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Intrathecal Oxotremorine Affects Formalin-Induced Behavior and Spinal Nitric Oxide Synthase Immunoreactivity in Rats

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PRZEWŁOCKA, B., J. MIKA, F. CAPONE, H. MACHELSKA AND F. PAVONE. *Intrathecal oxotremorine affects formalin-induced behavior and spinal nitric oxide synthase immunoreactivity in rats.* PHARMACOL BIOCHEM BEHAV **62**(3) 531–536, 1999.—The present research was undertaken to investigate, by behavioral and immunohistochemical methods, the effects of intrathecal (ITH) injection of the muscarinic agonist oxotremorine on the response to the long-lasting nociceptive stimulus induced by injection of formalin into the rat hind paw. Formalin injection induced a biphasic, pain-induced behavioral response (paw jerks), as well as an increase in the number of nitric oxide (NO) synthase-labeled neurons in laminae I–III, IV, and X, but not in laminae V–VI. Oxotremorine (0.1–10 ng, ITH) inhibited paw-jerk frequency in both phases of formalin-induced behavior. The immunohistochemical results showed that ITH-injected oxotremorine differently affected the level of NO synthase in lumbar part of the spinal cord: no change or increase after the dose of 1 ng, and a significant reduction of nitric oxide synthase neurons after the higher dose (10 ng). These results evidenced a role of cholinergic system in the modulation of tonic pain and in nitric oxide synthase expression at the spinal cord level, which further suggests that these two systems could be involved in phenomena induced by long-lasting nociceptive stimulation. © 1999 Elsevier Science Inc.

Oxotremorine Cholinergic antinociception Formalin pain Spinal nociception Nitric oxide synthase Rats

DATA derived from several fields of investigation are accumulating about the involvement of cholinergic system in pain modulation, suggesting both a supraspinal descending cholinergic pathway and a local spinal interneuronal cholinergic system (32,33). Autoradiographic studies have shown significant densities of cholinergic binding in supraspinal regions involved in pain transmission, and in spinal cord localized both postsynaptically and presynaptically on the nerve terminals of the primary afferents (8,38,39). Pharmacological evidence shows that cholinergic, muscarinic receptor activation induces antinociception (13,41,43). The analgesic effects of cholinergic agonists, which are known also in clinical practice, are well documented in the experimental research on phasic acute pain induced by brief, reflexive stimuli, such as thermal stimulus used in tail-flick test (9,28). A closer resemblance to clinical pain is given by the animal models of tonic pain. Between them, the formalin model, which is predominantly used in rats and mice, involves moderate, long-lasting pain generated by tissue injury (3). Formalin induces pain-related behavioral responses, characterized by an early and a late phase. The first phase is caused predominantly by the activation of nociceptors due to the stimulus, while the second phase is due to the inflammatory reaction in the peripheral tissue and to functional changes in the central nervous system (30). Recently, we have demonstrated antinociceptive effects of oxotremorine after its peripheral administration in this chronic pain model (2).

A role of nitric oxide (NO) in the modulation of pain sensitivity emerges from a large body of evidence (6,23, 24,26); it has been observed that the NO synthase is distributed throughout the central nervous system, including regions involved in pain transmission: NO synthase has been found in

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cerebral cortex, thalamus, raphe nuclei, periaqueductal gray, and in spinal cord in laminae I–III and in lamina X (4,27,37). Immunohistochemical studies revealed no changes (36) or increase (11) of the NO synthase immunoreactivity in the superficial layers of the rat lumbar spinal cord following peripheral nociceptive stimulation. A possible interaction of cholinergic system and NO is suggested by several studies (1,12,15,22), and recently we have demonstrated that NO synthase inhibition can influence oxotremorine-induced antinociception following acute stimuli in mice and rats (19,29).

To better understand the mechanisms and the pathways involved in cholinergic-induced analgesia, the aim of the present study was to investigate the effects of intrathecal administration of the muscarinic agonist oxotremorine in the modulation of the response to the long-lasting nociceptive stimulus induced by injection of formalin into the rat hind paw. Moreover, because involvement of NO in cholinergic-induced analgesia was suggested, and increase in NO synthase immunoreactivity in chronic pain was observed, we also evaluated the effect of formalin on NO synthase immunoreactivity and its possible modulation by oxotremorine.

METHOD

Animals

Male Wistar rats (300–400 g) from Laboratory Animals Breeding House (Rembertów, Poland) were used. The rats were housed in single cages lined with sawdust, on a standard light–dark cycle (0800–2000 h), with food and water ad lib. The experiment was carried out according to the protocol approved by the Ethical Commission of the Institutes.

Surgical Preparation

The rats were implanted with chronic intrathecal (ITH) cannulas under pentobarbital (Sigma, St. Louis, MO) anesthesia. The rat head was fixed in stereotactic table and an incision was made in the atlanto-occipital membrane. A catheter (PE10, Clay Adams, Sparks, MD) was carefully introduced to the subarachnoid space at the rostral level of the lumbar enlargement of the spinal cord according to Yaksh and Rudy (42). Studies were curried out 5–14 days after surgery. Drugs were dissolved in distilled water and injected in a volume of 5 μl (single injection) or 10 μl (coadministration) followed by 10 μl of distilled water to flush the catheter.

Formalin Test

The rats were lightly anesthetized with ether and 100 μl of 10% formalin (Sigma, St.Louis, MO) solution was subcutaneously injected into the dorsal surface of the left hind paw. The rat was then placed in a wire cage for observation of the formalin-injected paw. Pain-related behavior was quantified by counting the incidence of spontanous flinching, shaking, and jerking of the injected paw. All these behaviours are named as jerks in the text. Paw jerks were continuously counted for each individual animal for 60 min and finally scored for two characteristic time points: 0–5 (first phase) and 20–40 min (second phase) after formalin administration. The rats were injected ITH with 0.1, 0.5, 1, and 10 ng oxotremorine free base (Sigma, St. Louis, MO) 15 min before formalin administration. Control animals were injected in the same way with distilled water and tested in the same time schedule as experimental groups; six to eight animals per group was tested.

Immunohistochemistry

At the time of 24 h after formalin injection three to four rats from each group were anesthetized with pentobarbital (Sigma) (50 mg/kg, intraperitoneally) and perfused intracardially with freshly prepared cold (4°C) 4% paraformaldehyde in 0.1-M Sorensen buffer (pH = 7.4). For the control group rats (n = 2–3) were injected with distilled water and then subjected to the same procedure as formalin-injected rats. The spinal cords were removed, dissected, and postfixed for 2 h, cryoprotected in 15% sucrose, and frozen on dry ice. Sections (30 μm thick) (maximum 30 slices from one spinal cord) were cut from lumbar segments L4 to L5 on a Shandon cryostat. After short incubation with Triton-X the sections were incubated with the primary antibody to the rat neuronal NO synthase (Santa Cruz Biotechnology Inc.; Burlingame, CA) (1:270 dilution with 0.1% fetal calf serum in PBS) for 24 h at 5°C. After rinsing with PBS, sections were incubated with biotinylated secondary antibody (Vectastein kit; 1:270 dilution with 0.3% Triton X in PBS) for 1 h at room temperature. After rinsing with PBS they were developed 10 min or less (monitoring the intensity of the reaction) in diaminobenzidine–H_2O_2 (DAB) solution (DAB: 0.05% diaminobenzidine in 0.05 M PO_4 + 0.003% H_2O_2). After rinsing with PBS the sections were mounted on gelatin-coated slides, dried, dehydrated with absolute alcohol followed by xylene, and coverslipped with permount. For control experiments, immunohistochemistry for NO synthase was performed following incubation of the sections with control peptide corresponding to amino acids 1400–1419 of the carboxy terminus of rat NO synthase 1, supplied together with other reagents in a kit for measuring NO synthase immunoreactivity. NO synthase immunoreactivity was not detected in any of the control sections. The number of the neurons labelled by NO

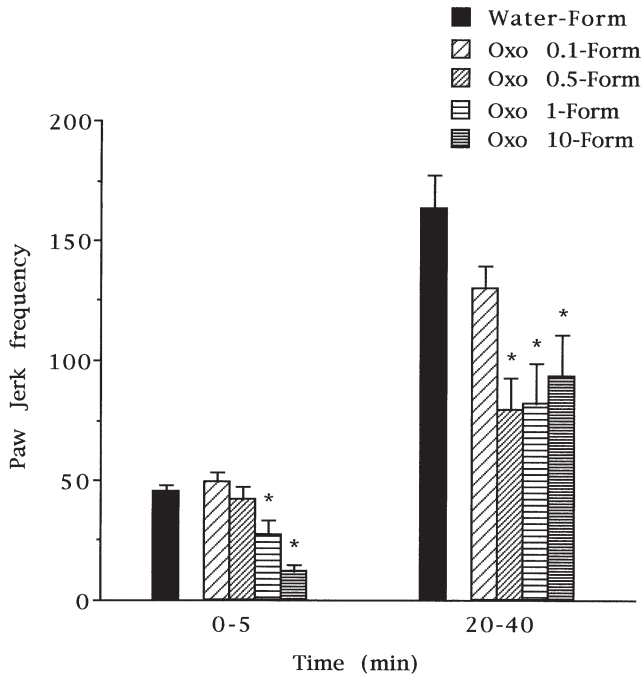

FIG. 1. Effects of different doses of oxotremorine (0.1–10 ng/5μl) injected intrathecally on number of paw jerks during the first (0–5 min) and the second (20–40 min) phases of formalin-induced behavior. *$p < 0.05$ vs. formalin injected rats.

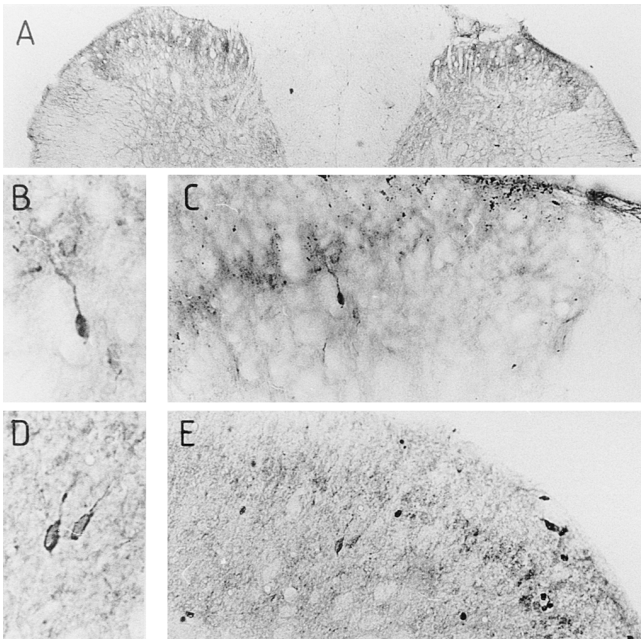

FIG. 2. Photomicrographs of NO synthase-positive cells in a dorsal horn section of a rat lumbar spinal cord. (A) Low magnification of a dorsal horn showing the NO synthase-positive cells. (B and C) Laminae I–III with a single NO synthase-positive cell in the control section of the lumbar spinal cord, higher and lower magnification, respectively. (D and E) Laminae I–III with NO synthase-positive cells in section of the lumbar spinal cord after formalin injection, higher and lower magnification, respectively.

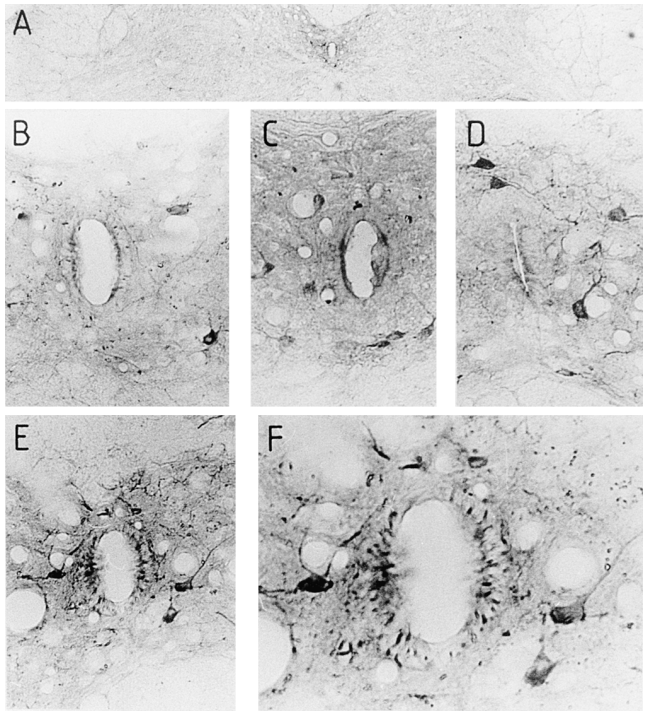

FIG. 3. Photomicrographs of NO synthase-positive cells around the central canal in a section of a rat lumbar spinal cord. (A) Low magnification of a lamina X showing the NO synthase-positive cells around the central canal. (B) Higher magnification of lamina X with NO synthase-positive cells around the central canal in control section. (C) Lamina X with NO synthase-positive cells in formalin-treated rats. (D) Lamina X with NO synthase-positive cells in rats treated with 1 ng (ITH) of oxotremorine before formalin. (E and F) Lamina X with NO synthase-positive cells in rats treated with 10 ng (ITH) of oxotremorine before formalin, lower and higher magnification, respectively.

synthase was counted in ipsi- and contralateral side to the formalin-injected paw of 10 slices obtained from two to three rats in laminae I–III, IV, V–VI, and X of the lumbar spinal cord.

Data Analysis

The results were statistically assessed by the analysis of variance (ANOVA). Specific comparisons were carried out using Duncan's (behavioral results) or Bonferroni (immunohistochemistry) multiple-range test.

RESULTS

Effects of ITH-Injected Oxotremorine on Formalin-Induced Paw-Jerk Frequency

Injection of formalin induced pain-related biphasic behavioral responses: for example, spontaneous flinching, shaking, and jerking of the injected paw. Early phase developed within the initial 5 min after injection and is followed by late phase starting form 20 min after treatment (Fig. 1)

Oxotremorine (0.1–10 ng) dose-dependently inhibited paw-jerk frequency in the first phase, $F(4, 47) = 8.92$, $p < 0.001$, of formalin-induced behavior, while in the second phase the effect was not dose dependent, statistically significant decrease, $F(4, 47) = 7.001$, $p < 0.001$, of the paw-jerk frequency was observed (Fig. 1).

Effects of Formalin and Oxotremorine on the NO Synthase Immunoreactivity in the Rat Spinal Cord

Formalin injection resulted in significant increase in number of neurons positively labeled with NO synthase (Figs. 2 and 3). The increase was observed in lamina I–III, $F(2, 77) = 21.06771$, $p < 0.001$, IV, $F(2, 77) = 19.17134$, $p < 0.001$, and X, $F(2, 77) = 7.513216$, $p < 0.001$. In laminae V–VI the effect was not observed (data not shown). In laminae I–III and IV the increase was much more pronounced in the ipsilateral side in comparison with contralateral side. The increase observed in lamina X was the same in both sides (Fig. 4).

In laminae I–III, oxotremorine decreased the formalin-induced increase in number of NO synthase-positive neurons; however, only after the higher dose, $F(3, 116) = 34{,}48665$, $p < 0.001$. After the lower dose there was a tendency to increase the number of neurons, but only in the ipsilateral side, $F(3, 96) = 18{,}16859$, $p < 0.001$ (Fig. 4). In lamina IV the lower dose of oxotremorine significantly increased the number of NO synthase-labeled neurons in both sides, $F(3, 96) = 9.636453$, $p < 0.001$, while the higher dose significantly decreased the number of neurons, $F(3, 116) = 15{,}70967$, $p < 0.001$, the effect being more pronounced in the ipsilateral side (Fig. 3). In lamina X the formalin-induced increase of NO synthase labelled neurons was counteracted by both doses of oxotremorine, $F(3, 96) = 6{,}767782$, $p < 0.001$, and $F(3, 116) = 16{,}85082$, $p < 0.001$, for the lower and the higher dose, respectively), the effects being more pronounced in the contralateral side (Figs. 3 and 5).

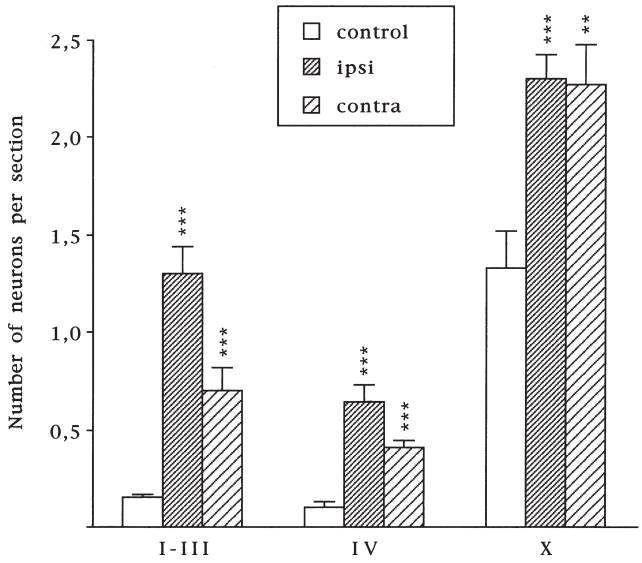

FIG 4. Effects of formalin on the number of NO synthase positive neurons in the laminae I–III, IV, and X of the spinal cord in ipsi- and contralateral side to the formalin injection. $^{**}p > 0.02$, $^{***}p > 0.01$ vs. control group.

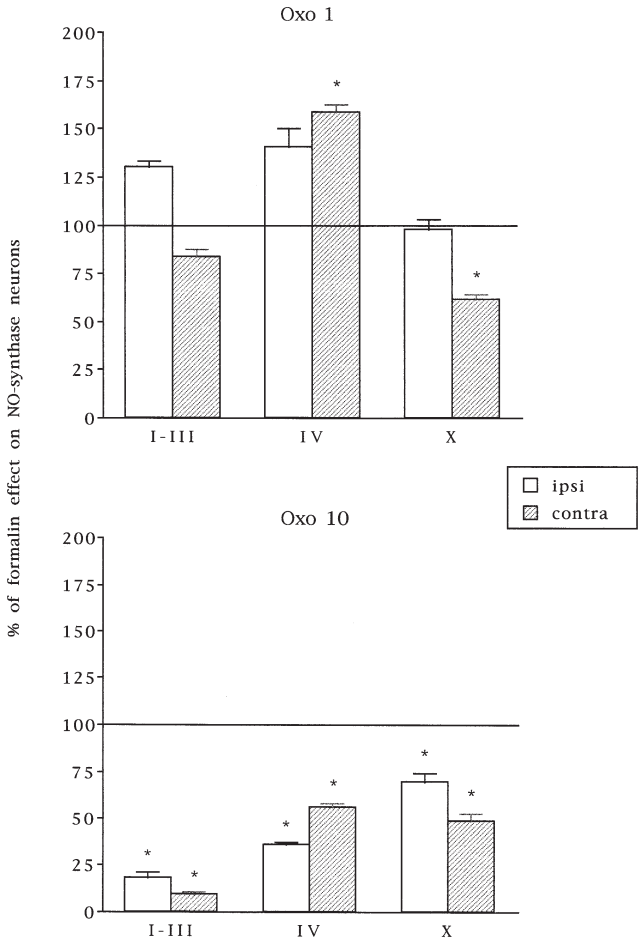

FIG. 5. Effects of two doses of oxotremorine (1 and 10 ng/5μl ITH) on the formalin-induced increase in the number of NO synthase-positive neurons in the laminae I–III, IV, and X of the spinal cord, in ipsi- and contralateral side to the formalin injection. The values are presented as a percent of the formalin effect, but statistical analysis was carried out on the raw data. Lines correspond to the 100% of the values of NO synthase positive neurons after formalin shown in Fig. 4. $^{*}p > 0.05$ vs. the corresponding formalin-treated group.

DISCUSSION

The results of the present research extend the knowledge of the analgesic properties of the muscarinic agonist oxotremorine, showing that intrathecal administration of this cholinergic drug induces antinociceptive effects in both phases of the formalin-induced behavioral response. It means that oxotremorine can act as spinal analgesic not only after acute noxious stimuli, but also in the formalin model of chronic pain, which is considered a model of postsurgical pain in humans. This is an important point because it is known that the neural pathways involved in the modulation and transmission of noxious stimuli, as well as the effects of drugs on nociception can be different, depending on the nature of the stimulus, its duration and site of application.

A great number of studies have recently demonstrated the involvement of NO in control of pain at the spinal level. It was found that inhibition of NO synthase in the spinal cord produced antinociception (10,20,31) and ITH-administered NO donors induced hyperalgesia (17,25,34). These pharmacological and electrophysiological data are supported by biochemical studies showing enhanced release of substance P and calcitonin gene-related peptide, neuropeptides principally involved in nociceptive transmission, by the NO donor sodium nitroprusside (7). Moreover, NO synthase inhibitors reduce the expression of c-*fos*, induced by noxious mechanical stimuli (18). Furthermore, NO synthase immunoreactivity is present in neurons involved in nociceptive transmission, in brain structures (27), and in spinal neurons (4). Our results of the immunohistochemical studies demonstrating a significant increase in the NO synthase immunoreactivity in the laminae I–III, IV, and X of the dorsal horn after formalin, especially in the ipsilateral side, show that NO immunoreactivity could be a marker of neuronal activation by pain. In other models of chronic pain, such as that induced by carrageenan administration, unilateral hind paw inflammation produced bilateral increase in NADPH–diaphorase histochemical staining in the rat lumbar spinal cord (35), but no increase in the number of NO synthase-stained neurons (36). However, in a previous report in which, as in our experiments, NO synthase immunoreactivity was measured 24 h following unilateral hindpaw injection of formalin, an increase in the number of NO synthase immunoreactive neurons in the dorsal horn of the rat lumbar spinal cord was observed (11). In this previous study the increase in NO synthase immunoreactivity was demonstrated only in the superficial laminae of the ipsilateral side of the spinal cord, while our results also show an increase in the contralateral side and in lamina X. The twice higher formalin concentration (10%) used in our experiments could explain the stronger effect observed, which further confirm the possible utilization of NO synthase activity as a marker of nociceptive stimulation. According to this, it was observed (21) that protracted noxious stimulation produced by peripheral formalin injection enhanced, in microdialysated rats, spinal release of glutamate, which is known to be followed by activation of NO synthase.

Another point emerging from the present results is that the dose of 10 ng of oxotremorine impaired the formalin-induced

increase of NO synthase immunoreactivity in the dorsal horn of the rat lumbar spinal cord supporting the antinociceptive effect observed at the behavioral level. We demonstrated the effects of the muscarinic cholinergic stimulation in the formalin model of pain both at behavioral and biochemical levels: the analgesic effect of oxotremorine on paw-jerk frequency and the antagonism of formalin-induced increase of neurons labeled for NO synthase.

Both doses of oxotremorine used in our study induce antinociception in the two phases of the formalin model, but their influence on NO synthase is different. In contrast to the higher dose, the lower one potentiates the formalin effect, especially in lamina IV. It has to be considered that the level of NO synthase was measured 24 h after formalin injection and probably the lower dose, with a shorter action, is not able to antagonize the continuous painful stimulus from the inflammed paw, antagonism that occurs after the higher dose. However, it cannot be excluded that oxotremorine potentiates to some extend the activity of NO system, as evidenced by the experiments in the cultured rat sensory neurons where the increase of cGMP after oxotremorine was observed (1). This effect could be a reason for the different results obtained by other authors studying interactions between NO and the cholinergic system: the antinociceptive role exerted by NO on supraspinal and peripheral cholinergic-mediated analgesia (5,6,14,40). In addition there is some evidence that NO may induce pronociceptive effects at low concentrations and antinociceptive effects at higher concentrations (16).

In summary, our behavioral study demonstrates the spinal antinociceptive effect of oxotremorine in the formalin model of chronic pain. In addition, immunohistochemical study show that prolonged peripheral nociceptive stimulation results in an increase of spinal NO synthase immunoreactivity, this effect being modulated by ITH-injected oxotremorine. The present results further support possible involvement of the NO pathway in antinociception induced by stimulation of spinal muscarinic receptors.

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