Research report

Differential antagonism of endomorphin-1 and endomorphin-2 spinal antinociception by naloxonazine and 3-methoxynaltrexone

Shinobu Sakurada a,*, Takafumi Hayashi a, Masayuki Yuhki a, Tsutomu Fujimura b, Kimie Murayama b, Akihiko Yonezawa a, Chikai Sakurada c, Mitsuhiro Takeshita d, James E. Zadina e, Abba J. Kastin e, Tsukasa Sakurada c

a Department of Physiology and Anatomy, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Sendai 981-8558, Japan
b Division of Biochemical Analysis, Central Laboratory of Medical Sciences, Juntendo University School of Medicine, 2-1-1 Hongo, Tokyo 113-8421, Japan
c Department of Biochemistry, Daichi College of Pharmaceutical Sciences, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan
d Department of Pharmaceutics, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Sendai 981-8558, Japan
e Veterans Affairs Medical Center and Tulane University School of Medicine, New Orleans, LA, USA

Accepted 25 July 2000

Abstract

To determine the role of spinal mu-opioid receptor subtypes in antinociception induced by intrathecal (i.t.) injection of endomorphin-1 and -2, we assessed the effects of β-funaltrexamine (a selective mu-opioid receptor antagonist) naloxonazine (a selective antagonist at the mu-opioid receptor) and a novel receptor antagonist (3-methoxynaltrexone) using the paw-withdrawal test. Antinociception of i.t. endomorphins and [D-Ala2, MePhe4, Gly(ol)3]enkephalin (DAMGO) was completely reversed by pretreatment with β-funaltrexamine (40 mg/kg s.c.). Pretreatment with a variety of doses of i.t. or s.c. naloxonazine 24 h before testing antagonized the antinociception of endomorphin-1, -2 and DAMGO. Judging from the ID50 values of naloxonazine, the antinociceptive effect of endomorphin-2 was more sensitive to naloxonazine than that of endomorphin-1 or DAMGO. The selective morphine-6β-glucuronide antagonist, 3-methoxynaltrexone, which blocked endomorphin-2-induced antinociception at each dose (0.25 mg/kg s.c. or 2.5 ng i.t.) that was inactive against DAMGO, did not affect endomorphin-1-induced antinociception but shifted the dose–response curve of endomorphin-2 3-fold to the right. These findings may be interpreted as indicative of the existence of a novel mu-opioid receptor subtype in spinal sites, where antinociception of morphine-6β-glucuronide and endomorphin-2 are antagonized by 3-methoxynaltrexone. The present results suggest that endomorphin-1 and endomorphin-2 may produce antinociception through different subtypes of mu-opioid receptor. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Opioid receptors

Keywords: Naloxonazine; β-Funaltrexamine; 3-Methoxynaltrexone; Endomorphin-1; Endomorphin-2

1. Introduction

Recently two novel peptides have been isolated from bovine brain [41] that have high affinity and selectivity for the mu-opioid receptor, and have been termed endomorphin-1 (Tyr-Pro-Trp-Phe-NH2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH2). Furthermore, both peptides have been isolated from human brain [6] and immuno-reactivity for endomorphin-1 and endomorphin-2 were widely and densely distributed throughout human and rat brain whereas endomorphin-2-immunoreactivity is present in the superficial laminae of the spinal cord dorsal horn where primary afferent nociceptors terminate. These findings indicate that endomorphin-2 plays a physiological role in regulating nociceptive information in the spinal cord [16–18]. Both endomorphin-1 and endomorphin-2 significantly increase nociceptive thresholds after both spinal and supraspinal administration [4,31,35,41] which are blocked by mu-receptor-specific antagonists, naloxone and

*Corresponding author. Fax: +81-22-275-2013.
E-mail address: s-sakura@tohoku-pharm.ac.jp (S. Sakurada).
β-Funaltrexamine [41]. Neither supraspinal endomorphin-1 or -2 elicited antinociception in CXBK mice which are also insensitive to morphine [4].

There is biochemical and pharmacological evidence supporting the existence of mu-opioid receptor subtypes [5,20,40] which are localized in spinal and supraspinal structures involved in the modulation of nociception [19]. At least two mu-opioid receptor subtypes have been proposed: μ₁ and μ₂ opioid receptor subtypes [23,24]. β-Funaltrexamine irreversibly antagonizes both μ₁ and μ₂ opioid receptors and inhibits both supraspinal and spinal antinociception, whereas naloxonazine selectively antagonizes the μ₁-opioid receptor. It has been suggested that these receptor subtypes have different physiological roles, with μ₁-opioid receptors mediating supraspinal antinociception, whereas μ₂-opioid receptors mediate spinal antinociception measured by the tail-flick test.

Recent report suggests that heroin and morphine-6β-glucuronide in the family of mu opioids both act through a novel third mu-opioid receptor subtype, distinct from those mediating morphine’s actions. 3-methoxynaltrexone selectively competes for [3H]morphine-6β-glucuronide binding antagonizes the antinociceptive actions of heroin and morphine-6β-glucuronide without interfering with mu (morphine and DAMGO), delta- or kappa-opioid receptor agonist-induced antinociception [2].

The objective of the present study was to determine whether antinociceptive activities of i.t. administered endomorphin-1 and endomorphin-2 are mediated through mu-opioid receptor subtypes, μ₁, μ₂, or a novel mu-opioid receptor in the mouse paw-withdrawal test.

2. Materials and methods

2.1. Animals

Adult male ddY mice weighing 22–25 g were housed in a light- and temperature-controlled room (lights on 09:00–21:00 h; 24°C) and had free access to food and water. The experiments were performed with the approval of the Committee of Animal Experiments in Tohoku Pharmaceutical University.

2.2. Injection procedure

The procedure for intrathecal (i.t.) injections was adapted from the method of Hylden and Wilcox [11] with a constant injection volume of 2 μl/mouse. For i.t. administration, a 29-gauge needle connected to a Hamilton microsyringe was inserted directly between L5 and L6, and the drug was administered at a rate of 2 μl/10 s.

2.3. Drugs

Endomorphin-1, -2 and 3-methoxynaltrexone were synthesized in our laboratory. β-Funaltrexamine and naloxonazine were purchased from Research Biochemical International (Natick, MA), and [d-Ala², MePhe³, Gly(ol)⁴]enkephalin (DAMGO) was from Sigma (St. Louis, MO). Endomorphins and DAMGO were dissolved in sterile artificial cerebrospinal fluid (CSF) containing 7.4 g NaCl, 0.19 g KCl, 0.19 g MgCl₂, 0.14 g CaCl₂/1000 ml and 3-methoxynaltrexone (2.5 ng/2 μl) was dissolved in 0.1% Tween in CSF. β-Funaltrexamine (40 mg/kg, s.c.) and naloxonazine (6.91, 10.4, 15.6, 23.3, 35, 39.4, 43.7, 52.5, 65.7 and 78.7 mg/kg, s.c.) were dissolved in saline and injected s.c. in a volume of 0.1 ml/10 g body weight 24 h before testing. Under these conditions, β-funaltrexamine (40 mg/kg, s.c.) antagonizes both μ₁- and μ₂-mediated antinociception, and naloxonazine’s actions of s.c. 35 mg/kg are relatively selective for μ₁-receptors [13]. 3-Methoxynaltrexone (0.125, 0.1875, 0.25, 0.375, 0.5, 0.75 and 1.0 mg/kg, s.c.) was dissolved in 0.1% Tween-80 solution in saline and administered in a volume of 0.1 ml/10 g body weight. 3-Methoxynaltrexone antagonizes heroin-induced antinociception at doses (2.5 ng i.t. or 0.25 mg/kg s.c.) that are inactive against morphine- and DAMGO-induced antinociception [2,34].

2.4. Assessment of nociceptive threshold

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 use of 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 hind paw. The reaction time to remove the paw from the source of noxious radiant heat was measured. The intensity of the light beam was adjusted so that baseline reaction time was 2–4 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. The average threshold of the control response before injections of compounds was determined by two consecutive measurements each separated by 10 min. No animal was used more than once. To prevent experimenter bias, observers were uninformed of the dose of the endomorphins, DAMGO and 3-methoxynaltrexone being injected, and were uninformed of whether 3-methoxynaltrexone, naloxonazine or β-funaltrexamine was given as a pretreatment. After determination of pre-drug values, animals were injected. Antinociceptive activity for each animal was calculated with the following equation and represented as percent of maximum possible effect (% MPE)= [(P2−P1)/(10−P1)]×100, where P1 and P2 are pre-drug and post-drug response times (in seconds), respectively.
2.5. Data analysis and statistics

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$_{50}$ values and their 95% confidence limits (95% CL) for the antinociceptive effect of the endomorphins and DAMGO were computed according to the method of Litchfield and Wilcoxon [14] using Programs 11 and 47 of the Pharmacological calculations system of Tallarida and Murray [37].

3. Results

3.1. Potency and time course of i.t. injection of endomorphin-1 and -2

The time course of antinociceptive activity for i.t. endomorphin-1, -2 and DAMGO is shown in Fig. 1. Groups of 10 mice were tested for antinociception at 1, 5, 10, 15, 20, 30 and 45 min. Endomorphin-1 and -2 at 1 min post-injection produced dose-dependent antinociception with ED$_{50}$ values of 0.14 (0.06–0.32) and 0.24 (0.12–0.49) nmol, respectively (figure not shown).

Endomorphin-1 and -2 after 5 min of i.t. injection induced antinociception with ED$_{50}$ values of 1.9 (0.59–6.11) and 2.6 (1.51–4.47) nmol, respectively (Fig. 1A,B). The two peptides had equal potency as seen from ED$_{50}$ values. The ED$_{50}$ value for i.t. DAMGO was 14.0 (8.52–22.99) pmol at the 5–10 min peak time of antinociception (Fig. 1C).

3.2. Antagonistic effects of s.c. β-funaltrexamine and naloxonazine on antinociception induced by endomorphins

Groups of 10 mice received either β-funaltrexamine (40 mg/kg, s.c.) or naloxonazine (35 mg/kg, s.c.) 24 h before i.t. injection of endomorphins or DAMGO. Pretreatment of β-funaltrexamine markedly decreased the antinociceptive responses in both endomorphins and DAMGO treatment groups compared with non-pretreatment groups, confirming the involvement of mu-opioid receptors in the response of the three opioid peptides. Endomorphin-1- and DAMGO-induced antinociception were insensitive to naloxonazine (35 mg/kg, s.c.), whereas endomorphin-2 responses were completely antagonized by a lower dose (23.3 mg/kg, s.c.) of the mu$_1$-opioid receptor antagonist. Different dose responses for naloxonazine against a fixed dose of endomorphins (5 nmol) or DAMGO (20 pmol) confirmed that the antinociception induced by endomorphin-2 was more sensitive to the antagonist than that induced by endomorphin-1 or DAMGO (Fig. 2). ID$_{50}$ values for s.c. naloxonazine on endomorphin-1-, -2- and DAMGO-induced antinociception were 48 (39.91–57.73) mg/kg, 12 (9.65–14.93) mg/kg and 60 (51.58–69.79) mg/kg, respectively.

3.3. Effects of pretreatment with i.t. injected naloxonazine on spinal endomorphin-induced antinociception

The antagonistic effects of naloxonazine on antinociception induced by equipotent doses of i.t. endomorphins and DAMGO were examined. Groups of mice received graded doses of naloxonazine 24 h before i.t. injection of endomorphins or DAMGO. Naloxonazine at a dose of 4 µg significantly antagonized the antinociceptive activity produced by i.t. endomorphin-2 without affecting the an-
Effects of naloxonazine and β-funaltrexamine on i.t. endomorphin-1-, endomorphin-2- and DAMGO-induced antinociception in the mouse paw-withdrawal test. Graded doses of naloxonazine (NLZ) and β-funaltrexamine (β-FNA, 40 mg/kg) were administered s.c. 24 h before i.t. administration of endomorphin-1, endomorphin-2 and DAMGO. Antinociceptive effects were measured 5 min after i.t. administration of the endomorphins or 10 min after i.t. DAMGO. Each column represents the mean S.E.M. for 10 mice. **P<0.01 compared to each agonist alone.

3.4. Inhibition of endomorphin-induced antinociception by 3-methoxynaltrexone

The selective morphine-6-glucuronide antagonist, 3-methoxynaltrexone (0.25 mg/kg, s.c.), which blocked endomorphin-2-antinociception at a dose that was inactive against DAMGO did not affect endomorphin-1-induced antinociception (Fig. 4A). Pretreatment with 0.25 mg/kg of 3-methoxynaltrexone markedly decreased 60% of the antinociceptive response to i.t. endomorphin-2 without influencing endomorphin-1- and DAMGO-induced antinociception (Fig. 4A).

At 5 and 10 min after injection, the response to i.t. endomorphin-2 was markedly decreased by coadministration of 2.5 ng of 3-methoxynaltrexone (Fig. 4C). The ED₅₀ value of endomorphin-2 after 5 min of i.t. coadministration was 7.2 (4.25–12.20) nmol. The dose–response curve of endomorphin-2 shifted 3-fold to the right (Fig. 4C). However, 3-methoxynaltrexone (2.5 ng) in combination with endomorphin-1 and DAMGO was ineffective at attenuating the responses to i.t. administration of both endomorphin-1 and DAMGO.

4. Discussion

Both endomorphin-1 and -2 induced dose-dependent antinociception after spinal administration in the paw-withdrawal test. The peak effects of endomorphins-induced antinociception occurred rapidly, within 1 min of the i.t. injection, and disappeared at 10–15 min after injection. The present results of i.t. injected endomorphins are in agreement with those of Stone et al. [36] and Sakurada et al. [31] who reported that the antinociceptive effect of the endomorphins is short-lasting and is absent 15 min following i.t. injection as assayed by the tail-lick test and the tail-pressure test. Endomorphins are 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 [35].

We used a variety of doses of i.t. and s.c. naloxonazine and 3-methoxynaltrexone to determine sensitivity to antagonists of mu opioid subtypes involved in the antinociceptive responses to the endomorphins and DAMGO. β-Funaltrexamine irreversibly antagonizes both μ₁- and μ₂-opioid receptors [27] and inhibits both supraspinal and spinal antinociception, whereas naloxonazine selectively antagonizes μ₁-opioid receptors and inhibits sup-
Fig. 3. Effects of naloxonazine on endomorphin-1, endomorphin-2 and DAMGO-induced antinociception in the mouse Paw withdrawal test. Groups of mice received the indicated dose of naloxonazine in combination with endomorphin-1 (5 nmol, i.t.), endomorphin-2 (5 nmol, i.t.) and DAMGO (20 pmol, i.t.). Graded doses of naloxonazine (NLZ, white column) were administered i.t. 24 h before i.t. administration of the three peptides. **P<0.01 compared to each agonist alone.

It should be noted that a reasonable naloxonazine dose to obtain a relative μ₁ selectivity in mice would be 35 mg/kg (s.c.) [13]. Antinociception of endomorphin-1 and -2 (0.078–0.625 nmol) i.t. administered 1 min after injection was not significantly antagonized by pretreatment with naloxonazine (unpublished), while the antinociceptive activity of endomorphin-2 after 5 min of i.t. injection was completely blocked by pretreatment with a reasonable dose of 35 mg/kg s.c. naloxonazine to obtain a relative μ₁ selectivity in the paw-withdrawal test. Moreover, endomorphin-2 was significantly antagonized by pretreatment with a low dose of naloxonazine (10.37 mg/kg s.c.), an irreversible μ₁-opioid receptor antagonist, suggesting that endomorphin-2 may be a highly selective agonist at the μ₁-opioid receptor subtype of μ-opioid subtypes receptors. Pretreatment with naloxonazine at each dose of 35 or 43.8 mg/kg s.c. did not block the antinociceptive response of DAMGO, a μ-opioid agonist, to thermal stimulus of the paw-withdrawal test after i.t. injection, whereas higher doses of naloxonazine (52.5, 65.6 or 78.8
mg/kg s.c.) significantly attenuated DAMGO-induced antinociception (Fig. 2), indicating that at high doses naloxonazine loses much of its selectivity mu opioid receptors.

Intrathecal naloxonazine did not block the antinociceptive effects of DAMGO and endomorphin-1 at a dose of 4 mg which significantly antagonized endorphin-2-induced antinociception. DAMGO and endomorphin-1 are extremely insensitive to antagonism by pretreatment with i.t. naloxonazine judging from the ID values of naloxonazine. These results suggest that a reasonable dose of i.t. administered naloxonazine to get a relative mu selectivity in mice would be 4 μg/mouse.

As seen from the ID values of naloxonazine, endomorphin-2 was relatively sensitive to naloxonazine whereas endomorphin-1 was relatively insensitive to antagonism by both i.t. and s.c. pretreatment with naloxonazine. This indicates that endomorphin-1 can act as a predominantly mu-opioid receptor agonist and endomorphin-2 as a mu-opioid receptor agonist in the current test paradigm. As in our previous report with the tail-pressure test, naloxonazine (35 mg/kg s.c.) did not completely abolish the action of i.t. endomorphin-2. The reason for this difference may be related to different nociceptive assays.

An alternative explanation for the differential effectiveness of the mu antagonists against the various agonists is that endomorphin-2 has lower efficacy than endomorphin-1 or DAMGO. Irreversible antagonists have long been used to estimate efficacy [3], and lower efficacy agonists are more susceptible than higher efficacy agonists to a reduction in maximal effects by irreversible antagonists [12]. Thus, the β-funaltrexamine and naloxonazine results could reflect different receptor subtypes or different efficacies. The lack of a selective irreversible antagonist for subtypes other than the mu receptor limits the ability to distinguish between these possibilities. Studies with GTP-γ-S binding assays, however, have failed to detect significant differences in efficacy between endomorphin-1 and endomorphin-2 [8,10].

Reversible antagonists, by contrast, have provided evidence that different agonists act through different receptors. Evidence for the presence of separate mu and delta receptors in the vas deferens, for example, included the demonstration that naloxone antagonized the effects of morphine more potently than those of enkephalin [15]. In the present study, endomorphin-2 was more susceptible than DAMGO and endorphin-1 to antagonism by the reversible antagonist, 3-methoxynaltrexone. Indeed, as shown in Fig. 4C, 2.5 ng of the antagonist produced a >3-fold shift in the dose–response curve for endomorphin-2, indicating that this dose is well above the Ke for endomorphin-2 (the dose of antagonist that requires a doubling of the agonist for the same effect). By contrast, the same dose had no effect on the endomorphin-1 dose–response curve. These results support the idea that endomorphin-2 acts through a site distinct from endomorphin-1 and DAMGO. The discovery that the antinociception induced by morphine-6β-glucuronide, heroin, and 6-acetylmorphine (a heroin metabolite) can be antagonized by 3-methoxynaltrexone at doses which are inactive against morphine has proven to be a means to distinguish the different antinociceptive mechanisms of alkaloids within the mu-opioid receptor agonist family [2,39]. The present study is the first to show that 3-methoxynaltrexone can also distinguish the actions of different peptide mu agonists. 3-methoxynaltrexone selectively blocked the antinociception of endomorphin-2 far more effectively than that of endorphin-1 or DAMGO. Thus, based on antagonism by 3-methoxynaltrexone, endomorphin-2, but not endorphin-1 or DAMGO, has behavioral and pharmacological similarity to morphine-6β-glucuronide and heroin.

A single gene encoding the mouse mu-opioid receptor (MOR-1) has been cloned [28,38] and seven splice variants have been identified [1,21,22,42]. Morphine produces antinociception by activating mu-opioid receptors encoded by the MOR-1 gene. In antisense mapping studies [29,30], the antisense oligodeoxynucleotides targeting exon 1 of MOR-1 effectively reduce the antinociceptive effect produced by morphine without affecting the action of morphine-6β-glucuronide, an extremely potent mu-opioid receptor agonist. By contrast, antisense probes targeting exons 2 and 3 reduce the antinociceptive effect produced by morphine-6β-glucuronide, but not that by morphine even though both opioids belong to the mu-opioid family. Indeed, i.c.v. morphine-6β-glucuronide produces antinociceptive effects in a MOR-1 exon 1 knockout mouse in which morphine and DAMGO are inactive [34].

More recently, five new MOR-1 exons (exon 6, 7, 8, 9 and 10) have been identified, and antisense mapping of these exons revealed that they were all involved in morphine antinociception without altering antinociception of morphine-6β-glucuronide [21,22]. Thus, the antisense experiments support the idea of distinct sites, but the newly discovered exons are not a component of the receptor responsible for morphine-6β-glucuronide antinociception [21,22]. Both endorphin-1 and endorphin-2 bind with high affinity to each of the new splice variants of mu receptor [21,22].

The original division of mu- and mu-receptor subtypes was characterized primarily on the basis of pharmacological studies using naloxonazine. Similarly, the more recent use of 3-methoxynaltrexone revealed binding characteristics of morphine-6β-glucuronide and heroin that were distinct from morphine, suggesting a possible novel subtype. The currently known splice variants of the mu receptor, however, do not appear to account for the different subtypes or binding states reflected by sensitivity to naloxonazine or 3-methoxynaltrexone. Thus, additional isoforms or alternative physical states of the mu receptor may yet be discovered that account for the distinct patterns
of antagonism shown by these compounds. Regardless, the present results demonstrate that endomorphin-2 interacts with mu receptors in a manner that is similar to morphine-6β-glucuronide and pharmacologically distinguishable from morphine as well as other peptides, including DAMGO and the closely related peptide endomorphin-1. Thus, the endomorphins can serve a unique role in understanding the complex agonist–receptor interactions at the mu receptor and its potential isoforms, subtypes, and physical states.

References


