tyr-D-Arg-Phe-β-Ala (Sakurada et al., 2000b) but not that induced by DAMGO or endomorphin-1 (Sakurada et al., 2002; Hung et al., 2002), indicating that D-Pro^2-endomorphin-2 is an antagonist which selectively blocks the antinociception induced by μ_1-opioid receptor agonists in the spinal cord. D-Pro^2-endomorphin-2 acts as a selective μ_1-opioid receptor antagonist like naloxonazine. On the other hand, D-Pro^2-endomorphin-1 attenuated the antinociception induced by i.t.-administered endomorphin-1 and DAMGO but not endomorphin-2, suggesting that D-Pro^2-endomorphin-1 may act as a selective μ_2-opioid receptor antagonist (Sakurada et al., 2002).

We found in the present study that D-Pro^2-Tyr-W-MIF-1, an analogue of Tyr-W-MIF-1 containing D-Pro in position 2, inhibited the antinociception induced by Tyr-W-MIF-1, endomorphin-1, and DAMGO, but not endomorphin-2, deltorphin II, or U-50,488H, in a dose-dependent manner. The present results clearly suggest that D-Pro^2-Tyr-W-MIF-1 is a selective antagonist for μ_2-opioid receptor. Interestingly, the antinociception of Tyr-W-MIF-1 was significantly attenuated at the doses of 0.1-0.4 nmol (Fig. 3a and 3b), doses which did not affect endomorphin-1-, endomorphin-2- or DAMGO-induced antinociception (Fig. 3a and 3b). A higher dose (0.8 nmol) of D-Pro^2-Tyr-W-MIF-1 significantly attenuated the antinociception with endomorphin-1 or DAMGO without affecting the antinociception with endomorphin-2, deltorphin II, or U-50,488H (Fig. 3a and 3b, Table 3). The finding that the antinociception

induced by Tyr-W-MIF-1 can be antagonized by D-Pro^2-Tyr-W-MIF-1 at doses which are inactive against endomorphin-1 and DAMGO indicates that it could be used to distinguish the different antinociceptive mechanism within the μ_2-opioid receptor agonists. We previously reported that D-Pro^2-endomorphin-1 shows the antagonistic property for μ_2-opioid receptor. Like a D-Pro^2-Tyr-W-MIF-1, the i.t. co-administration of D-Pro^2-endomorphin-1 significantly attenuated the antinociception induced by endomorphin-1, DAMGO, and Tyr-W-MIF-1 (Sakurada et al., 2002; unpublished observation). However, the antagonistic property of D-Pro^2-endomorphin-1 against Tyr-W-MIF-1 is characteristically similar to those against endomorphin-1 and DAMGO, suggesting that unlike D-Pro^2-Tyr-W-MIF-1, D-Pro^2-endomorphin-1 can not discriminate the antinociception induced by these μ_2-opioid receptor agonists. The present study is the first to show that D-Pro^2-Tyr-W-MIF-1 can also distinguish the actions of different peptidic μ_2-opioid receptor agonists. D-Pro^2-Tyr-W-MIF-1 selectively blocked the antinociception of Tyr-W-MIF-1 far more effectively than that of endomorphin-1 and DAMGO, while the antinociception induced by endomorphin-2 was not reduced by co-administered with D-Pro^2-Tyr-W-MIF-1 (Fig. 3a and 3b). The differential antagonistic sensitivity of D-Pro^2-Tyr-W-MIF-1 on inhibition of the thermal nociceptive response by μ_2-opioid receptor agonists led us to speculate that the μ_2-opioid receptors could be subdivided into a subclass of the μ_2-opioid receptor which is relatively insensitive to D-Pro^2-Tyr-W-MIF-1 and a subclass of the μ_2-opioid receptor which is extremely sensitive to D-Pro^2-Tyr-W-MIF-1, whereas D-Pro^2-endomorphin-1 failed to separate the different subclasses of μ_2-opioid receptors.

D-Pro^2-Tyr-W-MIF-1 selectively blocked the antinociceptive effect of i.t. administration of Tyr-W-MIF-1, whereas the antinociceptive effect of DAMGO or endomorphin-1, which are

insensitive to naloxonazine, was not inhibited at same dose at which the antinociception caused by Tyr-W-MIF-1 was eliminated. These results also indicate that D-Pro^2-Tyr-W-MIF-1 may be a useful tool to discriminate between the antinociceptive effects of μ_2-opioid receptor agonists which act via the different subclasses of the μ_2-opioid receptor.

References

Erchegyi J, Kastin AJ, and Zadina JE (1992) Isolation of a novel tetrapeptide with opiate and antiopiate activity from human brain cortex: Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1). *Peptides* 13:623-631.

Erchegyi J, Zadina JE, Qiu XD, Kersh DC, Ge LJ, Brown MM, and Kastin AJ (1993) Structure-activity relationships of analogs of the endogenous brain peptides Tyr-MIF-1 and Tyr-W-MIF-1. *Pept Res* 6:31-38.

Gergen KA, Zadina JE, Kastin AJ, and Paul D (1996a) Intrathecal Tyr-W-MIF-1 produces potent, naloxone-reversible analgesia modulated by α_2-adrenoceptors. *Eur J Pharmacol* 298:235-239.

Gergen KA, Zadina JE, and Paul D (1996b) Analgesic effects of Tyr-W-MIF-1: a mixed μ_2-opioid receptor agonist/μ_1-opioid receptor antagonist. *Eur J Pharmacol* 316: 33-38.

Hackler L, Kastin AJ, Erchegyi J, and Zadina JE (1993) Isolation of Tyr-W-MIF-1 from bovine hypothalami. *Neuropeptides* 24:159-164.

Hahn EF, Carroll-Buatti M, and Pasternak GW (1982) Irreversible opiate agonist and antagonists: the 14-hydroxydihydromorphine azines. *J Neurosci* 2:572-576

Hung KC, Wu HE, Mizoguchi H, Sakurada S, Okayama T, Fujimura T, Murayama K, Sakurada T, Fujimoto JM, and Tseng LF (2002) D-Pro^2-Endomorphin-1 and D-Pro^2-endomorphin-2, respectively, attenuate the antinociception induced by endomorphin-1 and endomorphin-2 given intrathecally in the mouse. *J Pharmacol Exp Ther* 303:874-879.

Hylden JLK and Wilcox GL (1980) Intrathecal morphine in mice: a new technique. *Eur J Pharmacol* 67:313-316.

Ling GSF, Simantov R, Clark JA, and Pasternak GW (1986) Naloxonazine actions in vivo. *Eur J Pharmacol* 129:33-38.

Moskowitz AS and Goodman RR (1985) Autoradiographic distribution of Mu_1 and Mu_2 opioid binding in the mouse central nervous system. *Brain Res* 360:117-129.

Reed GW, Olson GA, and Olson RD (1994) The Tyr-MIF-1 family of peptides. *Neurosci Biobehav Rev* 18:519-525.

Sakurada S, Zadina JE, Kastin AJ, Katsuyama S, Fujimura T, Murayama K, Yuki M, Ueda H, and Sakurada T (1999) Differential involvement of μ-opioid receptor subtypes in endomorphin-1- and -2-induced antinociception. *Eur J Pharmacol* 372:25-30.

Sakurada S, Hayashi T, Yuhki M, Fujimura T, Murayama K, Yonezawa A, Sakurada C, Takeshita M, Zadina JE, Kastin AJ, and Sakurada T (2000a) Differential antagonism of endomorphin-1 and endomorphin-2 spinal antinociception by naloxonazine and 3-methoxynaltrexone. *Brain Res* 881:1-8.

Sakurada S, Takeda S, Sato T, Hayashi T, Yuki M, Kutsuwa M, Tan-No K, Sakurada C, Kisara K, and Sakurada T (2000b) Selective antagonism by naloxonazine of antinociception by Tyr-D-Arg-Phe-β-Ala, a novel dermorphin analogue with high affinity at μ-opioid receptors. *Eur J Pharmacol* 395:107-112.

Sakurada S, Hayashi T, Yuhki M, Orito T, Zadina JE, Kastin AJ, Fujimura T, Murayama K, Sakurada C, Sakurada T, Narita M, Suzuki T, Tan-no K, and Tseng LF (2001) Differential antinociceptive effects induced by intrathecally administered endomorphin-1 and endomorphin-2 in the mouse. *Eur J Pharmacol* 427:203-210.

Sakurada S, Watanabe H, Hayashi T, Yuhki M, Fujimura T, Murayama K, Sakurada C, and Sakurada T (2002) Endomorphin analogues containing D-Pro^2 discriminate different μ-

opioid receptor mediated antinociception in mice. *Br J Pharmacol* 137:1143-1146.

Sato T, Sakurada S, Takahashi N, Sakurada T, Tan-No K, Wako K, and Kisara K (1999) Contribution of spinal μ_1-opioid receptors to morphine-induced antinociception. *Eur J Pharmacol* 369:183-187.

Tseng LF, Narita M, Suganuma C, Mizoguchi H, Ohsawa M, Nagase H, and Kampine JP (2000) Differential antinociceptive effects of endomorphin-1 and endomorphin-2 in the mouse. *J Pharmacol Exp Ther* 292:576-583.

Wolozin BL and Pasternak GW (1981) Classification of multiple morphine and enkephalin binding sites in the central nervous system. *Proc Natl Acad Sci USA* 78:6181-6185.

Zadina JE, Kastin AJ, Ge LJ, and Brantl V (1990) Hemorphins, cytochrophins and human beta-casomorphins bind to antiopiate (Tyr-MIF-1) as well as opiate binding sites in rat brain. *Life Sci* 47:PL25-PL30.

Zadina JE, Kastin AJ, Kenigs V, Bruno C, and Hackler L (1993) Prolonged analgesia after intracerebroventricular Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2). *Neurosci Lett* 155:220-222.

Zadina JE, Kastin AJ, Ge LJ, and Hackler L (1994a) Mu, delta, and kappa opiate receptor binding of Tyr-MIF-1 and of Tyr-W-MIF-1, its active fragments, and two potent analogs. *Life Sci* 55:PL461-PL466.

Zadina JE, Kastin AJ, Hackler L, Ge LJ, Kenigs V, Gergen KA, Messenger KA, and Chang SL (1994b) Opiate receptor binding, guinea pig ileum activity, and prolonged analgesia induced by the brain peptide Tyr-W-MIF-1 and two potent analogs. *Regul Pept* 54:341-342.

Zadina JE, Hackler L, Ge LJ, and Kastin AJ (1997) A potent and selective endogenous agonist for the μ-opiate receptor. *Nature* 386:499-502.

Footnotes

a) Financial support

This work was supported by a Grant-in-Aid for Scientific Research (C) (KAKENHI 12672220) from the Japan Society for the Promotion of Science.

b) Person to receive reprint requests

Shinobu Sakurada, Ph.D.

Department of Physiology and Anatomy

Tohoku Pharmaceutical University

4-4-1 Komatsushima, Aoba-ku

Sendai 981-8558, Japan

Legends for figures

Fig. 1. Tyr-W-MIF-1-induced antinociception in the mouse paw-withdrawal test. (a) Time course of antinociception induced by i.t. administration of Tyr-W-MIF-1. Groups of mice were treated with i.t. various doses of Tyr-W-MIF-1 (2.0-16 nmol) or ACSF, and the antinociception induced by Tyr-W-MIF-1 was measured 5, 10, 15, 20 30, and 45 min after the treatment. Each value represents the mean ± S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a two-way ANOVA followed by the Bonferroni's test. The F value of the two-way ANOVA for Tyr-W-MIF-1 (2.0, 4.0, 8.0, and 16 nmol) in comparison with ACSF were $F[1,126] = 6.932$ ($p < 0.01$), $F[1,126] = 39.17$ ($p < 0.001$), $F[1,126] = 68.17$ ($p < 0.001$), and $F[1,126] = 135.1$ ($p < 0.001$), respectively. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. ACSF. (b) The antinociception induced by various i.t. doses of Tyr-W-MIF-1 injected at peak effect time. Groups of mice were treated with various i.t. doses of Tyr-W-MIF-1 (2.0-16 nmol), and the antinociception induced by Tyr-W-MIF-1 was measured 10 min after the treatment. Each value represents the mean ± S.E.M. for 10 mice. The ED_{50} value with its 95% confidence interval was calculated with the computer-assisted curve-fitting program GraphPad Prism.

Fig. 2. Effect of naloxonazine on the antinociception induced by i.t. injection of Tyr-W-MIF-1, DAMGO, endomorphin-1 (EM-1), or endomorphin-2 (EM-2) in the mouse paw-withdrawal test. Groups of mice were pretreated with various i.t. doses of naloxonazine (1.4-44.2 nmol) or ACSF 24 h before the i.t. administration of Tyr-W-MIF-1 (16 nmol), DAMGO (20 pmol), EM-1 (5.0 nmol), or EM-2 (5.0 nmol). The antinociception induced by i.t. administration of Tyr-W-MIF-1, DAMGO, EM-1, and EM-2 were measured 10 min, 5 min, 5 min, and 5 min after the treatment,

respectively. Both (a) the antinociceptive effects (% MPE) of the agonists and (b) the inhibitory effect (%) of naloxonazine against the agonists are represented the mean ± S.E.M. for 10 mice. (a) The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Dunnett's test. The F values of the one-way ANOVA for Tyr-W-MIF-1, DAMGO, EM-1, and EM-2 were $F[4,45] = 11.55$ ($p < 0.001$), $F[4,45] = 18.07$ ($p < 0.001$), $F[4,45] = 19.97$ ($p < 0.001$), and $F[5,54] = 12.85$ ($p < 0.001$), respectively. *$p < 0.05$ and ***$p < 0.001$ vs. ACSF. (b) The ID_{50} values with their 95% confidence intervals were calculated with the computer-assisted curve-fitting program GraphPad Prism. For the statistical significance of the differences between groups, the entire curves were compared using the F-test, according to the instruction provided with GraphPad Prism. The F values for Tyr-W-MIF-1 against DAMGO, EM-1 and EM-2 were 121.1 ($p < 0.001$), 1278 ($p < 0.001$) and 607.5 ($p < 0.001$), respectively. The F values for EM-2 against DAMGO and EM-1 were 70.35 ($p < 0.001$) and 209.9 ($p < 0.001$), respectively.

Fig. 3. Effect of D-Pro^2-Tyr-W-MIF-1 on the antinociception induced by i.t. administration of DAMGO, endomorphin-1 (EM-1), endomorphin-2 (EM-2), and Tyr-W-MIF-1 in the mouse paw-withdrawal test. Groups of mice were co-administered various i.t. doses of D-Pro^2-Tyr-W-MIF-1 (0.025-1.2 nmol) with Tyr-W-MIF-1 (16 nmol), DAMGO (20 pmol), EM-1 (5.0 nmol), or EM-2 (5.0 nmol), and the antinociception induced by Tyr-W-MIF-1, DAMGO, EM-1 and EM-2 were measured 10 min, 5 min, 5 min, and 5 min after the treatment, respectively. Both (a) the antinociceptive effects (% MPE) of the agonists and (b) the inhibitory effect (%) of D-Pro^2-Tyr-W-MIF-1 against the agonists are represented the mean ± S.E.M. for 10 mice. (a) The statistical significance of the differences between groups was assessed with a one-way ANOVA followed

by the Dunnett's test. The F values of the one-way ANOVA for Tyr-W-MIF-1, DAMGO, EM-1, and EM-2 were $F[7,72] = 16.06$ ($p < 0.001$), $F[7,72] = 9.222$ ($p < 0.001$), $F[7,72] = 9.478$ ($p < 0.001$), and $F[7.72] = 0.9996$ (ns), respectively. *$p < 0.05$ and ***$p < 0.001$ vs. agonist alone. (b) The ID_{50} values with their 95% confidence intervals were calculated with the computer-assisted curve-fitting program GraphPad Prism. For the statistical significance of the differences between groups, the entire curves were compared using the F-test, according to the instruction provided with GraphPad Prism. The F values for Tyr-W-MIF-1 against DAMGO and EM-1 were 68.52 ($p < 0.001$) and 45.53 ($p < 0.001$), respectively.

JPET Fast Forward. Published on November 23, 2004 as DOI: 10.1124/jpet.104.075697
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

Effects of β-funaltrexamine, nor-binaltorphimine and naltrindole on the antinociception induced by Tyr-W-MIF-1 in the mouse paw-withdrawal test

Treatment	% MPE
Tyr-W-MIF-1 (16 nmol)	
+ ACSF (24 h)	79.55 ± 6.17
Tyr-W-MIF-1 (16 nmol)	
+ β-funaltrexamine (4.0 nmol, 24 h)	16.84 ± 4.06***
Tyr-W-MIF-1 (16 nmol)	
+ nor-binaltorphimine (4.0 nmol, 24 h)	81.11 ± 6.81
Tyr-W-MIF-1 (16 nmol)	
+ ACSF (5 min)	81.49 ± 8.26
Tyr-W-MIF-1 (16 nmol)	
+ naltrindole (0.033 nmol, 5 min)	79.06 ± 7.37

Groups of mice were pretreated i.t. with μ-opioid receptor antagonist β-funaltrexamine (4.0 nmol), κ-opioid receptor antagonist nor-binaltorphimine (4.0 nmol) or ACSF 24 h before, or with δ-opioid receptor antagonist naltrindole (0.033 nmol) or ACSF 5 min before the i.t. administration of Tyr-W-MIF-1 (16 nmol), and the antinociception induced by Tyr-W-MIF-1 was measured 10 min after the treatment. Each value represents the mean ± S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Newman-Keuls's test. The F value of the one-way ANOVA was $F[4,45] = 18.05$ ($p < 0.001$). ***$p < 0.001$ vs. ACSF (24-h pretreatment).

JPET Fast Forward. Published on November 23, 2004 as DOI: 10.1124/jpet.104.075697
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 2

The inhibiting effects of naloxonazine or D-Pro^2-Tyr-W-MIF-1 on the antinociception induced by DAMGO, endomorphin-1, endomorphin-2, and Tyr-W-MIF-1 in the mouse paw-withdrawal test

	Naloxonazine	
Treatment	ID_{50} (nmol)	Hill slope
DAMGO (20 pmol)	13.66 (10.35-18.04)	1.77 (0.90-2.64)
Endomorphin-1 (5.0 nmol)	15.79 (14.99-16.63)	1.73 (1.58-1.89)
Endomorphin-2 (5.0 nmol)	4.58 (3.82-5.49)	1.10 (0.85-1.35)
Tyr-W-MIF-1 (16 nmol)	35.34 (33.69-37.07)	2.52 (2.21-2.83)
	D-Pro^2-Tyr-W-MIF-1	
Treatment	ID_{50} (nmol)	Hill slope
DAMGO (20 pmol)	0.98 (0.82-1.18)	1.64 (1.05-2.23)
Endomorphin-1 (5.0 nmol)	0.75 (0.63-0.89)	1.79 (1.17-2.40)
Endomorphin-2 (5.0 nmol)	NA	NA
Tyr-W-MIF-1 (16 nmol)	0.21 (0.15-0.28)	1.16 (0.76-1.55)

Groups of mice were pretreated various i.t. doses of naloxonazine (1.4-44.2 nmol) 24 h prior or co-administered various i.t. doses of D-Pro^2-Tyr-W-MIF-1 (0.025-1.2 nmol) with DAMGO (20 pmol), endomoprhin-1 (5.0 nmol), endomoprhin-2 (5.0 nmol), or Tyr-W-MIF-1 (16 nmol), and the antinociception induced by DAMGO, endomorphin-1, endomorphin-2 and Tyr-W-MIF-1 were measured 5 min, 5 min, 5 min, and 10 min after the treatment, respectively. The ID_{50} values and hill slope values with their 95% confidence intervals were calculated with the

computer-assisted curve-fitting program GraphPad Prism. NA: not available.

TABLE 3

Effect of D-Pro^2-Tyr-W-MIF-1 on U-50,488H- and deltorphin II-induced antinociception in the mouse paw-withdrawal test

Treatment	% MPE
U-50,488H (30 nmol)	83.33 ± 6.15
U-50,488H (30 nmol) + D-Pro^2-Tyr-W-MIF-1 (0.025 nmol)	83.82 ± 5.05
U-50,488H (30 nmol) + D-Pro^2-Tyr-W-MIF-1 (0.05 nmol)	88.59 ± 3.54
U-50,488H (30 nmol) + D-Pro^2-Tyr-W-MIF-1 (0.1 nmol)	88.99 ± 4.22
U-50,488H (30 nmol) + D-Pro^2-Tyr-W-MIF-1 (0.2 nmol)	86.47 ± 5.18
U-50,488H (30 nmol) + D-Pro^2-Tyr-W-MIF-1 (0.4 nmol)	87.63 ± 3.76
U-50,488H (30 nmol) + D-Pro^2-Tyr-W-MIF-1 (0.8 nmol)	86.79 ± 4.88
U-50,488H (30 nmol) + D-Pro^2-Tyr-W-MIF-1 (1.2 nmol)	86.49 ± 4.86
Deltorphin II (4 nmol)	78.20 ± 7.57
Deltorphin II (4 nmol) + D-Pro^2-Tyr-W-MIF-1 (0.025 nmol)	85.37 ± 5.82

Deltorphin II (4 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.05 nmol)	84.24 ± 5.87
Deltorphin II (4 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.1 nmol)	87.54 ± 4.29
Deltorphin II (4 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.2 nmol)	85.00 ± 5.88
Deltorphin II (4 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.4 nmol)	88.03 ± 4.40
Deltorphin II (4 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (0.8 nmol)	87.78 ± 5.50
Deltorphin II (4 nmol)	
+ D-Pro^2-Tyr-W-MIF-1 (1.2 nmol)	88.07 ± 4.86

Groups of mice were co-administered various i.t. doses of D-Pro^2-Tyr-W-MIF-1 (0.025-1.2 nmol) and 30 nmol of U-50,488H or 4 nmol of deltorphin II, and the antinociception induced by U-50,488H or deltorphin II was measured 10 or 5 min after the treatment, respectively. Each value represents the mean ± S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Dunnett's test.

Fig. 1a

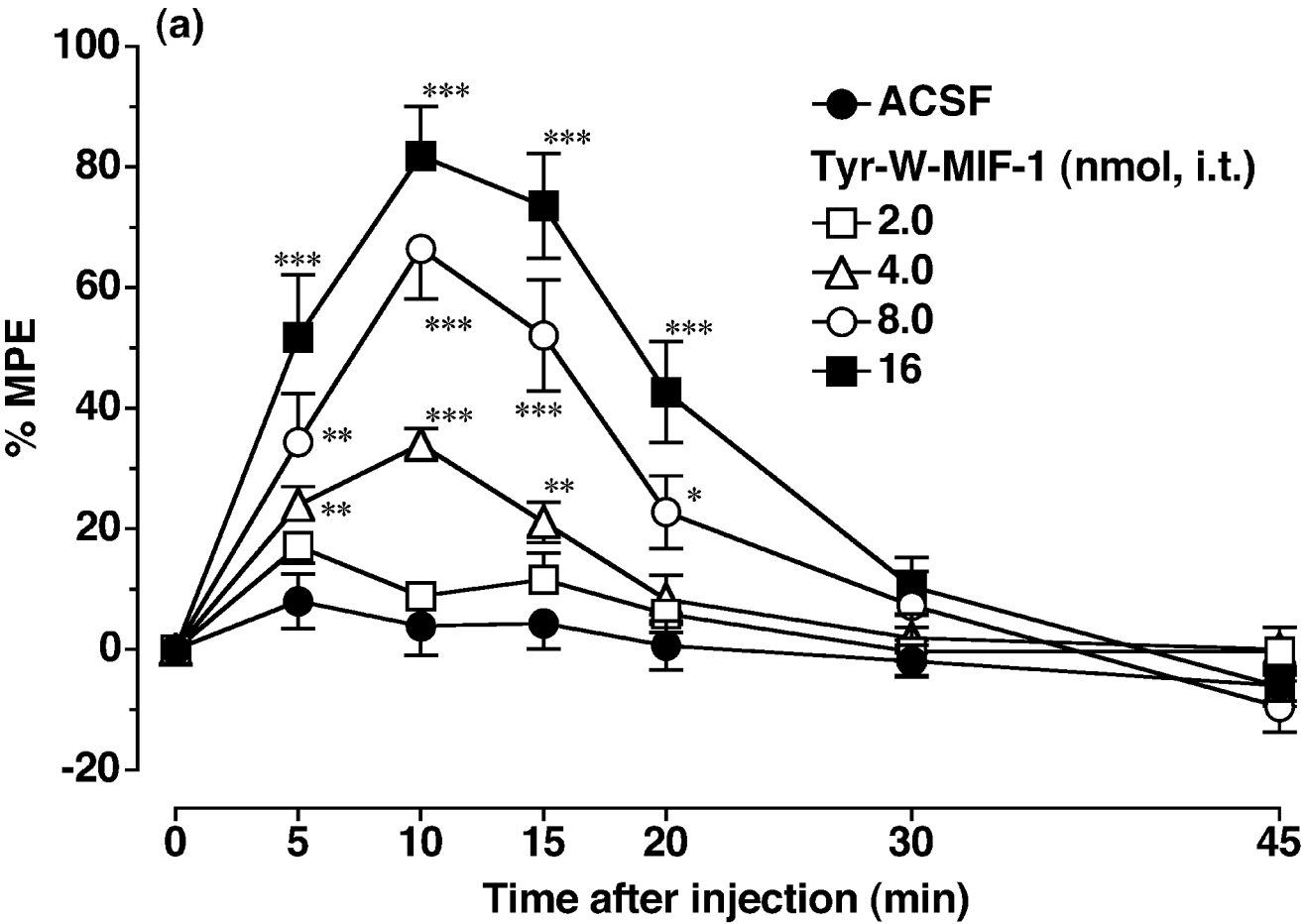

(a)
100
80
60
40
20
0
-20
% MPE
ACSF
Tyr-W-MIF-1 (nmol, i.t.)
2.0
4.0
8.0
16
***
**
*
0
5
10
15
20
30
45
Time after injection (min)

Fig. 1b

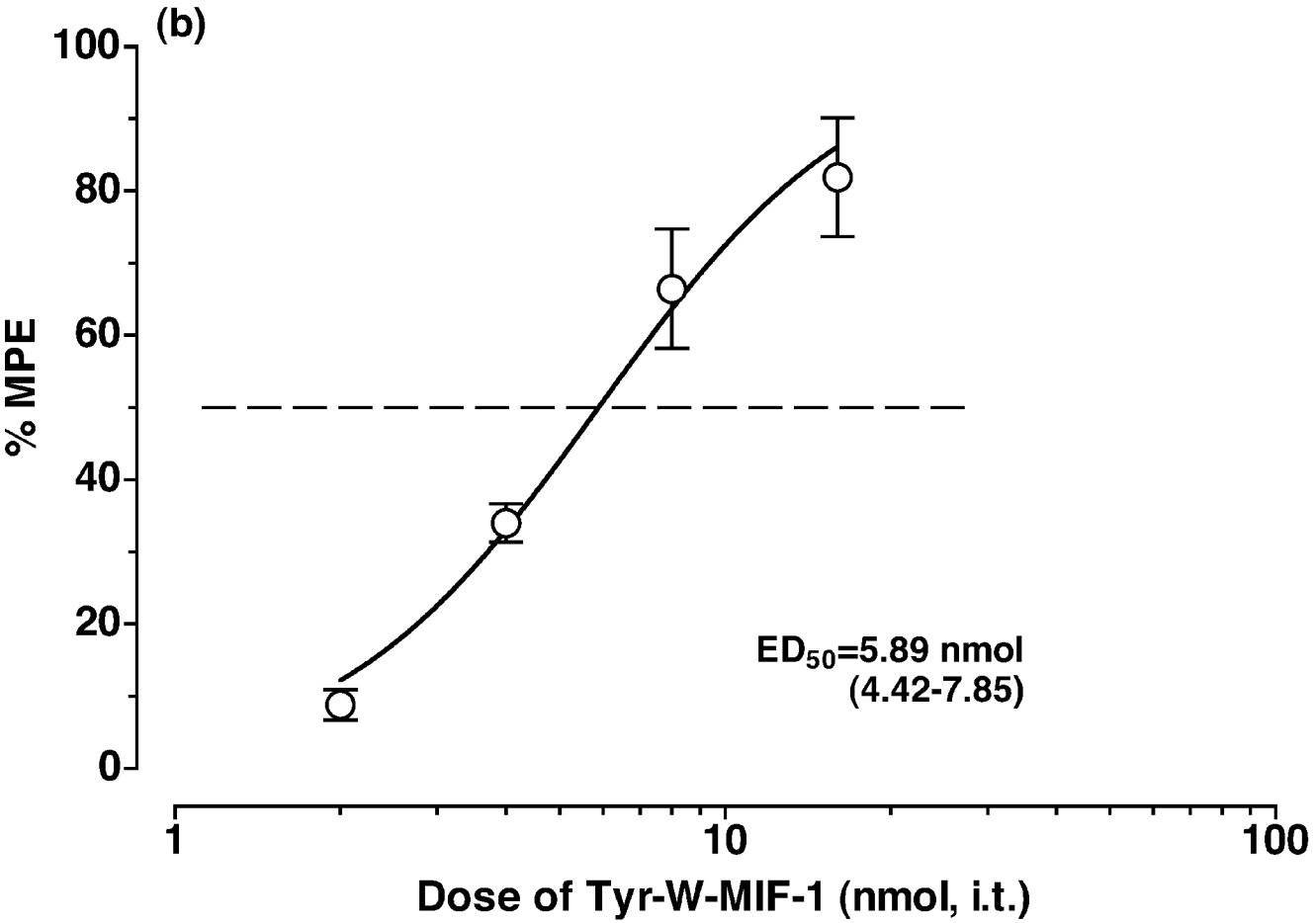

(b)
100
80
60
40
20
0
% MPE
ED50=5.89 nmol
(4.42-7.85)
1
10
100
Dose of Tyr-W-MIF-1 (nmol, i.t.)

Fig. 2a

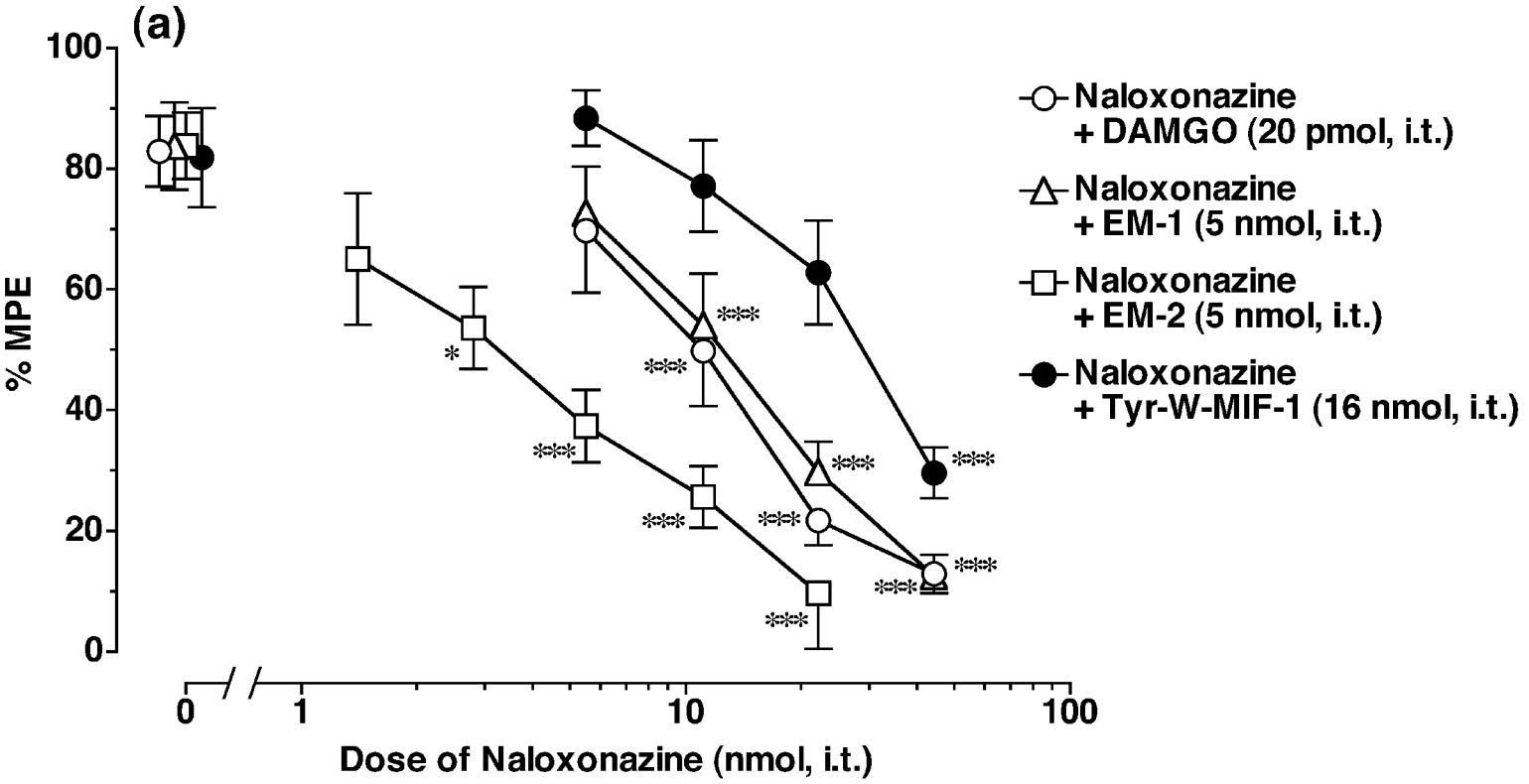

(a)
100
80
60
40
20
0
% MPE
0
1
10
100
Dose of Naloxonazine (nmol, i.t.)
Naloxonazine
+ DAMGO (20 pmol, i.t.)
Naloxonazine
+ EM-1 (5 nmol, i.t.)
Naloxonazine
+ EM-2 (5 nmol, i.t.)
Naloxonazine
+ Tyr-W-MIF-1 (16 nmol, i.t.)
*
***

Fig. 2b

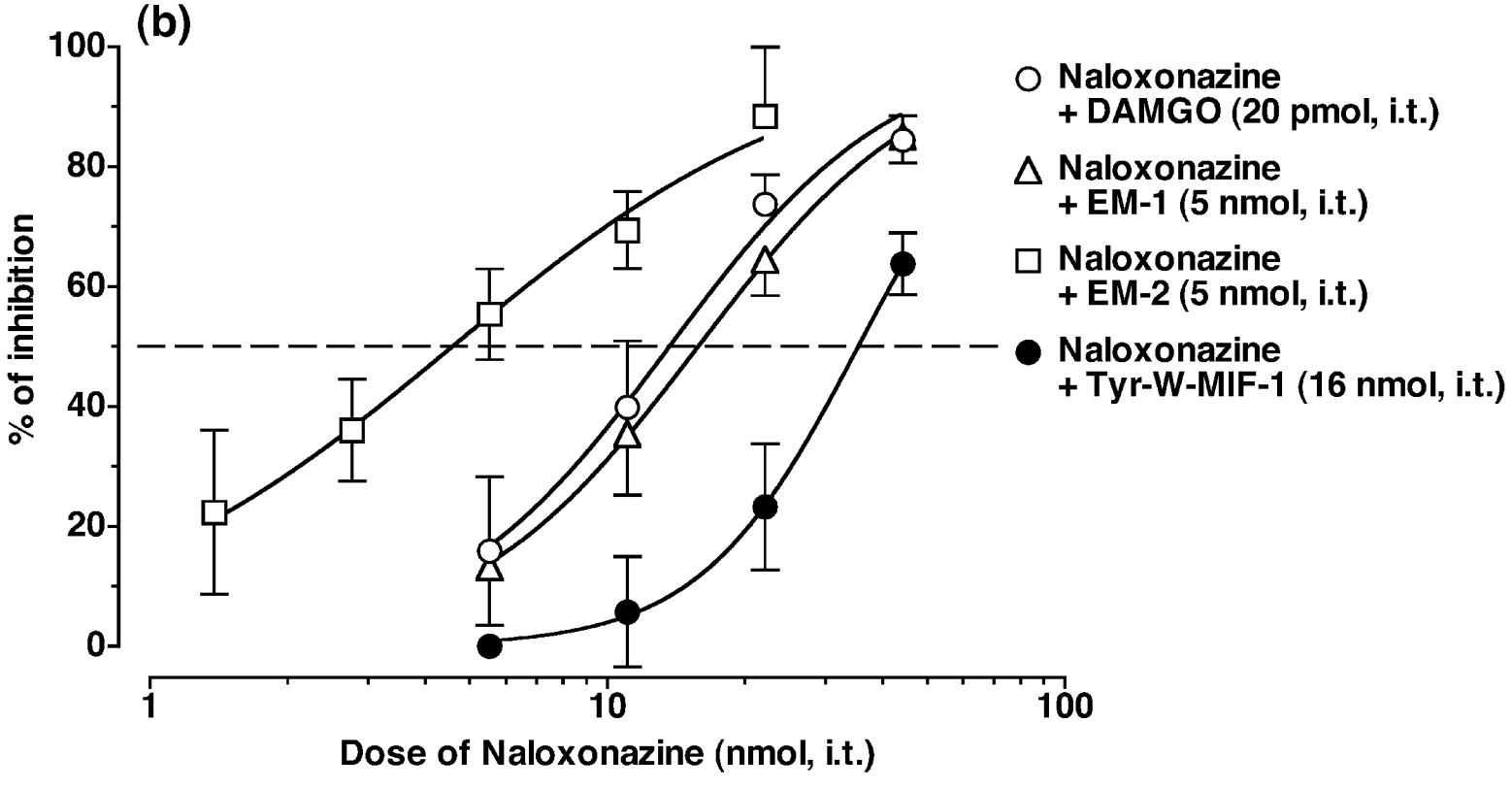

(b)
100
80
60
40
20
0
% of inhibition
1
10
100
Dose of Naloxonazine (nmol, i.t.)
Naloxonazine
+ DAMGO (20 pmol, i.t.)
Naloxonazine
+ EM-1 (5 nmol, i.t.)
Naloxonazine
+ EM-2 (5 nmol, i.t.)
Naloxonazine
+ Tyr-W-MIF-1 (16 nmol, i.t.)

Fig. 3a

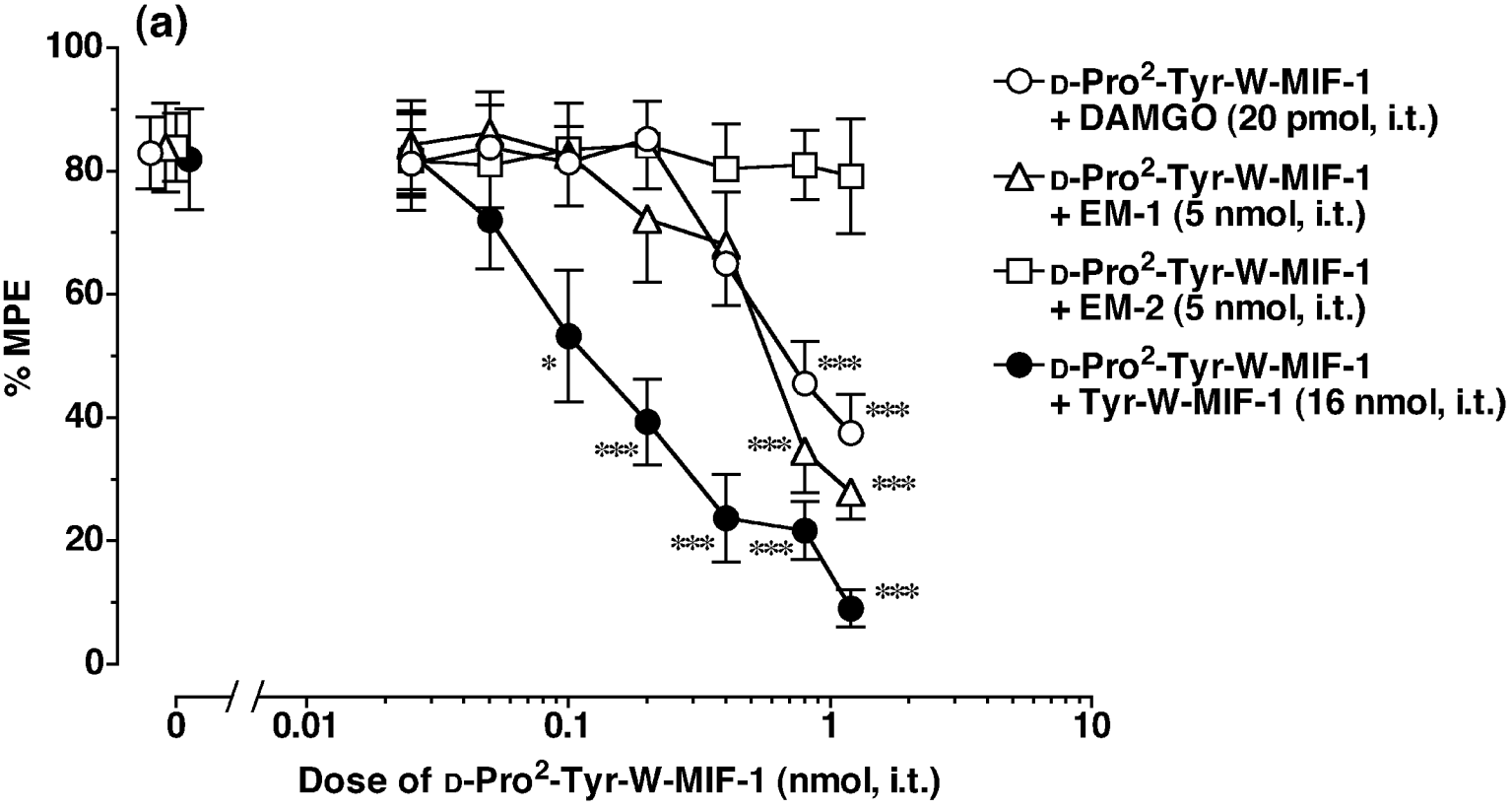
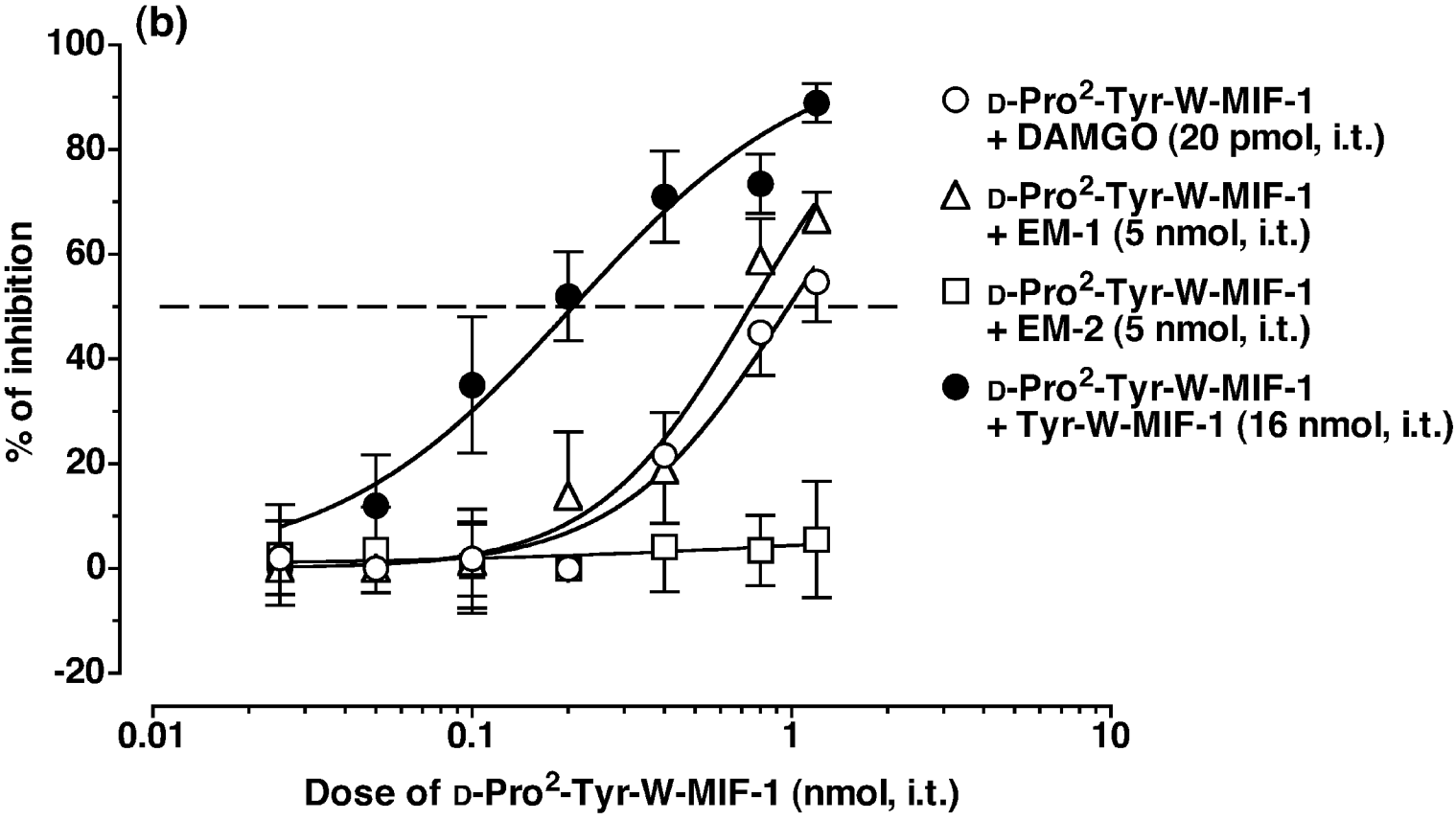

(a)
100
80
60
40
20
0
% MPE
0
0.01
0.1
1
10
Dose of D-Pro2-Tyr-W-MIF-1 (nmol, i.t.)
D-Pro2-Tyr-W-MIF-1 + DAMGO (20 pmol, i.t.)
D-Pro2-Tyr-W-MIF-1 + EM-1 (5 nmol, i.t.)
D-Pro2-Tyr-W-MIF-1 + EM-2 (5 nmol, i.t.)
D-Pro2-Tyr-W-MIF-1 + Tyr-W-MIF-1 (16 nmol, i.t.)
*
***

Fig. 3b


(b)
100
80
60
40
20
0
-20
% of inhibition
0.01
0.1
1
10
Dose of D-Pro2-Tyr-W-MIF-1 (nmol, i.t.)
D-Pro2-Tyr-W-MIF-1 + DAMGO (20 pmol, i.t.)
D-Pro2-Tyr-W-MIF-1 + EM-1 (5 nmol, i.t.)
D-Pro2-Tyr-W-MIF-1 + EM-2 (5 nmol, i.t.)
D-Pro2-Tyr-W-MIF-1 + Tyr-W-MIF-1 (16 nmol, i.t.)