Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 504 (2004) 177–183

www.elsevier.com/locate/ejphar

Increased release of immunoreactive dynorphin A_{1-17} from the spinal cord after intrathecal treatment with endomorphin-2 in anesthetized rats

Randy J. Leitermann, Maia Terashvili, Hirokazu Mizoguchi, Hsiang-En Wu, Franklin Chen, Andrew Clithero, Leon F. Tseng*

Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI 53226, USA

Received 22 July 2004; received in revised form 30 September 2004; accepted 4 October 2004
Available online 28 October 2004

Abstract

We previously demonstrated pretreatment with antiserum against dynorphin A_{1-17} attenuates endomorphin-2-induced analgesia and antianalgesia, suggesting that these endomorphin-2 effects are mediated by the release of dynorphin A_{1-17}. Lumbar–cisternal spinal perfusion was used to measure the release of immunoreactive dynorphin A_{1-17} into spinal perfusates from urethane-anesthetized rats following endomorphin-2 or endomorphin-1 treatment within the perfusion solution. Treatment with endomorphin-2 (5–50 nmol) for 3 min caused a dose-dependent increase of immunoreactive dynorphin A_{1-17} in spinal perfusates, with a maximal increase detected between 24 and 48 min after endomorphin-2 treatment, while levels returned to baseline within 60 min. Endomorphin-2-induced release of immunoreactive dynorphin A_{1-17} was attenuated by pretreatment with μ-opioid receptor antagonist naloxone or 3-methoxynaltrexone. Endomorphin-1 induced a slight increase in immunoreactive dynorphin$_{1-17}$ as well, but only at the highest dose used (50 nmol). Our results suggest that endomorphin-2 stimulated a specific subtype of μ-opioid receptor to induce the release of immunoreactive dynorphin A_{1-17} in spinal cords of rats.

Keywords: Endomorphin-2; Dynorphin A_{1-17}; Spinal cord

1. Introduction

The opioid tetrapeptides, endomorphin-1 and endomorphin-2, first isolated from the mammalian brain, both contain high affinity and selectivity for the μ-opioid receptor, suggesting that they are endogenous ligands for this receptor (Zadina et al., 1997). Receptor binding studies show that endomorphin-1 and endomorphin-2 potently compete for the μ-opioid receptor binding site, but not the δ- or κ_1-opioid receptor binding site (Goldberg et al., 1998; Spetea et al., 1998). In addition, autoradiography studies using both endomorphin-1 and endomorphin-2 show a staining pattern similar to that reported for other μ-opioid receptor ligands. In the G-protein binding assay, endomorphin-1 and endomorphin-2 both cause an increase in stimulation of [^{35}S]GTPγS binding in both the mouse spinal cord and brain (Narita et al., 1998, 2000). This increase is blocked by co-incubation with μ-opioid antagonist, β-funaltrexamine or D-Phe-Cys-D-Tyr-Orn-Thr-Pen-Thr-NH_2 (CTOP), but not by δ- or κ-opioid antagonists, naltrindole or nor-binaltorphimine, respectively. In vivo studies using endomorphin-1 or endomorphin-2 correlate closely with in vitro studies. Intrathecal (i.t.) treatment with endomorphin-1 or endomorphin-2 produces potent antinociception, which can be blocked by i.t. pretreatment with μ-opioid antagonist, naloxone or D-Phe-Cys-Tyr-D-Try-Orn-Thr-Pen-Thr-NH_2 (CTOP) (Stone et al., 1997; Ohsawa et al., 2001). These findings strongly indicate that both endomorphin-1 and endomorphin-2 are selective agonist ligands for the μ-opioid receptor.

Although endomorphin-1 and endomorphin-2 are both selective for the μ-opioid receptor, they likely stimulate

* Corresponding author. Tel.: +1 414 456 5686; fax: +1 414 456 6507.
E-mail address: ltseng@mcw.edu (L.F. Tseng).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.10.006

different subtypes of μ-opioid receptor to produce their analgesic effect. A μ_1 specific opioid receptor antagonist, naloxonazine, is more sensitive at blocking the antinociception produced by i.t. or intracerebroventricular administered endomorphin-2 than endomorphin-1, while general μ_1 and μ_2 opioid antagonist, β-funaltrexamine, blocks both endomorphin-1 and endomorphin-2 equally well (Sanchez-Blanquez et al., 1999; Sakurada et al., 2000). Similarly, morphine-6β-glucuronide antagonist, 3-methoxynaltrexone, a novel member of the μ-opioid family, is able to block antinociception produced by i.t. endomorphin-2 at a dose that has no effect on endomorphin-1 (Sakurada et al., 2000). We have previously shown that i.t. treatment with antisense oligodeoxynucleotides against specific exons of the μ-opioid receptor clone leads to a differential loss of antinociception produced by endomorphin-1 and endomorphin-2 (Wu et al., 2002b). Additional evidence supporting different subtypes of receptors is observed when endomorphin-1 and endomorphin-2 analgesic mechanisms are studied. The antinociception induced by endomorphin-2, unlike endomorphin-1 or other μ-opioid receptor agonists, is partially blocked by i.t. pretreatment with antiserum against dynorphin A_{1-17} or κ-opioid receptor antagonist, nor-binaltorphimine, demonstrating an additional component in the endomorphin-2 produced antinociception involving the release of dynorphin A_{1-17} acting on κ-opioid receptors in the spinal cord (Ohsawa et al., 2000, 2001).

Dynorphin A_{1-17} is a unique endogenous opioid peptide that is not only involved in antinociceptive actions, but also contains nociceptive properties (for review, see Smith and Lee, 1988; Millan, 1999; Laughlin et al., 2001). Recently, we have shown that i.t. pretreatment with both analgesic and subanalgesic doses of endomorphin-2 in mice inhibits morphine-induced antinociception through a dynorphin A_{1-17} mediated mechanism (Wu et al., 2003). Pretreatment with antiserum against dynorphin A_{1-17}, to neutralize dynorphin A_{1-17} action, eliminates the inhibitory effect induced by endomorphin-2 against morphine-produced analgesia and reinforces the potency of morphine. This mechanism is termed antianalgesia (Fujimoto and Rady, 1989; Rady et al., 1991). The antianalgesia induced by endomorphin-2 is believed to be mediated through a novel μ-opioid receptor, causing the release of dynorphin A_{1-17} in the spinal cord, which acts through a non-opioid/non-*N*-methyl-D-aspartate receptor to produce the antianalgesic action (Wu et al., 2003). The antinociceptive effect of endomorphin-2 in mice is also partially mediated by release of dynorphin A_{1-17} in the spinal cord, but which act through κ-opioid receptors to contribute to the antinociceptive effect of endomorphin-2 (Ohsawa et al., 2000; 2001). The purpose of our current study was designed to support our previous pharmacological mouse data by obtaining biochemical evidence that immunoreactive dynorphin A_{1-17} was released in the cerebrospinal fluid (CSF) of rats following endomorphin-2 treatment and to obtain a time course for this release. Pretreatment with different μ-opioid antagonists was also used to confirm the role of μ-opioid receptors in endomorphin-2 stimulated release of dynorphin A_{1-17}. Endomorphin-1 stimulated release of dynorphin A_{1-17} was also examined.

2. Materials and methods

2.1. Animals

Male CD rats weighing 300–350 g (Charles River Breeding Laboratories, Wilmington, MA) were used. Animals were housed two 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. All experiments were approved by and conformed to the guidelines of the Medical College of Wisconsin Animal Care Committee.

2.2. Spinal cord perfusion procedure

Lumbar–cisternal intrathecal perfusion technique, as described previously (Suh and Tseng, 1990; Suh et al., 1992), was used to collect the release of dynorphin A_{1-17} in the spinal perfusate of rats. Rats were administered methylatropine bromide (5 mg/kg, i.p.), anesthetized with urethane (1.5 g/kg, i.p.), and mounted in a stereotaxic apparatus (David Kopf Instrument, Tujinga, CA). A tracheotomy tube was inserted to help breathing. Body temperature was maintained at 37 °C with a heating pad connected to a rectal thermometer (Harvard Apparatus). The cisternal membrane was opened and PE-10 tubing was inserted 10.5 cm down the subarachnoid space so that the tip was located in the vicinity of the lumbar spinal segments. This PE-10 tubing was connected to inflow tubing, while PE-50 outflow tubing was placed on the surface of the opened cisternal space. Both tubes were placed in separate channels of a peristaltic pump (Rainin Instrament, Woburn, MA) and perfused with artificial CSF at a rate of 50 μl/min. Fractions of solution were collected every 12 min (600 μl/sample) by a microfractionator. Initially, rats were perfused with artificial CSF for 30–45 min before two baseline fractions were collected. After fractions were collected, they were boiled for 5 min to denature the enzymes that degrade dynorphin A_{1-17}. Triton X-100 (Sigma, St. Louis, MO) was added to each sample to make a final concentration of 0.02%. Fractions were then frozen until analyzed by enzyme linked immunosorbent assay (ELISA). The artificial CSF has a pH of 7.4 and consists of NaCl (145.67 mM), KCl (2.60 mM), $MgCl_2 \cdot 6H_2O$ (0.90 mM), $CaCl_2 \cdot 2H_2O$ (1.26 mM), $Na_2HPO_4 \cdot 7H_2O$ (0.50 mM), $NaHCO_3$ (4.19 mM), bacitracin (0.20 mg/ml), bovine serum albumin (0.12 mg/ml), thiorphan (0.10 mg/ml), and aprotinin (0.50 TIU/ml) (Sigma).

2.3. ELISA procedure

Immunoreactive Dyn in spinal cord perfusates was measured by an antigen competitive ELISA. One hundred microliters of rabbit anti-serum against dynorphin A_{1-17} was added to each well of goat anti-rabbit precoated plates (Pierce, Rockford, IL) and incubated for 50 min, while shaking. After three washes with wash buffer (1.6% NaCl, 0.04% KCl, 0.544% $Na_2HPO_4(7H_2O)$, and 0.048% KH_2PO_4), 50 μl of various amounts of dynorphin A_{1-17} (standard) or perfusate samples in artificial CSF was added and incubated at room temperature for 1 h. One hundred microliters of biotin-conjugated dynorphin A_{1-17} (Phoenix Pharmaceuticals, Belmont, CA) and 100 μl of avidin-conjugated horse radish peroxidase (Pierce) in dilution buffer (wash buffer with .01M EDTA and 0.5% BSA) were added in separate steps with washes in between and incubated for 1 h. The color reaction was developed with 100 μl of a Chromogen (3,3′ ,5,5′ Tetramethylbenzidine) and hydroperoxide enzyme substrate (Hope Laboratories, Belmont, CA) for 1 h, stopped with 100 μl 2N HCl, and measured spectrophotometrically at an optical density of 450 nm on a microplate reader (Labsystems Multiskan Plus, Fisher Scientific, Pittsburgh, PA). Standard curves (0–16 ng/ml) were plotted net optical density versus the dynorphin A_{1-17} concentration standards. The antiserum against dynorphin A_{1-17} was produced by immunization of New Zealand white rabbits according to the method described in previous publications, and the potencies and cross-immunoreactivity of these antisera have been characterized (Tseng and Collins, 1993; Wu et al., 2002a). No cross-reactivity occurred between the dynorphin A_{1-17} antiserum and endomorphin-1 or endomorphin-2.

2.4. Drug preparation and administration

Endomorphin-1 and endomorphin-2 were obtained from Calbiochem (La Jolla, CA). Naloxone hydrochloride (naloxone) and 3-methoxynaltrexone hydrochloride were obtained from Sigma. All drugs were dissolved in the artificial CSF and delivered by lumbar–cisternal pulse perfusion. After baseline fractions were collected, artificial CSF containing endomorphin-1 or endomorphin-2 was pulse perfused at time 0 min for a total of 3 min and then artificial CSF alone was perfused for the remainder of the experiment.

2.5. Statistical analysis

The data are expressed as the mean and S.E.M. Statistical analysis of difference between groups was assessed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-test or by one-way ANOVA followed by Dunnett's post-test. Statistical significance was determined by using GraphPad Prism and StatMate software (version 3.0, GraphPad Software, San Diego, CA).

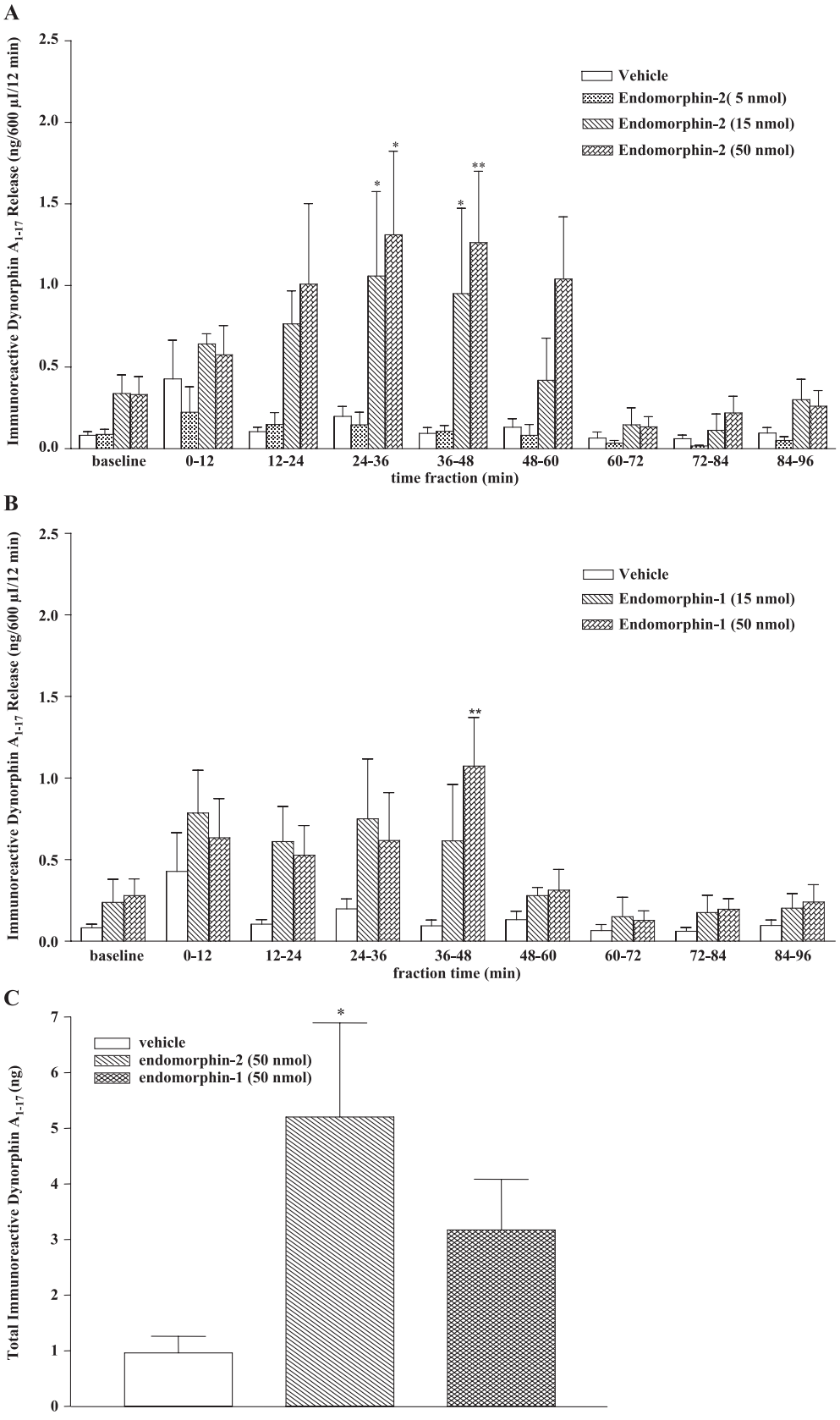
3. Results

3.1. Effect of spinal treatment with endomorphin-2 or endomorphin-1 on the release of immunoreactive dynorphin A_{1-17} into the spinal perfusate of anesthetized rats

Lumbar–cisternal spinal pulse perfusion with endomorphin-2 (5–50 nmol) (Fig. 1A) for 3 min caused a dose-dependent increase in immunoreactive dynorphin A_{1-17} content in spinal perfusate fractions. Immunoreactive dynorphin A_{1-17} content increased shortly after treatment with 15 or 50 nmol endomorphin-2 for 3 min, reached a peak 24–48 min later and returned to baseline levels within 60–72 min after treatment with endomorphin-2. Treatment with 5 nmol of endomorphin-2 showed no appreciable change in immunoreactive dynorphin A_{1-17} content from control levels. Immunoreactive dynorphin A_{1-17} content from control rats perfused with only artificial CSF remained near the baseline level for the entire perfusion. Total amount of immunoreactive dynorphin A_{1-17} detected in the perfusate during the 60-min period after the initial 3 min treatment with endomorphin-2 (50 nmol) (Fig. 1C) was significantly higher than control levels during the same time period. Treatment with the same doses of endomorphin-1 (15–50 nmol) (Fig. 1B) as endomorphin-2, produced a relatively smaller increase in immunoreactive dynorphin A_{1-17} with a significant increase only occurring in one fraction (36–48 min) and only at the 50-nmol dose. Total amount of immunoreactive dynorphin A_{1-17} detected in the perfusate during the 60-min period after the initial 3 min treatment with endomorphin-1 (50 nmol) (Fig. 1C) was not significantly different from control levels. Higher doses of endomorphin-1 were not studied. Our results also showed that the ranges of difference between means for vehicle and various doses of endomorphin-treated groups at various time points under the power of 0.9 with 0.05 two-tailed statistical difference are 0.129–0.946, 0.335–1.737, 0.241–1.711, 0.243–1.237, and 0.244–1.145 for comparisons between vehicle and 5 nmol endomorphin-2, vehicle and 15 nmol endomorphin-2, vehicle and 50 nmol endomorphin-2, vehicle and 15 nmol endomorphin-1, and vehicle and 50 nmol endomorphin-1, respectively.

3.2. Effect of spinal pretreatment with μ-opioid antagonists on the endomorphin-2 induced release of immunoreactive dynorphin A_{1-17} into spinal perfusate fractions of anesthetized rats

Lumbar–cisternal spinal pulse perfusion with μ-opioid antagonist, naloxone (10 ng) or 3-methoxynaltrexone (25 ng) for 10 min before and co-treated with endomorphin-2 for 3 min, completely blocked the increase of immunoreactive dynorphin A_{1-17} release induced by endomorphin-2 treatment (Fig. 2). Pretreatment with naloxone (10 ng) or 3-methoxynaltrexone (25 ng) alone for 13 min did not


A
Immunoreactive Dynorphin A1-17 Release (ng/600 µl/12 min)
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
Endomorphin-2(5 nmol)
Endomorphin-2 (15 nmol)
Endomorphin-2 (50 nmol)
*
*
*
**
baseline
0-12
12-24
24-36
36-48
48-60
60-72
72-84
84-96
time fraction (min)
B
Immunoreactive Dynorphin A1-17 Release (ng/600 µl/12 min)
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
Endomorphin-1 (15 nmol)
Endomorphin-1 (50 nmol)
**
baseline
0-12
12-24
24-36
36-48
48-60
60-72
72-84
84-96
fraction time (min)
C
Total Immunoreactive Dynorphin A1-17 (ng)
7
6
5
4
3
2
1
0
vehicle
endomorphin-2 (50 nmol)
endomorphin-1 (50 nmol)
*

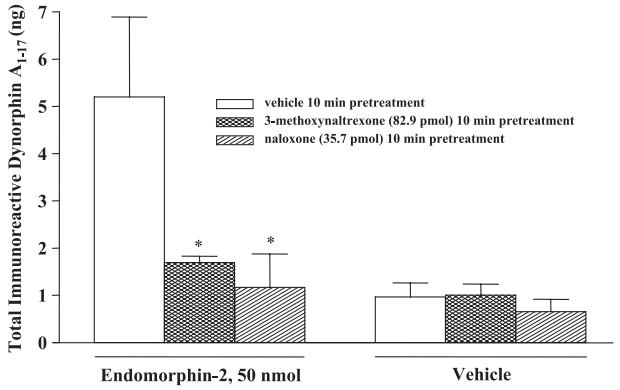


Fig. 2. After an initial perfusion with artificial CSF for 40 min, groups of rats were pretreated i.t. by pulse perfusion with vehicle (artificial CSF), μ-opioid antagonist 3-methoxynaltrexone (82.9 pmol) or naloxone (35.7 pmol) for 10 min, and then co-administered with vehicle or endomorphin-2 (50 nmol) for another 3 min. Fractions of spinal perfusate were collected (600 μl/fraction) every 12 min. The amount of immunoreactive dynorphin $A_{1–17}$ in each fraction was analyzed by an ELISA technique. Columns represent mean total immunoreactive dynorphin $A_{1–17}$ release (ng) within the first 5 fractions (60 min) following pretreatment with μ-opioid antagonist or vehicle and vertical bar represents standard error (n=4–5). Statistical differences between vehicle or endomorphin-2 treated groups were assessed by one-way ANOVA followed by Dunnett's post-test. *P<0.05.

cause any change in content of immunoreactive dynorphin $A_{1–17}$ from baseline levels.

4. Discussion

Previously, we have provided pharmacological evidence that dynorphin $A_{1–17}$ is released in the spinal cord of mice following i.t. treatment with endomorphin-2. Analgesic doses of endomorphin-2 produce potent antinociception that is partially blocked by pretreatment with antiserum against dynorphin $A_{1–17}$ or with κ-opioid receptor antagonist, nor-binaltorphimine, suggesting that the endomorphin-2-produced antinociception is mediated in part by the release of dynorphin $A_{1–17}$ acting on κ-opioid receptors in the spinal cord (Ohsawa et al., 2001). Both analgesic and subanalgesic doses of i.t. endomorphin-2 produce antianalgesia against various opioid agonists by a mechanism involving the release of dynorphin $A_{1–17}$ in the spinal cord (Wu et al., 2003). This antianalgesia is also eliminated by pretreatment with antiserum against dynorphin $A_{1–17}$, but not by pretreatment with nor-binaltorphimine, suggesting that released dynorphin $A_{1–17}$ does not act through κ-opioid receptors. Our present study found direct biochemical evidence showing an increase in release of immunoreactive dynorphin $A_{1–17}$ in spinal perfusate samples of rats that were i.t. treated with endomorphin-2 for 3 min.

On the contrary, endomorphin-1 produced antinociception in mice is not blocked by treatment with nor-binaltorphimine or dynorphin $A_{1–17}$ antiserum, indicating dynorphin $A_{1–17}$ is not involved in this system, nor does endomorphin-1 induce antianalgesic effects similar to equivalent doses of endomorphin-2 (Ohsawa et al., 2001; Wu et al., 2003). We found in the present study that intrathecal treatment with endomorphin-1 was much less effective than endomorphin-2 in increasing the release of dynorphin $A_{1–17}$. The highest dose of endomorphin-1 used (50 nmol) is considered sufficiently high, because endomorphin-1 is at least twofold more potent than endomorphin-2 when administered intrathecally in various antinociceptive tests (Goldberg et al., 1998; Przewlocka et al., 1999; Ohsawa et al., 2001; Tseng, 2002).

The increase in release of immunoreactive dynorphin $A_{1–17}$ into spinal perfusates after a 3-min pulse perfusion with endomorphin-2 developed slowly and continued to increase, where it reached a peak between 24 and 48 min. Immunoreactive dynorphin $A_{1–17}$ content returned to baseline levels within 60 min. Interestingly, the time course for release of immunoreactive dynorphin $A_{1–17}$ into the spinal perfusate following endomorphin-2 treatment correlates very closely with the time course for endomorphin-2 to induce antianalgesia against opioid agonists shown previously in the mouse spinal cord (Wu et al., 2003). Wu et al. (2003) showed a 45-min pretreatment with small doses of endomorphin-2 before analgesic doses of morphine produce a strong attenuation of morphine antinociception. Pretreatment with endomorphin-2 for 30 or 60 min produces less attenuation, while pretreatment for 15, 90, or 120 min had no effect. Our current finding thus provides direct biochemical evidence that endomorphin-2 does cause a release of dynorphin $A_{1–17}$ in the spinal cord of rats, which may contribute to the inhibitory effect endomorphin-2 has against various opioid agonists. However, the reason is not clear why there is a lag in time between endomorphin-2 treatment and peak dynorphin $A_{1–17}$ release and development of antianalgesia. Possible supraspinal involvement in the release of dynorphin $A_{1–17}$ following endomorphin-2 treatment cannot be ruled out at this time and should be looked into further.

The time course for the dynorphin $A_{1–17}$ release after endomorphin-2 treatment in the present study does not

Fig. 1. Rats were initially intrathecally (i.t.) perfused with artificial cerebrospinal fluid (CSF) for 55 min followed by pulse perfusion with endomorphin-2 (5–50 nmol) (A, C) or endomorphin-1 (15–50 nmol) (B, C) from time 0 to 3 min, and were then perfused with artificial CSF for the remainder of the perfusion. Fractions of spinal perfusate were collected (600 μl/fraction) every 12 min. The amount of immunoreactive dynorphin $A_{1–17}$ released by endomorphin-2 or endomorphin-1 was analyzed by an enzyme linked immunosorbent assay (ELISA) technique. Control groups consisted of perfusion with only artificial CSF (vehicle) for the entire experiment. Baseline fractions were collected before any drug was added. Columns represent the mean immunoreactive dynorphin $A_{1–17}$ release (ng/600 μl/12 min fraction) (A, B) or mean total release (ng) within the first 5 fractions (0–60 min) (C) and vertical bar represents standard error (n=4–5). Statistical differences between vehicle and endomorphin-2 or endomorphin-1 treated groups were assessed with either two-way ANOVA followed by Bonferroni's post-test (A, B) or one-way ANOVA followed by Dunnett's post-test (C). *P<0.05, **P<0.01.

correlate as well with endomorphin-2-produced analgesia. Endomorphin-2 administered intrathecally produces rapid antinociception in numerous analgesic tests, producing a peak analgesic effect in 10–15 min in the tail-flick and paw-pressure tests in rats and within 2–5 min when given intrathecally in the tail-flick test in mice (Stone et al., 1997; Horvath et al., 1999; Przewlocka et al., 1999; Ohsawa et al., 2001; Tseng, 2002). We found during the present study that a gradual release of dynorphin A_{1-17} occurred shortly after endomorphin-2 treatment, which may only in part contribute to the analgesic effect of endomorphin-2. As stated previously, κ-opioid receptor antagonist, nor-binaltorphimine, or antiserum directed against dynorphin partially inhibits i.t. endomorphin-2 produced antinociception in mice, suggesting endomorphin-2-produced antinociception may only to some extent be caused by released dynorphin A_{1-17} acting on κ-opioid receptors in the spinal cord (Ohsawa et al., 2001).

The neuronal mechanism involved in the release of immunoreactive dynorphin A_{1-17} by endomorphin-2 is mediated through μ-opioid receptors. Pretreatment with the non-specific μ-opioid antagonist, naloxone, or novel morphine-6β-glucuronide μ-opioid receptor antagonist, 3-methoxynaltrexone, inhibited the increase in immunoreactive dynorphin A_{1-17} release in spinal fractions when compared to samples in endomorphin-2 perfused alone. Earlier work shows endomorphin-2-produced antinociception in the spinal cord is inhibited by naloxone or 3-methoxynaltrexone, as has endomorphin-2-induced antianalgesia (Stone et al., 1997; Sakurada et al., 2000; Sakurada et al., 2001; Wu et al., 2003). Since 3-methoxynaltrexone does not block morphine, D-Ala^2, *N*-Me-Phe^4, Gly-ol^5-enkephalin (DAMGO), or endomorphin-1 induced antinociception at the same doses that block endomorphin-2, it is suggested that endomorphin-2 acts through a novel opioid receptor subtype different from which morphine, DAMGO, and endomorphin-1 stimulate (Brown et al., 1997; Sakurada et al., 2000). We propose that this novel subtype may also be involved in the release of immunoreactive dynorphin A_{1-17} following endomorphin-2 i.t. treatment. Previously, our lab has shown evidence that endomorphin-1 and endomorphin-2 may stimulate different subtypes of μ-opioid receptors. Antisense oligodeoxynucleotides against exon-1 or -4 of the μ-opioid receptor clone attenuate the antinociception produced by both endomorphin-1 and endomorphin-2, while those against exon-8 only attenuate the antinociception produced by endomorphin-1, but not endomorphin-2, suggesting different splice variants may be involved in endomorphin-1 and endomorphin-2 analgesia (Wu et al., 2002b). D-Pro^2-endomorphin-1 administered intrathecally blocks antinociception produced by i.t endomorphin-1, but not endomorphin-2, while D-Pro^2-endomorphin-2 blocks antinociception produced by endomorphin-2, and a much lesser extent, endomorphin-1 (Hung et al., 2002).

In conclusion, we have for the first time provided direct biochemical evidence that following lumbar–cisternal pulse perfusion with endomorphin-2, or to a lesser extent, endomorphin-1, an increase in immunoreactive dynorphin A_{1-17} content was found in the spinal perfusate of rats. The stimulated increase in release in immunoreactive dynorphin A_{1-17} by perfusion with endomorphin-2 was blocked by pretreatment with naloxone or 3-methoxynaltrexone, suggesting stimulation of μ-opioid receptors by endomorphin-2 was required for release of dynorphin A_{1-17} in the spinal cord.

Acknowledgements

This work was supported by U.S. Public Health Service Grant DA 12588 from the National Institute on Drug Abuse (NIDA), National Institutes of Health. A preliminary report of these results was present at the 33rd Annual Meeting of the Society for Neuroscience, New Orleans, LA, November 8–12, 2003.

References

Brown, G.P., Yang, K., King, M.A., Rossi, G.C., Leventhal, L., Chang, A., Pasternak, G.W., 1997. 3-Methoxynaltrexone, a selective heroin/morphine-6β-glucuronide antagonist. FEBS Lett. 412, 35–38.

Fujimoto, J.M., Rady, J.J., 1989. Intracerebroventricular physostigmine-induced analgesia: enhancement by naloxone, β-funaltrexamine and nor-binaltorphimine and antagonism by dynorphin A (1–17). J. Pharmacol. Exp. Ther. 251, 1045–1052.

Goldberg, I.E., Rossi, G.C., Letchworth, S.R., Mathis, J.P., Ryan-Moro, J., Leventhal, L., Su, W., Emmel, D., Bolan, E.A., Pasternak, G.W., 1998. Pharmacological characterization of endomorphin-1 and endomorphin-2 in mouse brain. J. Pharmacol. Exp. Ther. 286, 1007–1013.

Horvath, G., Szikszay, M., Tomboly, C., Benedek, G., 1999. Antinociceptive effects of intrathecal endomorphin-1 and endomorphin-2 in rats. Life Sci. 65, 2635–2641.

Hung, K.-C., Wu, H.-E., Mizoguchi, H., Sakurada, S., Okayama, T., Fujimura, T., Murayama, K., Sakurada, T., Fujimoto, J.M., Tseng, L.F., 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.

Laughlin, T.M., Larson, A.A., Wilcox, G.L., 2001. Mechanisms of induction of persistent nociception by dynorphin. J. Pharmacol. Exp. Ther. 299, 6–11.

Millan, M.J., 1999. The induction of pain: an integrative review. Prog. Neurobiol. 57, 1–164.

Narita, M., Mizoguchi, H., Oji, G.E., Tseng, E.L., Suganuma, C., Nagase, H., Tseng, L.F., 1998. Characterization of endomorphin-1 and -2 on [^{35}S] GTPγS binding in the mouse spinal cord. Eur. J. Pharmacol. 351, 383–387.

Narita, M., Mizoguchi, H., Narita, M., Dun, N.J., Hwang, B.H., Endoh, T., Suzuki, T., Nagase, H., Suzuki, T., Tseng, L.F., 2000. G protein activation by endomorphins in the mouse periaqueductal gray matter. J. Biomed. Sci. 7, 221–225.

Ohsawa, M., Mizoguchi, H., Narita, M., Chu, M., Nagase, H., Tseng, L.F., 2000. Differential mechanisms mediating descending pain controls for antinociception induced by supraspinally administered endomorphin-1 and endomorphin-2 in the mouse. J. Pharmacol. Exp. Ther. 294, 1106–1111.

Ohsawa, M., Mizoguchi, H., Narita, M., Nagase, H., Kampine, J.P., Tseng, L.F., 2001. Differential antinociception induced by spinally adminis-

tered endomorphin-1 and endomorphin-2 in the mouse. J. Pharmacol. Exp. Ther. 298, 592–597.

Przewlocka, B., Mika, J., Labuz, D., Toth, G., Przewlocki, R., 1999. Spinal analgesic action of endomorphins in acute, inflammatory, and neuropathic pain in rats. Eur. J. Pharmacol. 367, 189–196.

Rady, J.J., Fujimoto, J.M., Tseng, L.F., 1991. Dynorphins other than dynorphin A(1–17) lack spinal antianalgesic activity but do act on dynorphin A(1–17). J. Pharmacol. Exp. Ther. 259, 1073–1080.

Sakurada, S., Hayashi, T., Yuhki, M., Fujimura, T., Murayama, K., Yonezawa, A., Sakurada, C., Takeshita, M., Zadina, J.E., Kastin, A.J., Sakurada, T., 2000. Differential antagonism of endomorphin-1 and endomorphin-2 spinal antinociception by naloxonazine and 3-methoxynaltrexone. Brain Res. 881, 1–8.

Sakurada, S., Hayashi, T., Yuhki, M., Orito, T., Zadina, J.E., Kastin, A.J., Fujimura, T., Murayama, K., Sakurada, C., Sakurada, T., Narita, M., Suzuki, T., Tan-no, K., Tseng, L.F., 2001. Differential antinociceptive effects induced by intrathecally administered endomorphin-1 and endomorphin-2 in the mouse. Eur. J. Pharmacol. 427, 203–210.

Sanchez-Blanquez, P., DeAntonio, I., Rodriguez-Diaz, M., Garzon, J., 1999. Antisense oligodeoxynucleotide targeting distinct exons of the cloned μ-opioid receptor distinguish between endomorphin-1 and morphine supraspinal antinociception in mice. Antisense Nucleic Acid Drug Dev. 9, 253–260.

Smith, A.P., Lee, N.M., 1988. Pharmacology of dynorphin. Ann. Rev. Pharmacol. Toxicol. 28, 123–140.

Spetea, M., Monory, K., Tomboly, C., Toth, G., Tzavara, E., Benyhe, S., Hanoune, J., Borsodi, A., 1998. In vitro binding and signaling profile of the novel mu opioid receptor agonist endomorphin-2 in rat brain membranes. Biochem. Biophys. Res. Commun. 250, 720–725.

Stone, L.S., Fairbanks, C.A., Laughlin, T.M., Nguyen, H.O., Bushy, T.M., Wessendorf, M.W., Wilcox, G.L., 1997. Spinal analgesic actions of the new endogenous opioid peptides endomorphin-1 and -2. NeuroReport 8, 3131–3135.

Suh, H.H., Tseng, L.F., 1990. Intrathecal administration of thiorphan and bestatin enhances the antinociception and release of met-enkephalin induced by β-endorphin intraventricularly in anesthetized rats. Neuropeptides 16, 91–96.

Suh, H.H., Collins, K.A., Tseng, L.F., 1992. Intrathecal cholecystokinin octapeptide attenuates the antinociception and release of immunoreactive met-enkephalin induced by intraventricular β-endorphin in the rat. Neuropeptides 21, 131–137.

Tseng, L.F., Collins, K.A., 1993. Spinal involvement of both dynorphin A and met-enkephalin in the antinociception induced by intracerebroventricularly administered bremazocine but not morphine in the mouse. J. Pharmacol. Exp. Ther. 266, 1430–1438.

Tseng, L.F., 2002. Recent advances in the search of the μ-opioidergic system: the antinociceptive properties of endomorphin-1 and endomorphin-2 in the mouse. Jpn. J. Pharmacol. 89, 216–220.

Wu, H.-E., Hung, K.-C., Mizoguchi, H., Nagase, H., Tseng, L.F., 2002a. Roles of endogenous opioid peptides in modulation of nocifensive response to formalin. J. Pharmacol. Exp. Ther. 300, 647–654.

Wu, H.-E., Mizoguchi, H., Terashvili, M., Leitermann, R.J., Hung, K.-C., Fujimoto, J.M., Tseng, L.F., 2002b. Spinal pretreatment with antisense oligodeoxynucleotides against exon-1, -4, or -8 of μ-opioid receptor clone leads to differential loss of spinal endomorphin-1- and endomorphin-2-induced antinociception in the mouse. J. Pharmacol. Exp. Ther. 303, 867–873.

Wu, H.-E., Sun, H.-S., Darpolar, M., Leitermann, R.J., Kampine, J.P., Tseng, L.F., 2003. Dynorphinergic mechanism mediating endomorphin-2-induced antianalgesia in the mouse spinal cord. J. Pharmacol. Exp. Ther. 307, 1135–1141.

Zadina, J.E., Hackler, L., Ge, L.-J., Kastin, A.J., 1997. A potent and selective endogenous agonist for the μ-opioid receptor. Nature 386, 499–502.