Differential Antinociception Induced by Spinally Administered Endomorphin-1 and Endomorphin-2 in the Mouse

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ABSTRACT

We have previously demonstrated that the antinociception induced by either endomorphin-1 or endomorphin-2 given supraspinally is mediated by the stimulation of μ-opioid receptors. However, the antinociception induced by endomorphin-2 given supraspinally contains additional components, which are mediated by the spinal release of dynorphin A (1–17) acting on κ-opioid receptors and the spinal release of [Met5]enkephalin acting on δ-opioid receptors in the spinal cord. The present studies were performed to determine whether there are any differential effects on the tail-flick inhibition induced by endomorphin-1 and endomorphin-2 given intrathecally (i.t.) in mice. Endomorphin-1 or endomorphin-2 given i.t. inhibited the tail-flick response in a dose-dependent manner. The tail-flick inhibition induced by endomorphin-1 was blocked by i.t. pretreatment with μ-opioid receptor antagonist d-Phe-Cys-Tyr-d-Try-Orn-Thr-Pen-Thr-NH₂ (CTOP), but not κ-opioid receptor antagonist nor-binaltorphimine (nor-BNI), δ-opioid receptor antagonist 7-benzylidene naltrexamine (BNTX), or δ₂-opioid receptor antagonist naltriben (NTB). In contrast, the tail-flick inhibition induced by endomorphin-2 given i.t. was blocked by i.t. pretreatment with CTOP or nor-BNI, but not BNTX or NTB. Intrathecal pretreatment with antisera against dynorphin A (1–17), but not antisera against [Met5]enkephalin, [Leu5]enkephalin, or β-endorphin, blocked the tail-flick inhibition induced by i.t.-administered endomorphin-2. None of these antisera attenuated the i.t.-administered endomorphin-1-induced tail-flick inhibition. It is concluded that the tail-flick inhibition induced by endomorphin-1 and endomorphin-2 given spinally is mediated by the stimulation of μ-opioid receptors. However, the tail-flick inhibition induced by spinally injected endomorphin-2 contains an additional component, which is mediated by the spinal release of dynorphin A (1–17) acting on κ-opioid receptors in the spinal cord. We propose that there are at least two different subtypes of μ-opioid receptors for endomorphin-1 and endomorphin-2 to produce antinociception in the spinal cord.

Two new peptides, endomorphin-1 and endomorphin-2, have been recently isolated from mammalian brain. These two peptides activate μ-opioid receptors with high affinity and selectivity, raising the possibility that they are two endogenous μ-opioid receptor ligands (Zadina et al., 1997). In receptor binding assays, both endomorphin-1 and endomorphin-2 bind to opioid μ₁- and μ₂-receptor sites potently (Goldberg et al., 1998). Neither compound has appreciable affinities for opioid δ- and κ₁ receptors. Endomorphins were found in the regions of the brain and spinal cord, which are also rich in μ-opioid receptors (Martin-Schild et al., 1997, 1998; Zadina et al., 1997; Pierce et al., 1998; Schreff et al., 1998). Endomorphin-1 is more widely and more densely distributed throughout the brain than endomorphin-2, whereas endomorphin-2 is more prevalent in the spinal cord than endomorphin-1. The greatest density of endomorphin-2 fibers is located in superficial laminae of the spinal dorsal horn and the nucleus of the spinal trigeminal tract (Martin-Schild et al., 1999). Since endomorphin-2 immunoreactivity is diminished by the dorsal rhizotomy and colocalized with calcitonin gene-related peptide or Substance P, it seems likely that endomorphin-2 is present in the primary sensory afferent neurons (Martin-Schild et al., 1997, 1998; Pierce et al., 1998).

In behavioral experiments, intrathecal (i.t.) or i.c.v. injection of endomorphin-1 or endomorphin-2 produces potent analgesia, which is blocked by the pretreatment with the μ-opioid receptor antagonists nalofoxone or β-funaltrexamine (Stone et al., 1997; Tseng et al., 2000a). In the μ-opioid receptor knockout mice or μ-opioid receptor-deficient CXBK mice, neither endomorphin-1 nor endomorphin-2 produce any significant antinociceptive effects (Tseng et al., 1998; Mizoguchi et al., 1999). In [35S]guanosine-5'-O-(3-thio) triphosphate binding assay, neither endomorphin-1 nor en-
endomorphin-2 produce any activation of G protein in the spinal cord (Narita et al., 1998) and in the pons/medulla (Mizoguchi et al., 1999) membrane obtained from the μ-opioid receptor knockout mice. These findings strongly indicate that the μ-opioid receptors play an essential role in mediating endomorphin-1- and endomorphin-2-induced antinociception and G protein activation.

We have previously demonstrated that, like morphine or [d-Ala2,N-Me-Phe4,Gly-ol1]-enkephalin, both endomorphin-1 and endomorphin-2 given supraspinally produce their antinociception by the stimulation of μ-opioid receptors, because these antinociceptive effects induced by endomorphin-1 and endomorphin-2 given i.c.v. are blocked by the i.c.v. pretreatment with μ-opioid receptor antagonist β-funaltrexamine. In addition, blockade of α2-adrenoceptors and 5-HT receptors in the spinal cord by i.t. injection of yohimbine and methysergide, respectively, blocks effectively the tail-flick inhibition induced by i.c.v.-administered endomorphin-1 and endomorphin-2. However, the antinociception induced by endomorphin-2 given supraspinally contains additional components, which are mediated by the spinal release of dynorphin A (1–17) acting on κ-opioid receptors and the spinal release of [Met5]enkephalin acting on δ-opioid receptors in the spinal cord (Ohsawa et al., 2000). This is evidenced by the finding that the tail-flick inhibition induced by i.c.v.-administered endomorphin-2, but not endomorphin-1 is blocked by i.t. pretreatment with antiserum against dynorphin A (1–17) or [Met5]enkephalin or opioid κ- and δ-receptor antagonist nor-binaltorphimine (nor-BNI) and naltriben (NTB), respectively (Ohsawa et al., 2000). Present studies were then designed to determine whether there are also any differential antinociceptive effects of endomorphin-1 and endomorphin-2 given spinally.

Materials and Methods

Animals. Male ICR mice weighing 25 to 30 g (Charles River Breeding Laboratories, Wilmington, MA) were used for the studies. All experiments were approved by and conformed to the guidelines of the Medical College of Wisconsin Animal Care Committee. Animals were housed five per cage in a room maintained at 22 ± 0.5°C with an alternating 12-h light/dark cycle. Food and water were available ad libitum. Animals were used only once in all experiments.

Drugs and Antisera. Endomorphin-1 (Tyr-Pro-Arg-Phe-NH2), endomorphin-2 (Tyr-Pro-Phe-Phe-NH2), Zadina et al., 1997), NTB (Portoghese et al., 1992), 7-benzylidene naltrexamine (BNTX, Portoghese, 1991), and nor-BNI were synthesized in H. Nagase’s laboratory (Pharmaceutical Research Laboratories, Kamakura, Japan). Another drug used was b-Phe-Cys-Try-b-Try-Omn-Thr-Phe-Thr-NH2 (CTOP; Peninsula Laboratory International, Belmont, CA). The antisera against dynorphin A (1–17), [Met5]enkephalin, [Leu5]enkephalin, and β-endorphin were produced by immunization of male New Zealand White rabbits according to the method described previously and the potencies and the cross-immunoreactivities of these antisera have been characterized (Tseng and Collins, 1993; Tseng et al., 2000a).

Assessment of Antinociceptive Response. Antinociceptive response was determined with the tail-flick test (D’Amour and Smith, 1941). For the measurement of the latency of the tail-flick response, mice were gently held with one hand with the tail positioned in the apparatus (Model TF6; EMDEV Instrument Co., Maidens, VA) for radiant heat stimulation. The tail-flick response was elicited by applying radiant heat to the dorsal surface of the tail. The intensity of the heat stimulus was adjusted so that the animal flicked its tail within 3 to 5 s. The latency of the tail-flick response was measured before (T0) and at various times after (T1) i.t. injections of endomorphins. The inhibition of the tail-flick response by endomorphins was expressed as a percentage of the maximum possible effect, which was calculated as [(T1 – T0)/(T2 – T0)] × 100, where the cut-off time, T2, was set at 10 s for the tail-flick response.

Intrathecal Injection. Intrathecal injection was made according to the procedure of Hylden and Wilcox (1980) using a 25-guage needle. Injection volume was 5 μl. Mice were pretreated i.t. with the selective opioid receptor antagonists nor-BNI and NTB 24 h prior to, or CTOP, BNTX, or NTB 10 min prior to i.t. challenge with endomorphins. The doses of the receptor antagonists used in the present study were determined based on the information obtained from the previous studies that these doses of antagonists are sufficient to completely block the antinociception induced by respective selective opioid receptor agonists (Tseng et al., 1997). Antiserum against dynorphin A (1–17), [Met5]enkephalin, [Leu5]enkephalin, or β-endorphin was given 60 min before i.t. administration of the endomorphins (Ohsawa et al., 2000).

Statistical Analysis. The data are expressed as the mean with S.E.M. Comparisons of data were made with a one-way analysis variance following by the Student’s t test (comparisons between two groups) and Bonferroni/Dunn probability test (comparisons between two groups for the positive response rate).

Results

Tail-Flick Response to i.t. Administration of Endomorphin-1 and Endomorphin-2. Groups of mice were injected i.t. with different doses of endomorphin-1 or endomorphin-2, and the tail-flick response was measured 5, 10, 15, and 20 min after injection. Intrathecal injection of endomorphin-1 or endomorphin-2 dose dependently caused an increase of the inhibition of the tail-flick response. The inhibition reached its peaks 5 min after injection, rapidly declined, and returned to the preinjection level 20 min after injection (Fig. 1). The duration of the tail-flick inhibition induced by endomorphin-1 and endomorphin-2 was about the same. The dose-response curves of the tail-flick inhibition induced by i.t.-administered endomorphin-1 and endomorphin-2 observed at 5 min after i.t. injection are shown in Fig. 2. The ED50 values (95% confidence limit) for endomorphin-1 and endorphin-2 for the tail-flick inhibition were estimated to be 1.71 (1.39–2.11) and 3.58 (2.96–4.33), respectively.

Effects of i.t. Pretreatment with CTOP, nor-BNI, BNTX, or NTB on Tail-Flick Inhibition Induced by i.t.-Administered Endomorphin-1 and Endomorphin-2. The tail-flick inhibition induced by the i.t.-administered endomorphin-1 (10 μg) or endomorphin-2 (10 μg) was significantly attenuated by i.t. pretreatment with 50 ng of CTOP. A higher dose of CTOP (150 ng) almost completely blocked the tail-flick inhibition induced by endomorphin-1 or endomorphin-2 given i.t. Intrathecal pretreatment with nor-BNI (3–30 μg) dose dependently attenuated the inhibition of the tail-flick response induced by i.t.-administered endomorphin-2 (10 μg). However, nor-BNI even at a high dose of 30 μg only partially blocked the endomorphin-2-induced tail-flick inhibition (Fig. 4). On the other hand, the same i.t. pretreatment with nor-BNI (10 and 30 μg) did not significantly affect the tail-flick inhibition induced by i.t.-administered endomorphin-1 (10 μg) (Fig. 3). Intrathecal pretreatment with BNTX (1 μg) or NTB (3 μg) did not block the inhibition of the tail-flick response induced by either endomorphin-1 or endomorphin-2 (10 μg) given i.t. (Figs. 3 and 4). The doses of the antagonists used have been previously reported to com-
pletely block the antinociception induced by respective selective opioid agonists (Tseng et al., 1997).

As shown in Fig. 5A, i.t. pretreatment with nor-BNI (10 μg) did not affect the inhibition of the tail-flick response induced by various doses of endomorphin-1 given i.t. and caused no change of the dose-response curve for the endomorphin-1-induced tail-flick inhibition. However, the same treatment with nor-BNI (10 μg) significantly attenuated the tail-flick inhibition induced by endomorphin-2 given i.t. and caused the shift of the dose-response curve for i.t.-injected endomorphin-2-induced tail-flick inhibition to the right by 2.29-fold (Fig. 5B).

Effects of i.t. Pretreatment with Antiserum against Dynorphin A (1–17), [Met⁵]enkephalin, [Leu⁶]enkephalin, or β-Endorphin on Tail-Flick Inhibition Induced by i.t.-Administered Endomorphin-1 and Endomorphin-2. Dynorphin A (1–17) has been proposed to be the endogenous opioid ligand for κ-opioid receptors. The finding that antinociception induced by endomorphin-2 was blocked by the κ-opioid receptor antagonist nor-BNI suggests that endomorphin-2 may release dynorphins, which subsequently act on κ-opioid receptor to produce antinociception. The effect of i.t. pretreatment with an antisera against dynorphin A (1–17), [Met⁵]enkephalin, [Leu⁶]enkephalin, or β-endorphin on the tail-flick inhibition induced by endomorphin-1 and endomorphin-2 were studied. Intrathecal pretreatment with an antiserum against dynorphin A (1–17) (10–300 μg) dose dependently attenuated the tail-flick inhibition induced by endomorphin-2 (10 μg) (Fig. 7). However, dynorphin A (1–17) antiserum even at a high dose of 300 μg only partially, but significantly, attenuated the endomorphin-2-induced tail-flick inhibition. In contrast, the same i.t. pretreatment with antiserum against dynorphin A (1–17) (100 and 300 μg) did not affect the tail-flick inhibition induced by endomorphin-1 (10 μg) given i.t. (Fig. 6). The tail-flick inhibition induced by endomorphin-1 (10 μg) or endomorphin-2 (10 μg) given i.t. was not affected by i.t. pretreatment with an antiserum against [Met⁵]enkephalin, [Leu⁶]enkephalin, or β-endorphin (Figs. 6 and 7).

Discussion

We have previously demonstrated that the inhibition of the tail-flick response induced by either endomorphin-1 or endomorphin-2 given i.c.v. is blocked by i.c.v. pretreatment with a selective μ-opioid receptor antagonist β-funaltrexamine, indicating that the antinociception induced by endomorphin-1 and endomorphin-2 given supraspinally is mediated selectively by the stimulation of μ-opioid receptors (Tseng et al., 2000a). Also, blockade of α₂-adrenoceptors and 5-HT receptors in the spinal cord by i.t. pretreatment with yohimbine and methysergide, respectively, attenuates the antinociception induced by endomorphin-1 or endomorphin-2, indicating that these two peptides given supraspinally release noradrenaline and 5-HT in the spinal cord for the production of antinociception. In the present study, antinociception induced by either endomorphin-1 or endomorphin-2 injected intrathecally was completely blocked by i.t. pretreatment with CTOP. This finding indicates that the spinally administered endomorphin-1- or endomorphin-2-induced antinociception is also mediated by the stimulation of μ-opioid recep-

Fig. 2. Dose-response curves for the inhibition of the tail-flick response induced by i.t. injection of endomorphin-1 and endomorphin-2. Groups of mice were administered an i.t. injection of different doses of endomorphin-1 or endomorphin-2, and the tail-flick responses were measured 5 min after the injection. Each value represents the mean ± S.E. for 10 mice.
tors in the spinal cord. Earlier, Stone et al. (1997) reported that endomorphin-1 or endomorphin-2 given i.t. dose dependently produces antinociception, which is blocked by naloxone given i.t., in the tail-flick test. The importance of μ-opioid receptors for endomorphin-1 and endomorphin-2 to produce antinociception is also supported by our previous studies. Both endomorphin-1 and endomorphin-2 do not activate G proteins in the spinal cord and pons/medulla tissues of the μ-opioid receptor knockout mice (Narita et al., 1998; Mizoguchi et al., 1999) and both peptides given i.c.v. fail to produce any antinociception in μ-opioid receptor knockout mice (Mizoguchi et al., 1999).

However, the antinociception induced by endomorphin-2, but not endomorphin-1, contains an additional component, which is mediated by the stimulation of κ-opioid receptors at the supraspinal and spinal sites. This is evidenced by the findings in our previous studies that the antinociception induced by i.c.v.-administered endomorphin-2, but not endomorphin-1, is blocked by the i.c.v. or i.t. pretreatment with κ-opioid receptor antagonist nor-BNI (Ohsawa et al., 2000; Tseng et al., 2000a). We found in the present study that antinociception induced by endomorphin-2, but not endomorphin-1 given i.t. was also attenuated by i.t. pretreatment.

![Figure 3](image3.png)

**Fig. 3.** Effects of the blockade of μ-, δ-, δ₂-, and κ-opioid receptors by i.t. pretreatment with CTOP, BNTX, NTB, and nor-BNI, respectively, on the inhibition of the tail-flick responses induced by i.t. injection of endomorphin-1. Groups of mice were administered an i.t. injection of nor-BNI (10 and 30 μg) 24 h or CTOP (50 and 150 ng), BNTX (1 μg), or NTB (3 μg) 10 min before i.t. challenge with endomorphin-1 (10 μg). The tail-flick responses were measured 5 min after the injection of endomorphin-1. Each column represents the mean ± S.E. for 10 mice. *P < 0.05; **P < 0.01 compared with the saline-injected mice.

![Figure 4](image4.png)

**Fig. 4.** Effects of the blockade of μ-, δ-, δ₂-, and κ-opioid receptors by i.t. pretreatment with CTOP, BNTX, NTB, and nor-BNI, respectively, on the inhibition of the tail-flick responses induced by i.t. injection of endomorphin-2. Groups of mice were administered an i.t. injection of nor-BNI (0.1–30 μg) 24 h or CTOP (50 and 150 ng), BNTX (1 μg), or NTB (3 μg) 10 min before i.t. challenge with endomorphin-2 (10 μg). The tail-flick responses were measured 5 min after the injection of endomorphin-2. Each column represents the mean ± S.E. for 10 mice. *P < 0.05; **P < 0.01 compared with the saline-injected mice.

![Figure 5](image5.png)

**Fig. 5.** Effects of i.t. pretreatment with 10 μg of nor-BNI on the dose-response curve for the inhibition of the tail-flick responses induced by i.t. injection of endomorphin-1 (A) and endomorphin-2 (B) in the mouse. Groups of mice were administered an i.t. injection of nor-BNI (10 μg) 24 h before i.t. challenge with endomorphin-1 or endomorphin-2. The tail-flick responses were measured 5 min after the injection of endomorphin-1 and endomorphin-2. Each point represents the mean ± S.E. for 10 mice. The potency ratio (95% confidence limit) of i.t.-injected endomorphin-1- and endomorphin-2-induced antinociception in saline-treated group versus nor-BNI-treated group was 1.01 (0.41–2.52) and 2.29 (1.74–3.11), respectively.
Dynorphin A (1–17) has been proposed to be a neurotransmitter for κ-opioid receptors. The possibility that i.t.-administered endorphin-2-induced antinociception is mediated by the spinal release of dynorphin A (1–17), which subsequently stimulates κ-opioid receptors for producing antinociception, was then explored. It was found that i.t. pretreatment with an antiserum against dynorphin A (1–17) attenuated the antinociception induced by i.t.-injected endorphin-2, but not endorphin-1. The results of the present study are also consistent with our previous finding that the antinociception induced by endorphin-2 given i.c.v. was also attenuated by i.c.v. pretreatment with antiserum against dynorphin A (1–17) (Tseng et al., 2000a), indicating that antinociception induced by endorphin-2 given either supraspinally or spinally is mediated by the same dynorphinergic mechanism. Thus, activation of μ-opioid receptors by endorphin-2 initially induces the release of dynorphin A (1–17), which subsequently acts on κ-opioid receptors for the production of antinociception. We propose that there are two subtypes of μ-opioid receptors that are involved in endorphin-1- and endorphin-2-induced antinociception. One subtype of μ-opioid receptors is stimulated by both endorphin-1 and endorphin-2 and another subtype of μ-opioid is solely stimulated by endorphin-2 and is involved in the release of dynorphin A (1–17) acting on κ-opioid receptors for the production of antinociception.

The antinociception induced by i.c.v.-injected endorphin-2 also contains another component, which is mediated by the spinal release of [Met6]enkephalin acting on δ-opioid receptors in the spinal cord. This view is supported by the finding that i.t. pretreatment with antisera against [Met6]enkephalin or δ-opioid receptor antagonist NTB attenuated the tail-flick inhibition induced by endorphin-2 given i.c.v. (Ohsawa et al., 2000). However, we found in the present study that i.t. pretreatment with antiserum against [Met6]enkephalin or NTB failed to affect the tail-flick inhibition induced by endorphin-2 given i.t., indicating that antinociception induced by endorphin-2 and δ-opioid receptors in the spinal cord are not involved in spinally administered endorphin-2-induced antinociception.

Others from different laboratories also reported the different antinociceptive effects induced by endorphin-1 and endorphin-2. Systemic pretreatment with µ-opioid receptor antagonist naloxonazine attenuates the antinociceptive induced by endorphin-2, but not endorphin-1 given i.t. or i.c.v., suggesting that antinociception induced by endorphin-2, but not endorphin-1 is mediated by the stim-
ulation of naloxonazine-sensitive \( \mu \)-opioid receptors (Sakurada et al., 1999, 2000). Pretreatment with different antisense oligodeoxynucleotides (ODNs) against different G protein subunits was also able to differentiate antinociceptive effects induced by endorphin-1 and endorphin-2. Intrathecal pretreatment with antisense ODN against G protein subunit \( \mathrm{G}_{\alpha_{\text{o}}/\text{i}} \) protein attenuates the antinociception induced by i.t.-administered endorphin-2, but not endorphin-1, while i.t. pretreatment with antisense ODN against G protein subunits of \( \mathrm{G}_{\alpha_{1}, \text{o}_2} \), or \( \mathrm{G}_{\text{a}_{3}, \text{o}_2} \) does not affect the antinociception induced by either endorphin-1 or endorphin-2 (Sánchez-Blázquez et al., 1999). It is most likely that the differential antinociceptive effects observed are mediated by the stimulation of different subtypes of \( \mu \)-opioid receptors.

In conclusion, the antinociception induced by both endorphin-1 and endorphin-2 given spinally is mediated by the stimulation of \( \mu \)-opioid receptors in the spinal cord. However, endorphin-2-induced antinociception also contains an additional component, which is mediated by the release of dynorphin A (1–17) acting on \( \delta \)-opioid receptors in the spinal cord. It is most likely that different subtypes of \( \mu \)-opioid receptors are involved in endorphin-1- and endorphin-2-induced antinociception in the spinal cord.

References


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