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Antinociception by a peripherally administered novel endomorphin-1 analogue containing β-proline

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Abstract

We previously described a novel endomorphin-1 analogue (Tyr-L-β-Pro-Trp-Phe-NH_2; Endo1-β-Pro) more resistant to enzymatic hydrolysis than endomorphin-1 that acts as a μ-opioid receptor agonist. In this study we report that Endo1-β-Pro, s.c. injected in the mouse, is an effective antinociceptive agent in the tail flick (ED_{50} = 9.2 mg/kg) and acetic acid-induced abdominal constriction (ED_{50} = 1.2 mg/kg) tests. Moreover, s.c. Endo1-β-Pro significantly decreases, in the mouse, the gastrointestinal propulsion measured as transit of an orally administered charcoal meal (ED_{50} = 10.0 mg/kg). Subcutaneous β-funaltrexamine or a high dose of the μ_1-opioid receptor-selective antagonist naloxonazine (50 mg/kg) prevents the antinociceptive and antitransit action of Endo1-β-Pro; moreover, these effects are partially blocked by i.c.v. naloxone or by i.p. naloxone methiodide, this latter does not readily cross the blood–brain barrier. On the contrary, the κ-opioid receptor antagonist nor-binaltorphimine or the δ-opioid receptor antagonist naltrindole are ineffective Thus, Endo1-β-Pro may act, preferentially, through central and peripheral μ_2-opioid receptors to produce antinociception and to inhibit gastrointestinal transit. Endo1-β-Pro is among the first endomorphin-1 analogues showing antinociceptive activity after systemic administration. This compound will be extremely useful for exploring the pharmacological profile of endomorphins in vivo and confirms the potential therapeutic interest of endomorphin derivatives as novel analgesic agents.

Keywords: Endomorphin-1; Antinociception; Gastrointestinal motility; μ-Opioid receptor; Naloxonazine; Naloxone methiodide

1. Introduction

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) are peptides isolated from the mammalian brain which bind to μ-opioid receptors with high affinity and selectivity (Zadina et al., 1997; Horvath, 2000; Okada et al., 2002; Abbadie et al., 2002). Neither compound has appreciable affinity for δ- and κ-opioid receptors (Horvath, 2000). i.c.v. or i.t. injection of endomorphins produces potent antinociception, blocked by μ-opioid receptor antagonists, abolished in μ-opioid receptor knockout mice (Horvath, 2000), and lacking relevant rewarding properties (Wilson et al., 2000). Pharmacological and biochemical evidences support the hypothesis of the existence of at least two μ-opioid receptor subtypes (Pasternak and Wood, 1986). It has been suggested that μ_1-opioid receptors are blocked by β-funaltrexamine and naloxonazine whereas μ_2-opioid receptors' action is antagonized by β-funaltrexamine or a high dose (>35 mg/kg s.c.) of naloxonazine (Sakurada et al., 1999). Antinociception induced by endomorphin-1 seems to be mediated by μ_2-opioid receptors whereas endomorphin-2 may act, preferentially, through μ_1-opioid receptors (Sakurada et al., 1999). Endomorphins, i.c.v. or i.t. administered, display antinociceptive activity in different assays which are routinely employed to evaluate the pain threshold in laboratory animals exposed to an acute or inflammatory pain (Horvath, 2000) and appear to relieve neuropathic pain, which is assumed to be less sensitive to traditional opioid receptor agonists (Przewlocki and Przewlocka, 2001).

We previously described a novel endomorphin-1 analogue (Tyr-L-β-Pro-Trp-Phe-NH_2; Endo1-β-Pro), obtained by substituting proline with its β-amino acid homologue, which was more resistant to enzymatic hydrolysis than endomorphin-1; it bound with high affinity to μ-opioid

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receptors ($K_i = 2.1$ nM) and concentration-dependently inhibited forskolin-stimulated cyclic AMP formation, thus behaving as a μ-opioid receptor agonist (Cardillo et al., 2002). Here we describe the antinociceptive activity of Endo1-β-Pro administered peripherally in the mouse evaluated by the tail flick and the acetic acid-induced abdominal constriction tests. We have investigated any involvement of μ_1- and μ_2-opioid receptors by administering the selective μ_1-opioid receptor antagonist naloxonazine (Pasternak and Wood, 1986). Finally, to determine whether antinociception produced by peripheral Endo1-β-Pro was central or peripheral in nature, we evaluated the antagonist effect of naloxone methiodide, a quaternary derivative which does not readily cross the blood–brain barrier (Craft et al., 1995). Pharmacological characterization of Endo1-β-Pro was extended to evaluate any effect elicited by this compound on gastrointestinal propulsion measured as transit of an orally administered charcoal meal in the mouse. In fact opioids, including i.c.v. endomorphin-1 (Goldberg et al., 1998), inhibit gastrointestinal transit (for a review, see Schulz et al., 1979; Manara and Bianchetti, 1985; Burks et al., 1988).

2. Materials and methods

2.1. Drugs

Endo1-β-Pro was synthesized in our laboratories as previously described (Cardillo et al., 2002). Endomorphin-1, β-funaltrexamine hydrochloride, naloxonazine dihydrochloride and naloxone hydrochloride were purchased from Sigma/RBI (Natick, MA, USA). Naloxone methiodide, naltrindole hydrochloride and nor-binaltorphimine dihydrochloride were purchased from Tokris Cookson (Bristol, UK).

2.2. Animals and treatments

Adult male CD-1 mice (Charles River, Calco, Como, Italy) weighing 25–30 g were used. They were housed in a light- and temperature-controlled room (light on 08:00–20:00 h; 24 °C) and had free access to food and water. This research was conducted in compliance with the guidelines of the Animal Care and Use Committee of the University of Bologna and conformed to the International Association for the Study of Pain (IASP) guidelines on ethical standards for investigations of experimental pain in animals. i.c.v. injections were done in a volume of 2 μl of sterile artificial cerebrospinal fluid (CSF; 7.4 g NaCl, 0.19 g KCl, 0.19 g $MgCl_2$, 0.14 g $CaCl_2$/1000 ml) under ether anaesthesia as previously described (Haley and McCormick, 1957). The anaesthetic did not affect antinociceptive measurements up to 60 min later (data not shown). For s.c. or i.p. injections, the compounds were dissolved in saline (vehicle) and injected in a volume of 0.1 ml/10 g body weight.

2.3. Tests of antinociception

Nociception was evoked by immerging the mouse's tail in hot water (52 ± 0.5 °C) and measuring the latency to withdrawal. Before treatment, each mouse was tested and the latency to tail flick recorded (control latency, CL). Animals not flicking their tails within 5 s were not used (≈ 7% of mice employed in this study); baseline readings were 3.7 ± 0.4 s (mean ± S.E.M.; $n = 90$). The test was repeated 5, 10, 15, 30, 45 and 60 min after drug administration and the latency to tail flick was defined as the test latency (TL); a cut-off of 10 s was adopted. The antinociceptive response was expressed as % of maximum possible effect (MPE), calculated by the following equation: $MPE = 100 \times (TL - CL)/(10 - CL)$. Antinociception was also evaluated, in different groups of mice, by counting stretching or writhing responses during a 10-min period, after i.p. injection of acetic acid (0.1 ml/10 g of a 0.6% solution in water). Endo1-β-Pro (0.5–3.0 mg/kg) or saline (10 ml/kg) were injected s.c. 10 min prior to acetic acid solution. The control group was treated with saline and then with the acetic acid solution. In order to calculate the ED_{50}, the formula for computing an average percent of antinociception was: 100 − (average writes in the drug-treated group/average writes in the control group × 100). A temporal summary of pretreatment and treatment conditions of experiments carried out to evaluate the nociceptive threshold is shown in Fig. 1. Under these conditions, the action of opioid receptor antagonists has been shown effective in mice (Sakurada et al., 1999; Neilan et al., 2001).

2.4. Measurement of gastrointestinal transit

Gastrointestinal transit was assessed using the charcoal meal test in different groups of mice. Mice were fasted 18 h before the experiments, except that they had free access to water; then, they were treated with Endo1-β-Pro or saline. An

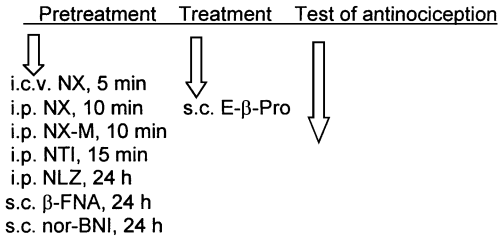

Fig. 1. Temporal summary of pretreatment and treatment conditions of experiments carried out to evaluate the nociceptive threshold. In the tail flick assay, the nociceptive threshold was evaluated 5 min before treatment and the test was repeated 5, 10, 15, 30, 45 and 60 min after Endo1-β-Pro. In the acetic acid-induced abdominal constriction test, the number of stretching or writhing responses was registered during a 10-min period after i.p. injection of acetic acid solution. Endo1-β-Pro was administered 10 min in advance. Mice were pretreated with the following opioid antagonists: naloxone hydrochloride (NX), naloxone methiodide (NX-M), naloxonazine (NLZ), β-funaltrexamine (β-FNA), nor-binaltorphimine (nor-BNI) and naltrindole (NTI).

aqueous charcoal suspension (10 ml/kg of a 10% activated charcoal suspension in 5% gum acacia) was given orally to each mouse, 10 min after saline or Endo1-β-Pro administration. Opioid receptor antagonists were administered before Endo1-β-Pro as indicated in the legend to Fig. 5. Mice were killed by cervical dislocation under ethyl ether anaesthesia 20 min after receiving the charcoal, the intestine was carefully removed and the omentum separated avoiding stretching. The total length of the small intestine from pylorus to ileocaecal junction as well as the length travelled by the charcoal was measured. The propulsive activity of the gut was calculated as the percentage of the distance travelled by the charcoal relative to the total length of the small intestine. The inhibitory effect of Endo1-β-Pro on gastrointestinal transit (GIT) was expressed as percent inhibition of transit in drug-treated animals (drug GIT) compared with the mean transit obtained in a group of vehicle-treated mice (vehicle GIT; $n = 8$) and calculated by the following equation: % inhibition = 100 × (vehicle GIT-drug GIT)/(vehicle GIT).

2.5. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical significance of the data was estimated by a mixed two-factor analysis of variance (ANOVA) or by one-way ANOVA and Dunnett's post hoc test. A level of probability of 0.05 or less was accepted as significant. The ED_{50} values and their 95% confidence limits (CL_{95}) were generated from dose–response curves using a computer program based on the method of Litchfield and Wilcoxon (Tallarida and Murray, 1987).

3. Results

3.1. Antinociceptive action of Endo1-β-Pro in the mouse

Endo1-β-Pro (5–20 mg/kg s.c.) resulted in a dose-related antinociceptive response in the tail flick assay; two-way ANOVA with repeated measures showed significant interactions between time and dose ($P < 0.01$) for the following intervals: (a) Endo1-β-Pro 5 mg/kg, 15 min; (b) Endo1-β-Pro 10 mg/kg, 5–30 min; (c) Endo1-β-Pro 20 mg/kg, 5–45 min; (d) NX + Endo1-β-Pro 20 mg/kg, 10 and 15 min (Fig. 2A). Antinociceptive action of Endo1-β-Pro was reduced by i.c.v. naloxone (10 nmol/mouse; Fig. 2A) whereas endomorphin-1 (at the doses up to 30 mg/kg s.c.) did not cause any antinociception (data not shown). The effect peaked 10 after injection ($ED_{50} = 9.2$ mg/kg; 95% CL_{95}: 7.4–11.4) (Fig. 2B). As shown in Fig. 3A, Endo1-β-Pro-induced antinociception was partially blocked by i.p. administered naloxone methiodide (30 mg/kg, given 10 min before) and fully antagonized by a higher dose of naloxonazine (50 mg/kg s.c.), injected 24 h before; on the contrary, lower doses of naloxonazine (10 mg/kg s.c., data not shown; or 35 mg/kg s.c., Fig. 3A) were ineffective as well as lower doses of naloxone methiodide (10 and 20 mg/kg i.p.; data not shown). β-Funaltrexamine (40 mg/kg s.c.; 24 h before) blocked the antinociception elicited by Endo1-β-Pro (20 mg/kg s.c.) (Fig. 3B). The κ-opioid receptor antagonist nor-binaltorphimine (32 mg/kg, s.c.; 24 h before) or the δ-opioid receptor antagonist naltrindole (10 mg/kg i.p. 15 min before) did not affect antinociception elicited by Endo1-β-Pro (20 mg/kg s.c.) (Fig. 3B). When administered alone, none of the antagonists altered the nociceptive threshold (data not shown).

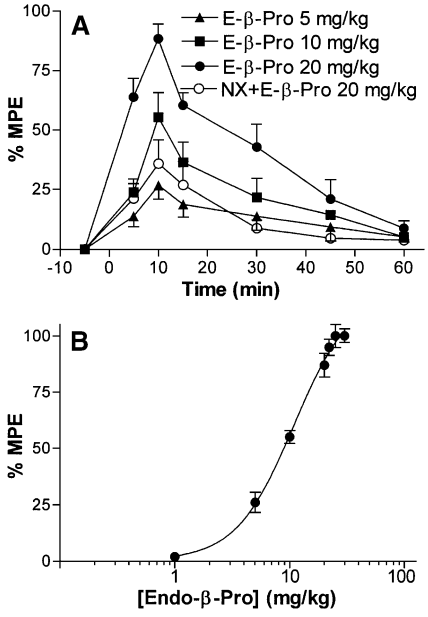

Fig. 2. Antinociception produced by Endo1-β-Pro (E-β-Pro) in the mouse tail flick assay. Antinociception was expressed as % of maximum possible effect (% MPE) as reported under Materials and methods. Each group represents the mean ± S.E.M. of six to eight mice. (A) Time-course of the antinociceptive action and antagonism by i.c.v. injection of naloxone (NX; 10 nmol/mouse, 5 min before Endo1-β-Pro) dissolved in artificial CSF. Control mice treated with CSF alone, or naloxone-treated mice, did not show any significant change of nociceptive threshold (for the sake of clarity these data are not shown). (B) Dose–response curve for s.c. Endo1-β-Pro (1–30 mg/kg) injected 10 min before.

Endo1-β-Pro (0.5–3 mg/kg), s.c. injected 10 min in advance, reduced the abdominal constrictions elicited by acetic acid in a dose-related manner (Fig. 4). The ED_{50} (1.2 mg/kg; CL_{95}: 0.2–8.2) was lower than that in the tail flick test. As shown in Fig. 5A, i.c.v. naloxone (10 nmol/mouse) or i.p. naloxone methiodide (30 mg/kg i.p.) partially blocked antinociception by Endo1-β-Pro (2 mg/kg s.c.); moreover, a higher dose of naloxonazine (50 mg/kg s.c.) blocked the action of this peptide whereas lower (10 mg/kg s.c.; data not shown and 35 mg/kg s.c., Fig. 5A) of naloxonazine were ineffective as well as lower of naloxone methiodide (10 and 20 mg/kg i.p.; data not shown). β-Funaltrexamine (40 mg/kg s.c.) prevented Endo1-β-Pro-induced antinociception (Fig. 5B); on the contrary, the κ-opioid receptor antagonist nor-binaltorphimine (32 mg/kg, 24 h before) or the δ-opioid receptor antagonist naltrindole (10 mg/kg i.p. administered 15 min before Endo1-β-Pro) were ineffective (Fig. 5B).

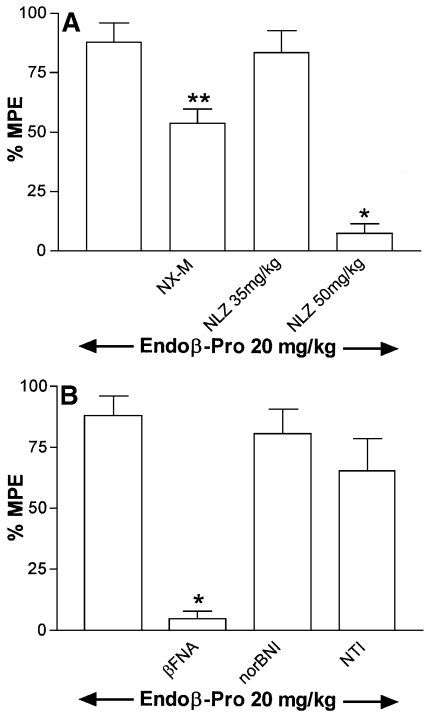

Fig. 3. Antagonism of antinociception elicited by s.c. Endo1-β-Pro (20 mg/kg) in the tail flick assay by different opioid antagonists. (A) Antagonism by naloxone methiodide (NX-M; 30 mg/kg, i.p. administered 10 min before Endo1-β-Pro) or naloxonazine (NLZ; 35 and 50 mg/kg i.p. administered 24 h before Endo1-β-Pro). (B) Antagonism by β-funaltrexamine (βFNA; 40 mg/kg, s.c. administered 24 h before Endo1-β-Pro), nor-binaltorphimine (norBNI; 32 mg/kg, s.c. administered 24 h before Endo1-β-Pro) and naltrindole (NTI; 10 mg/kg, i.p. administered 15 min before Endo1-β-Pro). Nociceptive threshold was evaluated 10 min after injection of Endo1-β-Pro. Data are the mean ± S.E.M. of six to eight mice/group. Antinociception was expressed as % of maximum possible effect (% MPE) as reported under Materials and methods. $^{**}P<0.05$; $^{*}P<0.01$ vs. mice treated with Endo1-β-Pro alone (Dunnett's multiple comparison test after ANOVA).

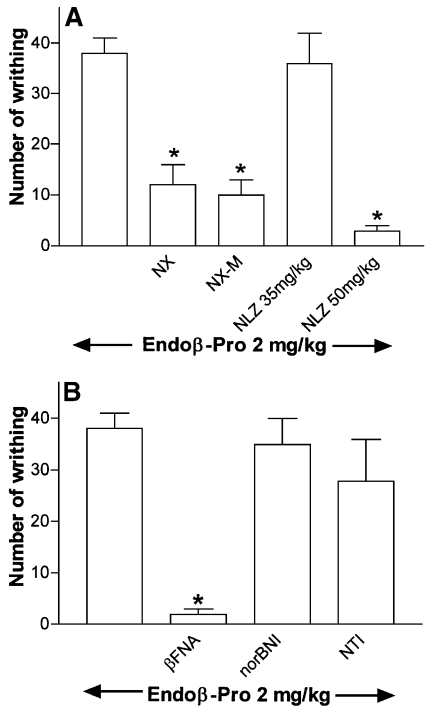

Fig. 5. Antagonism of antinociception elicited by s.c. Endo1-β-Pro (2 mg/kg) in the acetic acid-induced abdominal constriction test by different opioid antagonists. (A) Antagonism by naloxone (NX; 10 nmol/mouse, i.c.v. admnistered 5 min before Endo1-β-Pro) dissolved in artificial CSF; naloxone methiodide (NX-M; 30 mg/kg, i.p. administered 10 min before Endo1-β-Pro); or naloxonazine (NLZ; 35 and 50 mg/kg i.p. administered 24 h before Endo1-β-Pro). (B) Antagonism by β-funaltrexamine (βFNA; 40 mg/kg, s.c. administered 24 h before Endo1-β-Pro), nor-binaltorphimine (norBNI; 32 mg/kg, s.c. administered 24 h before Endo1-β-Pro) and naltrindole (NTI; 10 mg/kg, i.p. administered 15 min before Endo1-β-Pro). Data are the mean ± S.E.M. of six to eight mice/group. $^{*}P<0.01$ vs. mice treated with Endo1-β-Pro alone (Dunnett's multiple comparison test after ANOVA).

3.2. Effect Endo1-β-Pro on gastrointestinal transit

Endo1-β-Pro, s.c. administered in mice, inhibited gastrointestinal propulsion of a charcoal meal in a dose-related manner (ED_{50} = 10.0 mg/kg; 95% CL_{95}: 9.6–10.3). The highest dose used (20 mg/kg) reduced gastrointestinal transit of a charcoal meal by 94% (Fig. 6A). The non-selective opioid receptor antagonist naloxone (10 nmol/mouse, i.c.v.) or naloxone methiodide (30 mg/kg, i.p.), partially decreased Endo1-β-Pro-induced inhibition of upper gastrointestinal transit (Fig. 6B); however, lower doses of naloxone methiodide (10 and 20 mg/kg i.p.) were ineffective (data not shown). β-Funaltrexamine (40 mg/kg s.c.) and naloxonazine (50 mg/kg s.c.), injected 24 h before, blocked the action of this peptide whereas lower of naloxonazine (10 and 35 mg/kg s.c., data not shown) were ineffective. Naloxone hydrochloride (10 mg/kg i.p.), injected 10 min before, prevented the action of this peptide; on the contrary, the κ-opioid receptor antagonist nor-binaltorphimine (32 mg/kg, s.c. injected 24 h before) or the δ-opioid receptor antagonist naltrindole (10 mg/kg, i.p. administered 15 min before) did not affect inhibition of gastrointestinal propulsion by Endo1-β-Pro (20 mg/kg s.c.) (data not shown). When administered alone, none of

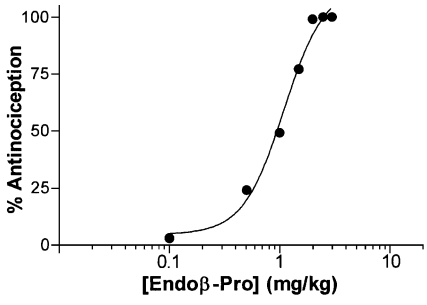

Fig. 4. Dose–response curve for s.c. Endo1-β-Pro in the acetic acid-induced abdominal constriction test. Data are the mean ± S.E.M. of six to eight mice/group. Percent of antinociception was calculated as reported under Materials and methods.

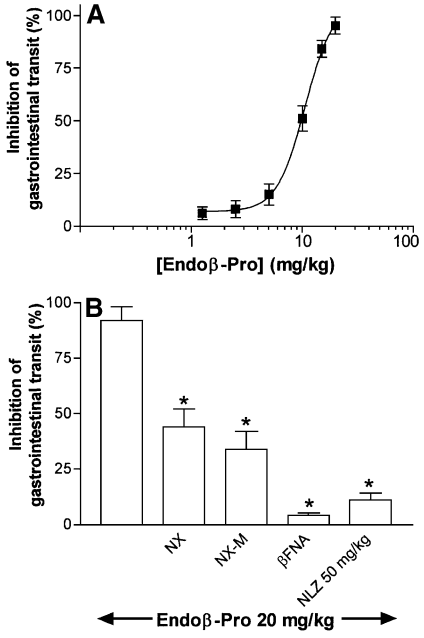

Fig. 6. Effect of Endo1-β-Pro on upper gastrointestinal transit of a charcoal meal in mice. (A) Dose–response curve for s.c. Endo1-β-Pro (1.25–20 mg/kg). (B) Antagonism of antitransit effect of a single dose of Endo1-β-Pro (20 mg/kg, s.c.) by naloxone (NX; 10 nmol/mouse, i.c.v. administered 5 min before Endo1-β-Pro) dissolved in artificial CSF; naloxone methiodide (NX-M; 30 mg/kg, i.p. administered 10 min before Endo1-β-Pro); β-funaltrexamine (β-FNA; 40 mg/kg, s.c. administered 24 h before Endo1-β-Pro) or naloxonazine (NLZ; 50 mg/kg i.p. administered 24 h before Endo1-β-Pro). Data are the mean ± S.E.M. of six to eight mice/group and are expressed as % change in gastrointestinal transit versus vehicle-treated mice as reported under Materials and methods. *$P < 0.01$ vs. mice treated with Endo1-β-Pro alone (Dunnett's multiple comparison test after ANOVA).

the antagonists altered the gastrointestinal transit in charcoal-treated mice (data not shown).

4. Discussion

The novel endomorphin-1 analogue Endo1-β-Pro, contrary to endomorphin-1 (Horvath, 2000), displayed a significant antinociceptive activity in the mouse following peripheral administration. The majority of opioid peptides undergo a rapid enzymatic degradation, particularly in blood, by exo- and endopeptidases; for this reason and for their low permeation across the blood–brain barrier, they cannot reach the central nervous system in an amount sufficient to elicit analgesia following peripheral administration (Egleton et al., 1998). Endomorphins appear to be vulnerable to rapid degradation by peptidases (Tomboly et al., 2002). Dipeptidyl peptidase IV, a membrane-bound serine proteinase, has been proposed to participate in endomorphins' degradation (Horvath, 2000). In a previous study (Cardillo et al., 2002), we have demonstrated that Endo1-β-Pro displayed enhanced enzymatic hydrolysis resistance. Therefore, this property may stabilize it towards peptidases in vivo and thus, prolonging the half-life, may also allow it to reach the brain. However, how it can penetrate the brain is unclear. It has been shown that numerous small peptides slowly cross the blood–brain barrier by simple diffusion and/or by saturable transport systems (Kastin et al., 1999). Therefore, these systems could also contribute to brain penetration of Endo1-β-Pro. Recently, Hau et al. (2002) have reported that cationization of endomorphin-2 by guanidino addition, increased peptide's half-life, blood–brain barrier transport and analgesic profile. Therefore, appropriate structural modifications of endomorphins may modify their physicochemical properties and, thus, brain penetration and their pharmacological properties following peripheral administration.

Antinociception elicited by Endo1-β-Pro would not affect behavioural responses; in fact, in behavioural observations carried out on separate groups of mice, this compound (1–20 mg/kg s.c.) did not cause any significant increase of spontaneous locomotor activity, nor circling behaviour, Straub tail or grooming were observed (Spampinato et al., unpublished data). In agreement with these observations, Denis Soignier et al. (2000) and Ukai et al. (2000) have reported that i.c.v. injection of endomorphin-1 (10 μg) did not change locomotor activity in mice. Moreover, Bujdoso et al. (2001) have found that only the lowest doses of endomorphin-1 (0.25–1.0 μg i.c.v.) increased locomotor and rearing activity in mice, resulting in a bell-shaped dose–response curve.

Previous radioligand binding studies have confirmed a μ-opioid receptor affinity for Endo1-β-Pro; moreover, this peptide was capable to block forskolin-stimulated cAMP production in SH-SY5Y cells acting through μ-opioid receptors (Cardillo et al., 2002). The selective opioid antagonists, employed in this study, confirmed the specificity of Endo1-β-Pro activity in vivo for μ-opioid receptors. Antinociception elicited by Endo1-β-Pro, as well as that induced by central administration of endomorphin-1 (Sakurada et al., 1999), was extremely insensitive to antagonism by pretreatment with naloxonazine. This latter compound has been shown to block preferentially μ_1-opioid receptors rather than μ_2-opioid receptors (Sakurada et al., 1999). Thus Endo1-β-Pro, as well as endomorphin-1, may act predominantly as μ_2-opioid receptor agonist and it does not interact significantly with κ- or δ-opioid receptors. On the contrary, antinociception induced by endomorphin-2 is sensitive to μ_1-opioid receptors and is mediated, at least partially, by κ-opioid receptors (Tseng et al., 2000). Therefore, we can suggest that Endo1-β-Pro maintains the binding profile of endomorphin-1.

The antinociceptive activity of systemically administered Endo1-β-Pro was partially blocked by both i.c.v. naloxone and i.p. naloxone methiodide, this latter compound does not readily cross the blood–brain barrier (Craft et al., 1995); thus, implicating both central and peripheral mechanisms of action. Several studies have shown that i.c.v. naloxone blocks analgesia elicited by opioid receptor agonists mainly binding to central receptors (Yeung and Rudy, 1980; Porreca

et al., 1981). However, the rapid egress of this compound from the brain (Berkowitz et al., 1975) could allow it to block peripheral opioid receptors. Central mechanisms may involve supraspinal and spinal μ-opioid receptors (Horvath, 2000; Przewlocki and Przewlocka, 2001). Peripheral mechanisms through which opioids produce antinociception are still unclear. Several studies have shown that opioids display antinociceptive properties mediated through peripheral μ- and k-opioid receptors located on primary afferent neurons and that their activation may inhibit the release of excitatory, proinflammatory mediators from peripheral nerve endings (Yaksh and Nozaki-Taguchi, 1999). Moreover, opioids have been shown to decrease activity of cutaneous nociceptors (Andreev et al., 1994) following inflammation. Similarly, opioids produce peripherally-mediated antinociception in rodents, evaluated by the tail flick test (Kolesnikov and Pasternak, 1999), or ascertained through visceral models of pain (for a review, see Stein, 1993). Craft et al. (1995), for instance, have reported that the systemic administration of the μ-opioid peptide agonist [D-Ala^2,NMePhe^4,Gly-ol]enkephalin (DAMGO) prevented abdominal licking in a rat model of visceral pain.

Endo1-β-Pro was more effective to block abdominal constrictions elicited by acetic acid than tail withdrawal from hot water. This action is in agreement with data showing that peripheral administration of μ-opioid receptor agonists in the mouse is more effective in visceral models of pain while the tail-flick test is less sensitive (Hayes et al., 1987). It has been suggested that this effect could be due to a more efficient receptor–effector coupling and/or to a higher receptor density in the physiological structures which mediate antinociception in these tests (Hayes et al., 1987). Moreover, it should be considered that peripherally administered opioid receptor agonists may easily reach peripheral opioid receptors controlling visceral pain (Reichert et al., 2001).

Endo1-β-Pro caused a dose-dependent reduction of the gastrointestinal transit in the mouse, ascertained by the charcoal meal assay. This method evaluates the motor activity of the gastrointestinal tract and reflects a combination of gastric emptying and intestinal transit effects; thus, present data do not establish a distinctive site (gastric or intestinal) of action of Endo1-β-Pro, but a combination of both as well as has been widely demonstrated for other opioid agonists (for a review, see Manara and Bianchetti, 1985). The inhibitory effect of Endo1-β-Pro on gastrointestinal transit is mediated by μ-opioid receptors since it was blocked in mice pre-treated with the selective μ-opioid receptor antagonist β-funaltrexamine, a competitive, non-reversible antagonist that binds covalently to μ-opioid receptors (Jiang et al., 1990; Mjanger and Yaksh, 1991). These receptors appear to be located at central and peripheral level (Schulz et al., 1979; Burks et al., 1988). We have investigated the peripheral component using the antagonist naloxone methiodide which does not cross the blood–brain barrier (Lewanowitsch and Irvine, 2002). In mice pre-treated with this opioid receptor antagonist, the antitransit effect of Endo1-β-Pro was only partially blocked. Thus Endo1-β-Pro, as well as other opioid agonists (Schulz et al., 1979; Burks et al., 1988), acts on gastrointestinal propulsive activity through two distinct mechanisms. One arises in the central nervous system (Goldberg et al., 1998) whereas the other is due to a peripheral action on the gut. The dose of naloxone methiodide required to block antinociception and antitransit effects of Endo1-β-Pro (30 mg/kg i.p.) was higher than the dose of naloxone hydrochloride (10 mg/kg). This is in agreement with previous studies showing that naloxone methiodide has a lower potency than naloxone hydrochloride at opioid receptors (Bianchetti et al., 1983; Lewanowitsch and Irvine, 2002).

Endo1-β-Pro is among the first endomorphin-1 analogues showing antinociceptive activity after systemic administration. This compound will be extremely useful for exploring the pharmacological profile of endomorphins in vivo and confirms the potential therapeutic interest of endomorphin derivatives as novel analgesic agents.

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References

Abbadie, C., Rossi, G.C., Orciuolo, A., Zadina, J.E., Pasternak, G.W., 2002. Anatomical and functional correlation of the endomorphins with mu opioid receptor splice variants. Eur. J. Neurosci. 16, 1075–1082.

Andreev, N., Urban, L., Dray, A., 1994. Opioids suppress spontaneous activity of polymodal nociceptors in the rat paw sin induced by ultraviolet irradiation. Neuroscience 58, 793–798.

Berkowitz, B.A., Ngai, S.H., Hempstead, J., Spector, S., 1975. Disposition of naloxone: use of a new radioimmunoassay. J. Pharmacol. Exp. Ther. 195, 499–504.

Bianchetti, A., Nisato, D., Sacilotto, R., Dragonetti, M., Picerno, N., Tarantino, A., Manara, L., Angel, L.M., Simon, E.J., 1983. Quaternary derivatives of narcotic antagonists: stereochemical requirements at the chiral nitrogen for in vitro and in vivo activity. Life Sci. 33 (Suppl. 1), 415–418.

Bujdoso, E., Jaszberenyi, M., Tomboly, C., Toth, G., Telegedy, G., 2001. Effects of endomorphin-1 on open-field behaviour and on the hypothalamic–pituitary–adrenal system. Endocrine 14, 221–224.

Burks, T.F., Fox, D.A., Hirning, L.D., Shook, J.E., Porreca, F., 1988. Regulation of gastrointestinal function by multiple opioid receptors. Life Sci. 43, 2177–2181.

Cardillo, G., Gentilucci, L., Qasem, A.R., Sgarzi, F., Spampinato, S., 2002. Endomorphin-1 analogues containing β-proline are μ-opioid receptor agonists and display enhanced enzymatic hydrolysis resistance. J. Med. Chem. 45, 2571–2578.

Craft, R.M., Henley, S.R., Haaseth, R.C., Hruby, V.J., Porreca, F., 1995. Opioid antinociception in a rat model of visceral pain: systemic versus local drug administration. J. Pharmacol. Exp. Ther. 275, 1535–1542.

Denis Soignier, R., Vaccarino, A.L., Brennan, A.M., Kastin, A.J., Zadina, J.E., 2000. Analgesic effects of endomorphin-1 and endomorphin-2 in the formalin test in mice. Life Sci. 76, 907–912.

Egleton, R.D., Abbruscato, T.J., Thomas, S.A., Davis, T.P., 1998. Transport

of opioid peptides into the central nervous system. J. Pharm. Sci. 87, 1433–1439.

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.

Haley, T.J., McCormick, W.G., 1957. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. Br. J. Pharmacol. 12, 12–15.

Hau, V.S., Huber, J.D., Campos, C.R., Lipkowski, A.W., Misicka, A., Davis, T.P., 2002. Effect of guanidino modification and proline substitution on the in vitro stability and blood–brain barrier permeability of endomorphin II. J. Pharm. Sci. 91, 2140–2149.

Hayes, A.G., Sheehan, M.J., Tyers, M.B., 1987. Differential sensitivity of models of antinociception in the rat, mouse and guinea-pig to μ- and κ-opioid receptor agonists. Brit. J. Pharmacol. 91, 823–832.

Horvath, G., 2000. Endomorphin-1 and endomorphin-2: pharmacology of the selective endogenous μ-opioid receptor agonists. Pharmacol. Ther. 88, 437–463.

Jiang, Q.L., Heyman, J.S., Sheldon, R.J., Koslo, R.J., Porreca, F., 1990. *Mu* antagonist and *kappa* agonist properties of β-funaltrexamine (β-FNA) in vivo: long-lasting spinal analgesia in mice. J. Pharmacol. Exp. Ther. 252, 1006–1011.

Kastin, A.J., Pan, W., Maness, L.M., Banks, W.A., 1999. Peptides crossing the blood–brain barrier: some unusual observations. Brain Res. 848, 96–100.

Kolesnikov, Y., Pasternak, G.W., 1999. Topical opioids in mice: analgesia and reversal tolerance by a topical *N*-methyl-D-aspartate antagonist. J. Pharmacol. Exp. Ther. 290, 247–252.

Lewanowitsch, T., Irvine, R.J., 2002. Naloxone methiodide reverses opioid-induced respiratory depression and analgesia without withdrawal. Eur. J. Pharmacol. 445, 61–67.

Manara, L., Bianchetti, A., 1985. The central and peripheral influences of opioids on gastrointestinal propulsion. Annu. Rev. Pharmacol. Toxicol. 25, 249–273.

Mjanger, E., Yaksh, T.L., 1991. Characteristics of dose-dependent antagonism by β-funaltrexamine of the antinociceptive effects of intrathecal μ agonists. J. Pharmacol. Exp. Ther. 258, 544–549.

Neilan, C.L., Nguyen, T.M.-D., Schiller, P.W., Pasternak, G.W., 2001. Pharmacological characterization of the dermorphin analog [Dmt^1] DALDA, a highly potent and selective μ-opioid peptide. Eur. J. Pharmacol. 419, 15–23.

Okada, Y., Tsuda, Y., Bryant, S.D., Lazarus, L.H., 2002. Endomorphins and related opioid peptides. Vitam. Horm. 65, 257–279.

Pasternak, G.W., Wood, P.L., 1986. Multiple mu opiate receptors. Life Sci. 38, 1888–1898.

Porreca, F., Cowan, A., Tallarida, R.J., 1981. Time course of antagonism of morphine antinociception by intracerebroventricularly administered naloxone in the rat. Eur. J. Pharmacol. 76, 55–59.

Przewlocki, R., Przewlocka, B., 2001. Opioids in chronic pain. Eur. J. Pharmacol. 429, 79–91.

Reichert, J.A., Daughters, R.S., Rivard, R., Simone, D.A., 2001. Peripheral and preemptive opioid antinociception in a mouse visceral pain model. Pain 89, 221–227.

Sakurada, S., Zadina, J.E., Kastin, A.J., Katsuyama, S., Fujimura, T., Murayama, K., Yuki, M., Ueda, H., Sakurada, T., 1999. Differential involvement of μ-opioid receptor subtypes in endomorphin-1- and -2-induced antinociception. Eur. J. Pharmacol. 372, 25–30.

Schulz, R., Wuster, M., Herz, A., 1979. Centrally and peripherally mediated inhibition of intestinal motility by opioids. Naunyn-Schmiedeberg's Arch. Pharmacol. 308, 255–260.

Stein, C., 1993. Peripheral mechanisms of opioid analgesia. Anesth. Analg. 76, 183–191.

Tallarida, R.J., Murray, R.B., 1987. Manual of Pharmacological Calculation with Computer Programs. Springer-Verlag, New York, NY.

Tomboly, C., Peter, A., Toth, G., 2002. In vitro quantitative study of the degradation of endomorphins. Peptides 23, 1573–1580.

Tseng, L.F., Narita, M., Suganuma, C., Mizoguchi, H., Ohsawa, M., Nagase, H., Kampine, J.P., 2000. Differential antinociceptive effects of endomorphin-1 and endomorphin-2 in the mouse. J. Pharmacol. Exp. Ther. 292, 576–583.

Ukai, M., Watanabe, Y., Kameyama, T., 2000. Effects of endomorphin-1 and -2, endogenous μ-opioid receptor agonists, on spontaneous alternation performance in mice. Eur. J. Pharmacol. 395, 211–215.

Wilson, A.M., Soignier, R.D., Zadina, J.E., Kastin, A.J., Nores, W.L., Olson, R.D., Olson, G.A., 2000. Dissociation of analgesic and rewarding effects of endomorphin-1 in rats. Peptides 21, 1871–1874.

Yaksh, T.L., Nozaki-Taguchi, N., 1999. Characterization of the antihyperalgesic action of a novel peripheral Mu-opioid receptor agonist Loperamide. Anesthesiology 90, 225–234.

Yeung, J.C., Rudy, T.A., 1980. Sites of antinociceptive action of systemically injected morphine: involvement of supraspinal loci as revealed by intracerebroventricular injection of naloxone. J. Pharmacol. Exp. Ther. 215, 626–632.

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