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Characterization of muscarinic receptor subtypes in the rostral ventrolateral medulla and effects on morphine-induced antinociception in rats

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Abstract

The present study investigated the role of muscarinic receptor subtypes in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha of the rat rostral ventrolateral medulla in morphine-induced antinociception. The antinociceptive effects of morphine were evoked by systemic administration or microinjection into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. Administration of morphine produced antinociception for hot plate and tail immersion responses to noxious heat stimuli. These effects were antagonized by prior exposure to naloxone and inhibited by mecamylamine pretreatment. Morphine-induced antinociception was significantly inhibited by atropine in a dose-dependent manner. Muscarinic toxin-1 and pirenzepine inhibited morphine-induced antinociception for both the hot plate and tail immersion tests. At a dose of 5 nmol/site, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) also inhibited morphine-induced antinociception, although low doses of this drug did not significantly affect hot plate test response latency when morphine was systemically administered. These results suggest that the antinociceptive effects induced by morphine in part involve the muscarinic M_1 and M_3 receptors of the rat nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha.

Keywords: Morphine; Antinociception; Nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha; Muscarinic antagonist; (Rat)

1. Introduction

Activation of cholinergic neurons in the central nervous system produces antinociception and analgesia in a variety of animals, including humans (Zhuo and Gebhart, 1990, 1991; Iwamoto and Marion, 1993a; Hood et al., 1995). Pharmacological experiments have shown that the microinjection of acetylcholine or carbachol into specific brainstem nuclei can produce antinociception and can be reversed by muscarinic receptor antagonists (Brodie and Proudfit, 1984; Yaksh et al., 1985). Moreover, the intrathecal administration of muscarinic agonists also has an antinociceptive effect on the response to noxious heat stimulation via the muscarinic M_1, M_2 or M_3 receptors (Iwamoto and Marion, 1993b; Naguib and Yaksh, 1997). Thus, the cholinergic pathway in the central nervous system may be involved in endogenous pain control.

The rostral ventrolateral medulla, which includes the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha and the lateral reticular nucleus, modulates aspects of antinociception and the cardiovascular systems via cholinergic neurons (Ossipov and Gebhart, 1986; Zhuo and Gebhart, 1990; Kubo et al., 1997). Anatomically, the rostral ventrolateral medulla receives cholinergic projections primarily from the pedunculopontine tegmental nucleus (Mitani et al., 1988; Yasui et al., 1990). In addition, other studies have shown the existence of a descending cholinergic system from the rostral ventrolateral medulla to the spinal cord and the presence of small to medium-sized cholinergic neurons and choline acetyltransferase mRNA in small cells of the nucleus reticularis gigantocellularis/nucleus reticularis paragigantocellularis (Jones et al., 1986; Lauterborn et al., 1993). These observations suggest that the cholinergic system plays an impor-

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tant role in the rostral ventrolateral medulla including the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha.

The interaction of morphine with cholinergic neurons in the central nervous system is well known. The midbrain periaqueductal gray, rostral ventral medullary nucleus raphe magnus and the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha are the supraspinal structures involved in morphine-induced analgesia (Satoh et al., 1979; Azami et al., 1982). In addition, evidence exists that shows the descending cholinergic system, spinal cholinergic receptors and cholinesterase to be involved in the mechanisms of opioid analgesia (Naguib and Yaksh, 1994; Fang and Proudfit, 1996; Hood et al., 1997). Moreover, various combinations of morphine and anticholinesterase result in an increased antinociceptive effect (Beilin et al., 1997). Our findings that either systemically administered or rostral ventrolateral medulla-injected morphine enhances the release of acetylcholine in the rostral ventrolateral medulla, including nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha, and that morphine locally injected into the rostral ventrolateral medulla induces an antinociceptive effect also support the notion of a relationship between cholinergic neurons and morphine antinociception (Taguchi et al., 1999). Thus, the present study investigated the possible role of cholinergic receptor subtypes in the rostral ventrolateral medulla, in nociception induced by morphine systemically administered or injected in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha, using non-selective and muscarinic M_1, M_2 or M_3 receptor antagonists.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 250–350 g were housed individually under automatically controlled environmental conditions and a 12-h light–dark cycle with free access to food and water. All animals were quarantined in centralized animal facilities for at least seven days upon arrival. Each animal was used only once in the present study. Experiments were carried out according to the guidelines for animal care and use published by the National Institutes of Health.

2.2. Preparation for microinjection

After an acclimation period, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. A midline incision was made on

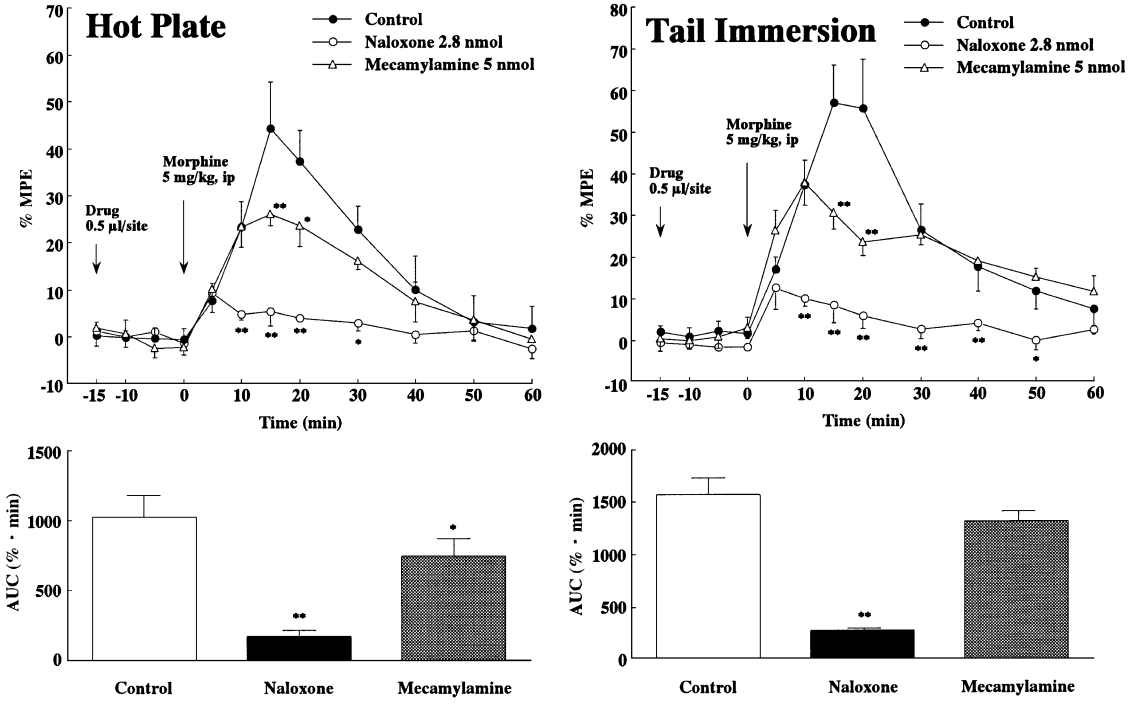

Fig. 1. Time course of intraperitoneally administered (5 mg/kg) morphine-induced antinociceptive effects, and effects of naloxone (2.8 nmol/site) and mecamylamine (5 nmol/site) injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. Naloxone almost completely reversed the hot plate and tail immersion responses, and mecamylamine also significantly depressed morphine-induced antinociception. The effects of naloxone and mecamylamine are further shown in the lower graphs, which display the AUC as an indicator of morphine-induced antinociception. The AUC was calculated from the data in the upper graphs between 0 and 60 min after morphine administration. All points represent mean response latencies of five to seven animals and error bars express S.E. (*$P<0.05$, **$P<0.01$ compared with control).

the scalp, then a burr hole was drilled for a 27-gauge stainless-steel cannula (guide cannula) that was fixed using dental acrylic and was anchored to the skull using stainless-steel screws. The 30-gauge stainless-steel cannula used for drug injection had its tip extending 4 mm beyond the tip of the guide cannula into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha, according to the atlas of Paxinos and Watson (1986). The coordinates of the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha were as follows: bregma − 11.5 mm, lateral 0.9 mm and depth − 10.0 mm. To confirm that no abnormalities had resulted from the procedure, the animals were observed for 1 week after surgery and prior to drug administration.

2.3. Drug administration

The following drugs were microinjected: morphine hydrochloride (Sankyo), naloxone hydrochloride (Endo Lab.), nicotinic receptor antagonist, mecamylamine hydrochloride (Nacalai), the specific muscarinic receptor antagonist, atropine sulfate (Tokyokasei); the selective muscarinic M_1 receptor antagonist, muscarinic toxin-1 (Alomone Labs); the potently muscarinic M_1 but also muscarinic M_4 receptor antagonist, pirenzepine hydrochloride; the selective muscarinic M_2 receptor antagonist, methoctramine tetrahydrochloride; and the muscarinic M_3 receptor antagonist, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; Research Biochemicals International). All drugs were dissolved immediately before use in sterile physiological saline and filtered through a 0.2-μm pore-sized membrane. The injection cannula was connected to a Hamilton microsyringe with PE-10 polyethylene tubing and was filled with either saline or a drug solution. These solutions (0.5 μl each) were injected over 60 s into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. After infusion of saline or test drugs, the injection cannula was carefully withdrawn.

2.4. Effect of muscarinic receptor antagonist on morphine-induced antinociception

The hot plate and tail immersion tests were conducted to assess the nociceptive response. In the hot plate test, the animals were placed onto a heated plate set at 55 °C and the latency of paw licking or jumping was measured. A cut-off time of 30 s was established to avoid tissue injury. In the tail immersion test, the animals were held while their tails were immersed in water at 52 °C. The interval between tail immersion and the withdrawal or jerk response was timed. A cut-off time of 9 s was established to minimize tissue damage. The average of hot plate and

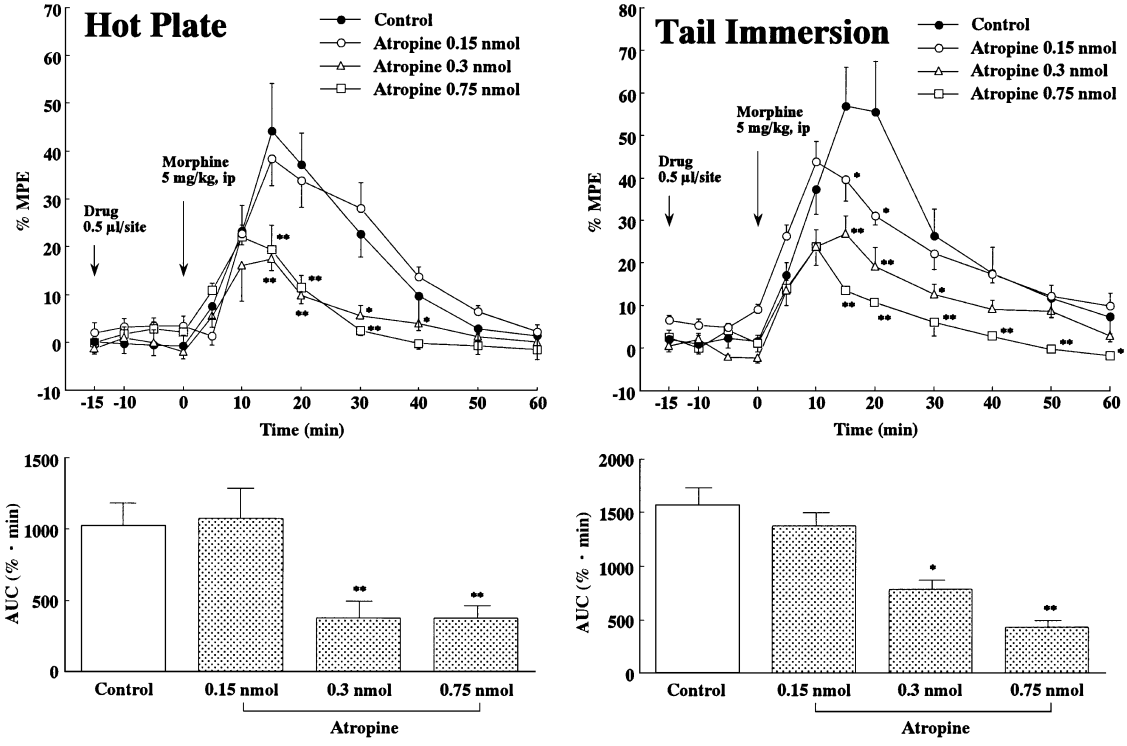

Fig. 2. Effects of various doses of atropine (0.15, 0.3 and 0.75 nmol/site) injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha on morphine-induced antinociception. Atropine significantly and dose-dependently depressed antinociception induced by systemically administered morphine. The effects of atropine on morphine-induced antinociception are presented in the lower graphs, which depict the AUC, an indicator of morphine-induced antinociception. The AUC was calculated from the data in the upper graphs between 0 and 60 min after morphine administration. All points represent mean response latencies of five to seven animals and error bars express S.E. (*$P<0.05$, **$P<0.01$ compared with control).

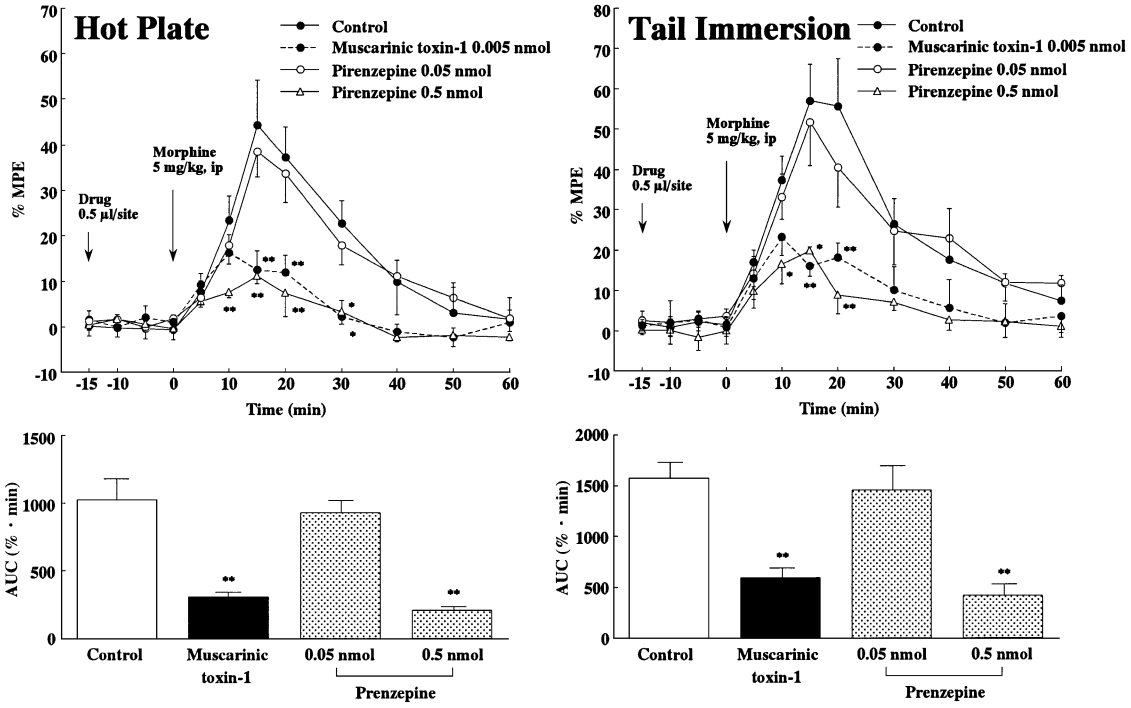

Fig. 3. Effects of muscarinic toxin-1 and pirenzepine injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha on intraperitoneally administered (5 mg/kg) morphine-induced antinociception. Treatment with muscarinic toxin-1 (0.005 nmol/site) and a high dose of pirenzepine (0.5 nmol/site) inhibited hot plate and tail immersion responses. The effects of muscarinic toxin-1 and pirenzepine are further demonstrated in the lower graphs, which display the AUC as an indicator of morphine-induced antinociception. The AUC was calculated from the data in the upper graphs between 0 and 60 min after morphine administration. All points represent mean response latencies of five to seven animals and error bars express S.E. (*$P<0.05$, **$P<0.01$ compared with control).

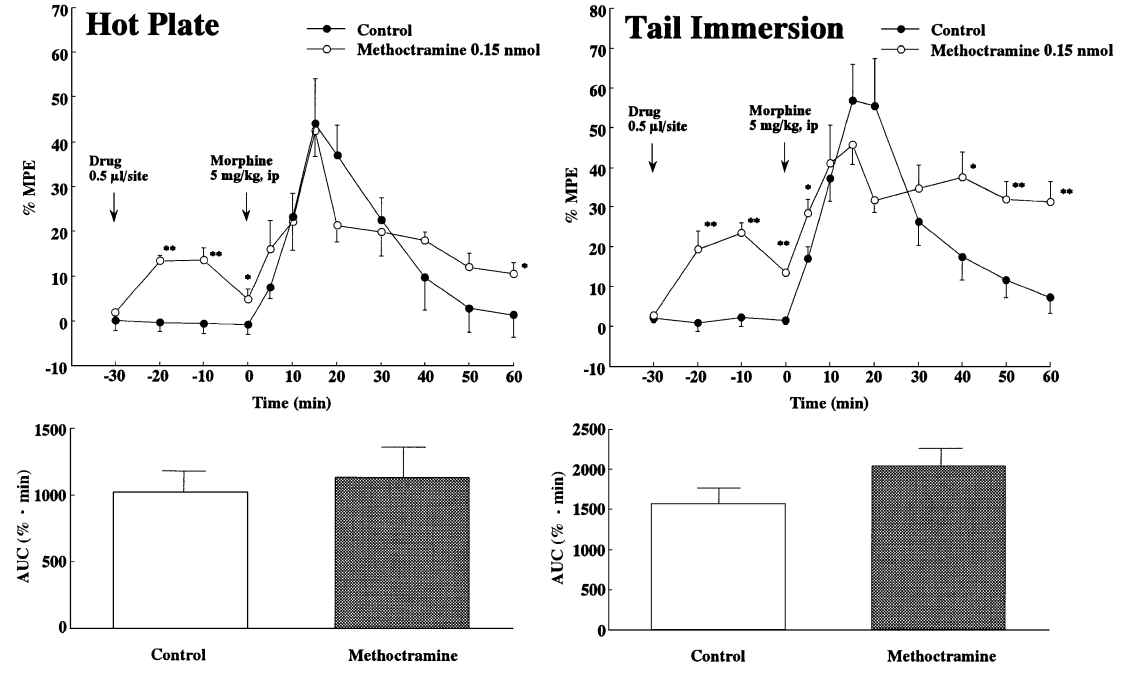

Fig. 4. Effects of methoctramine (0.15 nmol/site) injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha on intraperitoneally administered (5 mg/kg) morphine-induced antinociception. Administration of methoctramine into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha induced significant antinociception for both hot plate and tail immersion responses. The effects of methoctramine on the AUC, an indicator of morphine-induced antinociception, are displayed in the lower graphs. The AUC was calculated from the data in the upper graphs between 0 and 60 min after morphine administration. Methoctramine did not alter maximal levels or $AUC_{(0-60\ min)}$ and this effect changed little over the +60 min testing period. All points represent mean response latencies of six animals and error bars express S.E. (*$P<0.05$, **$P<0.01$ compared with control).

tail immersion response latency values from three consecutive trials before drug administration was used as the baseline.

Ten minutes after the baseline response latencies were obtained, vehicle, naloxone, mecamylamine, atropine or muscarinic receptor antagonists were injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha, and test response latency was recorded every 5 min. Immediately after the third test (defined as $t=0$), the animals were given morphine intraperitoneally (5 mg/kg) or through an injection cannula into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha (3 nmol/site). Test latencies were determined at +5 min and at 5-min intervals for +20 min. Thereafter, latency was measured at 10-min intervals for +60 min.

2.5. Location of the injection site

The animals were deeply anesthetized with sodium pentobarbital after experimentation. Correct placement of the microinjections was verified in each animal by injecting methylene blue into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. The animals were then killed with an overdose of sodium pentobarbital. The brain of each animal was removed, fixed overnight in 10% formalin and cryostat sections were prepared.

2.6. Analysis of the data

The hot plate and tail immersion responses of each rat were converted to percent maximal possible effect (%MPE), according to the following formula;

$$\%\mathrm{MPE} = \frac{\text{post-drug latency} - \text{pre-drug latency}}{\text{cut-off time} - \text{pre-drug latency}} \times 100$$

The effects of the drugs in the hot plate and tail immersion tests were also demonstrated by the area under the curve (AUC) from $t=0$ to +60 min after morphine administration. The data are expressed as means ± standard error (S.E.) for each group. The significance of the difference between the groups was analyzed by repeated measurement two-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test or non-paired (Student's or Aspin–Welch's) *t*-test. A value of $P<0.05$ was regarded as statistically significant.

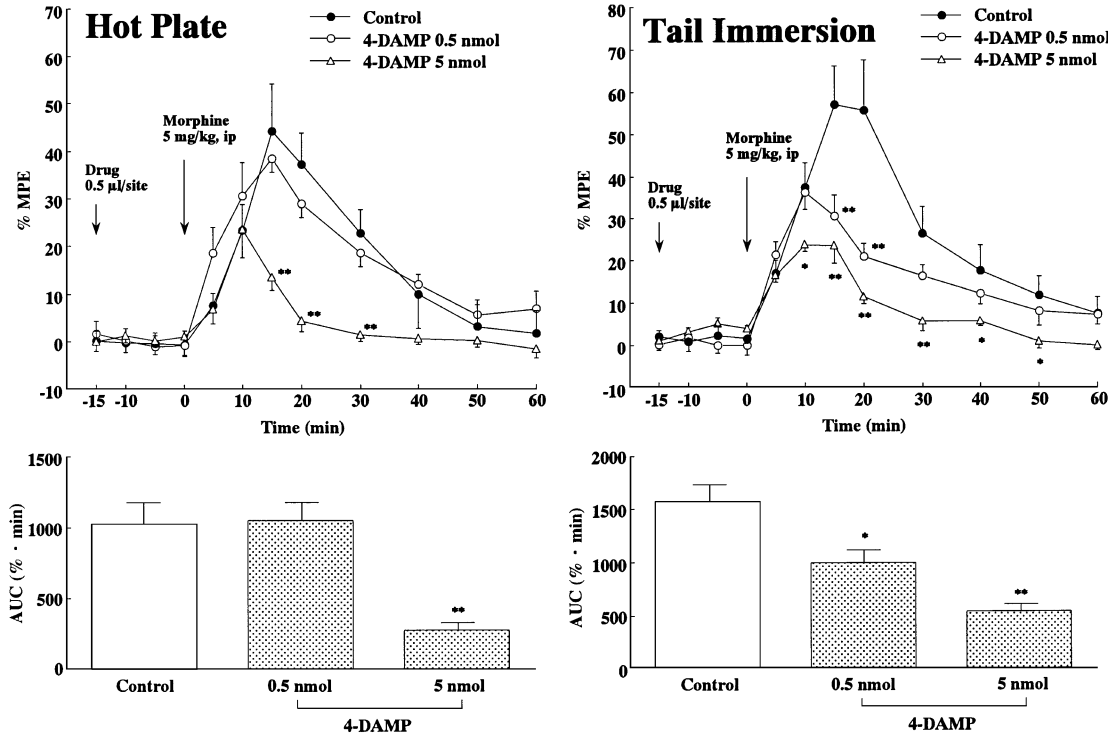

Fig. 5. The effects of 4-DAMP injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha on intraperitoneally administered (5 mg/kg) morphine-induced antinociception. No significant differences were observed in hot plate response latencies between the control group and the lower-dose 4-DAMP (0.5 nmol/site) group. In contrast, antinociceptive effects of morphine were moderately inhibited by low-dose 4-DAMP in the tail immersion test. At a high dose of 4-DAMP (5 nmol/site), inhibitory effects on antinociception were equal during hot plate and tail immersion tests. The effects of 4-DAMP on the AUC, that represents morphine-induced antinociception, are shown in the lower graphs. The AUC was calculated from the data in the upper graphs between 0 and 60 min after morphine administration. All points represent mean response latencies of five to eight animals and error bars express S.E. (*$P<0.05$, **$P<0.01$ compared with control).

3. Results

3.1. Effects of naloxone, mecamylamine and atropine on antinociception induced by intraperitoneally administered morphine

Figs. 1 and 2 show time courses of the antinociceptive effects of intraperitoneally administered morphine (5 mg/kg) and show the effects of naloxone, mecamylamine and atropine microinjected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha on hot plate and tail immersion responses. Morphine induced an antinociceptive response in the control group (saline injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha). Maximal %MPE during the hot plate (44.3 ± 10.0%) and tail immersion (57.0 ± 9.0%) tests occurred 15–20 min after morphine administration. Thereafter, the peak level decreased progressively with time and reached the baseline value at +60 min.

Prior exposure of the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha to microinjected naloxone (2.8 nmol/site) resulted in almost complete antagonism of the antinociceptive effect of morphine as demonstrated in both the hot plate and tail immersion tests. Mecamylamine (5 nmol/site) moderately inhibited morphine-induced antinociception, and atropine (0.15, 0.3 and 0.75 nmol/site) and also inhibited antinociception in a dose-dependent manner, but these effects were weaker than that of naloxone. A 0.15 nmol/site dose of atropine did not affect morphine-induced antinociception in the hot plate test. Morphine-induced antinociception was decreased in response to 0.3 and 0.75 nmol/site atropine (maximum %MPE at 0.3 nmol/site: 17.6 ± 2.5%; at 0.75 nmol/site: 21.1 ± 6.1%). Similar results were obtained for the tail immersion experiments except for 0.15 nmol/site atropine, which inhibited the effects of morphine. The maximal %MPE in the 0.15, 0.3 and 0.75 nmol/site atropine-treated groups was 43.8 ± 4.8%, 26.8 ± 4.3% and 23.9 ± 4.5%, respectively.

The $AUC_{(0-60\ min)}$ value, representing the antinociceptive effect of morphine, was significantly reduced for the hot plate response but not the tail immersion response in rats pretreated with microinjected mecamylamine. Naloxone antagonized the antinociceptive effect of morphine in both the hot plate and tail immersion tests as shown by $AUC_{(0-60\ min)}$ analysis (Fig. 1). Analysis of the $AUC_{(0-60\ min)}$ for atropine (0.15, 0.3 and 0.75 nmol/site) indicated dose-dependent antinociception for both the hot plate and tail immersion responses (Fig. 2).

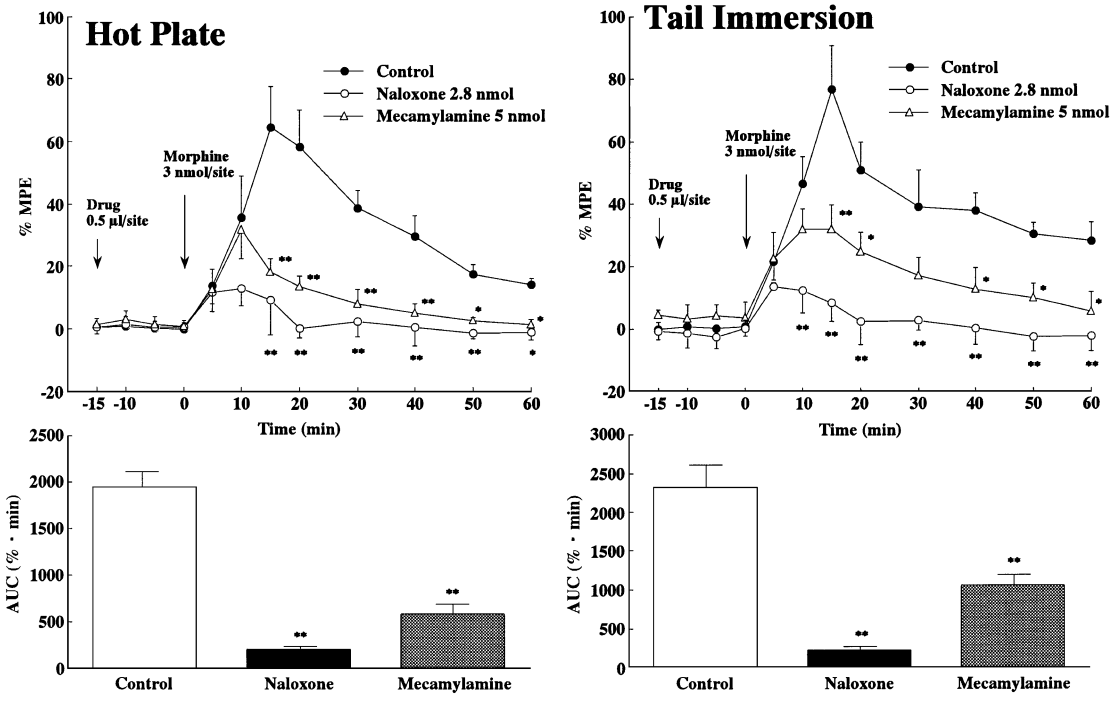

Fig. 6. Time course of antinociceptive effects induced by injection of morphine (3 nmol/site) into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha, and effects of naloxone (2.8 nmol/site) and mecamylamine (5 nmol/site) injected into the same location. Naloxone almost completely reversed hot plate and tail immersion responses. Mecamylamine also significantly inhibited morphine-induced antinociception. The effects of naloxone and mecamylamine on the AUC, representing morphine-induced antinociception, are shown in the lower graphs. The AUC was calculated from the data in the upper graphs collected 0–60 min after morphine microinjection. All points represent mean response latencies of six to eight animals and error bars express S.E. (*$P<0.05$, **$P<0.01$ compared with control).

3.2. Effect of pirenzepine, muscarinic toxin-1, methoctramine and 4-DAMP on antinociception induced by intraperitoneally administered morphine

Prior exposure to pirenzepine (0.05 nmol/site) did not alter morphine-induced antinociception until +60 min after testing was initiated. However, at a dose of 0.5 nmol/site, pirenzepine significantly inhibited the antinociceptive effect of intraperitoneally administered morphine. The maximal levels of %MPE were 11.2 ± 1.6% and 19.9 ± 0.9% for the hot plate and tail immersion tests, respectively. Muscarinic toxin-1 (0.005 nmol/site) inhibited morphine-induced antinociception to nearly the same degree as did 0.5 nmol/site pirenzepine (Fig. 3). Analysis of the $AUC_{(0-60\ min)}$ value indicated a significant antinociceptive effect of microinjected pirenzepine (0.5 nmol/site) but not pirenzepine (0.05 nmol/site). Microinjected muscarinic toxin-1 (0.005 nmol/site) reduced the $AUC_{(0-60\ min)}$ values of morphine-induced antinociception in both the hot plate and tail immersion tests (Fig. 3).

Prior exposure to microinjected methoctramine (0.15 nmol/site) induced moderate, but significant antinociception in the period before morphine administration (maximal levels of %MPE for the hot plate test: 13.6 ± 4.9%, $P<0.01$; tail immersion test: 23.5 ± 4.7%, $P<0.01$). After systemic administration of morphine, the highest values for %MPE in the methoctramine-injected animals during the hot plate (42.7 ± 5.0%) and tail immersion (45.8 ± 8.5%) tests did not differ significantly from the controls (Fig. 4). Similarly, $AUC_{(0-60\ min)}$ values in methoctramine (0.5 nmol/site)-treated rats did not differ significantly from the controls. In the tail immersion test, the antinociceptive effect of morphine remained at a high level throughout the 60-min testing period (Fig. 4).

Test response latencies did not differ from those of the control group during the hot plate test in response to 0.5 nmol/site 4-DAMP. However, morphine-induced antinociception was moderately inhibited by this dose of 4-DAMP during the tail immersion test (maximal level of %MPE: 36.1 ± 3.9%). At 5 nmol/site 4-DAMP, antinociception was depressed to the same degree in both the hot plate and tail immersion tests (maximal level of %MPE, hot plate test: 23.7 ± 6.2%, tail immersion test: 23.9 ± 1.7%; Fig. 5). The $AUC_{(0-60\ min)}$ value, representing the antinociceptive effect of morphine in rats pretreated with microinjected 4-DAMP (0.5 nmol/site), was significantly reduced in the tail immersion test but not in the hot plate test. However, 4-DAMP (5 nmol/site) inhibited the antinociceptive effect of morphine in both the hot plate and tail immersion responses (Fig. 5).

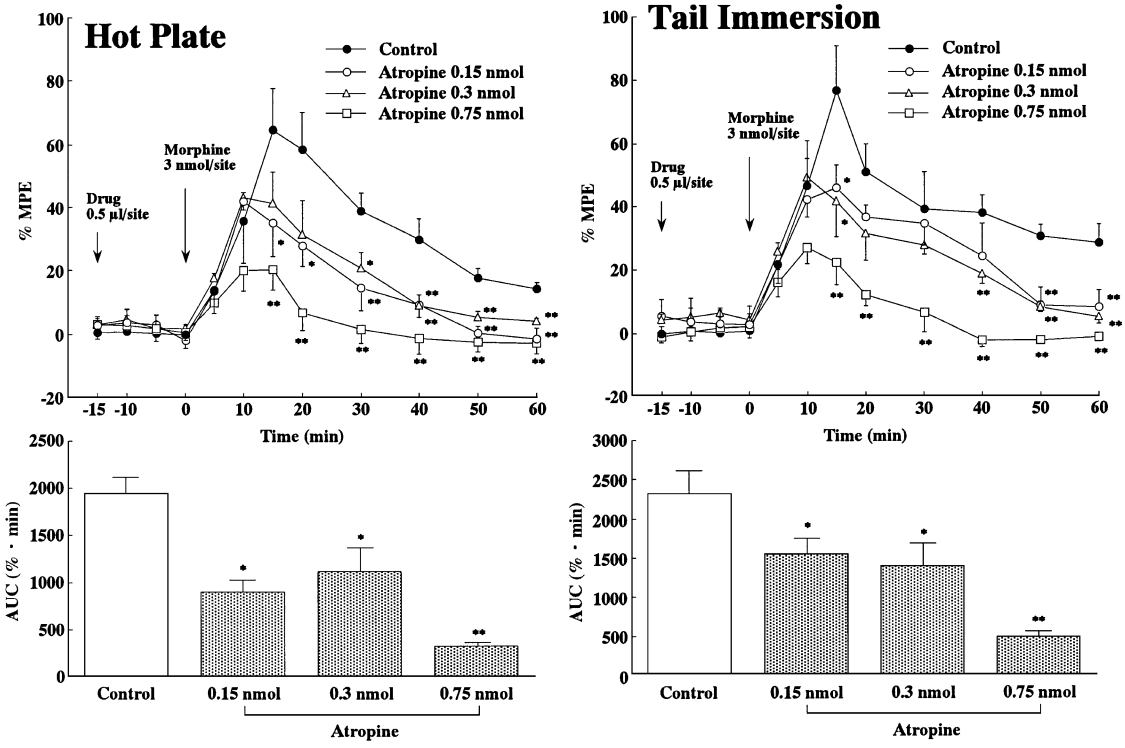

Fig. 7. Effects of various doses of atropine (0.15, 0.3 and 0.75 nmol/site) injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. Atropine significantly and dose-dependently depressed antinociception by morphine. The effects of atropine on the AUC, that represents morphine-induced antinociception, are shown in the lower graphs. The AUC was calculated from the data in the upper graphs collected 0–60 min after morphine microinjection. All points represent mean response latencies of six to eight animals and error bars express S.E. (*$P<0.05$, **$P<0.01$ compared with control).

3.3. Effects of naloxone, mecamylamine and atropine on antinociception induced by morphine microinjected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha

In the hot plate and tail immersion tests, microinjection of morphine (3 nmol/site) induced a higher antinociceptive response than did intraperitoneal administration. Injected morphine maximally inhibited the hot plate and tail immersion response at +15 min (maximal levels of %MPE, hot plate test: 64.6 ± 13.1%; tail immersion test: 76.6 ± 14.3%). The antinociceptive effect of morphine tended to decrease over the course of the hot plate and tail immersion tests, although latency remained high until +60 min after administration (Fig. 6).

Naloxone (2.8 nmol/site) inhibited the subsequent morphine-induced antinociceptive response and %MPE peaked at less than 20% in both the hot plate and tail immersion tests. Mecamylamine (5 nmol/site) moderately inhibited morphine-induced antinociception (maximal levels of %MPE, hot plate test: 31.9 ± 9.1%; tail immersion test: 31.9 ± 6.7%; Fig. 6), and atropine dose-dependently inhibited morphine-induced antinociception in both the hot plate and tail immersion tests (Fig. 7). However, 0.15 and 0.3 nmol/site atropine only moderately inhibited the hot plate response (maximal levels of %MPE at 0.15 nmol/site: 42.0 ± 2.8%; at 0.3 nmol/site: 43.1 ± 1.6%) and the tail immersion response (maximal levels of %MPE at 0.15 nmol/site: 46.0 ± 7.1%; at 0.3 nmol/site: 49.0 ± 11.8%). At a dose of 0.75 nmol/site, atropine clearly inhibited morphine-induced antinociception. Maximal levels of %MPE during the hot plate and tail immersion tests were 20.4 ± 6.4% and 27.0 ± 5.0%, respectively (Fig. 7).

Microinjection of either mecamylamine or naloxone significantly reduced the antinociceptive effect of microinjected morphine ($AUC_{(0-60\ min)}$) in rats subjected to the hot plate and tail immersion tests (Fig. 6). Analysis of the $AUC_{(0-60\ min)}$ for microinjected atropine identified dose-dependent antinociception for both the hot plate and tail immersion responses (Fig. 7).

3.4. Effects of muscarinic toxin-1, pirenzepine and 4-DAMP on antinociception induced by morphine microinjected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha

In both the hot plate and tail immersion tests, muscarinic toxin-1 (0.005 nmol/site) clearly depressed morphine-induced antinociception. Maximal levels of %MPE during the hot plate and tail immersion tests were 20.1 ± 6.0% and

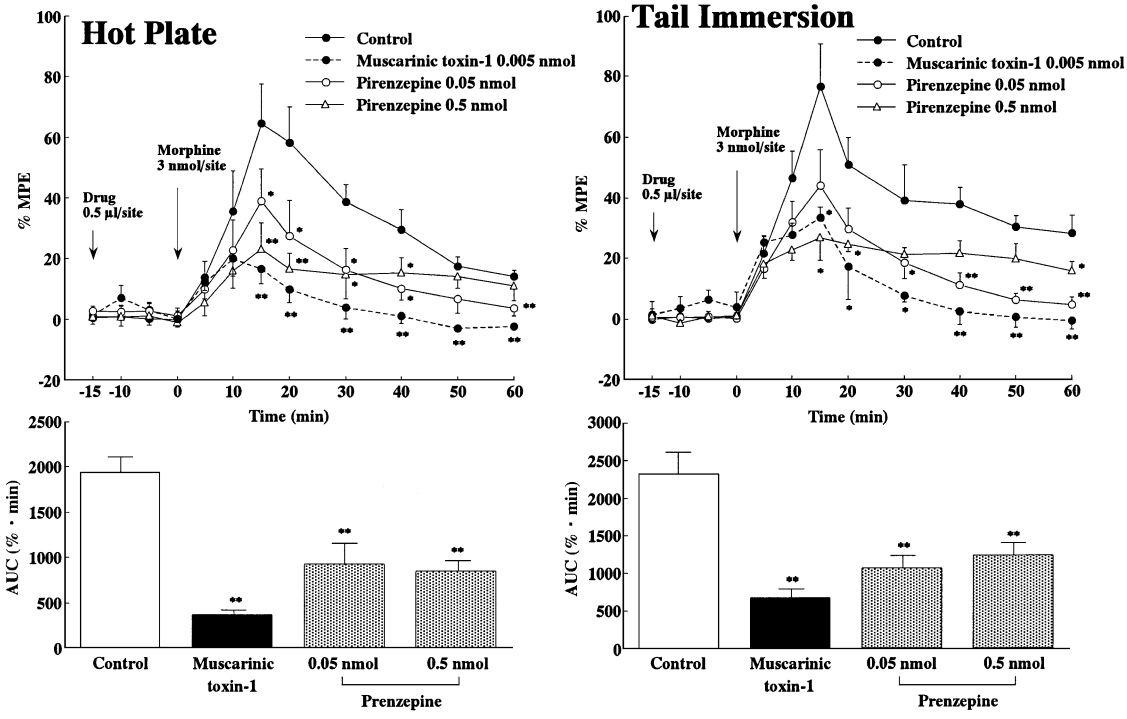

Fig. 8. Effects of muscarinic toxin-1 and pirenzepine injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha on antinociception induced by morphine (3 nmol/site) administered at the same location. Pirenzepine (0.05 nmol/site) potently inhibited morphine-induced antinociception in hot plate and tail immersion tests even at lower doses. Muscarinic toxin-1 (0.005 nmol/site) and high doses of pirenzepine (0.5 nmol/site) inhibited antinociception more potently than did low doses of pirenzepine. Maximal levels of %MPE changed only slightly throughout +60 min of testing with a high-dose treatment with pirenzepine. The effects of muscarinic toxin-1 and pirenzepine on the AUC, representing morphine-induced antinociception, are shown in the lower graphs. The AUC was calculated from the data in the upper graph collected 0–60 min after morphine microinjection. All points represent mean response latencies of six to eight animals and error bars express S.E. (*$P<0.05$, **$P<0.01$ compared with control).

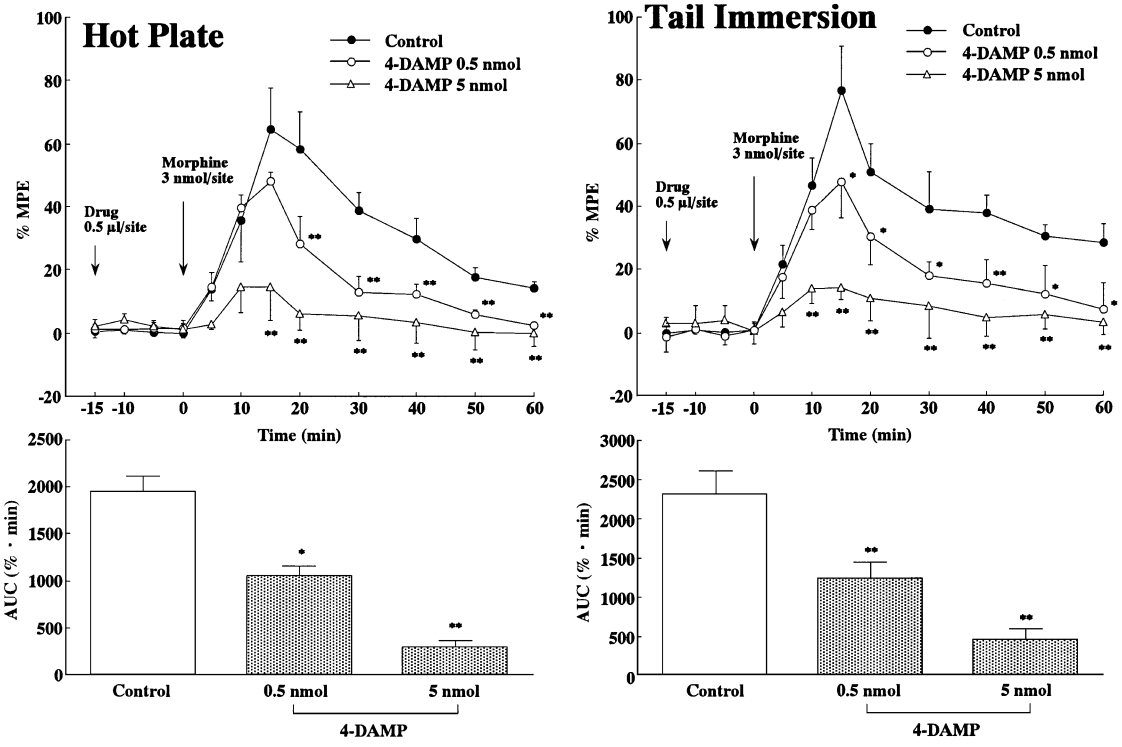

Fig. 9. Effects of 4-DAMP injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha on antinociception induced by morphine (3 nmol/site) administered at the same location. The lower dose of 4-DAMP (0.5 nmol/site) moderately inhibited morphine-induced antinociception, whereas the higher dose of 4-DAMP (5 nmol/site) was more potent. The effects of 4-DAMP on the AUC, representing morphine-induced antinociception, are shown in the lower graphs. The AUC was calculated from the data in the upper graphs collected 0–60 min after morphine microinjection. All points represent mean response latencies of six to eight animals and error bars express S.E. (*$P<0.05$, **$P<0.01$ compared with control).

33.5 ± 3.5%, respectively. On the other hand, pirenzepine (0.05 nmol/site) inhibited antinociception more efficiently after direct administration of morphine into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha rather than after intraperitoneal administration. Although a high dose of pirenzepine (0.5 nmol/site) inhibited antinociception more than the low dose of pirenzepine, the maximal levels of %MPE (hot plate test: 23.3 ± 8.7%; tail immersion test: 27.0 ± 7.6%) changed little until +60 min after testing began (Fig. 8). $AUC_{(0-60\ min)}$ analysis suggested that microinjection of muscarinic toxin-1 and pirenzepine significantly reduced the antinociceptive effect of microinjected morphine in both the hot plate and tail immersion tests (Fig. 8).

At 0.5 nmol/site, 4-DAMP had a greater inhibitory effect on antinociception induced by direct administration of microinjected morphine into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha than on intraperitoneally induced antinociception in the hot plate test (maximal levels of %MPE: 48.2 ± 3.0%). At 5 nmol/site, 4-DAMP completely inhibited antinociception (maximal levels of %MPE, hot plate test: 14.6 ± 8.1%; tail immersion test: 14.2 ± 3.6%). Response latencies of both 4-DAMP-treated groups returned to the baseline at the end of the testing period (Fig. 9). $AUC_{(0-60\ min)}$ analysis revealed that microinjection of 4-DAMP significantly reduced the antinociceptive effect of microinjected morphine in both the hot plate and tail immersion tests (Fig. 9).

3.5. Location of the injection site

Fig. 10 shows histological verification of the microinjection site within the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha regions.

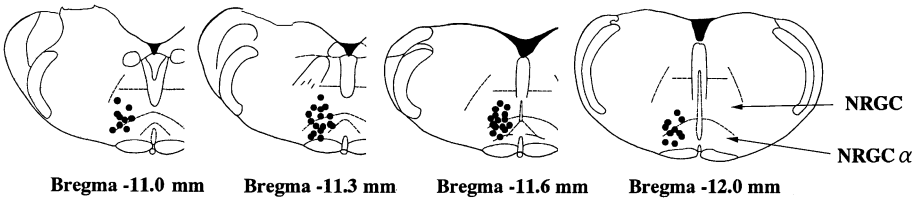

Fig. 10. Location of nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha microinjection sites. Location of all injection sites was confirmed within the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha regions by methylene blue injection.

4. Discussion

In the present study, both systemic administration and microinjection of morphine into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha induced antinociception in the hot plate and tail immersion tests. This effect was clearly reversed in a dose-dependent manner by a preceding microinjection of atropine, a selective antagonist of the muscarinic cholinergic receptor. Muscarinic receptor agonists and nicotine injected into the rostral ventral medulla, periaqueductal gray or nucleus raphe magnus produce an antinociceptive response, which is antagonized by local application of muscarinic and nicotinic receptor antagonists (Brodie and Proudfit, 1984; Iwamoto, 1989, 1991; Guimaraes et al, 2000) or by an intrathecal injection of muscarinic M_1 and M_2 antagonists (Iwamoto and Marion, 1993a). Moreover, microinjection of morphine and electrical stimulation of the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha inhibits the spinal nociceptive reflex and the spinal dorsal horn neuron response to peripheral stimulation (Satoh et al., 1979; Azami et al., 1982; Sandkuhler and Gebhart, 1984; Zhuo and Gebhart, 1990). These results indicate that stimulated cholinoreceptors in the brainstem are involved in antinociception and analgesia. The present study found that atropine and mecamylamine inhibited the subsequent antinociception induced by both systemic administration and microinjection of morphine. However, the inhibition induced by atropine was about 6.6–33.3 times more potent than that induced by mecamylamine. Thus, the antinociceptive effects of morphine are mediated by interactions between muscarinic receptors and nicotinic receptor sites in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha.

Taguchi et al. (1999) found that local application of morphine into the rostral ventrolateral medulla, using in vivo microdialysis, increases hot plate and tail immersion responses and enhances acetylcholine release. However, it is well known that morphine has a marked inhibitory effect on the motility of the gastrointestinal tract in humans as well as experimental animals (Ill, 1989). The inhibitory effect of morphine is mainly due to reduced acetylcholine release from enteric cholinergic neurons (Szerb, 1982). Likewise, morphine inhibits the release of acetylcholine from longitudinal muscle in myenteric plexus preparations of the guinea pig ileum (Nishiwaki et al., 2000). These discrepancies may be related to the route, dose of morphine administration and to the acetylcholine release methods applied (e.g., in vivo and in vitro). Moreover, these results suggest a difference in morphine-mediated regulation of cholinergic neurons between central and peripheral nervous systems. However, we also observed that naloxone, an opioid receptor antagonist, antagonized morphine-induced antinociception. Therefore, our findings indicate that both systemic administration and microinjection of morphine induced antinociception in part by activation of the cholinergic system in the rat nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha.

The effects of morphine on cholinergic systems in the brain are well known and considerable interest has been expressed in the potential role of acetylcholine in the analgesic mechanisms of morphine (Beilin et al., 1997; Hood et al., 1997). Muscarinic antagonists injected into the spinal cord inhibited the analgesic effects of morphine in rats (Chiang and Zhuo, 1989). Muscarinic M_1 receptors are located mainly in the central nervous system (Eglen and Whiting, 1986; Smith et al., 1989) and their antagonist, pirenzepine, inhibits hot plate and tail flick antinociception induced by a muscarinic M_1 receptor agonist applied to the rostral ventral medulla (Iwamoto and Marion, 1994). Naguib and Yaksh (1994) suggested that muscarinic antinociception is mediated via muscarinic M_1 and/or M_3 receptors in the spinal cord. In contrast, the antinociception induced by the highly muscarinic M_1-selective receptor agonists appears to be independent of the muscarinic M_1 receptor (Sheardown et al., 1997).

In the present study, the muscarinic M_1 receptor antagonist, pirenzepine, inhibited morphine-induced antinociception in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. Pirenzepine binds to m1 receptor proteins with a 6- to 35-fold higher affinity than to m2, m3, m4 or m5 receptors (Dorje et al., 1991). Distinguishing between muscarinic M_1 and M_4 receptors is difficult because the selectivity for M_1 is only six-fold higher than that for M_4. To distinguish which of the receptor subtypes is involved in the antinociception of morphine requires an antagonist that has a high degree of selectivity for only one subtype. Muscarinic toxin-1 has been isolated from the venom of the green mamba, *Dendroaspis angusticeps*, and has 100-fold higher affinity for muscarinic M_1 than for M_4 receptors and no affinity for other subtypes (Max et al., 1993a,b). Because of its selectivity, this toxin has been used to localize muscarinic M_1 receptors at various sites, including the pain-processing region of the rat brainstem (Adem et al., 1997). In the present study, muscarinic toxin-1, a potently selective muscarinic M_1 receptor antagonist, required a 100-fold lower concentration than pirenzepine to inhibit the antinociceptive effects of both systemically administered and microinjected morphine. Therefore, inhibition of morphine-induced hot plate and tail immersion antinociception by muscarinic toxin-1 demonstrates a role for the muscarinic M_1 receptor in muscarinic antinociception in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha.

The presynaptic muscarinic M_2 autoreceptors are present in the rat rostral ventral medulla (Iwamoto, 1991). Iwamoto and Marion (1994) reported that although microinjection of the muscarinic M_2 receptor antagonist, methoctramine, into the rostral ventral medulla produces an antinociceptive effect, presynaptic muscarinic M_2 receptors are not implicated in muscarinic antinociception. The present study found that the antinociceptive effects of

methoctramine injected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha were similar. Blockade of presynaptic muscarinic M_2 receptors in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha by methoctramine may activate acetylcholine receptors by negative feedback onto postsynaptic muscarinic M_1 receptors, and stimulation of the muscarinic M_1 receptors by acetylcholine would increase the hot plate and tail immersion threshold. On the other hand, methoctramine microinjected into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha significantly prolonged the antinociceptive effects of morphine. Systemic administration and microinfusion of morphine increases the release of acetylcholine into the extracellular space of the rostral ventrolateral medulla, including the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha (Taguchi et al, 1999). Methoctramine may enhance nociception via blockade of presynaptic muscarinic M_2 autoreceptors in the cholinergic neurons of nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. Thus, the activation of muscarinic M_2 autoreceptors may have prolonged the morphine-induced antinociception in the hot plate and tail immersion tests. Our data suggest that the antinociception induced by both systemic administration and microinjection of morphine is not mediated by muscarinic M_2 receptors. However, an unknown mechanism may contribute to the prolongation of morphine-induced antinociception in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha muscarinic M_2 receptors.

Dawson et al. (1991) reported that muscarinic M_3 receptors are involved in the mouse tail-flick response to noxious stimulation. Recently, Honda et al. (2000) demonstrated that intrathecal injection of the muscarinic M_3 receptor antagonist, 4-DAMP, inhibited formalin-induced nociception dose-dependently. In the present study, the muscarinic M_3 receptor antagonist, 4-DAMP, inhibited morphine-induced antinociception in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. However, in the tail immersion test, a low dose of 4-DAMP inhibited antinociception induced by systemic morphine administration, whereas in the hot plate test, the same low dose of 4-DAMP failed to inhibit the response to intraperitoneally administered morphine. Thus, the hot plate response suggested that low-dose 4-DAMP does not interfere with systemically administered morphine-induced antinociception, which is mediated by muscarinic M_3 receptors in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. The role of the muscarinic M_3 receptor subtype in processing nociceptive information in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha cannot be discounted since the high-dose group showed apparent inhibition of morphine-induced antinociception. In fact, pre- and postsynaptic muscarinic M_3 receptors are present in the rat brain (Doods et al., 1987; Buyukuysal et al., 1998). Our results may be explained by supposing that 4-DAMP at a low dose acts only on presynaptic muscarinic M_3 receptors, whereas a high dose acts not only at presynaptic muscarinic M_3 receptors but also at postsynaptic muscarinic receptors in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. Our findings indicate that the effects of 4-DAMP on the morphine-induced antinociception are mediated via muscarinic M_3 receptors in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. In addition, muscarinic M_4 receptors in the spinal cord were previously reported to be involved in muscarinic antinociception of thermal nociceptive responses (Ellis et al., 1999). Further work is required to elucidate the morphine-induced antinociception for the muscarinic subtypes.

In conclusion, our results indicate that the muscarinic M_1 receptor antagonists, muscarinic toxin-1 and pirenzepine inhibited the antinociception that was induced by both systemic administration and microinjections of morphine into the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha. The hot plate and tail immersion responses to methoctramine treatment and the hot plate response to low-dose 4-DAMP were not affected by antinociception induced with systemically administered morphine. However, a high dose of 4-DAMP inhibited the antinociception. Thus, the muscarinic M_1 and M_3 receptors in the nucleus reticularis gigantocellularis/nucleus reticularis gigantocellularis alpha antagonize antinociceptive effects of both systemically administered and microinjected morphine. These results extend the findings of a previous report

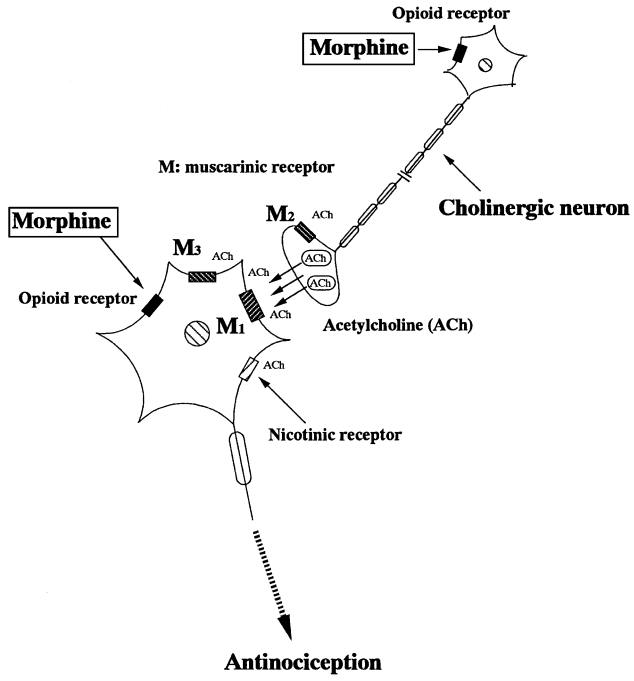

Fig. 11. Model illustrating the hypothetical participation of rostral ventrolateral medulla muscarinic receptors in the modulation of antinociception produced by administration of morphine.

(Taguchi et al, 1999) and support the hypothesis that morphine-induced antinociception is modulated by the activation of cholinergic mechanisms in the rostral ventrolateral medulla of rats (Fig. 11).

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