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SPECIAL REPORT

Endomorphin analogues containing D-Pro^2 discriminate different μ-opioid receptor mediated antinociception in mice

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The antagonistic actions of D-Pro^2-endomorphins on inhibition of the paw withdrawal response by endomorphins were studied in mice. D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2, injected intrathecally (i.t.), had no significant effect on the nociceptive thermal threshold alone. When D-Pro^2-endomorphin-1 (0.05–0.1 pmol) was injected simultaneously with i.t. endomorphin-1 (5.0 nmol) or endomorphin-2 (5.0 nmol), antinociception induced by endomoprhin-1 was reduced significantly, whereas endomorphin-2-induced antinociception was not affected by D-Pro^2-endomorphin-1. Antinociception induced by i.t. endomorphin-2 (5.0 nmol) was reduced significantly by its analogue, D-Pro^2-endomorphin-2 (100 pmol), but not by D-Pro^2-endomorphin-1. D-Pro^2-endomorphin-1. D-Pro^2-endomorphin-1 also antagonized the antinociceptive effect of i.t. DAMGO, a μ-opioid receptor agonist, whereas D-Pro^2-endomorphin-2 failed to reduce the effect of DAMGO. These results suggest that endomorphin analogues containing D-Pro^2 are able to discriminate the antinociceptive actions of μ_1- and μ_2-opioid receptor agonists at the spinal cord level.
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Keywords: D-Pro^2-endomorphin-1; D-Pro^2-endomorphin-2; endomorphins; opioid receptor antagonists; antinociception; intrathecal injection; spinal cord

Abbreviations: CSF, cerebrospinal fluid; i.c.v., intracerebroventricular(ly); i.t., intrathecal(ly); DAMGO, [D-Ala^2, MePhe^4, Gly(ol)^5]enkephalin

Introduction

The newly isolated endogenous opioid tetrapeptides, endomorphin-1 and endomorphin-2, have high affinity and selectivity for μ-opioid receptors (Zadina *et al*., 1997). Neither endomorphins had appreciable affinity for δ- and κ-opioid receptors. These endomorphins are found in the brain and spinal cord where high densities of μ-opioid receptors occur. Endomorphin-1-like immunoreactivity is more prominent in the brain, whereas endomorphin-2-like immunoreactivity is more prevalent in the spinal cord (Martin-Schild *et al*., 1999). Thus, endomorphin-1 and -2 are the putative endogenous ligand for the morphine-preferring μ-opioid receptors. Distinct pharmacological properties of endomorphins have been reported in several behavioural experiments in rodents (Zadina *et al*., 1997; Stone *et al*., 1997; Sakurada *et al*., 1999; 2000a; Horvath *et al*., 1999; Przewlocka *et al*., 1999). Antinociception induced by endomorphin-1 and endomorphin-2 given intrathecally (i.t.) or intracerebroventricularly (i.c.v.) is selectively blocked by pre-treatment with the μ-opioid receptor antagonists, naloxone or β-funaltrexamine (Zadina *et al*., 1997; Sakurada *et al*., 1999; 2000a), indicating that they are mediated by the stimulation of μ-opioid receptors. Furthermore, pre-treatment with the μ_1-opioid receptor antagonist, naloxonazine, attenuates antinociceptive effects induced by i.t. administered endomorphin-2 but not by endomorphin-1, suggesting that endomorphin-2-induced antinociception may be mediated by the stimulation of μ_1-opioid receptors (Sakurada *et al*., 1999; 2000a). The antinociceptive effect of i.t. DAMGO, a selective μ-opioid agonist, is insensitive to naloxonazine and sensitive to β-funaltrexamine, which binds μ_1- and μ_2-opioid receptors (Pick *et al*., 1991). The *in vivo* and *in vitro* studies of D-Pro^2-endomorphin-2, an enzyme-resistant analogue of endomorphin-2, have shown that the D-Pro^2 substitution in endomorphin-2 is more potent than endomorphin-2 in significantly increasing tail-flick latencies when injected i.c.v. in rats, since D-Pro^2-endomorphin-2 is totally resistant to the action of dipeptidyl peptidase IV (Shane *et al*., 1999). In contrast, the pharmacological activity of D-Pro^2-endomorphins is less potent than that of parent tetrapeptides, as drastic loss of activity in the guinea-pig ileum and opioid receptor binding assays occur in the presence of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 (Paterlini *et al*., 2000; Okada *et al*., 2000).

The purpose of the present study is to determine whether D-Pro^2-endomorphins discriminate μ_1- and/or μ_2-opioid receptor mediation of antinociception induced by three different μ-opioid receptor agonists, endomorphin-1, -2 and DAMGO at the spinal cord level.

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Methods

Adult male ddY mice weighing 22–25 g were housed in a light- and temperature-controlled room (light on 0900 to 2100 h; 23°C) and had free access to food and water. The experiments were performed with the approval of the Committee of Animal Experiments at Tohoku Pharmaceutical University. Endomorphin-1, -2 and D-Pro^2-endomorphins were synthesized in our laboratory. DAMGO was purchased from Sigma (St. Louis, MO, U.S.A.). Endomorphin-1 (5 nmol), endomorphin-2 (5 nmol) and D-Pro^2-endomorphin-1 (0.03–1.0 pmol), D-Pro^2-endomorphin-2 (25–100 pmol) and DAMGO (20 pmol) were dissolved in sterile artificial cerebrospinal fluid (CSF) containing 7.4 g NaCl, 0.19 g KCl, 0.19 g $MgCl_2$, 0.14 g $CaCl_2$ 1000 ml^{-1}. For i.t. administration, a 29-gauge needle connected to Hamilton microsyringe was inserted directly between L5 and L6, and each peptide was administered at a rate of 2 μl 10^{-1}. Endomorphins and DAMGO in combination with D-Pro^2-endomorphins were also co-administered i.t. in a volume of 2 μl.

The antinociceptive activity of opioid peptides against the response to a thermal stimulus was assessed by the mouse paw withdrawal test. Antinociceptive thresholds were determined by an automated tail-flick unit (BM kiki, Tokyo). 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 radiant heat source was positioned under the glass floor directly beneath the hindpaw. The heat stimulus intensity was determined by the reaction time of the removal of the paw from a source of noxious radiant heat. The intensity of the light beam was adjusted so that baseline reaction time was 2.5–3.5 s. The light beam was focused on the same plantar spot of the hind paw in all animals. To prevent tissue damage, trials were terminated automatically if the mouse did not lift the paw within 10 s. Baseline latencies were determined before experimental treatment for all animals as the mean of two trials. The measurements of hindpaw withdrawal were determined by an experimenter. To prevent experimenter bias, observers were uninformed of the dose of each compound being injected. After determination of pre-drug values, animals were injected. Antinociceptive activity for each animal was calculated with the following equation and represented as per cent of maximum possible effect (% MPE) = (P2 − P1/10 − P1) × 100, where P1 and P2 are pre-drug and post-drug responsive time (in seconds), respectively.

Statistical significance of the data was estimated with a mixed two-factor analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A level of probability of 0.05 or less was accepted as significant. The ED_{50} or ID_{50} values and their 95% confidence limits (95% CL) for the antinociceptive or antagonistic effect of compounds examined were computed according to our previous report (Sakurada *et al*., 1999).

Results

The i.t. injection of endomorphin-1 (5 nmol), -2 (5 nmol) and DAMGO (20 pmol) produced a marked antinociceptive effect as assayed by the paw withdrawal test (Figure 1). Endomorphin-1 and -2 at a dose of 5 nmol caused almost equipotent antinociception in intensity and duration. The antinociceptive effect reached a peak at 1 min after injection of both endomorphins, rapidly declined, and returned to the pre-injection level in 20 min. The ED_{50} values for endomorphin-1 and -2 were 0.14 and 0.24 nmol, respectively. The ED_{50} value for i.t. DAMGO was 14.0 pmol at the 5 min peak time of antinociception. These pharmacological properties of endomorphins and DAMGO confirm our previous reported data (Sakurada *et al*., 2000a). Single injection of D-Pro^2-endomorphin-1 (0.03–1.0 pmol) or D-Pro^2-endomorphin-2 (25–100 pmol) was without affecting the paw withdrawal response (data not shown).

The antagonistic effect of D-Pro^2-endomorphin-1 or D-Pro^2-endomorphin-2 on antinociception induced by i.t. endomorphin-1 and -2 at a dose of 5.0 nmol was examined. As seen in Figure 1, the antinociceptive effect of endomorphin-1 (5 nmol) at 1 or 5 min after i.t. injection was inhibited significantly by co-administration of D-Pro^2-endomorphin-1 (0.08 and 0.1 pmol). The ID_{50} values of D-Pro^2-endomorphin-1 on endomorphin-1-induced antinociception were 0.13 pmol and 0.058 pmol at 1 and 5 min after i.t. co-injection, respectively (Table 1). No further antagonism in antinociception of endomorphin-1 at a dose of 5 nmol was seen by 0.25 or 1.0 pmol of D-Pro^2-endomorphin-1 (Figure 2). The i.t. co-injection of D-Pro^2-endomorphin-2 (25–100 pmol) produced no significant effect on inhibition of the paw withdrawal response induced by i.t. endomorphin-1 (5.0 nmol). Artificial CSF (2 μl) alone, injected i.t., had no apparent effect on inhibition of the paw withdrawal response.

Co-administration of D-Pro^2-endomorphin-2 (50 and 100 pmol) at 1 or 5 min after i.t. injection produced a dose-dependent antagonism on inhibition of the paw withdrawal response by endomorphin-2 (Figure 1). The maximum antagonistic effect on endomorphin-2-induced antinociception was observed at 100 pmol of D-Pro^2-endomorphin-2, (Figure 2). The ID_{50} values for D-Pro^2-endomorphin-2 on inhibition of the paw withdrawal response by endomorphin-2 were 60.1 pmol and 70.0 pmol at 1 and 5 min after i.t. co-injection, respectively (Table 1). Antinociception induced by endomorphin-2 was unaffected by D-Pro^2-endomorphin-1 (0.03–1.0 pmol).

DAMGO, injected i.t. at a dose at 20 pmol, was antinociceptive in the paw withdrawal test. DAMGO-induced antinociception was antagonized significantly by D-Pro^2-endomorphin-1 (0.01–0.08 pmol) at 10 min post-co-injection, but not by D-Pro^2-endomorphin-2. The ID_{50} values for D-Pro^2-endomorphin-1 on DAMGO-induced antinociception were 0.022 pmol and 0.019 pmol at 5 and 10 min after i.t. co-injection, respectively.

Discussion

Spinal administration of endomorphin-1 and -2 at a dose of 5 nmol induced equipotent antinociception. The present results of i.t. administered endomorphins are in agreement with those of Stone *et al*. (1997) and Sakurada *et al*. (1999; 2000a) who reported that the antinociceptive effect of the endomorphins is short-lasting and is absent 15–20 min following i.t. injection, as assayed by the tail-flick test, paw withdrawal test and tail-pressure test. Endomorphins are

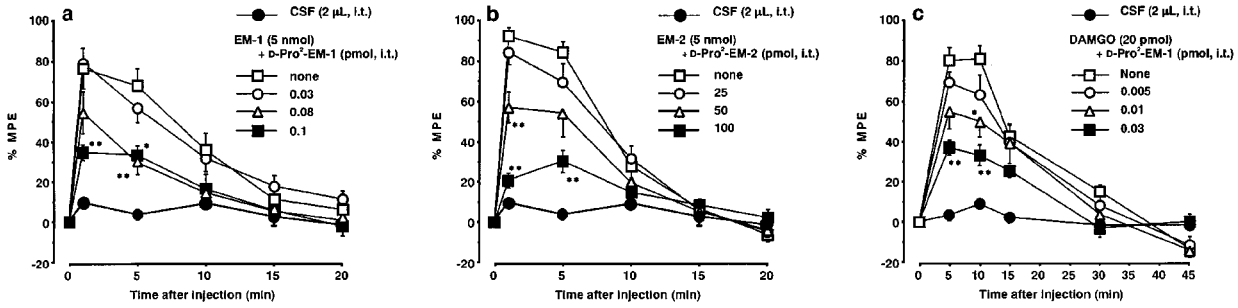

Figure 1 The time course of antagonistic effects of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 on antinociception induced by i.t. endomorphin-1 (a), endomorphin-2 (b) and DAMGO (c) in the mouse paw withdrawal test. Endomorphins and DAMGO were co-administered i.t. with D-Pro^2-endomorphins. Each point in the time-course effect represents the mean ± s.e.mean of 10 mice. $^{**}P<0.01$, $^{*}P<0.05$, compared with each agonist alone. EM-1: endomorphin-1; EM-2: endomorphin-2.

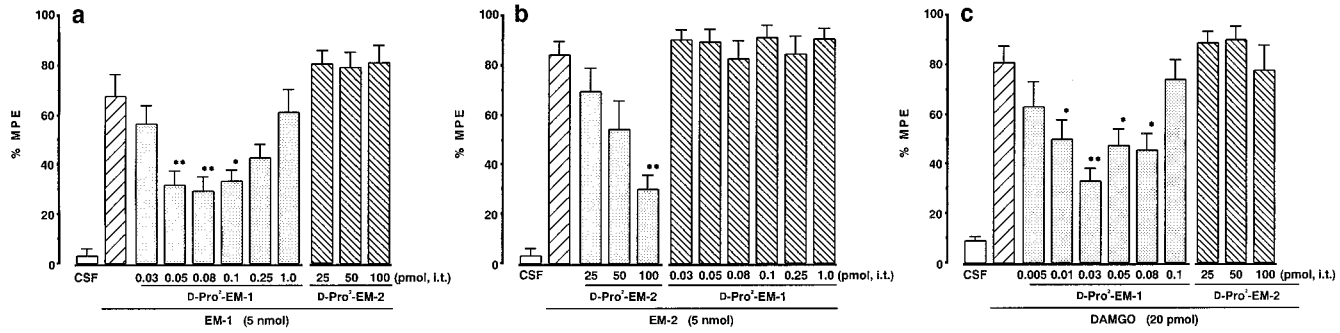

Figure 2 Effects of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 on antinociception induced by i.t. endomorphin-1 (a), endomorphin-2 (b) and DAMGO (c) in the mouse paw withdrawal test. Each column represents the mean ± s.e.mean of 10 mice. Measurements were taken 5 and 10 min following i.t. endomorphins and DAMGO, respectively $^{**}P<0.01$, $^{*}P<0.05$, compared with each agonist alone. EM-1: endomorphin-1; EM-2: endomorphin-2.

Table 1 Antagonistic effect of D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 on antinociception induced by endomorphin-1, -2 and DAMGO in mice

Agonists (dose)	*Time after injection* (min)	ID_{50} (pmol) *D-Pro²-EM-1*	ID_{50} (pmol) *D-Pro²-EM-2*
EM-1	1	0.13 (0.072–0.237)	–
(5 nmol)	5	0.058 (0.038–0.089)	–
EM-2	1	–	60.1 (39.95–90.41)
(5 nmol)	5	–	70.0 (40.4–121.0)
DAMGO	5	0.022 (0.01–0.046)	–
(20 pmol)	10	0.019 (0.008–0.044)	–

Values in parenthesis are 95% confidence limits. Each agonist was co-injected i.t. with D-Pro^2-endomorphin-1 or D-Pro^2-endomorphin-2. –, Significantly not antagonized; EM-1: endomorphin-1; EM-2: endomorphin-2.

small peptides that consist of only four amino acids, making them vulnerable to rapid degradation by peptidases. Dipeptidyl peptidase IV is a membrane bound serine proteinase proposed to be involved in the inactivation of endomorphins. Endomorphin-2-induced antinociception is modulated by the proteolytic enzyme, dipeptidyl peptidase IV such that dipeptidyl peptidase inhibitor itself, produces an opioid-sensitive antinociception, and enhances endomorphin-2-induced antinociception. An enzyme-resistant analogue of endomorphin-2, D-Pro^2-endomorphin-2 produces more potent and longer-lasting opioid sensitive antinociception after i.c.v. administration (Shane *et al*., 1999). The diastereoisomer of endomorphin-1, D-Pro^2-endomorphin-1 possesses much lower potency than that of the parent peptide in the guinea-pig ileum assay, and is not an antagonist at either the μ- or κ-opioid receptors, as it is unable to shift the dose–response curve to either morphine or ethylketazocine (Paterlini *et al*., 2000).

There is biochemical and pharmacological evidence supporting the existence of μ-opioid receptor subtypes, which are localized in spinal and supraspinal structures involved in the modulation of nociception (Wolozin & Pasternak, 1981; Moskowitz & Goodman, 1985). At least two μ-opioid receptor subtypes have been proposed; μ_1- and μ_2-. 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 receptor. It is noteworthy that 35 mg kg^{-1} (s.c.) of naloxonazine is a reasonable dose to selectively block μ_1-opioid receptors in mice (Ling *et al*., 1986). The antinoci-

ceptive effect of endomorphin-2 is completely blocked by pretreatment with naloxonazine at the dosage (35 mg kg^{-1} s.c.) which shows the selectivity to μ_1-opioid receptor in the paw withdrawal test, suggesting that endomorphin-2 may be a selective agonist for μ_1-opioid receptor. Pretreatment with naloxonazine at the dosage of 35 mg kg^{-1} (s.c.) does not block the antinociception induced by endomorphin-1 or DAMGO, whereas higher doses of naloxonazine (52.5 or 65.6 mg kg^{-1}, s.c.) significantly attenuate endomorphin-1 induced antinociception, indicating that at high dosage, naloxonazine may lose its selectivity for μ_1-opioid receptor (Sakurada *et al*., 2000b). This means that endomorphin-1 or DAMGO can act as a predominantly μ_2-opioid receptor agonist and endomorphin-2 as a μ_1-opioid receptor agonist. Thus, endomorphin-1 has similarity of antinociception to DAMGO on lack of antagonism by naloxonazine. However, the selective μ_2-opioid antagonist has not yet been found.

We found in the present study that the antinociceptive effect of endomorphin-1 was inhibited by co-administration of D-Pro^2-endomorphin-1 but not by D-Pro^2-endomorphin-2. Antagonistic action of D-Pro^2-endomorphin-1 on endomorphin-1-induced antinociception reached a maximum effect at 0.08 pmol and declined with increases in doses. Similarly, the bell-shaped pattern in the dose–response of D-Pro^2-endomorphin-1 was observed in the case of DAMGO-induced antinociception. These results led to speculate that D-Pro^2-endomorphin-1 at higher doses may have an inhibitory action on endomorphin-1 degradation by peptidases. On the other hand, D-Pro^2-endomorphin-2 at a dose of 100 pmol significantly eliminated the antinociceptive activity produced by i.t. endomorphin-2 without affecting the antinociception of endomorphin-1 and DAMGO. The present study is the first to show that endomorphin analogues, D-Pro^2-endomorphin-1 and D-Pro^2-endomorphin-2 can distinguish the action of endomorphin-1 from that of endomorphin-2, suggesting possibility that D-Pro^2-endomorphin-1 may act as a μ_2-opioid receptor antagonist and D-Pro^2-endomorphin-2 as a μ_1-opioid receptor antagonist.

D-Pro^2-endomorphin-1 selectively blocked the antinociceptive effect of i.t. administered DAMGO, as well as endomorphin-1, whereas antinociception of Tyr-D-Arg-Phe-β-Ala (Sakurada *et al*., 2000a), the selective μ_1-opioid receptor agonist was inhibited by co-administration of D-Pro^2-endomorphin-2 but not D-Pro^2-endomorphin-1 (unpublished data). These results also indicate that these two D-Pro^2-endomorphins may be a useful tool to discriminate between the antinociceptive effects of μ_1- and μ_2-opioid receptor agonists.

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