**D-Pro²-Endomorphin-1 and D-Pro²-Endomorphin-2, Respectively, Attenuate the Antinociception Induced by Endomorphin-1 and Endomorphin-2 Given Intrathecally in the Mouse**

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**ABSTRACT**

First, the antinociception with the tail-flick test of D-Pro²-endomorphin-1 and D-Pro²-endomorphin-2 given i.t. was compared with that produced by endomorphin-1 and -2 in male CD-1 mice. High doses of D-Pro²-endomorphin-1 (0.2–0.4 pmol) and D-Pro²-endomorphin-2 (300–800 pmol) given i.t. produced antinociception with low intrinsic activity [about 25% maximum possible effect (MPE)] compared with that of endomorphin-1 (16.4 nmol) and endomorphin-2 (35 nmol) (>90% MPE). Second, coadministration of a low dose of D-Pro²-endomorphin-1 (0.1 pmol), which given alone did not affect the tail-flick latencies, markedly attenuated the antinociception induced by endomorphin-1 (16.4 nmol) but not by endomorphin-2 (35 nmol). Similarly, coadministration of a low dose of D-Pro²-endomorphin-2 (200 pmol), which given alone did not affect the tail-flick latencies, significantly attenuated the antinociception induced by endomorphin-2 (35 nmol) and, to a much lesser extent, endomorphin-1 (16.4 nmol). It is concluded that D-Pro²-endomorphin-1 and D-Pro²-endomorphin-2 at high doses were partial opioid receptor agonists to produce antinociception, and at low doses were opioid receptor antagonists to block selectively the antinociception induced by endomorphin-1 and endomorphin-2, respectively. Furthermore, our results are consistent with the view that the antinociception induced by endomorphin-1 and endomorphin-2 is mediated by the stimulation of different subtypes of μ-opioid receptors.

Endomorphin-1 and endomorphin-2 are two endogenous tetrapeptides isolated from the bovine frontal cortex (Zadina et al., 1997) and human brain (Hackler et al., 1997). These peptides are the first endogenous peptides to be proposed to have high affinity and selectivity for μ-opioid receptors. Receptor-binding assays and immunocytochemical studies reveal that endomorphin-1 and endomorphin-2 potently compete with μ₁- and μ₂-receptors and that they are widely located at the sites in the brain and spinal cord abundant in μ-opioid receptors (Martin-Schild et al., 1997, 1998, 1999; Goldberg et al., 1998; Pierce et al., 1998; Shreff et al., 1998; Wu et al., 1999). Through the stimulation of μ-opioid receptors, endomorphin-1 and endomorphin-2 inhibit the electrical activity of rostral ventrolateral medulla neurons or spinal substantia gelatinosa neurons (Chu et al., 1999; Wu et al., 1999). The release of endomorphin-2 from the spinal cord can be achieved by electrical stimulation (Williams et al., 1999). The administration of endomorphin-1 and endomorphin-2 given i.c.v. and i.t. produces potent antinociceptive responses that are blocked by μ-opioid receptors antagonists naloxone, β-funaltrexamine, and D-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) (Zadina et al., 1997; Stone et al., 1997; Narita et al., 1998; Tseng et al., 2000). Neither endomorphin-1 nor endomorphin-2 activates μ-opioid receptor-coupled G proteins in μ-opioid receptor knockout mice, and the antinociception induced by endomorphin-1 and endomorphin-2 is attenuated in heterozygous knockout mice and virtually abolished in homozygous knockout mice (Mizoguchi et al., 1999). These findings support the view that antinociception induced by the endomorphin-1 and endomorphin-2 is mediated by the stimulation of μ-opioid receptors.

**ABBREVIATIONS:** CTOP, D-Phe-Cys-Tyr-o-Trp-Orn-Thr-Pen-Thr-NH₂; β-FNA, β-funaltrexamine; nor-BNI, nor-binaltorphimine; MPE, maximum possible effect; ANOVA, analysis of variance; D-Pro-EM-1, D-Pro²-endomorphin-1; D-Pro-EM-2, D-Pro²-endomorphin-2.
However, more recent results illustrate that different subtypes of $\mu$-opioid receptors may be involved in antinociceptive effects induced by endomorphin-1 and endomorphin-2. For example, Sakurada et al. (1999) reported that $\mu_1$-opioid receptor antagonist naloxoxazine was more effective in blocking the antinociceptive effects induced by endomorphin-2 than endomorphin-1 in mice. The antinociception induced by endomorphin-1 is blocked by $\mu$-opioid receptor antagonists CTOP or $\beta$-funaltrexamine ($\beta$-FNA) but not by $\kappa$-opioid antagonist nor-binaltorphimine (nor-BNI). On the other hand, the antinociception induced by endomorphin-2 is blocked by CTOP or $\beta$-FNA and also by nor-BNI (Tseng et al., 2000; Ohsawa et al., 2001). The findings are taken to indicate that two different subtypes of $\mu$-opioid receptors are involved in endomorphin-1- and endomorphin-2-induced antinociception.

**Materials and Methods**

**Animals.** Male CD-1 mice (Charles River Laboratories, Inc., Wilmington, MA), weighing 25 to 30 g were used. Animals were housed five per group in a room maintained at 22 ± 0.5°C with an alternating 12-h light/dark cycle. Food and water were available ad libitum.

**Drugs.** Endomorphin-1 (Tyr-Pro-Trp-Phe-NH$_2$) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH$_2$) were purchased from Calbiochem (La Jolla, CA). The peptides were dissolved in sterile saline solution (0.9% NaCl solution) containing 10% hydroxypropyl-$\beta$-cyclodextrin for i.t. injection. D-Pro$_2$-Endomorphin-1 (Tyr-D-Pro-Trp-Phe-NH$_2$) and D-Pro$_2$-endomorphin-2 (Tyr-D-Pro-Phe-Phe-NH$_2$) were obtained from Dr. T. Sakurada (Department of Biochemistry, Daiichi College of Pharmaceutical Science, Fukuoka, Japan). These D-Pro$_2$-endomorphins were first completely dissolved in dimethyl sulfoxide and then 0.9% sodium chloride solution was added to a final concentration of dimethyl sulfoxide at 1%.

**Assessment of Antinociceptive Response.** The antinociceptive response was assessed with the thermal tail-flick test (D’Amour and Smith, 1941). Mice were gently held with the tail positioned in the tail-flick apparatus (model TF6; EMDIE Instrument Co., Maidens, VA) for radiant heat stimulation of the dorsal surface of the tail. The intensity of the heat stimulus was adjusted to cause the animal to flick its tail within 3 to 4 s the baseline of latency. After measuring the latency, different groups of mice were treated with endomorphin-1, endomorphin-2, or vehicle given i.t., and the tail-flick responses were then measured at different times after injection. The data were expressed as percentage of maximum possible effect (%MPE), which was calculated as $[(T_0 - T_p)/(T_p - T_0)] \times 100$. $T_0$ and $T_p$ were predrug and postdrug latency, respectively, and $T_p$ was the cutoff time that was set at 10 s to minimize tissue damage.

**Drug Administration Protocol.** The i.t. injection (5 μl) was performed according to the procedure of Hylden and Wilcox (1980) using a 25-μl Hamilton syringe with a 30-gauge needle. Groups of mice were injected i.t. with various doses of endomorphin-1 (0.82–16.4 nmol), endomorphin-2 (1.75–35.0 nmol), D-Pro$_2$-endomorphin-1 (0.03–0.4 pmol), D-Pro$_2$-endomorphin-2 (50–800 pmol), or vehicle, and the tail-flick tests were performed at 2.5, 5, 7.5, 10, 15, and 20 min thereafter. The effects of D-Pro$_2$-endomorphin-1 and D-Pro$_2$-endomorphin-2 on the tail-flick inhibition induced by endomorphin-1 and endomorphin-2 were studied. Groups of mice were coadministered i.t. with various doses of D-Pro$_2$-endomorphin-1 (0.03–0.4 pmol) or D-Pro$_2$-endomorphin-2 (50–800 pmol) with endomorphin-1 (16.4 nmol) or endomorphin-2 (35 nmol) and the tail-flick response was measured thereafter. In another experiment, groups of mice were coadministered i.t. with D-Pro$_2$-endomorphin-1 (0.1 pmol) or D-Pro$_2$-endomorphin-2 (200 pmol) with various doses of endomorphin-1 (0.82–16.4 nmol) or endomorphin-2 (1.75–35 nmol), and the tail-flick response was measured thereafter. To establish dose-response curves, at least four doses were used with 8 to 11 mice at each dose. For the calculation of the ED$_{50}$ values for endomorphin-1- and endomorphin-2-induced tail-flick inhibition, the antinociception was assessed using peak effect, which occurred at either 2.5 or 5 min after administration.

**Statistical Analysis.** The data were expressed as the mean with S.E.M. The maximal %MPE was used to graph dose-response curves for endomorphin-1 and endomorphin-2. GraphPad Prism software (version 3.0; GraphPad Software, San Diego, CA) was used to calculate dose-response curves, ED$_{50}$ values and their confidence intervals. A two-way ANOVA followed by Bonferroni’s post test was used to determine the time in which the attenuation of antinociception reached a maximum by D-Pro$_2$-endomorphin-1 or D-Pro$_2$-endomorphin-2 administration. A one-way ANOVA followed by Dunnett’s test was used to compare the difference for each group versus the control group.

**Results**

**Time Courses and Dose Effects of i.t. Administration of Endomorphin-1, Endomorphin-2, D-Pro$_2$-Endomorphin-1, and D-Pro$_2$-Endomorphin-2 on Tail-Flick Response.** Groups of mice were injected i.t. with different doses of endomorphin-1, endomorphin-2, D-Pro$_2$-endomorphin-1, D-Pro$_2$-endomorphin-2, or vehicle, and the tail-flick response was measured 2.5, 5, 7.5, 10, 15, and 20 min after injection. Intrathecal injection of endomorphin-1 at doses 0.82 to 16.4 nmol (Fig. 1A) and endomorphin-2 at doses 1.75 to 35.0 nmol (Fig. 1B) dose- and time-dependently produced inhibition of the tail-flick response. The inhibition of the tail-flick response induced by endomorphin-1 and endomorphin-2 developed rapidly, reached their peak at 5 min, declined rapidly, and returned to the preinjection level in 20 min. A dose 16.4 nmol of endomorphin-1 or 35 nmol of endomorphin-2 produced about 90% MPE. The antinociceptive ED$_{50}$ values of endomorphin-1 and endomorphin-2 are shown in Table 1. Endomorphin-1 was found to be 2.3-fold more potent than endomorphin-2 to produce the tail-flick inhibition.

D-Pro$_2$-Endomorphin-1 (0.1–0.4 pmol) (Fig. 2A) and D-Pro$_2$-endomorphin-2 (200–800 pmol) (Fig. 2B) given i.t. produced an apparent dose-dependent inhibition of the tail-flick response. However, at the three highest doses of each, there was a ceiling effect (about 25% MPE) where the increase in dose did not lead to a greater effect. It seemed that D-Pro$_2$-endomorphin-1 was about 2000-fold more potent than D-Pro$_2$-endomorphin-2 to produce the same ceiling effect. A small dose of D-Pro$_2$-endomorphin-1 (0.1 pmol) or D-Pro$_2$-endomorphin-2 (200 pmol) did not show any appreciable tail-flick inhibition.

**Effects of D-Pro$_2$-Endomorphin-1 and D-Pro$_2$-Endomorphin-2 on Tail-Flick Inhibition Induced by Endomorphin-1 and Endomorphin-2, Respectively.** The presence of low, ceiling antinociceptive action suggested that these analogs might be acting on the same respective opioid receptors as were endomorphin-1 and endomorphin-2. Then, it might be possible that either a positive or negative effect might be shown for these analogs to modify antinociception produced by endomorphin-1 and endomorphin-2. Groups of
mice were administered with various doses of D-Pro2-endo-
morphin-1 (0.03–0.4 pmol) together with an antinociceptive
dose of 16.4 nmol of endomorphin-1. Treatment with D-Pro2-
endomorphin-1 at 0.1 pmol, but not at other higher or lower
doses significantly attenuated the tail-flick inhibition in-
duced by endomorphin-1 (Fig. 3A).

A similar experiment was performed for the interaction of
D-Pro2-endomorphin-2 with endomorphin-2. D-Pro2-Endom-
orphin-2 at 100, 200, and 300 pmol attenuated the tail-flick
inhibition induced by endomorphin-2 (35 nmol) (Fig. 3B).

A crossover experiment then performed with the effective
doses of the analogs. As seen in Fig. 3A, 200 pmol of D-Pro2-
endomorphin-2 had a significant effect to reduce the tail-flick
inhibition induced by endomorphin-1. However, the effect did
not seem to be as great as it was against endomorphin-2 (Fig.
3B). When D-Pro2-endomorphin-1 was evaluated against
endomorphin-2-induced tail-flick inhibition, it had no signifi-
cant effect (Fig. 3B). Thus, the data indicated that there was
minimal crossover of the effects of D-Pro2-endomorphin-1 and
D-Pro2-endomorphin-2 relative to their selectivity of action
against endomorphin-1- and endomorphin-2-mediated an-
inociception.

In the preceding set of experiments, the analogs were given
at the same time as endomorphin-1 and endomorphin-2. In
the experiment given in Fig. 4, A and B, the time of treatment
with the analogs was given 0 (together), 5, and 10 min sep-
arately before the endomorphin-1 and endomorphin-2, and
tail-flick responses were then measured after the injection.
In each case, the respective analogs were most effective when
each was given at the same time (0 min) as the endomor-
phin-1 and endomorphin-2.

Effects of i.t. Treatment with a Fixed Dose of D-Pro2-
Endomorphin-1 and D-Pro2-Endomorphin-2 on Dose-
Response Curves for i.t. Endomorphin-1- and Endom-
orphin-2-Induced Tail-Flick Inhibition. Endomorphin-1
at doses 0.82 to 16.4 nmol or endomorphin-2 at doses 1.75 to
35 nmol given i.t. dose-dependently inhibited the tail-flick
response. The ED50 values, Hill slope function, and their 95%
confidence intervals are given in Table 1. Intrathecal coad-
ministration of 0.1 pmol of D-Pro2-endomorphin-1 with endo-
morphin-1 markedly attenuated the antinociceptive response
induced by endomorphin-1; the dose-response curve for en-
domorphin-1 was shifted to the right by 5.5-fold. The shift of
the dose-response curve for endomorphin-1-induced tail-flick
inhibition after D-Pro2-endomorphin-1 was not significantly
deviated from parallel (Fig. 5A; Table 1). Similarly, i.t. coad-
ministration of 200 pmol of D-Pro2-endomorphin-2 with endo-
morphin-2 markedly attenuated the antinociceptive re-
sponse induced by endomorphin-2; the dose-response curve
for endomorphin-2 was shifted to the right by 3.9-fold. The
shift of the dose-response curve for endomorphin-2-induced
tail-flick inhibition after D-Pro2-endomorphin-2 was not sig-
nificantly deviated from parallel (Fig. 5B; Table 1).

Discussion

As in our previous report (Ohsawa et al., 2001), i.t. admin-
istration of endomorphin-1 and endomorphin-2 produced an-
inociception with full intrinsic activity (>90% MPE). Endo-
morphin-1 was about 2.3-fold more potent than endomorphin-2. The analogs of endomorphins, D-Pro2-endomor-
phin-1 and D-Pro2-endomorphin-2, at high doses, also

![Fig. 1. Time course of changes in the tail-flick inhibition induced by i.t.-injected endomorphin-1 (EM-1; A) and endomorphin-2 (EM-2; B).](image)

**TABLE 1**
The antinociceptive potencies (ED50) and the slope function of dose-response curves for i.t. administration of endomorphin-1 and endomorphin-2 given alone or with D-Pro2-endomorphin-1 or D-Pro2-endomorphin-2

<table>
<thead>
<tr>
<th></th>
<th>ED50 (95% CI)</th>
<th>Hill Slope (95% CI)</th>
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<tbody>
<tr>
<td>Endomorphin-1</td>
<td>2.41 (1.84–3.15)</td>
<td>1.06 (0.74–1.37)</td>
</tr>
<tr>
<td>Endomorphin-2</td>
<td>5.60 (4.43–7.09)</td>
<td>1.44 (0.94–1.94)</td>
</tr>
<tr>
<td>Endomorphin-1 + 0.1 pmol D-Pro2-endomorphin-1</td>
<td>13.11 (7.14–24.08)</td>
<td>0.64 (0.23–1.05)</td>
</tr>
<tr>
<td>Endomorphin-2 + 200 pmol D-Pro2-endomorphin-2</td>
<td>21.63 (12.10–38.64)</td>
<td>0.74 (0.36–1.12)</td>
</tr>
</tbody>
</table>

CI, confidence interval.
produced antinociception, but with low intrinsic activity (about 25% MPE) and a ceiling effect. Thus, D-Pro²-endomorphin-1 and D-Pro²-endomorphin-2 were partial agonists compared with endomorphin-1 and endomorphin-2. The antinociceptive properties of D-Pro²-endomorphin-1 and D-Pro²-endomorphin-2 we found in the present studies in mice are consistent with the findings by others in rats (Shane et al., 1999; Krzanowska et al., 2000).

We found in the present studies that D-Pro²-endomorphin-1 and D-Pro²-endomorphin-2 at small doses are antagonists and antagonized the antinociception induced by endomorphin-1 and endomorphin-2, respectively. Coadministration of D-Pro²-endomorphin-2 with endomorphin-2 given i.t. attenuated the antinociception induced by endomorphin-2 and only slightly attenuated the antinociception induced by endomorphin-1. These results suggest that D-Pro²-endomorphin-2 seem to block the μ-opioid receptors predominantly stimulated by endomorphin-2 and to a lesser extent μ-opioid receptors stimulated by endomorphin-1. In addition, coadministration with D-Pro²-endomorphin-1 with endomorphin-1 attenuated the antinociception induced by endomorphin-1, but not endomorphin-2, indicating that D-Pro²-endomorphin-1 blocks μ-opioid receptors stimulated by endomorphin-1 only but not by endomorphin-2. The results of our present studies provide additional evidence to support the view that the antinociception induced by endomorphin-1 and endomorphin-2 is mediated by the stimulation of different subtypes of μ-opioid receptors.

D-Pro²-Endomorphin-1 and D-Pro²-endomorphin-2 blocked the antinociception induced by endomorphin-1 and endomorphin-2 only at small doses, but not at high doses. Only 0.1 pmol of D-Pro²-endomorphin and 100 to 300 pmol of D-Pro²-endomorphin-2, but not higher doses, blocked the antinociception induced by endomorphin-1 and endomorphin-2, respectively. Because D-Pro²-endomorphin-1 and D-Pro²-endomorphin-2 at high doses produced a weak antinociception with low intrinsic activity and ceiling effect, the failure for high doses of D-Pro²-endomorphin-1 or D-Pro²-endomorphin-2 to block the endomorphin-1- or endomorphin-2-induced antinociception is unexpected. The endomorphin-1 was found to be only
2.3-fold more potent than endomorphin-2 to produce the antinociception, but \( \text{D-Pro}^2 \)-endomorphin-1 was found to be at least 2000-fold more potent than \( \text{D-Pro}^2 \)-endomorphin-2 to produce the antinociception and to block the antinociception induced by endomorphin-1 and endomorphin-2, respectively (Figs. 2 and 3; Table 1). It is possible that \( \text{D-Pro}^2 \)-endomorphin-1 and \( \text{D-Pro}^2 \)-endomorphin-2 may, respectively, bind to different subtypes of \( \mu \)-opioid receptors to produce agonistic and antagonistic effects.

The view that different subtypes of \( \mu \)-opioid receptors are involved in endomorphin-1- and endomorphin-2-induced antinociception has been reported (Sakurada et al., 1999, 2000; Ohsawa et al., 2000; Wu et al., 2001). Pretreatment with \( \mu \)-opioid receptor antagonist naloxonazine is more effective in antagonizing the antinociception induced by endomorphin-2 than endomorphin-1. Also pretreatment with 3-methylnaltrexone, a morphine-6\( \beta \)-glucuronide antagonist, blocks the antinociception induced by endomorphin-2, but not endomorphin-1 (Sakurada et al., 1999, 2000). A unidirectional cross-tolerance between endomorphin-1 and endomorphin-2 in mice has been reported. Mice made tolerant to endomorphin-1 by i.c.v. pre-
treatment with endomorphin-1 exhibit nearly no cross-tolerance to endomorphin-2 to produce antinociception. On the other hand, mice made tolerant to endomorphin-2 exhibit partial cross-tolerance to endomorphin-1 (Wu et al., 2001). The antinociception induced by endomorphin-1 is blocked by -opioid receptor antagonists CTOP or -FNA but not by -opioid antagonist nor-BNI. On the other hand, the antinociception induced by endomorphin-2 is blocked by CTOP, -FNA, or nor-BNI (Tseng et al., 2000; Ohsawa et al., 2001). Furthermore, i.t. pretreatment with antiserum against dynorphin A(1-17) blocks the antinociception induced by endomorphin-2, but not endomorphin-1. Thus, the antinociception induced by endomorphin-1 and endomorphin-2 is mediated by the stimulation of different subtypes of -opioid receptors. The endomorphin-2-induced antinociception contains an additional component, which is mediated by the spinal release of dynorphin A(1-17) acting on -opioid receptors in the spinal cords (Ohsawa et al., 2001).

In opioid receptor binding assay in mouse brain homogenates, both endomorphin-1 and endomorphin-2 compete for both -1 and -2-receptor binding sites potently, consistent with the view that the antinociception induced by endomorphin-1 and endomorphin-2 is primarily mediated by the stimulation of -opioid receptors. However, the study was unable to identify the presence of different subtypes of -opioid receptors for endomorphin-1 and endomorphin-2 (Goldberg et al., 1998).

It is concluded that D-Pro endomorphin-1 and D-Pro endomorphin-2 at high doses are partial opioid agonists that produce weak antinociception and at low doses are antagonists to block selectively the antinociception induced by endomorphin-1 and endomorphin-2, respectively. Our results provide evidence that the antinociception induced by endomorphin-1 and endomorphin-2 is mediated by the stimulation of different subtypes of -opioid receptors.

References


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