Full Paper

The Spinal Muscarinic M₁ Receptors and GABA_A Receptors Contribute to the McN-A-343–Induced Antinociceptive Effects During Thermal Stimulation of Mice

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Received August 28, 2008; Accepted October 19, 2008

Abstract. The present study was undertaken to clarify how spinal muscarinic receptors are involved in the antinociceptive effects in thermal stimulation. Intrathecal (i.t.) injection of the muscarinic agonist McN-A-343 inhibited the tail-flick response to noxious thermal stimulation in a dose-dependent manner (31.5 – 63.0 nmol). This McN-A-343–induced antinociceptive effect was dose-dependently inhibited by intrathecal (i.t.) injection of a nonselective muscarinic receptor antagonist atropine, the selective muscarinic M₁ antagonist pirenzepine, or the M₄ antagonist himbacine. The inhibition of pirenzepine was greater than that of himbacine. In contrast, the selective muscarinic M₂ antagonist methoctramine did not inhibit the antinociceptive effects of McN-A-343. In addition, the McN-A-343–induced antinociceptive effect was attenuated by i.t. injection of the GABA_A antagonist bicuculline, but not by injection of the GABA_B antagonist CGP35348. These results suggest that McN-A-343 produces its antinociceptive effect on the response to thermal stimulation via spinal muscarinic M₁ receptors and, at least in part, through neuronal pathways involving spinal GABA_A receptors in mice.

Keywords: muscarinic M₁ receptor, McN-A-343, antinociception, spinal GABA_A receptor, spinal cord

Introduction

Nociceptive transmission can be modulated by spinal neurons. Cholinergic neurons in the spinal cord have been reported to produce an antinociceptive effect (1 – 5), while our previous studies showed a contribution of spinal muscarinic receptors to antinociception. For instance, the muscarinic M₃ receptor is involved in formalin-induced nociception (6), and the muscarinic M₁ receptor is involved in the antinociceptive effect caused by the intrathecal injection (i.t.) of clonidine in mice (7). We reported that intrathecal injection of the muscarinic receptor agonist McN-A-343 produced antinociceptive effects during thermal (8) and mechanical stimulation (9). Sullivan et al. (10) reported that the systematic administration of the muscarinic receptor agonist WAY-132984 produced antihyperalgesic and antiallodynic effects via muscarinic M₄ receptors in animal models of neuropathic pain. These findings suggest that cholinergic neurons and muscarinic receptors have important roles in pain signaling. However, the detailed mechanisms by which the cholinergic system influences pain inhibitory pathways remain poorly understood. Recently, it was demonstrated that the spinal antinociceptive system, involving spinal γ-aminobutyric acid (GABA) receptors, plays an important role in the regulation of the nociceptive response (11, 12) and that spinal GABA receptors mediate opioid agonist–induced antinociception during noxious thermal stimulation of mice (13). We demon-
strate here which subtype of spinal muscarinic receptor is involved in the inhibitory GABAergic systems in the spinal cord underlying the antinociceptive effects of the intrathecal administration of the muscarinic M<sub>1</sub>-receptor agonist McN-A-343.

**Materials and Methods**

**Animals**

Male mice (ddY strain; Kyudo, Kumamoto), weighing 25–30 g, were used throughout the experiment. Mice were housed at 23 ± 2°C with a 12/12 h light/dark cycle (light on at 07:00 h) and were given free access to commercial food and tap water. Experimental procedures were based on the Guidelines of the Committee for Animal Care and Use of Fukuoka University.

**Intrathecal (i.t.) injection of drugs**

A 28-gauge needle attached to a 25-μl Hamilton microsyringe was used for the i.t. injection of drugs. The needle was inserted into the intervertebral space between the fifth and sixth spinal vertebrae in conscious mice, as previously described (7, 14). Accurate placement of the needle was confirmed by a quick “flick” of the tail. Drugs administered via the i.t. route were administered slowly in a volume of 5 μl. Control mice received only saline or vehicle.

**Measurement of antinociceptive responses in tail-flick test**

The antinociceptive effects of drugs were measured in the tail flick test. Mice were held with a soft cloth to reduce unwanted stimuli, and radiant heat was applied to a spot about 1.5 cm from the tip of the tail using a commercially available thermal stimulator (Ugo Basile, Italy), and the latency to remove the tail from the noxious heat (tail-flick latency) was recorded. The intensity of the heat source was set such that baseline response time was between 2 and 3 s. Cut–off time was set to 10 s to minimize potential damage to the tail skin. The degree of the antinociceptive effect was expressed as percent maximum possible effect calculated as follows: %MPE = 100 × (post-drug latency – pre-drug latency) / (cut-off time – pre-drug latency).

**Locomotor activity and rota-rod testing**

To rule out the possibility of sedation and motor impairment induced by the i.t. injection of McN-A-343 in mice, an open-field test and a rota-rod treadmill test (diameter, 3 cm; Muromachi Kikai, Tokyo) were carried out, respectively. All behavioral tests were performed during the light portion of the circadian cycle (9:00 a.m. to 5:00 p.m.). A mouse was placed on the center of the floor in the open-field chamber (60 cm in diameter and 50 cm in height; the floor was divided into 19 blocks) and observed for 1 min. Locomotor activity (ambulation) was expressed as the numbers of blocks traversed. Each mouse was subjected to the rota-rod treadmill test once a day for a total period of three days. The treadmill was set to a rotating speed of 10 r.p.m. Mice that stayed on the treadmill rotating at 10 r.p.m. for 180 s were considered complete responders; their latencies were recorded as 180 s. The results were presented as the drop latency measured before and after the i.t. injection.

**Western blot analysis for the detection of spinal muscarinic M<sub>1</sub> receptors**

The lumbar parts of the spinal cords from mice were rapidly dissected out and frozen. Samples were homogenized in ice-cold homogenization buffer containing 250 mM sucrose, 150 mM NaCl, 2.5 mM EGTA, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, and 50 μg/ml leupeptin (pH 7.5) and then centrifuged for 1 h at 100,000 × g at 4°C. The supernatants were discarded. The pellets were homogenized in the homogenization buffer containing 1% Nonidet P-40, incubated on ice for 30 min, and then centrifuged at 100,000 × g for 1 h at 4°C. The muscarinic M<sub>1</sub> receptor proteins in the supernatants were detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Briefly, the amount of protein in the membrane fractions was assayed according to the method of Lowry et al. (15). Ten to twenty micrograms of protein per well was separated by SDS-PAGE using 8% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with blocking reagent (Western blocking reagent; Roche Diagnostics, Mannheim, Germany) and incubated with the following primary antibodies: a rabbit anti-muscarinic M<sub>1</sub> receptor antibody (1:1,000; Chemicon International, Temecula, CA, USA) and a mouse anti-β-actin antibody (1:10,000; Sigma Chemical Co., St. Louis, MO, USA) as the loading control. Blots were rinsed with Tris-buffered saline buffer containing Tween and bound antibodies were detected using horseradish-peroxidase–conjugated anti-mouse IgG (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Bound antibodies were visualized using the enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK) with autoradiography film.

**Drugs**

Methoctramine tetrahydrochloride and McN-A-343 (4-3-chlorophenyl-carbamoyloxy-2-butynyltrimethylammonium) were purchased from Research Biochemicals.
Pirenzepine hydrochloride, atropine sulfate, methoctramine tetrahydrochloride, bicuculline methiodide, CGP35348 (3-aminopropyl-di-ethoxy-methyl-phosphinic acid), and himbacine were purchased from Sigma. With the exception of himbacine, drugs were dissolved in sterile saline just before the experiment. Himbacine was dissolved in one drop of HCl and the pH of the solution was adjusted to 7.2 with NaOH; then the solution was diluted to the required volume with sterile saline.

Statistical analyses
Data are expressed as means ± S.E.M. Statistical analyses of data were performed using the unpaired two-tailed Student’s *t*-test. Multiple comparisons were analyzed for significance with one-way ANOVA followed by *post hoc* tests, such as Dunnett’s test or the Turkey-Kramer test. A value of *P* < 0.05 was taken to indicate a statistically significant difference.

Results

**Antinociceptive effects of i.t. injection of McN-A-343 on the response to noxious thermal stimulation in the tail-flick test**

The time course for the antinociceptive effect of i.t. injection of McN-A-343 is shown in Fig. 1A. McN-A-343 induced a significant increase in %MPE (antinociceptive effect), which reached a maximum within 15 min of i.t. injection. The McN-A-343–induced antinociceptive effect was dose-dependent (Fig. 1A). The McN-A-343–induced antinociceptive effects were significantly inhibited by i.t. injection of the non-selective muscarinic receptor antagonist atropine (Fig. 1B). Atropine alone had no effect on the tail-flick latency (data not shown). The dose of atropine was decided on the basis of our previous study (8).

**Effects of i.t. injection of McN-A-343 on motor function**

The intrathecal administration of McN-A-343 (63 nmol) did not induce significant changes in motor activity according to Hall’s open field test (Fig. 2A), and the same dose of McN-A-343 had no influence on the results of a rota-rod treadmill test (Fig. 2B). These data indicated that intrathecal McN-A-343 does not produce sedation or motor dysfunction.

**Effects of selective muscarinic M₁ and M₄-receptor antagonists on the McN-A-343–induced antinociceptive effect**

I.t. injections of the muscarinic M₁-receptor–selective antagonist pirenzepine inhibited the McN-A-343 (63 nmol)-induced increase in %MPE (antinociceptive effect) in a dose-dependent manner (Fig. 3A). I.t. injections of the selective M₄ antagonist himbacine also inhibited the McN-A-343 (63 nmol)-induced antinociceptive effect in a dose-dependent manner (Fig. 3B).

**A comparison of the dose-dependency curves for pirenzepine and himbacine to inhibit the McN-A-343–induced antinociceptive effect**

The relative potencies of pirenzepine and himbacine to inhibit the McN-A-343 (63 nmol)–induced increase in %MPE were compared (Fig. 3). Dose-dependency curves generated to show the %MPE values 15 min after the i.t. injection of drugs are shown in Fig. 3. The dose-dependency curve for himbacine was shifted to the right compared with that for pirenzepine (Fig. 4). These data indicated that the inhibition by himbacine was weaker than that by pirenzepine.
Effects of selective muscarinic M₂-receptor antagonists on the McN-A-343–induced antinociceptive effect

As shown in Fig. 5, the muscarinic M₂ antagonist methoctramine did not inhibit the McN-A-343 (63 nmol)-induced increase in %MPE. The dose for methoctramine was based on work in another laboratory that demonstrated specific blockade of muscarinic M₂ receptors by i.t. injection of methoctramine at 1 nmol in mice (16).

Western blot analysis for spinal muscarinic M₁ receptors

As shown in Fig. 6, a band with an approximate molecular mass of 65 kDa was predicted to correspond to muscarinic M₁-receptor (mACHR M₁) protein, as reported by Volpicelli-Daley et al. (17) in Western blot analysis.

Involvement of the GABAergic system in the McN-A-343–induced antinociceptive effect

To determine whether GABAergic systems are involved in the McN-A-343–induced antinociceptive effect, mice were treated with GABA-receptor antagonists. The McN-A-343 (i.t., 63 nmol)-induced increase in %MPE was attenuated by i.t. injection of the GABA_A antagonist bicuculline (Fig. 7A). However, i.t. injection of the GABA_B antagonist CGP35348 (1.3 nmol) did not significantly inhibit the McN-A-343–induced antinociceptive effect (Fig. 7B). The dose of CGP35348 was derived from the dose that produced specific blockade of GABA_B receptors in the spinal cords of rats (18), taking into consideration the difference in the body weights of mice, because i.t. injection of CGP35348 has not been reported in mice. Bicuculline (40 pmol, i.t.) or CGP35348 (1.3 nmol, i.t.) alone had
no effect on the tail-flick latency. However, i.t. injection of doses higher than 1.3 nmol of CGP35348 alone induced hyperalgesia (data not shown).

**Discussions**

In the present study, we examined the mechanisms underlying the antinociception induced by the intrathecal muscarinic agonist McN-A-343 in mice.

Intrathecal (i.t.) injection of McN-A-343 caused antinociceptive effects on the responses to noxious thermal stimulation in tail-flick tests. Co-injection of the muscarinic receptor antagonist atropine with McN-A-343 significantly inhibited the McN-A-343–induced antinociceptive effect. These results indicate that the McN-A-343–induced antinociceptive effect was mediated by muscarinic receptors in the spinal cord. On the other hand, intrathecal McN-A-343 had no effect on locomotion activity or motor function (Fig. 2). Therefore, we concluded that the McN-A-343–induced antinociceptive effect was not due to sedation or motor dysfunction.

Based on pharmacological and molecular cloning studies, muscarinic receptors have been classified into five subtypes, M₁, M₂, M₃, M₄, and M₅ (19); the existence of all receptor subtypes in the spinal cord has been reported (20). A physiological study by Davies et al. showed that McN-A-343 has selectivity for the muscarinic M₁ receptor (21), but Wang and el-Fakahany reported that McN-A-343 caused inhibition of cAMP formation via the muscarinic M₄ receptor in Chinese hamster ovary cells (22). Thus, we cannot rule out the possibility of the involvement of spinal muscarinic M₄ receptors in the McN-A-343–induced antinociceptive effect. On the other hand, Lazareno et al. reported that McN-A-343 did not activate the muscarinic M₄ receptors (23). Therefore, to determine which subtype of muscarinic receptor in the spinal cord is involved in the McN-A-343–induced antinociceptive effect, we examined the effects of i.t. injection of muscarinic M₁, M₂, and M₄ receptor antagonists on McN-A-343–induced antinociception.

I.t. injections of the muscarinic M₄-receptor–selective antagonist pirenzepine inhibited the McN-A-343–induced antinociceptive effect in a dose-dependent manner (Fig. 3A). It is reported that pirenzepine binds to the muscarinic M₁ receptor with a 6 – 35-fold higher affinity than to M₂, M₃, M₄, or M₅ receptors (24, 25). However, it is not easy to distinguish between muscarinic M₁ and M₄ receptors because pirenzepine has selectivity...
for the M₁ receptor only six-fold higher than that for the muscarinic M₄ receptor (24, 25). According to the review by Dhein (26), the pKᵢ value range, calculated as the negative logarithms of the affinity constant, for the muscarinic M₄ receptor is 7.1 – 8.1 for pirenzepine and 8.0 – 8.8 for the selective muscarinic M₄-receptor antagonist himbacine. Therefore, we used himbacine, which has higher affinity to muscarinic M₄ receptors, in this study.

I.t. injections of the selective M₄ antagonist himbacine also inhibited the McN-A-343–induced antinociceptive effects in a dose-dependent manner (Fig. 3B). However, the inhibition by himbacine was weaker than that induced by pirenzepine because the dose-dependent curve for himbacine was shifted to the right compared with that for pirenzepine (Fig. 4). By contrast, the selective muscarinic M₂ antagonist methoctramine did not inhibit the McN-A-343–induced antinociceptive effect (Fig. 5). The present pharmacological results support the possibility that spinal McN-A-343 produces its antinociceptive effect via muscarinic M₁ receptors.

To demonstrate the existence of spinal muscarinic M₁ receptors, we next carried out Western blot analysis. As shown in Fig. 6, Western blot analysis showed the existence of muscarinic M₁-receptor protein in mouse spinal cord. It has been reported that cholinergic inter-neurons exist in the spinal cord (27). Taken together, our present results suggest that spinal muscarinic M₁ receptors are involved in the intrathecal McN-A-343–induced antinociceptive effects on responses to noxious thermal stimuli in mice. On the other hand, inhibitory inter-neurons, such as GABAergic neurons, are involved in antinociceptive processing in the spinal cord (28). Indeed, stimulation of both GABAA receptors and GABAB receptors in the spinal dorsal horn has been shown to produce analgesia in primate models (29) and acute thermal nociception in the tail-flick test in mice (13). It has been reported that acetylcholine and muscarine activate GABA receptors in the spinal dorsal horn via release of the inhibitory neurotransmitter GABA (30, 31). In this study, the McN-A-343–induced antinociceptive effect was attenuated by i.t. injection of the GABA A receptor antagonist bicuculline, not by the GABA B antagonist CGP35348 (Fig. 7). Rashid and Ueda (32) reported that intrathecal nicotinic acetylcholine receptor agonists produce an antihyperalgesic effect through spinal GABAₐ receptors in a mouse model of neuropathic pain. The muscarinic M₁ receptor is coupled to Gq protein and activates phospholipase C leading to an increase in intracellular calcium, which is able to cause the release of GABA from GABAergic neurons. In human (33) and rat (34), autoradiographic studies have shown the existence of muscarinic M₁ receptors in the superficial laminas of the dorsal horn of spinal cord where the terminals of the primary afferents may be localized. In addition, it has been reported that GABA (35), its synthetic enzyme glutamate decarboxylase (GAD) (35), and GABAA receptors (36) also existed in the superficial laminas of the dorsal horn of spinal cord. These findings suggest that spinal muscarinic M₁ receptors seem to be involved in regulation of GABA release. Our results suggest the possibility that the activation of spinal muscarinic M₁ receptors may evoke release of GABA, which in turn may inhibit the transmission of noxious thermal stimuli through the activation of GABAA receptors in the mouse spinal cord. On the other hand, Chen and Pan (37) reported that activation of GABAB receptors was involved in the antinociceptive effect.
effects of the intrathecal non-selective muscarinic agonist muscarine and the cholinesterase inhibitor neostigmine in rats. However, they did not identify the muscarinic receptor subtype. Although we have no direct evidence, the muscarine-induced antinociception via GABA_B receptors may be caused by muscarinic receptor subtypes other than muscarinic M_1 receptors.

These findings show that McN-A-343 produces an antinociceptive effect on responses to thermal stimulation via spinal muscarinic M_1 receptors and, at least in part, through neuronal pathways involving spinal GABA_A receptors in mice.

Acknowledgments

We wish to thank Dr. Ichiro Harasawa for his helpful comments and suggestions. This work was supported in part by a Grants-in-Aid from The Ministry of Education, Culture, Sports, Science, and Technology of Japan (#19603022 and #19590265) and a grant from the Advanced Materials Institute and the Central Research Institute of Fukuoka University (#066006).

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