The Role of Spinal Muscarinic Acetylcholine Receptors in Clonidine-Induced Anti-nociceptive Effects in Rats

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We have examined the effects of intrathecal (i.t.) injection of the muscarinic acetylcholine receptor antagonist atropine on the clonidine-induced nociceptive effect in formalin-induced nociception in rats. The injection of 5% formalin into the hind paw caused biphasic nociceptive responses, and i.t. injection of clonidine inhibited both phases of the nociceptive response in a dose-dependent manner. Pretreatment with atropine (i.t.) only partially inhibited the nociceptive effect of clonidine. These results suggest that the nociceptive effect of clonidine in the rat formalin model may be at least partly mediated by muscarinic acetylcholine receptors in the spinal cord.

Key words nociception; spinal muscarinic acetylcholine receptor; clonidine

The spinal cord is a major site of transmission of nociception, and evidence suggests that the descending brainstem-spinal monoaminergic system plays an important role in the transmission of nociceptive information from primary afferent nerves in the dorsal horn of the spinal cord. Clinical and experimental studies have demonstrated that intrathecal (i.t.) injection of the α2 adrenoceptor agonist clonidine is effective in the treatment of pain. Recent reports show that muscarinic receptors in the dorsal horn of the spinal cord play an important role in the nociceptive effects of α2 adrenoceptor agonists. In fact, the presence of cholinergic neurons and muscarinic receptors in the spinal cord were also demonstrated. Together, these findings suggest that the interaction between monoaminergic neurons and cholinergic neurons in the spinal cord is important for the development of analgesia.

Muscarinic receptor agonists are also known to have an anti-nociceptive effect. Previously, we reported that the spinal muscarinic receptors, especially M1 muscarinic receptors, were involved in formalin-induced nociception and that an inhibitor of acetylcholinesterase, neostigmine caused significantly suppression of the formalin-induced nociceptive response. The formalin test in rodents is thought to be a good model for long-lasting pain of moderate intensity that resembles human chronic pain. Moreover, we reported that a muscarinic receptor antagonist atropine suppressed intrathecal (i.t.) clonidine-induced inhibition in mechanical transmission, but it did not affect clonidine-induced anti-nociception in noxious thermal transmission in the spinal cord. However, the mechanism of interaction between muscarinic receptors in the spinal cord and clonidine-induced anti-nociceptive effect in the formalin test is not fully understood.

In the present study, we therefore examined the role of spinal cord muscarinic receptors in the nociceptive effect of i.t. injection of clonidine in formalin-induced nociception.

Male Wistar rats weighing 290–320 g (Kyudo, Kumamoto) were used. The animals were kept in a room at 24±2 °C maintained on 12 h/12 h light/dark cycle (lights on at 7:00 a.m.) 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.

For injection of drugs, each rat was implanted with a chronic lumbar i.t. catheter under anesthesia, according to a method modified from Yaksh and Rudy. Briefly, rats were placed in a stereotaxic apparatus, and a polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, U.S.A.) filled with saline was inserted through an incision in the atlanto-occipital membrane and was advanced 8.5 cm caudally, to position its tip at the level of the lumbar enlargement. The rostral tip of the catheter was passed subcutaneously, externalized on top of the skull, and sealed with a stainless steel plug. Drug injections (10 µl) were carried out 7 d after the surgery. Each rat was placed in a clear plastic cage (36×31×18 cm) at least 30 min before the formalin injection to allow it to adapt to the new environment. A solution of 5% formalin in saline (50 µl) was injected into the plantar of the right hind paw, and the number of flinches (rapid paw shaking) of the injected paw was recorded as an indicator of pain-related behavior. The total number of hind paw flinches was determined during 12 five minute intervals for a total of 60 min, after injection of formalin. All experimental procedures were carried out between 10:00 h and 16:00 h. Only animals with normal motor function were used. Control rats received i.t. saline. Clonidine was injected i.t. 10 min before the injection of formalin. Muscarinic receptor antagonist atropine was injected intrathecally 10 min before i.t. injection of clonidine.

Studies on cardiovascular response were also performed. To measure blood pressure and heart rate, rats were anesthetized with urethane (1 g/kg i.p. Aldrich Chemical Co., Milwaukee, WI, U.S.A.), and the blood pressure and heart rate were measured directly through a cannula inserted into the femoral artery.

To confirm the placement of the i.t. catheters, 10 µl of 2% lidocaine or malachite green solution was injected i.t. at the end of the experiment. The animals became paralyzed in the hind limbs within 30 s of this local anesthetic injection. The dye was observed at the surface of the lumbar and thoracic regions of the spinal cord. Thus, we were sure that all drugs injected down the catheter were introduced into the lumbar subarachnoid space.

Clonidine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Atropine sulfate was purchased from Nacalai Tesque Inc. (Kyoto). All drugs were...
dissolved in saline for i.t. injection. Data are expressed as means±S.E.M. Statistical analysis of data was performed by Student’s t-test (unpaired) for comparison of the values from two groups and one way analysis of variance (ANOVA) followed by Dunnett’s test (comparisons with single control group) or Tukey’s test (comparison of two groups) for multiple comparisons.

As shown in Fig. 1A, 5% formalin injection into the right hind paw of rats caused biphasic flinching behavior in the injected paw. The first phase of flinching behavior was observed for 5 min after the injection, and then the second phase was shown in about 9 min after the injection and persisted for 50 min (Fig. 1A). The peak period of the second phase response was observed 25—35 min after the injection of formalin (Fig. 1A). i.t. injection of clonidine caused a dose-dependent reduction of flinching behavior during both phases of the nociceptive response to formalin. However, the anti-nociceptive effect of clonidine was the greatest during the second phase (Fig. 1B).

To determine the involvement of spinal muscarinic receptors in clonidine-induced anti-nociceptive effect in the formalin test, we next examined the effects of a muscarinic receptor antagonist, atropine, on the anti-nociceptive effect of clonidine. Pretreatment with the i.t. injection atropine (10, 20 nmol) partially inhibited the anti-nociceptive response to clonidine in the second phase (Fig. 2). In contrast, pretreatment with atropine had a tendency to inhibit the anti-nociceptive effect of clonidine in the first phase, however the effects were not statistically significant (Fig. 2). Injection of atropine (5—20 nmol) alone did not affect the formalin-induced nociceptive response (data not shown). In addition, neither clonidine nor atropine had any discernible effect on general behavior or motor function (data not shown).

Several reports have shown that clonidine-induced anti-nociceptive effects in mechanical3) and thermal stimulation11) or allodynia in a neuropathic pain model12) are suppressed by the i.t. injection of atropine. The finding that i.t. injection of neostigmine, an acetylcholinesterase inhibitor, reduces formalin-induced nociception in rats13) and mice9) is further evidence for an nociceptive effect of acetylcholine. Our preliminary experiment shows that neostigmine (25 ng, i.t.) decreased the pain-related behavior in the second phase, such as licking in mice. The neostigmine-induced anti-nociceptive

**Fig. 1.** Effect of i.t. Injection of Clonidine on Formalin-Induced Flinching Behaviors

The numbers of flinches are expressed as the sum of responses during 5 min intervals (A). Nociceptive responses in the first and second phases are expressed as the total number of flinches occurring at 0—5 min and 9—60 min after formalin injection into the hind paw (B). Clonidine was injected i.t. 10 min prior to injection of formalin into the hind paw. Data are expressed as mean±S.E.M. *p<0.05, **p<0.01, ***p<0.001 vs. saline.

**Fig. 2.** Effect of i.t. Injection of the Muscarinic Receptor Antagonist Atropine on i.t. Injection of Clonidine-Induced Anti-nociception

Nociceptive responses of the first and second phases are expressed as the total number of flinches occurring at 0—5 min and 9—60 min after formalin injection into the hind paw. Atropine or saline was injected i.t. 10 min prior to i.t. injection of clonidine (3 nmol). Data are expressed as mean±S.E.M.
effect was significantly reversed by a muscarinic antagonist atropine (100 ng, i.t.), but not by a nicotinic receptor antagonist mecamylamine (100 ng, i.t.). On the other hand, i.t. injection of a nicotinic agonist alone did not inhibit formalin-induced nociception. Thus, these results support the idea that spinal muscarinic receptors are involved in the anti-nociceptive effect of clonidine. However, muscarinic receptors in the spinal cord are partly involved in the ability of clonidine to inhibit formalin-induced nociceptive behaviors, since the anti-nociceptive effect of clonidine was partially inhibited by pretreatment with atropine.

Intrathecal injection of clonidine inhibited the mechanical allodynia in nerve ligation-induced neuropathic pain and the response induced by mechanical stimulation via muscarinic receptors in the spinal cord. But the muscarinic receptor antagonist atropine did not affect the inhibition induced by clonidine (i.t.) in nociceptive thermal response. It has been shown that mechanical transmission and mechanical allodynia are predominantly mediated by A-fiber affreents into the laminae III—VI of the spinal dorsal horn. On the other hand, thermal nociception is mainly transmitted through C-fiber afferents to the superficial laminae as well as in the III—VI laminae of the spinal cord. Hunskaar and Hole indicated that the first phase of formalin-induced nociceptive response is due to direct effects of formalin to the nociceptors, and that the second phase is due to inflammation by formalin. Moreover, the injection of formalin to paw resulted in expression of c-Fos protein in the superficial laminae as well as in the III—VI laminae of the spinal cord. Kantner et al. and Kuraishi et al. reported that the injection of formalin into the paw caused substance P and somatostatin release from the dorsal horn. Therefore, they suggested that substance P and somatostatin participate in the transmission of the formalin-induced nociceptive response. In vitro experiments, clonidine inhibited release of glutamate from the spinal synaptoneurosomes and substance P from the slice preparation of the spinal cord. Thus, it has been suggested that muscarinic receptors-independent anti-nociceptive response induced by clonidine in noxious thermal stimulation and in formalin stimulation may be caused by direct inhibition of neurotransmitter release from the C-fibers terminated in the superficial laminae of dorsal horn. On the other hand, the second phase of formalin-induced nociceptive response reflects the sensitization of wide dynamic range neurons in the laminae V of the dorsal horn. In addition, agonists of \( \alpha_2 \) adrenoceptors are thought to cause the postsynaptic hyperpolarization of wide dynamic range neurons of the dorsal horn. In addition, Klimscha et al. found that clonidine caused the release of acetylcholine from the spinal cord in sheep. Thus, it is suggested that the possibility that acetylcholine release induced by clonidine may inhibit in part the hyperpolarization of wide dynamic range neurons in the laminae V of the dorsal horn via the spinal muscarinic receptors.

Next, we examined the effect of clonidine on the cardiovascular system in anesthetized rats. i.t. injections of clonidine (3, 20 nmol) produced no changes in blood pressure and heart rate. For example, mean blood pressure (mmHg, n=3) was 86.8±3.0, 80.4±6.7 and 84.2±7.4 before, 15 and 30 min after clonidine (20 nmol) injection, respectively. Thus, the clonidine-induced anti-nociceptive effect may not be due to the hypotensive effects of this agent.

In conclusion, the present study shows that the anti-nociceptive effect of clonidine in the formalin-induced nociceptive response might be mediated in part by cholinergic activation in the spinal cord. In addition, the results of the present study suggest that combination of clonidine and acetylcholinesterase inhibitor expected to produce greater analgesia than higher alone, thereby reducing the dose requirements.

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