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Selective antagonism by naloxonazine of antinociception by Tyr-D-Arg-Phe-β-Ala, a novel dermorphin analogue with high affinity at μ-opioid receptors

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Abstract

To examine the role of μ-opioid receptor subtypes, we assessed the antinociceptive effect of H-Tyr-D-Arg-Phe-β-Ala-OH (TAPA), an analogue of dermorphin N-terminal peptide in mice, using the tail-flick test. Intracerebroventricularly (i.c.v.) or intrathecally (i.t.) injected TAPA produced potent antinociception with tail-flick as a thermal noxious stimulus. The selective μ_1-opioid receptor antagonist, naloxonazine (35 mg/kg, s.c.), or the selective μ-opioid receptor antagonist, β-funaltrexamine, 24 h before testing antagonized the antinociceptive effect of i.t. or i.c.v. TAPA on the response to noxious stimuli. Pretreatment with β-funaltrexamine completely antagonized the antinociception by both i.c.v. and i.t. administered TAPA and [D-Ala^2, Me-Phe^4, Gly(ol)^5]enkephalin (DAMGO). Especially in the tail-flick test, pretreatment with naloxonazine produced a marked rightward displacement of the i.t. TAPA dose–response curve for antinociception. Though DAMGO is a highly selective μ-opioid receptor agonist, pretreatment with naloxonazine partially blocked the antinociceptive response to DAMGO after i.c.v., but not after i.t. injection. These results indicate that TAPA can act as a highly selective μ_1-opioid receptor agonist (notable naloxonazine-sensitive receptor agonist) at not only the supraspinal level, but also the spinal level. These data also reveal different antinociceptive mechanisms for DAMGO and for TAPA.

Keywords: Dermorphin N-terminal tetrapeptide; Tail-flick test; Naloxonazine; β-Funaltrexamine; μ_1-Opioid receptor

1. Introduction

Dermorphin is a heptapeptide (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) derived from amphibian skin. Dermorphin selectively binds to μ-opioid receptors (De Castiglione and Rossi, 1985; Krumins, 1987) and displays potent opioid effects such as antinociception (Broccardo et al., 1981; Stevens and Yaksh, 1986). The N-terminal tetrapeptide of dermorphin is the minimum sequence for opioid activity but the fragment is less potent than the parent heptapeptide (Broccardo et al., 1981; Salvadori et al., 1982). However, replacement of the D-Ala^2 residue by D-Arg and of Gly^4 by β-Ala markedly enhances the potency of the tetrapeptide. The antinociceptive effect produced by intracerebroventricular (i.c.v.), intrathecal (i.t.) and s.c. administration of H-Tyr-D-Arg-Phe-β-Ala-OH (TAPA) was greater and of longer duration than that produced by morphine. Pretreatment with naloxone resulted in complete antagonism of the antinociceptive effects produced by administration of TAPA (Chaki et al., 1990).

TAPA binds with high affinity to [^{3}H][D-Ala^2, Me-Phe^4, Gly(ol)^5]enkephalin (DAMGO)-labeled μ-type sites in radioligand binding studies with rat membrane synapto-

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somes, whereas TAPA binds with no or negligible affinity to [^{3}H][D-Pen^{2,5}]enkephalin (DPDPE)-labeled d-opioid sites and [^{3}H](5α,7α,8β)-(+)-*N*-Methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8yl]benzeneacetamide (U69593)-labeled κ opioid sites. TAPA shows good enzymatic stability after 25 h of incubation with solubilized enzymes of mouse brain or spinal cord, in contrast to a rapid degradation of [Met^5]enkephalin (Chaki et al., 1990).

There is strong biochemical and pharmacological evidence for the existence of μ-opioid receptor subtypes (Pasternak, 1993; Elliott et al., 1994). The major subdivision of μ-opioid receptors, into μ_1 and μ_2 subtypes, is based on the binding of the μ-opioid receptor antagonist, naloxonazine, which binds irreversibly to μ_1-, but reversibly to μ_2-opioid receptors (Pasternak, 1993; Elliott et al., 1994). Furthermore, use of naloxonazine in vivo suggests that these receptor subtypes have different physiological roles, with μ_1-opioid receptors mediating supraspinal antinociception, while μ_2-opioid receptors mediate respiratory depression (Pasternak, 1993). Our recent reports have demonstrated that μ_1-opioid receptors are primarily involved in morphine-induced antinociception in tests involving formalin or the hot-plate method, whereas in the tail-pressure test, μ_2-opioid receptors may predominate (Sato et al., 1999). Furthermore, morphine produced antinociception through the μ_1- and μ_2-opioid receptor subtypes of μ-opioid receptors, after both central and spinal administration. These findings suggest the presence of μ_1-opioid receptors sensitive to naloxonazine in both spinal sites and supraspinal sites.

In the present study, the role of the μ-opioid receptor subtypes, μ_1 and μ_2, in the antinociceptive effect of TAPA on the responses to thermal noxious stimuli was examined using the irreversible μ_1-opioid receptor antagonist, naloxonazine.

2. Materials and methods

2.1. Animals

Male ddY mice weighing 20–25 g were used for all experiments. The animals were kept in a temperature-controlled room with a standard 12-h light–dark cycle (24 ± 0.5°C, 12-h dark–light cycle with lights on at 900 AM). Food and water were continuously available. Mice were tested only once. The experiments were performed with the approval of the Committee of Animal Experiments in Tohoku Pharmaceutical University.

2.2. Injection procedure

I.c.v. injections were made directly into the lateral ventricle according to the slightly modified method of Haley and McComick (1957). The method for i.t. injections was adapted from that of Hylden and Wilcox (1980). For the injections into the subarachnoid space, a 29-gauge needle, matched to a 50-μl Hamilton microsyringe, was directed into an intervertebral space at the level of the 5th and 6th lumbar vertebrae. All i.c.v. and i.t. injections were made in a volume of 5 μl in unanaesthetized mice.

2.3. Drugs

TAPA administered i.c.v. or i.t. was synthesized by the conventional solid phase method in our laboratory. TAPA and DAMGO (Sigma, St. Louis, MO, USA) were dissolved in sterile artificial cerebrospinal fluid (CSF) containing 126.6 mM NaCl, 2.5 mM KCl, 2.0 mM $MgCl_2$ and 1.3 mM $CaCl_2$. β-Funaltrexamine (40 mg/kg, s.c.) and naloxonazine (35 mg/kg, s.c.) were dissolved in saline and injected s.c. in a volume of 0.1 ml/10 g body weight

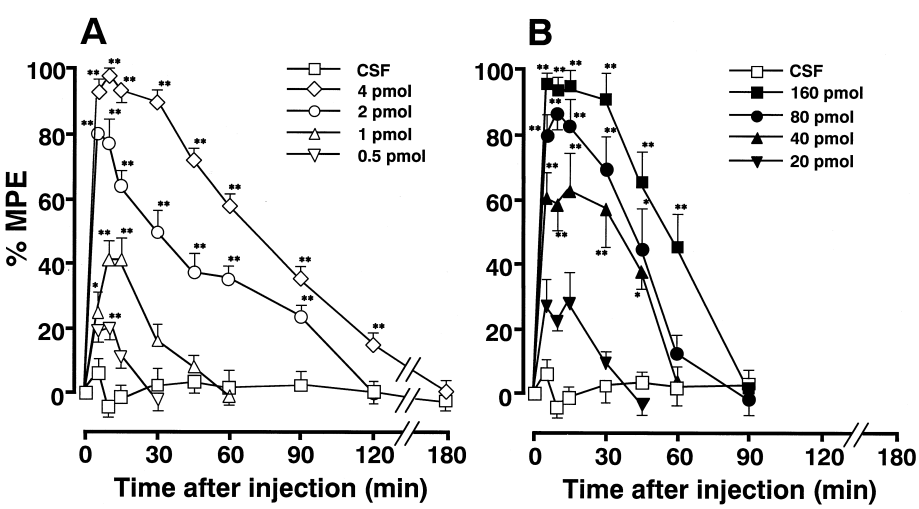

Fig. 1. Time course of effects of i.c.v. administered TAPA (A) and DAMGO (B) in the mouse tail-flick test. Antinociception was expressed as percent of maximum possible effect (% MPE) = 100 × (post-drug responsive latency − pre-drug responsive latency)/(10 − pre-drug responsive latency). Each data point represents the mean ± S.E.M. for 10 mice. $^{**}P < 0.01$ and $^{*}P < 0.05$, compared to the responsive value in the CSF-control group.

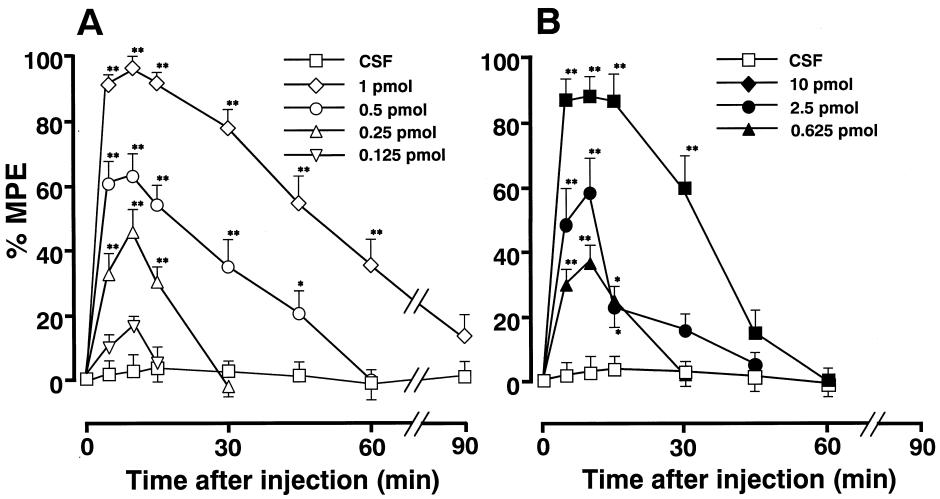

Fig. 2. Time course of effects of i.t. administered TAPA (A) and DAMGO (B) in the mouse tail-flick test. Antinociception was expressed as percent of maximum possible effect (% MPE) = 100 × (post-drug responsive latency − pre-drug responsive latency)/(10 − pre-drug responsive latency). Each data point represents the mean ± S.E.M. for 10 mice. $^{**}P < 0.01$ and $^{*}P < 0.05$, compared to the responsive value in the CSF-control group.

24 h prior to testing. Under these conditions, the actions of naloxonazine are relatively selective for μ_1-opioid receptors (Ling et al., 1986).

2.4. Assessment of nociceptive threshold

Antinociception was assessed using the tail-flick test. The tail-flick test was adapted from the classical design of D'Amour and Smith (1941). Nociceptive thresholds were determined by using an automated tail-flick unit (Ugo Basile, Italy). Tail-flick latencies were recorded as the time from the start of the heat stimulus to withdrawal of the tail. The intensity of the light beam was adjusted so that baseline readings were generally between 2.0 and 4.0 s. The light beam was focused on the same spot, about 1.0 cm from the tip of the tail, for all animals. A maximum latency of 10 s was imposed to minimize tissue damage. No animal was used more than once. To prevent experimenter bias, observers were uninformed of the dose of TAPA being injected, and were uninformed of whether naloxonazine was used as pretreatment when the modification of each agonist-induced antinociception was investigated. After determination of pre-drug values, animals were injected with TAPA or DAMGO. Antinociceptive activity for each animal was calculated from the following equation and expressed as a percent of the maximum possible effect: $\%\ \mathrm{MPE} = [(T_2 - T_1)/(10 - T_1)] \times 100$ where T_1 and T_2 are pre-drug and post-drug responsive latencies, respectively.

2.5. Data analysis and statistics

Statistical significance of the data was estimated with a mixed two-factor analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A level of probability of 0.05 or less was accepted as significant. The ED_{50} values and their 95% confidence limits (95% CL) for the antinociceptive effect of TAPA and DAMGO were computed according to the method of Litchfield and Wilcoxon (1949) using Programs 11 and 47 of the pharmacological calculations system of Tallarida and Murray (1987).

3. Results

3.1. Time courses of tail-flick response to i.c.v. or i.t. administration of TAPA and DAMGO

Groups of mice were given i.c.v. or i.t. CSF or different doses of TAPA or DAMGO. The antinociceptive activity of TAPA or DAMGO was estimated 5, 10, 15, 30, 45, 60, 90 and 120 min after i.c.v. or i.t. injections. The duration of the tail-flick inhibition induced by TAPA appeared to be longer than that of DAMGO (Figs. 1 and 2). ED_{50}

Table 1
ED_{50} values for TAPA and DAMGO antinociception to mechanical and thermal noxious stimuli in non-pretreated mice and in mice pretreated with naloxonazine

	Route	ED_{50} values (pmol)		(B)/(A)
		Non-pretreated (A)	Naloxonazine (B)	
TAPA				
Tail-flick test	i.c.v.	1.3 (0.87–1.80)	19.5 (15.11–23.90)	15.0
	i.t.	0.3 (0.20–0.47)	13.5 (9.29–19.61)	45.0
DAMGO				
Tail-pressure test[a]	i.c.v.	8.0 (4.15–15.40)	70.0 (34.63–141.51)	8.8
	i.t.	12.5 (6.23–25.07)	14.5 (7.62–27.59)	1.2
Tail-flick test	i.c.v.	36.0 (18.94–57.47)	130 (79.8–211.7)	3.6
	i.t.	3.0 (1.15–7.84)	2.8 (1.05–7.49)	0.9

[a] ED_{50} values of the tail-pressure test are cited from Sakurada et al. (1999), and were obtained 5 min after i.c.v. and i.t. administered DAMGO.

values for TAPA and DAMGO are shown in Table 1. Antinociception by i.c.v. or i.t. TAPA was found to be about 27.7- and 10-fold more potent than that by DAMGO for inhibiting tail-flick responses, respectively (Table 1).

3.2. Effects of naloxonazine or β-funaltrexamine on TAPA- and DAMGO-induced antinociception in the tail-flick test

The i.c.v. or i.t. administration of TAPA dose-dependently suppressed thermal nociception with ED_{50} of 1.3 (0.87–1.80) and 0.3 (0.20–0.47) pmol, respectively (Table 1). Pretreatment with naloxonazine markedly attenuated the antinociceptive actions of supraspinal and spinal TAPA (Fig. 3). Naloxonazine produced an approximately 15- and 40-fold shift to the right of the dose–response curve for i.c.v. and i.t., respectively (Table 1; Fig. 3).

Testing, 10 min after i.c.v. or i.t. injection of DAMGO, showed a dose-dependent inhibition of the tail-flick response to thermal stimulation. ED_{50} values for i.c.v. and i.t. administration of DAMGO were 36.0 (18.94–57.47) pmol and 3.0 (1.15–7.84) pmol, respectively (Table 1). Pretreatment with naloxonazine resulted in a significant shift of the dose–response curve for i.c.v. DAMGO approximately four fold to the right without altering its effectiveness toward i.t. DAMGO (Fig. 3). ED_{50} values for i.c.v. and i.t. DAMGO in conjunction with naloxonazine were 130 (79.8–211.7) pmol and 2.8 (1.05–7.49) pmol, respectively (Table 1).

Pretreatment with β-funaltrexamine antagonized the antinociception of both i.c.v. and i.t. administered DAMGO and TAPA (Fig. 3).

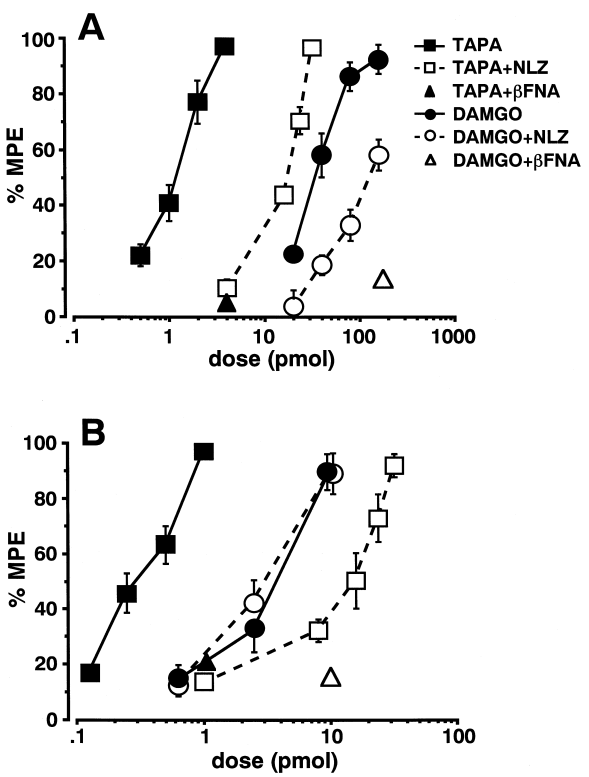

Fig. 3. Dose–response curves for the inhibition of the mouse tail-flick response induced by i.c.v. (A) and i.t. (B) administration of TAPA and DAMGO in non-pretreated groups and in mice pretreated with naloxonazine (NLZ) or β-funaltrexamine (β-FNA). Naloxonazine (35 mg/kg) and β-funaltrexamine (40 mg/kg) were administered s.c. 24 h before administration of each μ-opioid receptor agonist. Antinociceptive effect was measured 10 min after i.c.v. or i.t. administration of each μ-opioid receptor agonist.

4. Discussion

The main finding of the present study was that the antinociceptive effects of i.c.v. and i.t. TAPA, a dermorphin tetrapeptide analogue in the tail-flick test, were significantly antagonized by pretreatment with naloxonazine, an irreversible μ_1-opioid receptor antagonist, suggesting that TAPA may be a highly selective agonist at the μ_1-opioid receptor subtype of μ-opioid subtypes receptors.

Consistent with our previous finding that TAPA has good stability against degradation enzymes in the brain(Chaki et al., 1990), TAPA-induced antinociception is long-lasting as compared to that by morphine (Chaki et al., 1988; Sasaki et al., 1991). In addition, TAPA, with high selectivity for μ-opioid receptors (Chaki et al., 1988; Sasaki et al., 1991), is much more potent for producing antinociception than is DAMGO, a μ-opioid receptor agonist (Chaki et al., 1988; Sakurada et al., 1999).

In the present study, we used the μ-opioid receptor-selective antagonists, β-funaltrexamine and naloxonazine, to determine the subtype of receptor involved in the antinociceptive responses to TAPA and DAMGO. β-Funaltrexamine irreversibly antagonizes both μ_1- and μ_2-opioid receptors(Recht and Pasternak, 1987) and inhibits both supraspinal and spinal antinociception, whereas naloxonazine selectively antagonizes μ_1-opioid receptors and supraspinal antinociception, but does not antagonize spinal antinociception mediated through μ_2-opioid receptors (Ling et al., 1986; Heyman et al., 1988; Paul et al., 1989; Pick et al., 1991). Binding studies indicate that naloxonazine, injected s.c., produces a long-lasting and selective blockade of μ_1-binding sites (Ling et al., 1986). With respect to DAMGO-induced antinociception in the tail-flick test, i.c.v. DAMGO is highly sensitive to naloxonazine, whereas i.t. DAMGO is insensitive to naloxonazine and sensitive to β-funaltrexamine (Pick et al., 1991).

Naloxonazine did not completely abolish the action of i.c.v. DAMGO, whereas the DAMGO-induced antinociception was antagonized by β-funaltrexamine, indicating a potential residual action at μ_2-opioid receptors in the tail-pressure and tail-flick tests (Fig. 3; Sakurada et al., 1999). Nevertheless, TAPA was extremely sensitive to antagonism by naloxonazine. It is, therefore, probable that TAPA acts as a highly selective μ_1-opioid receptor agonist at the supraspinal level.

The antagonism of i.t. administered TAPA by naloxonazine was surprising because the antinociception induced by i.t. DAMGO in this and other experiments with both tail-flick and tail-pressure tests (Heyman et al., 1988; Paul et al., 1989; Sakurada et al., 1999) was not affected by the antagonist, indicating the greater sensitivity of TAPA to naloxonazine, especially in the tail-flick test. This difference implies that the actions of TAPA can be dissociated from those of DAMGO, even though the action of both compounds is within the μ-opioid family in the central nervous system.

Morphine produces antinociception by activating μ-opioid receptors encoded by the *MOR-1* gene. Although morphine-6β-glucuronide, heroin and 6-acetylmorphine are also considered μ-opioids, recent evidence suggests that they act through a distinct receptor mechanism (Schuller et al., 1999). An antisense setup and 3-methoxynaltrexone, a selective heroin and morphine-6β-glucuronide antagonist, could distinguish the differences in antinociceptive mechanisms between morphine and, heroin, morphine-6β-glucuronide and 6-acetylmorphine (Brown et al., 1997; Rossi et al., 1997).

Neither the physical basis for the putative μ_1- and μ_2-opioid receptors, nor the mechanism of action of naloxonazine, is fully understood. Studies with μ-opioid receptor knockout mice indicate that both μ_1- and μ_2-opioid receptors arise from the known sequence of the cloned μ-opioid receptor (Matthes et al., 1996). This indicates that splice variants of this receptor (other than the μ-opioid receptor-1B variant, (Zimprich et al., 1995)), or different physical states of the receptor, could be differentially sensitive to naloxonazine. Regardless, naloxonazine has proven to be a powerful tool for demonstrating differential effects of μ-opioid receptor family.

In conclusion, both TAPA and DAMGO injected i.c.v. and i.t. produced dose-related antinociceptive activity against a thermal stimulus. Pretreatment with naloxonazine markedly antagonized the antinociceptive effect of i.t. and i.c.v. TAPA, but not of i.t. DAMGO. However, a partial antagonism of i.c.v. DAMGO by naloxonazine was shown. The results indicate that TAPA may produce antinociception at μ_1-opioid receptor subtypes of μ-opioid receptor after both central and spinal administration.

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