Possible Involvement of Dynorphin A-(1–17) Release via \(\mu_1\)-Opioid Receptors in Spinal Antinociception by Endomorphin-2

Hirokazu Mizoguchi, Hiroyuki Watanabe, Takafumi Hayashi, Wataru Sakurada, Toshiki Sawai, Tsutomu Fujimura, Tsukasa Sakurada, and Shinobu Sakurada

Department of Physiology and Anatomy, Tohoku Pharmaceutical University, Sendai, Japan (H.M., H.W., T.H., W.S., T.Saw., S.S.); Division of Biochemical Analysis, Central Laboratory of Medical Sciences, Juntendo University School of Medicine, Tokyo, Japan (T.F.); and Department of Biochemistry, Daichi College of Pharmaceutical Sciences, Fukuoka, Japan (T.Sak.)

Received November 8, 2005; accepted January 3, 2006

ABSTRACT

The antinociception induced by i.t. or i.c.v. administration of endomorphins is mediated via \(\mu\)-opioid receptors. However, although endorphins do not have an appreciable affinity for \(\kappa\)-opioid receptors, pretreatment with the \(\kappa\)-opioid receptor antagonist norbinaltorphimine markedly reduces the antinociceptive response to i.c.v. or i.t. administered endomorphin-2 but not endomorphin-1. These results suggest that endomorphin-2 initially stimulates \(\mu\)-opioid receptors, which subsequently induce the release of dynorphins that act on \(\kappa\)-opioid receptors to produce antinociception. The present study was performed in mice to determine whether the release of dynorphins by i.t. administered endomorphin-2 is mediated through \(\mu\)-opioid receptors to produce antinociception. Intrathecal pretreatment with an antiserum against dynorphin A-(1–17), but not against dynorphin B-(1–13) or \(\alpha\)-neoendorphin, dose-dependently prevented the paw-withdrawal inhibition by endomorphin-2. The pretreatments with these antisera did not affect the endomorphin-1- or \([\alpha\text{-Ala}^2,\text{MePhe}^4,\text{Gly(ol)}^5]\)enkephalin-induced paw-withdrawal inhibition. The attenuation of endomorphin-2-induced antinociception by i.t. pretreatment with an antiserum against dynorphin A-(1–17) or s.c. pretreatment with norbinaltorphimine was blocked dose-dependently by s.c. pretreatment with the \(\mu\)-opioid receptor antagonist β-funaltrexamine or the \(\mu_1\)-opioid receptor antagonist naloxonazine at ultra-low doses that are ineffective against \(\mu\)-opioid receptor agonists. These results suggest that the spinal antinociception induced by endomorphin-2 is mediated through the stimulation of a distinct subtype of \(\mu_1\)-opioid receptor that induces the release of the endogenous \(\kappa\)-opioid peptide dynorphin A-(1–17) in the spinal cord.

Two endogenous opioid peptides, endomorphin-1 and endomorphin-2, have been recently discovered in the mammalian brain. These two peptides activate \(\mu\)-opioid receptors with high affinity and selectivity (Zadina et al., 1997; Goldberg et al., 1998; Gong et al., 1998; Hosohata et al., 1998). Immunoreactivities for these peptides have been localized in many areas of the central nervous system involved in pain processing, including the dorsal horn of the spinal cord, the caudal trigeminal nucleus, and the periaqueductal gray (Martin-Schild et al., 1997, 1998, 1999; Pierce et al., 1998). Some differences have been noted for the distribution of endomorphin-1 and endomorphin-2 in the brain and spinal cord. Endomorphin-1 is more abundant than endomorphin-2 in the brain, whereas endomorphin-2 dominates in the spinal cord (Martin-Schild et al., 1999). Both of these peptides display high affinity and selectivity for the \(\mu\)-opioid receptor; however, neither endomorphin has an appreciable affinity for \(\delta\)- or \(\kappa\)-opioid receptors (Zadina et al., 1997). Distinct pharmacological properties for the endomorphins have been reported in both electrophysiological (Chapman et al., 1997) and behavioral experiments (Stone et al., 1997; Zadina et al., 1997; Goldberg et al., 1998). The antinociception induced by endomorphin-1 and endomorphin-2 given i.t. or i.c.v. is selectively blocked by pretreatment with \(\mu\)-opioid receptor antagonists, naloxone, or \(\beta\)-funaltrexamine (Zadina et al., 1997; Goldberg et al., 1998; Sakurada et al., 1999), indicating that their antinociception is mediated by the stimulation of \(\mu\)-opioid receptors. Furthermore, pretreatment with the \(\mu_1\)-opioid receptor antagonist naloxonazine attenuates the antinocicep-

ABBREVIATIONS: DAMGO, \([\alpha\text{-Ala}^2,\text{MePhe}^4,\text{Gly(ol)}^5]\)enkephalin; ANOVA, analysis of variance; TAPA, H-Tyr-\(\alpha\)-Arg-Phe-\(\beta\)-Ala-OH.
tion induced by i.t. or i.c.v. administered endomorphin-2 but not endomorphin-1, suggesting that endomorphin-2-induced antinociception is mediated by the stimulation of \( \mu \)-opioid receptors (Sakurada et al., 1999, 2000a, 2001, 2002).

Although the antinociceptive effects induced by both endomorphin-1 and endomorphin-2 are mediated by the stimulation of \( \mu \)-opioid receptors, some differences in antinociceptive effects between endomorphin-1 and endomorphin-2 have been noted. The antinociception induced by supraspinally administered endomorphin-2, but not endomorphin-1, is blocked by i.c.v. pretreatment with the \( \kappa \)-opioid receptor antagonist norbinaltorphimine or an antiserum against dynorphin A-(1–17), suggesting that endomorphin-2 stimulates a different subtype of \( \mu \)-opioid receptor, which subsequently induces the release of dynorphins that act on \( \kappa \)-opioid receptors to produce antinociception (Tseng et al., 2000). The present behavioral pharmacological study was conducted to determine whether the release of dynorphin A-(1–17) by spinally administered endomorphin-2 is mediated through the stimulation of the \( \mu \)-opioid receptor.

### Materials and Methods

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

**Animals.** Male ddY mice weighing 22 to 25 g (SLC, Hamamatsu, Japan) were housed in a light- and temperature-controlled room (light on at 9:00 AM and off at 9:00 PM; 23°C). Food and water were available ad libitum. Animals were used only once.

**Drugs and Antisera.** Endomorphin-1 (Peptide Institute Inc., Osaka, Japan), endomorphin-2 (Peptide Institute Inc., \([\text{d-Ala}^2,\text{MePhe}^4,\text{Gly}(\text{ol})^5]\text{enkephalin (DAMGO)}) (Sigma Chemical Co., St. Louis, MO), naloxonazine (RBI, Natick, MA), \( \beta \)-funaltrexamine (RBD), norbinaltorphimine (RBD), antiserum against dynorphin A-(1–17) (Phoenix Pharmaceutical Inc., St. Joseph, MO), antiserum against dynorphin B-(1–13) (Bachem, San Carlos, CA), and antiserum against \( \alpha \)-neoendorphin (Bachem) were used. Endomorphins, DAMGO, and the antiserum were dissolved in sterile artificial cerebrospinal fluid containing 7.4 g of NaCl, 0.19 g of KCl, 0.19 g of MgCl2, and 0.14 g of CaCl2 in 1 liter. Naloxonazine, \( \beta \)-funaltrexamine, and norbinaltorphimine were dissolved in saline and injected s.c. in a volume of 0.1 ml/10 g b.wt. 24 h before testing.

**Assessment of Antinociceptive Response.** The antinociceptive response was assessed with the thermal paw-withdrawal test, using an automated tail-flick unit (BM kiki, Tokyo, Japan). Mice were adapted to the testing environment for at least 1 h before any stimulation. Each animal was restrained with a soft cloth to reduce visual stimuli, and a light beam as a noxious radiant heat stimulation was applied to the hind paw. The light beam focused on the plantar surface of the hind paw, and the latency for the paw-withdrawal response against the noxious radiant heat stimulation was measured. The intensity of the noxious radiant heat stimulation was adjusted so that the predrug latency for the paw-withdrawal response was 2.5 to 3.5 s. The antinociceptive effect was expressed as a percentage of the maximum possible effect, which was calculated with the following equation: \([T_1 - T_0] / (10 - T_0) \times 100\), where \( T_0 \) and \( T_1 \) are the predrug and postdrug latencies for the paw-withdrawal response, respectively. To prevent tissue damage to the paw, the noxious radiant heat stimulation was terminated automatically if the mouse did not lift the paw within 10 s. The measurement of the paw-withdrawal latency was performed by only one individual who was uninformed about the drug treatment for each mouse.

**Intrathecal Administration.** The i.t. administration was performed according to the procedure described by Hylden and Wilcox (1980) using a 10-μl Hamilton microsyringe with a 29-gauge needle. The injection volumes for opioid peptides and antisera were 2 and 4 \( \mu \)l, respectively.

**Statistical Analysis.** The data are expressed as the means ± S.E.M for 10 mice. The statistical significance of the differences between groups was assessed with a one-way analysis of variance (ANOVA) followed by either Dunnett’s test or the Bonferroni test or a two-way ANOVA followed by the Bonferroni test.

## Results

### Time Courses of the Paw-Withdrawal Inhibition Induced by Intrathecal Administration of Endomorphin-1, Endomorphin-2, and DAMGO.

Groups of mice were injected i.t. with endomorphin-1 (5 nmol), endomorphin-2 (5 nmol), DAMGO (20 pmol), or the vehicle, and the paw-withdrawal responses were measured at various times after the injection. The inhibitions of the paw-withdrawal responses after i.t. administration of endomorphin-1 or endomorphin-2 reached their peaks at 5 min, and paw-withdrawal responses returned to the preinjection level in 20 min (Fig. 1). On the other hand, the inhibition of the paw-withdrawal response after i.t. administration of DAMGO reached its peak 5 to 10 min after the injection, and the response returned to the preinjection level 30 min after the injection. Cerebrospinal fluid given i.t. did not produce any change in the latency of the paw-withdrawal response (Fig. 1).

### Effects of Subcutaneous Pretreatment with \( \beta \)-Funaltrexamine and Norbinaltorphimine on the Antinociception Induced by Intrathecal Administered Endomorphin-1, Endomorphin-2, and DAMGO.

Approximately equipotent doses of endomorphin-1, endomorphin-2, and DAMGO were chosen for the studies. Intrathecal injection of endomorphin-1 (5 nmol), endomorphin-2 (5 nmol), and DAMGO (20 pmol) produced 83.8, 80.8, and 72.2% of maximal possible effect at 5, 5, and 10 min after the injection, respectively (Fig. 1). Groups of mice were pretreated s.c. with the selective \( \mu \)-opioid receptor antagonist \( \beta \)-funaltrexamine (40 mg/kg) or the selective \( \kappa \)-opioid antagonist norbinaltorphimine (40 mg/kg).

![Fig. 1. Time course of the antinociceptive effects of endomorphin-1 (EM-1), endomorphin-2 (EM-2), and DAMGO in the mouse paw-withdrawal test.](https://example.com/fig1.png)
tive α-opioid receptor antagonist norbinaltorphimine (10 mg/kg) 24 h before the i.t. injection of endomorphin-1 (5 nmol), endomorphin-2 (5 nmol), or DAMGO (20 pmol). The inhibition of the paw-withdrawal response induced by endomorphin-1, endomorphin-2, and DAMGO was measured 5, 5, and 10 min after the treatment, respectively. The inhibition of the paw-withdrawal response induced by endomorphin-1, endomorphin-2, or DAMGO was completely inhibited by the pretreatment with β-funaltrexamine (Fig. 2). On the other hand, the inhibition of the paw-withdrawal response induced by endomorphin-2 was markedly reduced by the pretreatment with norbinaltorphimine, whereas the effects of endomorphin-1 and DAMGO were insensitive to norbinaltorphimine (Fig. 2).

Effects of Intrathecal Pretreatment with Antisera against Dynorphin A-(1–17), Dynorphin B-(1–13), and α-Neoeendorphin on the Antinoceception Induced by Intrathecal Administered Endomorphin-1, Endomorphin-2, and DAMGO. Groups of mice were pretreated i.t. with antisera (1:50 dilution) against dynorphin A-(1–17), dynorphin B-(1–13), or α-neoeendorphin for 15, 15, and 10 min before the i.t. injection of endomorphin-1 (5 nmol), endomorphin-2 (5 nmol), and DAMGO (20 pmol), respectively. The inhibition of the paw-withdrawal response induced by endomorphin-1, endomorphin-2, and DAMGO was measured 5, 5, and 10 min after the treatment, respectively. Intrathecal pretreatment with an antisera against dynorphin A-(1–17) attenuated the paw-withdrawal inhibition induced by endomorphin-2 in a dose-dependent manner (Fig. 3). However, the paw-withdrawal inhibition induced by endomorphin-2 was not affected by antisera against dynorphin B-(1–13) or α-neoeendorphin. The same pretreatment with these antisera did not affect the paw-withdrawal inhibition induced by endomorphin-1 or DAMGO (Fig. 3).

Effects of β-Funaltrexamine and Naloxonazine Injected Subcutaneous on the Attenuation of Endomorphin-2-Induced Paw-Withdrawal Inhibition by Subcutaneous Pretreatment with Norbinaltorphimine. Subcutaneous pretreatment with ultra-low doses of β-funaltrexamine (0.25–3 mg/kg) or naloxonazine (0.031–2 mg/kg) was ineffective against the paw-withdrawal inhibition induced by endomorphin-2 (5 nmol i.t.) (Table 1). In the following experiments, these ultra-low doses of β-funaltrexamine and naloxonazine were used without antisera.

**Fig. 3. Effects of antisera against dynorphin A-(1–17) (Anti-DynA), dynorphin B-(1–13) (Anti-DynB), or α-neoeendorphin (Anti-endo) on endomorphin-1 (EM-1), endomorphin-2 (EM-2), and DAMGO-induced antinoceception in the paw-withdrawal test. Anti-DynA, Anti-DynB, or Anti-endo was administered i.t. 15, 15, and 10 min before i.t. administration of EM-1 (5 nmol), EM-2 (5 nmol), and DAMGO (20 pmol), respectively. The antinoceceptive effects induced by EM-1, EM-2, and DAMGO were measured 5, 5, and 10 min after administration, respectively. Each value represents the mean ± S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with one-way ANOVA followed by the Bonferroni test. The F value of the one-way ANOVA for EM-2 was F(5, 54) = 10.85 (p < 0.01). **, p < 0.05; ***, p < 0.01 compared with EM-2 alone.**

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antinoceception</th>
<th>%MPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endomorphin-2 (5 nmol i.t.)</td>
<td>80.81 ± 5.30</td>
<td></td>
</tr>
<tr>
<td>Endomorphin-2 (5 nmol i.t.) + β-funaltrexamine (0.25 mg/kg s.c.)</td>
<td>86.80 ± 4.59</td>
<td></td>
</tr>
<tr>
<td>Endomorphin-2 (5 nmol i.t.) + β-funaltrexamine (0.5 mg/kg s.c.)</td>
<td>83.77 ± 5.32</td>
<td></td>
</tr>
<tr>
<td>Endomorphin-2 (5 nmol i.t.) + β-funaltrexamine (1 mg/kg s.c.)</td>
<td>83.45 ± 5.29</td>
<td></td>
</tr>
<tr>
<td>Endomorphin-2 (5 nmol i.t.) + β-funaltrexamine (2 mg/kg s.c.)</td>
<td>87.06 ± 4.95</td>
<td></td>
</tr>
<tr>
<td>Endopmorphin-2 (5 nmol i.t.) + naloxonazine (0.031 mg/kg s.c.)</td>
<td>84.27 ± 4.16</td>
<td></td>
</tr>
<tr>
<td>Endomorphin-2 (5 nmol i.t.) + naloxonazine (0.125 mg/kg s.c.)</td>
<td>90.69 ± 4.48</td>
<td></td>
</tr>
<tr>
<td>Endomorphin-2 (5 nmol i.t.) + naloxonazine (0.25 mg/kg s.c.)</td>
<td>76.71 ± 5.70</td>
<td></td>
</tr>
<tr>
<td>Endomorphin-2 (5 nmol i.t.) + naloxonazine (0.5 mg/kg s.c.)</td>
<td>87.05 ± 4.65</td>
<td></td>
</tr>
<tr>
<td>Endomorphin-2 (5 nmol i.t.) + naloxonazine (1 mg/kg s.c.)</td>
<td>76.65 ± 6.81</td>
<td></td>
</tr>
<tr>
<td>Endomorphin-2 (5 nmol i.t.) + naloxonazine (2 mg/kg s.c.)</td>
<td>76.38 ± 8.15</td>
<td></td>
</tr>
</tbody>
</table>

%MPE, percent maximal possible effect.
and naloxonazine were used. Groups of mice were pretreated s.c. with norbinaltorphimine (10 mg/kg) in combination with β-funaltrexamine (0.25–3 mg/kg) or naloxonazine (0.25–2 mg/kg) 24 h before i.t. treatment with endomorphin-2 (5 nmol). The inhibition of paw-withdrawal response induced by endomorphin-2 was measured 5 min after the treatment. Subcutaneous pretreatment with ultra-low doses of β-funaltrexamine at 1.0 to 3.0 mg/kg markedly reduced the attenuation of endomorphin-2-induced paw-withdrawal inhibition by s.c. pretreatment with norbinaltorphimine (Fig. 4). A similar phenomenon was observed after s.c. pretreatment with ultra-low doses of naloxonazine (1–4 mg/kg), which significantly diminished the inhibitory effect of norbinaltorphimine on the antinociception of endomorphin-2 (Fig. 5).

**Effects of β-funaltrexamine and Naloxonazine Injected Subcutaneous on the Attenuation of Endomorphin-2-Induced Paw-Withdrawal Inhibition by Intrathecal Pretreatment with an Antiserum against Dynorphin A-(1-17).** Groups of mice, pretreated s.c. with β-funaltrexamine (0.25–2.0 mg/kg s.c.) or naloxonazine (0.031–0.5 mg/kg) 24 h earlier, were injected i.t. with an antiserum against dynorphin A-(1–17) (1:50 dilution) 15 min before i.t. treatment with endo-morphin-2 (5 nmol). The inhibition of the paw-withdrawal response induced by endomorphin-2 was measured 5 min after the treatment. Subcutaneous pretreatment with ultra-low doses of β-funaltrexamine at 1 or 2 mg/kg markedly reduced the attenuation of endomorphin-2-induced paw-withdrawal inhibition by i.t. treatment with an antiserum against dynorphin A-(1–17) (Fig. 6). Likewise, s.c. pretreatment with ultra-low doses of naloxonazine from 0.031 to 0.5 mg/kg dose-dependently blocked the attenuation of endomorphin-2-induced paw-withdrawal inhibition by i.t. treatment with an antiserum against dynorphin A-(1–17) (Fig. 7).

![Fig. 4. Effect of β-funaltrexamine (β-FNA) on the attenuation of endomorphin-2 (EM-2)-induced antinociception by pretreatment with nor-binaltorphimine (nor-BNI). Both nor-BNI (10 mg/kg) and β-FNA (0.25–3.0 mg/kg) were administered s.c. 24 h before i.t. administration of EM-2 (5 nmol). The inhibition of the paw-withdrawal response induced by EM-2 was measured 5 min after the treatment. Each value represents the mean ± S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni test. The F value of the one-way ANOVA was F(6,63) = 8.527 (< 0.01). *p < 0.05; **p < 0.01 compared with EM-2 plus nor-BNI.](Image 4)

![Fig. 5. Effect of naloxonazine (NLZ) on the attenuation of endomorphin-2 (EM-2)-induced antinociception by pretreatment with nor-binaltorphimine (nor-BNI). Both nor-BNI (10 mg/kg) and NLZ (0.25–2.0 mg/kg) were administered s.c. 24 h before i.t. administration of EM-2 (5 nmol). The inhibition of paw-withdrawal response induced by EM-2 was measured 5 min after the treatment. Each value represents the mean ± S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni test. The F value of the one-way ANOVA was F(5,54) = 10.57 (< 0.01). **p < 0.01 compared with EM-2 plus nor-BNI.](Image 5)

![Fig. 6. Effect of β-funaltrexamine (β-FNA) on the attenuation of endomorphin-2 (EM-2)-induced antinociception by pretreatment with an antiserum against dynorphin A-(1–17) (Anti-DynA). β-FNA (0.25–2.0 mg/kg s.c.) and Anti-DynA (1:50 dilution i.t.) were administered 24 h and 15 min before i.t. administration of EM-2 (5 nmol), respectively. The inhibition of paw-withdrawal response induced by EM-2 was measured 5 min after the treatment. Each value represents the mean ± S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni test. The F value of the one-way ANOVA was F(4,45) = 8.527 (< 0.01). *p < 0.05; **p < 0.01 compared with EM-2 plus Anti-DynA.](Image 6)

**Discussion**

Our previous studies showed that inhibition of the paw-withdrawal response induced by endomorphin-1 or endomorphin-2 given i.t. is blocked by s.c. pretreatment with naloxone and the selective μ-opioid receptor antagonist β-funaltrexamine, indicating that the antinociception induced by both endomorphin-1 and endomorphin-2 given spinally is mediated by the stimulation of μ-opioid receptors (Sakurada et al., 2000a, 2001). The finding is consistent with evidence that endomorphin-1 and endomorphin-2 have a high affinity for μ-opioid receptors but have no appreciable affinity for either δ- or κ-opioid receptors (Zadina et al., 1997). Naloxonazine
irreversibly binds to $\mu_1$-opioid receptors and inhibits supraspinal antinociception (Ling et al., 1986; Paul et al., 1989; Pick et al., 1991). Recent behavioral pharmacological studies suggest the presence of $\mu_2$-opioid receptors that are sensitive to naloxonazine in spinal and supraspinal sites as assayed with the formalin test, hot-plate test, tail-pressure test, and tail-flick tests (Sato et al., 1999; Sakurada et al., 1999, 2000a,b). Autoradiographic studies show that $\mu_1$- and $\mu_2$-opioid receptors are localized in spinal and supraspinal structures involved in the modulation of nociception (Moskowitz and Goodman, 1985). Naloxonazine at a dose of 35 mg/kg (s.c.) has been used to selectively block $\mu_2$-opioid receptors in mice (Ling et al., 1986). The antinociceptive activity of endomorphin-2, but not endomorphin-1, given i.t. was completely blocked by pretreatment with this dose of naloxonazine (Sakurada et al., 2001), suggesting that spinal antinociception induced by endomorphin-2 and endomorphin-1 is mediated through the $\mu_1$-opioid receptor and the $\mu_2$-opioid receptor, respectively.

In the receptor binding assay, endomorphin-2 can bind both $\mu_1$- and $\mu_2$-opioid receptors but shows higher affinity for $\mu_1$-opioid receptors than for $\mu_2$-opioid receptors (Goldberg et al., 1998). In the mice pretreated s.c. with naloxonazine (35 mg/kg), an irreversible antagonist for $\mu_1$-opioid receptor, the dose-response curve of endomorphin-2 injected i.t. for antinociception is shifted to the right in a parallel manner (Sakurada et al., 1999). In contrast, endomorphin-2 given i.t. cannot produce the antinociception, even at higher doses in the mice pretreated s.c. with $\beta$-funtalrexamine, an irreversible antagonist for both $\mu_1$- and $\mu_2$-opioid receptors. These findings suggest that, although endomorphin-2 produces the spinal antinociception predominantly mediated through the $\mu_1$-opioid receptor, endomorphin-2 can produce the antinociception via the $\mu_2$-opioid receptor if the $\mu_1$-opioid receptor is occupied.

We found in a recent study that H-Tyr-D-Arg-Phe-$\beta$-Ala-OH (TAPA), a dermorphin tetrapeptide analog, is highly selective for the $\mu_1$-opioid receptor (Mizoguchi et al., 2004). More recently, we have demonstrated that the inhibitory effect induced by i.t. administered TAPA against i.t. capsaicin-elicited nociceptive responses is more potent than that against i.t. substance P-elicited nociceptive responses and was almost completely attenuated by s.c. pretreatment with naloxonazine, but not by i.t. coadministration of H-Tyr-D-Pro-Trp-Gly-NH$_2$ (D-Pro$^2$-Tyr-W-MIF-1), the $\mu_2$-opioid receptor antagonist (Watanabe et al., 2005), suggesting that TAPA may selectively act at $\mu_1$-opioid receptors localized presynaptically on excitatory amino acid- and neuropeptide-containing axon terminals in the dorsal horn (Watanabe et al., 2006). Likewise, antinociception induced by i.t. administered endomorphin-2 on capsaicin-induced nociceptive responses but not substance P-induced nociceptive responses was completely antagonized by s.c. pretreatment with naloxonazine (H. Watanabe, unpublished data). Endomorphin-2-like immunoreactivity is diminished by dorsal rhizotomy or exposure to capsaiacin and is colocalized with calcitonin gene-related peptide or substance P (Martin-Schild et al., 1997, 1998; Pierce et al., 1998). Our above results with endomorphin-2 on capsaicin-induced nociceptive responses are in agreement with the findings reported by Martin-Schild et al. (1997, 1998) and Pierce et al. (1998). These results suggest that the release of neurotransmitters from nociceptive primary afferents is regulated by endomorphin-2 in primary afferent terminals through the $\mu_1$-opioid receptors.

Prodynorphin produces three main peptides: $\alpha$-neoendorphin, dynorphin A-(1–17), and dynorphin B-(1–13). High levels of dynorphins are found in the brain as well as the dorsal horn of the spinal cord where modulation of nociceptive information occurs (Weber et al., 1982; Slater and Patel, 1983; Tan-No et al., 1997). Because dynorphin family members, such as dynorphin A-(1–17), dynorphin B-(1–13), and $\alpha$-neoendorphin, have a high affinity for the $\kappa$-opioid receptor, dynorphins have been suggested to be the endogenous ligands for $\kappa$-opioid receptors (Chavkin and Goldstein, 1981; Chavkin et al., 1982). Dynorphins have been shown to produce antinociception when administered i.t. (Han and Xie, 1982; Piercey et al., 1982). $\kappa$-Opioid and $\mu$-opioid receptor mRNA is intensely expressed in substance P-containing neurons (Sato and Minami, 1995). Therefore, $\kappa$- and $\mu$-opioid receptor agonists have been considered to act directly on the primary afferent terminals of substance P-containing neurons to presynaptically modulate the release of substance P (Jesse and Iversen, 1977; Zachariou and Goldstein, 1997). In the present study, we explored the possibility that i.t. administered endomorphin-2-induced antinociception is mediated by the spinal release of dynorphin A-(1–17) via $\mu$-opioid receptors. We found that i.t. pretreatment with an antiserum against dynorphin A-(1–17) or s.c. pretreatment with the $\kappa$-opioid receptor antagonist norbinaltorphimine attenuated the paw-withdrawal inhibition induced by i.t. administered endomorphin-2. However, i.t. pretreatment with an antiserum against dynorphin B-(1–13) or $\alpha$-neoendorphin did not block the endomorphin-2-induced antinociception, indicating that the effect of endomorphin-2 is selective for the release of dynorphin A-(1–17). This view is supported by the previous findings that the antinociception produced by spinally administered endomorphin-2 is attenuated by i.t. pretreatment with an antiserum against dynorphin.
A-(1–17) or the κ-opioid receptor antagonist norbinaltorphimine (Ohsawa et al., 2001; Sakurada et al., 2001).

It should be noted that the pharmacological ability of endomorphin-2 to release dynorphin A-(1–17) may be mainly involved in several discrepancies between endomorphin-1 and endomorphin-2 on their pharmacological effects, especially on the rewarding effect and locomotor enhancement. Endomorphin-1 given i.c.v. produces a remarkable rewarding and locomotor-enhancing effect in a dose-dependent manner (Bujdoso et al., 2001b; Narita et al., 2001). In contrast, endomorphin-2 given i.c.v. shows a bell-shaped dose-response curve for the rewarding and locomotor-enhancing effects and prominently produces an aversive rather than rewarding effect (Bujdoso et al., 2001a; Narita et al., 2001). As is well known, the rewarding and locomotor-enhancing effects of μ-opioid receptor agonists are mediated by the disinhibition of mesolimbic and nigrostriatal dopaminergic neurons via the activation of μ-opioid receptors located on the GABAergic neurons in the ventral tegmental area and substantia nigra, respectively (Narita et al., 2001). In the terminal of mesolimbic and nigrostriatal dopaminergic neurons on the nucleus accumbens and striatum, respectively, dynorphinergic neurons are localized to inhibit the release of dopamine. As we found for the spinal antinociception in the present study, unlike endomorphin-1, endomorphin-2 has a characteristic pharmacological ability to release dynorphin A-(1–17). The release of dynorphin A-(1–17) in the nucleus accumbens and striatum may be involved in the lack of the remarkable rewarding effect and locomotor enhancing effect of endomorphin-2. In fact, endomorphin-2 shows a remarkable rewarding effect when dynorphin A-(1–17) is blocked by the use of its antisera (Narita et al., 2001).

In the present study, s.c. pretreatment with ultra-low doses of β-funtrelxamine or naloxonazine that are ineffective against endomorphin-2 surprisingly prevented the attenuation of i.t. administered endomorphin-2-induced antinociception by i.t. pretreatment with an antisera against endomorphin-2 and s.c. pretreatment with norbinaltorphimine. These findings suggest that endomorphin-2 preferentially stimulates a distinct μ-opioid receptor (μ1-opioid receptor subtype-1), which is extremely sensitive to naloxonazine and subsequently induces the release of dynorphin A-(1–17) that acts on κ-opioid receptors to produce antinociception. However, if the μ1-opioid receptor subtype-1 is occupied by ultra-low doses of naloxonazine or β-funtrelxamine, endomorphin-2 should bind to another μ1-opioid receptor (μ1-opioid receptor subtype-2). Therefore, the component of antinociception induced by i.t. administered endomorphin-2 depends on the release of dynorphin A-(1–17), endomorphin-2-induced antinociception was not affected by the blockade of released dynorphin A-(1–17) after s.c. pretreatment with ultra-low doses of naloxonazine or β-funtrelxamine. This finding led us to speculate that, when ultra-low doses of naloxonazine or β-funtrelxamine interrupt endomorphin-2 binding to the μ1-opioid receptor subtype-1, which regulates the release of dynorphin A-(1–17) from dynorphinergic neurons that project to primary afferent terminals, endomorphin-2 at primary afferent terminals may act on the μ1-opioid receptor subtype-2, which is not involved in the release of dynorphin A-(1–17) (Fig. 8). Judging from the sensitivity to naloxonazine, we first propose here that there are two subtypes of μ1-opioid receptors that are involved in endomorphin-2-induced antinociception. One, an μ1-opioid receptor subtype-2, is sensitive to β-funtrelxamine (40 mg/kg s.c.) and naloxonazine (10–35 mg/kg s.c.) (Sakurada et al., 2000a) and is not involved in the release of dynorphin A-(1–17). The other, an μ1-opioid receptor subtype-1, is involved in the release of dynorphin A-(1–17) acting on κ-opioid receptors as an additional component of endomorphin-2-induced antinociception and is highly sensitive to both naloxonazine and β-funtrelxamine. The present study provides important evidence in the investigation of the spinal neuronal circuit for production of endomorphin-2-induced antinociception.

In conclusion, endomorphin-2 given spinaly produces the antinociception via simulation of multiple μ1-opioid receptors, μ1-opioid receptor subtype-1 and μ1-opioid receptor subtype-2. The μ1-opioid receptor subtype-1, which is extremely sensitive to naloxonazine and β-funtrelxamine, may be involved in the additional component of endomorphin-2-induced antinociception, which is mediated through the release of the endogenous κ-opioid peptide dynorphin A-(1–17).

References


Pick CG, Paul D, and Pasternak GW (1991) Comparison of naloxone and 


Address correspondence to: Dr. Shinobu Sakurada, Tohoku Pharmaceutical University, Department of Physiology and Anatomy, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan. E-mail: s-sakura@tohoku-pharm.ac.jp.