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Structure-constrained endomorphin analogs display differential antinociceptive mechanisms in mice after spinal administration

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ABSTRACT

We previously reported a series of novel endomorphin analogs with unnatural amino acid modifications. These analogs display good binding affinity and functional activity toward the μ opioid receptor (MOP). In the present study, we further investigated the spinal antinociceptive activity of these compounds. The analogs were potent in several nociceptive models. Opioid antagonists and antibodies against several endogenous opioid peptides were used to determine the mechanisms of action of these peptides. Intrathecal pretreatment with naloxone and β-funaltrexamine (β-FNA) effectively inhibited analog-induced analgesia, demonstrating that activity of the analogs is regulated primarily through MOP. Antinociception induced by analog 2 through 4 was not reversed by δ opioid receptor (DOP) or κ opioid receptor (KOP) antagonist; antibodies against dynorphin-A (1–17), dynorphin-B (1–13), and Leu^5/Met^5-enkephalin had no impact on the antinociceptive effects of these analogs. In contrast, antinociceptive effects induced by a spinal injection of the fluorine substituted analog 1 were significantly reversed by KOP antagonism. Furthermore, intrathecal pretreatment with antibodies against dynorphin-B (1–13) attenuated the antinociceptive effect of analog 1. These results indicate that the antinociceptive activity exerted by intrathecally-administered analog 1 is mediated, in part, through KOP with increased release of dynorphin-B (1–13). The chemical modifications used in the present study may serve as a useful tool to gain insight into the mechanisms of endomorphins activity.

1. Introduction

Endomorphin-1 (EM-1, H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, H-Tyr-Pro-Phe-Phe-NH_2) were isolated from the bovine brain and human cortex in 1997; the two peptides exhibit high affinity and selectivity for the μ opioid receptor (MOP) [1]. Although no mechanism for the endogenous synthesis of EMs has been identified [2,3], they are still of interest to peptide chemists/pharmacologists and are considered useful pharmacological tools. These two tetrapeptides exhibit pharmacological properties similar to those of morphine, producing potent antinociceptive activity in most pain models, with minimal undesired side effects [4,5]. It is known that endogenous opioid ligands are localized in many areas of the central nervous system for pain processing, including the dorsal horn of the spinal cord, trigeminal nucleus, and periaqueductal gray [6,7]. Studies have shown that EM-1 and EM-2 activity is mediated by MOP stimulation; intrathecal (i.t.) or intracerebroventricular (i.c.v) injection of EMs produced potent analgesia. Studies examining the mechanisms of antinociception have shown that other opioid receptors, such as the δ opioid receptor (DOP) and κ opioid receptor (KOP) may also participate in regulating the analgesic effects of EMs [8,9].

Opioid peptides are known to be poor penetrators of the blood–brain barrier and have low enzymatic stability, as well as other toxic effects; therefor, the application of EMs for clinical treatment of pain has been limited [2,10,11]. It is essential to clarify EM antinociception mechanisms to provide promising new analgesic candidates [12]. Extensive efforts have been made, and hundreds of EM-based compounds have been synthesized to gain insight into the structural requirements for bioavailability and activity of EMs [13]. β-Amino acids occur less frequently in nature than α-amino acids, which are found in endogenous peptides. Incorporation of β-amino acids into naturally occurring peptides has been shown to improve pharmacolo-

Abbreviations: DOP, δ opioid receptor; EM-1, endomorphin-1; EM-2, endomorphin-2; i.c.v., intracerebroventricular; i.p., intraperitoneal; i.t., intrathecal; KOP, κ opioid receptor; MOP, μ opioid receptor; MPE, maximum possible effect; s.c., subcutaneous

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Table 1
Opioid Receptor Binding Affinities and Functional Activity of EMs and Analogs.[a]

peptide	sequence	Binding Affinity			Functional Activity	
		K_i^{μ} (nM)	K_i^{δ}(nM)	selectivity K_i^{δ}/K_i^{μ}	EC_{50}(nM)	E_{max} (%)
EM-1	Tyr-Pro-Trp-Phe-NH_2	2.60 ± 0.21	6080 ± 640	2338	14.40 ± 0.62	83.13 ± 4
EM-2	Tyr-Pro-Phe-Phe-NH_2	3.20 ± 0.13	6420 ± 330	2006	11.80 ± 0.23	82.75 ± 4
1	Tyr-Pro-Trp-(4-FPh)Map-NH_2	13.7 ± 0.9	17040 ± 2050	1244	7.35 ± 1.02	81.45 ± 6
2	Tyr-Pro-Trp-(4-MeOPh)Map-NH_2	4.83 ± 0.91	10200 ± 1430	2112	10.91 ± 0.83	85.56 ± 3
3	Tyr-Pro-Trp-(Piperonyl)Map-NH_2	7.73 ± 1.02	18690 ± 1330	2418	10.70 ± 1.09	83.90 ± 5
4	Tyr-Pro-Trp-(1-Naphthyl)Map-NH_2	26.0 ± 3.5	84680 ± 10490	3264	72.30 ± 6.00	71.01 ± 4

[a] Values are cited from our previous report [17]. The potencies of the peptides were evaluated via cAMP accumulation assays using HEK293 cells expressing MOP.

gical activity and stability [14,15]. The C-terminal site (-Phe^4) of EMs plays an important role in mediating the activity of these peptides [16]. Previously, we reported a novel series of EM-1 analogs containing α-constrained β amino acids (Map), these analogs displayed better stability and in vitro activity than their parent peptide [17]. In the present study, we further examined their spinal analgesia activity after i.t. injection in several nociceptive mouse models. Table 1 shows the sequences and unnatural amino acids used in the present study [17]. In addition, specific opioid receptor antagonists and antibodies against endogenous opioid peptides were used to determine mechanisms of action and whether spinal administration of these analogs facilitates participation of other endogenous peptides in the pain relief process.

2. Methods and materials

2.1. Animals

Animals (Animal Center of Medical College of Lanzhou University, Gansu, People's Republic of China) were housed in a temperature-controlled environment (22 ± 1 °C) under standard 12 h light/dark conditions and received food and water ad libitum. Animals were used only once and received good care and humane treatment. The results of all studies involving animals are reported in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local ethics committee. Animal studies are reported in compliance with the ARRIVE guidelines [18]. All the protocols in this study were approved by the Ethics Committee of Lanzhou University (permit number: SYXK Gan 2009–0005), China. For antinociceptive effects, data points represent means ± SEM from experiments conducted on at least six mice. Randomization was performed by an individual other than the operator. In all experiments, mice were selected randomly from the pool and randomly divided into the groups. All the data were collected and analysed by two independent observers, who were blinded to the group assignment of samples or animals in in vivo experiments. This study complied with the replacement, refinement and reduction (the 3Rs). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Chemicals

In the present study, EMs and its analogs were synthesized by manual solution-phase methods as described in our previous reports [17,19]. Crude products were purified by semipreparative RP-HPLC and were 95–99% pure as determined by analytical RP-HPLC. The molecular weight of the peptide was confirmed by an electrospray ionization mass spectrometer (ESI-Q-TOF Maxis-4G, Bruker Daltonics, Germany).

The antibodies against dynorphin A (1–17) (anti-Dyn A antibody), dynorphin B (1–13) (anti-Dyn B antibody), [Met^5]enkephalin (anti-M-ENK antibody), and [Leu^5]enkephalin (anti-L-*E*NK antibody) were obtained from Abcam (Cambridge, UK). Naloxone hydrochloride, β-funaltrexamine hydrochloride (β-FNA), naltrindole isothiocyanate hydrochloride (NTI), and nor-binaltorphimine hydrochloride (nor-BNI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All compounds were dissolved in saline solution and stored at −20 °C.

2.3. Warm water tail-flick test

Male Kunming mice weighing 18–22 g were employed, various dose of analogues were injected i.t. and the warm water tail-flick responses were measured at different times. Nociception was evoked by immersing the mouse tail in warm water (50 ± 0.2 °C) and measuring the latency to withdrawal. Before treatment, each mouse was recorded the control latency (CL) to tail-flick, and those with a latency of approximately 3–5 s were selected. Less than 10% of animals were excluded from this assay. The latency to tail-flick was defined as the test latency (TL), the corresponding cut-off time was set at 10 s in order to minimize tissue damage. The antinociceptive response was expressed as the percent maximum possible effect (%MPE), calculated by the following equation: $\%MPE = 100 \times (TL - CL)/(10 - CL)$.

2.4. Formalin test

Male Kunming mice weighing 22–25 g were employed. Mice were pre-treated with test compounds 5 min before subcutaneous (s.c.) injection of 5% formalin solution (20 μL) into the right hind paw, and mice were immediately returned to a Plexiglas box (20 cm in height and 15 cm in diameter), under the floor at a 45° angle placed a mirror. The total time that the mice spent licking or biting the injected paw was recorded during an acute phase of short duration followed by a longer lasting tonic phase, the first-phase (0–5 min) and the second-phase (15–30 min) of the test. The nociceptive response was expressed as the second (sec), and 0.9% saline was used as control.

2.5. Writhing test

Male Kunming mice weighing 18–22 g were employed. Mice were pre-treated with test compounds 5 min before intraperitoneal (i.p.) injection of 1% acetic acid solution (10 mL/kg), which induce a contraction of the abdominal muscles together with a stretching of the hind limbs. Mice were immediately returned to a Plexiglas box (20 cm in height and 15 cm in diameter), and the number of writhes was cumulatively counted for 20 min.

2.6. Drug administration

The intrathecal (i.t.) administration was performed according to the procedure previously described [20]. A 29-gauge needle connected to a 10-μL Hamilton microsyringe was inserted directly between the L5 and L6 segments in mice. Puncture of the dura was indicated by a reflexive lateral flick of the tail or formation of an 'S' shape by the tail [21]. All the drugs were delivered slowly in an injection volume of 4 μL. The