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Differentiation of opioid receptor preference by [Dmt[1]]endomorphin-2-mediated antinociception in the mouse

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Abstract

The potent opioid [Dmt[1]]endomorphin-2 (Dmt-Pro-Phe-Phe-NH_2) differentiated between the opioid receptor subtypes responsible for the antinociception elicited by endomorphin-2 in mice. Antinociception, induced by the intracerebroventricular administration of [Dmt[1]]endomorphin-2 and inhibited by various opioid receptor antagonists [naloxone, naltrindole, β-funaltrexamine, naloxonazine], was determined by the tail-flick (spinal effect) and hot-plate (supraspinal effect) tests. The opioid receptor subtypes involved in [Dmt[1]]endomorphin-2-induced antinociception differed between these in vivo model paradigms: naloxone (non-specific opioid receptor antagonist) and β-funaltrexamine (irreversible μ_1/μ_2-opioid receptor antagonist) blocked antinociception in both tests, although stronger inhibition occurred in the hot-plate than the tail-flick test suggesting involvement of other opioid receptors. Consequently, we applied naloxonazine (μ_1-opioid receptor antagonist) that significantly blocked the effect in the hot-plate test and naltrindole (δ-opioid receptor antagonist), which was only effective in the tail-flick test. The data indicated that [Dmt[1]]endomorphin-2-induced spinal antinociception was primarily mediated by both μ_2- and δ-opioid receptors, while a supraspinal mechanism involved only μ_1/μ_2-subtypes.

Keywords: Dmt (2′,6′-dimethyl-L-tyrosine); Endomorphin; Antinociception; Spinal; Supraspinal; Antagonist

1. Introduction

The endogenous opioid peptides endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) isolated from mammalian brain have high affinity and selectivity for μ-opioid receptors with no appreciable affinity for δ- and κ- receptors (Zadina et al., 1997). Although endomorphin-1 and endomorphin-2 are structurally similar to other opioids containing the Tyr-Pro N-terminal region that have specificity for μ-opioid receptors, they are quite distinct from the known classical endogenous opioid peptides for μ-, δ- and κ-receptors with their N-terminal Tyr-Gly-Gly-Phe sequence. These natural ligands for μ-opioid receptor produce potent and prolonged analgesia in mice, which was reversed by the general opioid receptor antagonist naloxone (Stone et al., 1997; Zadina et al., 1997).

μ-Opioid receptors are pharmacologically divided into two subtypes: the μ_1-subtype appears to be more supraspinally selective, while the μ_2-subtype involves spinal effects (Bodnar et al., 1988; Heyman et al., 1988; Ling and

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Pasternak, 1983; Pasternak and Wood, 1986; Paul et al., 1989; Porreca et al., 1984; Wolozin and Pasternak, 1981). While both endomorphins were equipotent supraspinally, endomorphin-1 was significantly more potent spinally (Goldberg et al., 1998). In fact, Sakurada et al. (1999) reported that antinociception induced by endomorphin-1 seems to be mediated by the μ_2-opioid receptor, whereas endomorphin-2 may preferentially act through the μ_1-opioid receptor.

Studies on opioid peptides demonstrated that the introduction of 2′,6′-dimethyl-L-tyrosine (Dmt) in lieu of the common N-terminal Tyr residue in opioid ligands resulted in an exceptional improvement in receptor affinity and functional bioactivity in a wide variety of opioid peptides (Balboni et al., 2002; Bryant et al., 1998, 2003; Chandrakumar et al., 1992; Guerrini et al., 1996; Hansen et al., 1992; Okada et al., 1998, 1999, 2003a; Pitzele et al., 1994; Salvadori et al., 1995, 1997, 1999; Sasaki et al., 1999; Schiller et al., 2000). The substitution of Dmt for Tyr in endomorphin-2 ([Dmt^1]endomorphin-2) resulted in one of the most active peptides among several analogues containing an alkyl-modified aromatic ring of Tyr (Fujita et al., 2004; Li et al., in press; Okada et al., 2003b). Dmt increased μ-opioid receptor affinity and μ-opioid receptor bioactivity of endomorphin-2 by 5- and 83-fold, respectively (Okada et al., 2003b); and also elevated in vivo activity 1.5- to 3-fold as measured by the tail-flick and hot-plate tests, respectively (Li et al., in press). The δ-affinity and bioactivity of [Dmt^1]endomorphin-2, on the other hand, increased 2 to 3 orders of magnitude, thereby transforming the once highly selective ligand into a bivalent or bifunctional opioid mimetic substance lacking receptor selectivity (Fujita et al., 2004; Okada et al., 2003b).

In this study, we tested whether replacing Dmt for Tyr elicited a change in μ-opioid receptor subtypes and if δ-opioid receptors are involved in pain perception by [Dmt^1]endomorphin-2. A series of specific opiate receptor antagonists were employed, such as β-funaltrexamine for μ_1/μ_2-opioid receptor subtypes, naloxonazine for the μ_1-opioid receptor and the δ-opiate receptor antagonist naltrindole using the classic antinociceptive tail-flick and hot-plate tests to define which opioid receptor subtypes might be involved in antinociception.

2. Materials and methods

2.1. Animals

Male Swiss–Webster mice (20–25 g; Taconic Farms, Germantown, NY) were housed on a 12 h light/dark cycle with free access to food and water. This research was carried out according to protocols approved by the National Institute of Environmental Health Sciences' Animal Care and Use Committee (ACUC).

2.2. Drugs

Naloxone hydrochloride was obtained from Sigma (Louis, MO, USA), and naltrindole hydrochloride, naloxonazine dihydrochloride, β-funaltrexamine hydrochloride were purchased from Tocris (Ellsville, MO, USA). The opiate receptor antagonists were injected subcutaneously (s.c.): naloxone (10 mg/kg) and naltrindole (3 mg/kg) were administered 30 min before the test compound, while with naloxonazine (35 mg/kg) and β-funaltrexamine (40 mg/kg) the mice were treated 24 h prior to testing (Paul et al., 1989). The dose of naloxone and naltrindole was selected by their ability to completely block the effect of morphine (0.5 μg/mouse, i.c.v.) and deltorphin B (8 μg/mouse, i.c.v.), respectively. A highly purified sample of [Dmt^1]endomorphin-2 was synthesized as described previously (Okada et al., 2003b).

2.3. Intracerebroventricular injection

A 25 μl Hamilton microsyringe, fitted with disposable 27-gauge needle, was used for intracerebroventricular (i.c.v.) administration. The needle was inserted 2.3–3.0 mm deep as described (Laursen and Belknap, 1986). Briefly, the bregma was found by lightly rubbing the point of the needle over the skull until the suture was felt through the skin (1–3 mm rostral to a line drawn through the anterior base of the ears). The needle was inserted 2 mm lateral to the midline and the total volume injected was 4 μl. Shortly after testing, the animals were sacrificed according ACUC protocols: a slit was made along the midline of the scalp and mice exhibiting a needle tract 2 mm lateral from the bregma were counted as having been injected correctly.

2.4. Tail-flick test for spinal antinociception

A tail-flick instrument (Columbus Instruments, Columbus, OH) was used to study the spinal effect of the opioid compounds. Radiant heat was applied on the dorsal surface of the tail and the latency for removal of the tail from the onset of the radiant heat is defined as the tail-flick latency. The baseline of tail-flick latency was adjusted between 2 and 3 s (pre-response time) and a cut off time was set at 8 s to avoid external heat-related damage. The analgesic response was measured 10 min following i.c.v injection. The duration time for i.c.v. administration was 10 min and the test was terminated when tail-flick latency was close to the pre-response time.

2.5. Hot-plate test for supraspinal antinociception

Supraspinal effect was measured by placing the animals on an electrically heated plate (IITC MODEL 39D Hot Plate analgesia meter, Woodland Hills, CA) at 55±0.1 °C. Ten minutes after i.c.v. administration of the

opioid ligand, the hot-plate latency was measured as the interval between placement of mice onto the hot plate and observing movement consisting of either jumping, licking or shaking their hind paws with a baseline latency of 15 s and maximal cut off time of 30 s. The duration time for i.c.v. administration was 10 min and the test was terminated when hot-pate latency was close to the pre-response time.

2.6. Statistical analysis

Statistical significance of the data was estimated by one-way analysis of variance (ANOVA) followed by Dunnett's test using the computer software program JMP (SAS Institute Inc, Cary, NC). The data were considered significant at $P<0.05$. The area under the curve (AUC) was obtained by plotting the response time (s) on the ordinate and time (min) on the abscissa after administration of the compounds.

3. Results

3.1. Antinociception produced by [Dmt^1]endomorphin-2: tail-flick test

[Dmt^1]endomorphin-2 showed a dose-dependent antinociceptive response after i.c.v. administration in the tail-flick test, which was significant at a dose as low as 0.1 μg/mouse (Fig. 1A and B). The maximum response occurred at 10 min after administration and lasted at least an hour depending on the dose. Naloxone injected s.c. reversed the response of [Dmt^1]endomorphin-2 by 73% (Fig. 1C and D) and naltrindole effectively blocked 44% of the total effect similar to β-funaltrexamine (49%), while naloxonazine, whose mode of action is limited to μ_1-subtype and M6G receptors had no effect (Fig. 1E and F). The κ-opioid receptor antagonist, nor-binaltorphimine, was inactive against [Dmt^1]endomorphin-2-induced antinociception (data not shown). Opioid antagonists do not have any effect when administered alone (data not shown).

3.2. Antinociception produced by [Dmt^1]endomorphin-2: hot-plate test

Similar to the tail-flick test, the hot-plate test revealed a dose-dependent effect with [Dmt^1]endomorphin-2 on supraspinal antinociception with a maximum response at 10 min post injection (Fig. 2A and B). However, the compound was less active than the tail-flick test with significant antinociception beginning only at a dose of 1.0 μg/mouse. The effect of various opioid receptor antagonists on [Dmt^1]endomorphin-2-induced antinociception was also different than that in tail-flick test: while naloxone effectively reversed the effect of compound,

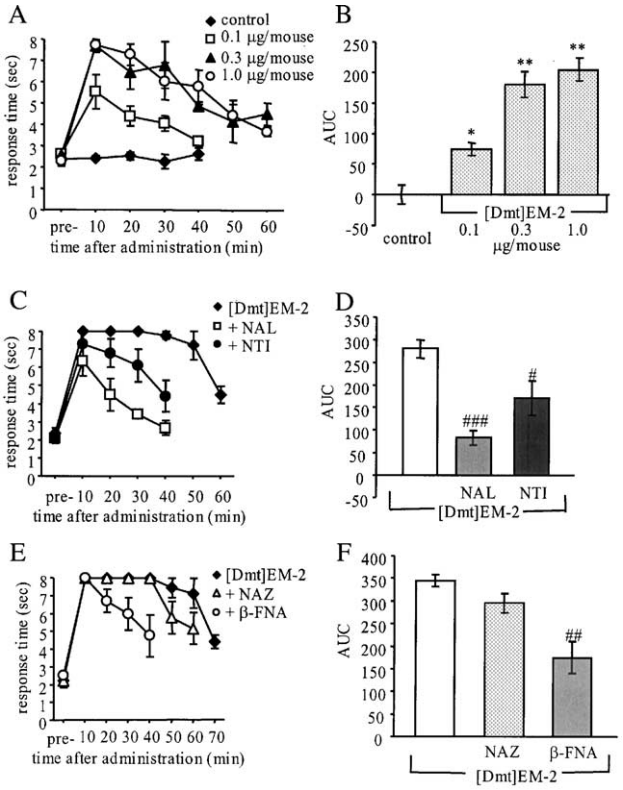

Fig. 1. Effect of [Dmt^1]endomorphin-2 ([Dmt]EM-2) in the tail-flick test after intracerebroventricular administration in mice. (A) Time course, (B) area under the curve (AUC), (C, D) effect of naloxone (NAL, 10 mg/kg s.c.) and naltrindole (NTI, 3 mg/kg s.c.), (E, F) effect of naloxonazine (NAZ, 35 mg/kg s.c.) and β-Funaltrexamine (β-FNA, 40 mg/kg s.c.). (C–F) [Dmt^1]endomorphin-2 was injected at a dose of 3 μg/mouse (i.c.v.). Each value is the mean with S.E.M. of 5–6 mice for (A) and (B) and 7–8 mice for (C) and (D). (*) Denotes values that are significantly different from saline-treated mice by Dunnett's test (**$P<0.01$, *$P<0.05$), (#) from [Dmt^1]endomorphin-2 group by Dunnett's test ($^{\#\#\#}P<0.001$, $^{\#\#}P<0.01$, $^{\#}P<0.05$).

naltrindole was inactive (Fig. 2C and D); on the other hand, β-funaltrexamine inhibited the activity 77% and naloxonazine exerted 45% antagonist activity (Fig. 2E and F). Nor-binaltorphimine was inactive (data not shown).

4. Discussion

Replacement of Tyr by Dmt at the N-terminus in endomorphin-2 not only enhanced the μ-opioid receptor affinity and bioactivity of endomorphin-2, but also dramatically increased the δ-opioid receptor affinity of the compound (Fujita et al., 2004; Okada et al., 2003b). In in vivo, [Dmt^1]endomorphin-2 displayed significant antinociception in mice following i.c.v. administration in tail-flick and hot-plate tests. Although both tail-flick and hot-plate tests are based on measuring the response of the animal to thermal stimuli, they involve different pain reflex mechanisms; i.e., tail-flick monitors a spinal reflex, while the hot-plate is used as a centrally integrated test

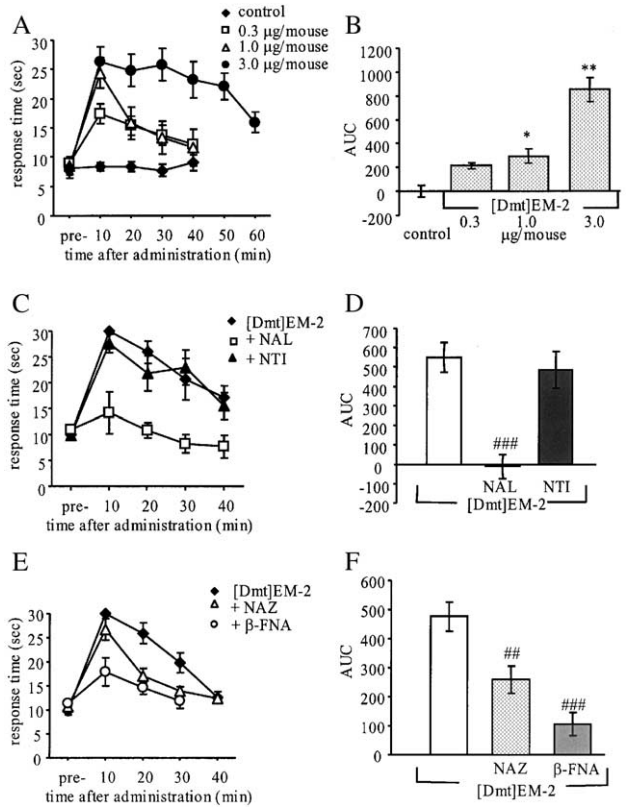

Fig. 2. Effect of [Dmt[1]]endomorphin-2 ([Dmt]EM-2) measured by the hot-plate test after intracerebroventricular administration in mice. (A) Time course, (B) area under the curve (AUC), (C, D) effect of naloxone (NAL, 10 mg/kg s.c.) and naltrindole (NTI, 3 mg/kg s.c.), (E, F) effect of naloxonazine (NAZ, 35 mg/kg s.c.) and β-Funaltrexamine (β-FNA, 40 mg/kg s.c.). (C–F) [Dmt[1]]endomorphin-2 was injected at a dose of 3 μg/mouse (i.c.v.). Each value is the mean with S.E.M. of 5–6 mice for (A) and (B) and 7–8 mice for (C) and (D). (*) Denotes values that are significantly different from saline-treated mice by Dunnett's test (**$P<0.01$, *$P<0.05$), and (#) from [Dmt[1]]endomorphin-2 group by Dunnett's test ($^{###}P<0.001$, $^{##}P<0.01$).

(Dewey et al., 1969; Schmauss and Yaksh, 1984). [Dmt[1]]endomorphin-2 acted spinally and supraspinally; however, the spinal effect was at least 10 times more potent than that observed with the supraspinal response. The bioactivity of [Dmt[1]]endomorphin-2 was reversible by the general opiate receptor antagonist naloxone, which is known to interact with opioid receptors in vivo (Sawynok et al., 1979).

Studies on the role of opioid receptors in the production of antinociception at the level of the supraspinal and spinal cord suggested that both μ- and δ-opioid receptors are involved in spinal mechanism (Heyman et al., 1988; Tung and Yaksh, 1982); however, data suggest that only μ_1-opioid receptors may be primarily responsible for supraspinal analgesia (Jinsmaa et al., 2004a,b; Ling and Pasternak, 1983). Sakurada et al. (1999) reported that endomorphin-2 was more supraspinally active and its activity mediated completely by μ-opioid receptors, preferentially the μ_1-subtype. On the other hand, since [Dmt[1]]endomorphin-2-induced antinociception was blocked partially by naltrindole in the tail-flick assay (44%) (Fig. 1), it suggested the involvement of δ-opioid receptors (Portoghese et al., 1988). Moreover, since naltrindole was an ineffective agent against [Dmt[1]]endomorphin-2-induced supraspinal antinociception, the results further indicate that the receptor types involved in these two neural tissues are distinguishable from each other as well as from the spectrum of activity elicited by endomorphin-2. While β-funaltrexamine and naltrindole significantly reversed the antinociception produced by [Dmt[1]]endomorphin-2 without inhibition by naloxonazine in the tail-flick test, antinociception was apparently mediated by a combination of the μ_2-opioid receptor and δ-opioid receptors rather than the μ_1-opioid subtype. On the other hand, that in the hot-plate test β-funaltrexamine and naloxonazine reversed the bioactivity of [Dmt[1]]endomorphin-2 by 77% and 45%, respectively, represents strong evidence for the involvement both μ_1/μ_2-opioid receptor subtypes in the supraspinal analgesic mechanism. There was no involvement of κ-opioid receptors in these tests, confirming the data with endomorphin-2 (Goldberg et al., 1998).

In mice, [Dmt[1]]endomorphin-2 apparently increased the duration of the biological action might involve several factors, including the enhanced opioid receptor affinity (Okada et al., 2003b), altering or selecting between opioid receptor subtypes known to be involved in producing analgesia, or increasing the stability against proteolytic degradation. In fact, endomorphin-1 and endomorphin-2 normally had a short half-life in which antinociception peaked at 5 min following i.c.v. administration and 1 min for intrathecal injections, and was absent by 15 min after either mode of injection (Sakurada et al., 1999; Stone et al., 1997). However, while the antinociception induced by [Dmt[1]]endomorphin-2 lasted at least an hour (depending on the specific test measurement and dose), the peak of activity occurred after 10 min which suggested that the enhanced potency of this analogue not only reflects the potencies observed in the in vitro analyses (ligand affinity and functional bioactivity) (Fujita et al., 2004; Okada et al., 2003b), but may also be considerably more protease resistant (Sasaki et al., 1999).

In conclusion, our results indicated that [Dmt[1]]endomorphin-2-induced antinociception is mediated by a combination of μ_2- and δ-opioid receptors in the spinal cord, while supraspinal antinociception was due solely to μ_1/μ_2-receptor subtypes. Dimethylation of Tyr enhanced μ-opioid receptor interaction in vitro (Salvadori et al., 1995; 1997, 1999; Okada et al., 2003a) and in vivo (Jinsmaa et al., 2004a;b) and brought about the reported changes in the interaction of endomorphin-2 with μ-opioid receptors. In other words, Dmt appeared responsible for enhancing the affinity, functional activity and antinociception of endomorphin-2 toward μ_2- and δ-opioid receptors, while simultaneously shifting the site of its action more spinally than supraspinally.

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