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Involvement of μ-opioid receptors in antinociceptive action of botulinum toxin type A

V. Drinovac [a], L. Bach-Rojecky [a], I. Matak [b], Z. Lacković [b,*]

[a] Department of Pharmacology, University of Zagreb, Faculty of Pharmacy and Biochemistry, Domagojeva 2, 10000 Zagreb, Croatia
[b] Laboratory of Molecular Neuropharmacology, Department of Pharmacology and Croatian Brain Research Institute, University of Zagreb School of Medicine, Šalata 11, 10000 Zagreb, Croatia

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ABSTRACT

Botulinum toxin A (BTX-A) is approved for treatment of chronic migraine and has been investigated in various other painful conditions. Recent evidence demonstrated retrograde axonal transport and suggested the involvement of CNS in antinociceptive effect of BTX-A. However, the mechanism of BTX-A central antinociceptive action is unknown. In this study we investigated the potential role of opioid receptors in BTX-A's antinociceptive activity.

In formalin-induced inflammatory pain we assessed the effect of opioid antagonists on antinociceptive activity of BTX-A. Naltrexone was injected subcutaneously (0.02–2 mg/kg) or intrathecally (0.07 μg/10 μl–350 μg/10 μl), while selective μ-antagonist naloxonazine was administered intraperitoneally (5 mg/kg) prior to nociceptive testing. The influence of naltrexone (2 mg/kg s.c.) on BTX-A antinociceptive activity was examined additionally in an experimental neuropathy induced by partial sciatic nerve transection. To investigate the effects of naltrexone and BTX-A on neuronal activation in spinal cord, c-Fos expression was immunohistochemically examined in a model of formalin-induced pain.

Antinociceptive effects of BTX-A in formalin and sciatic nerve transection-induced pain were prevented by non-selective opioid antagonist naltrexone. Similarly, BTX-A-induced pain reduction was abolished by low dose of intrathecal naltrexone and by selective μ-antagonist naloxonazine. BTX-A-induced decrease in dorsal horn c-Fos expression was prevented by naltrexone.

Prevention of BTX-A effects on pain and c-Fos expression by opioid antagonists suggest that the central antinociceptive action of BTX-A might be associated with the activity of endogenous opioid system (involving μ-opioid receptor). These results provide first insights into the mechanism of BTX-A's central antinociceptive activity.

1. Introduction

Botulinum toxin type A (BTX-A), an endopeptidase derived from *Clostridium botulinum*, cleaves SNAP-25 (Synaptosomal Associated Protein of 25 kDa), one of the proteins essential for neuroexocytosis (Blasi et al., 1993). Prevention of acetylcholine release in neuromuscular junction and autonomous synapses is the main feature of BTX-A poisoning. The same mechanism enables local application of BTX-A in low picomolar doses to be used in treatment of neuromuscular and autonomous disorders (Lim and Seet, 2010).

Apart from its effect on neuromuscular junction, recent preclinical and clinical studies reported the efficacy of BTX-A in reduction of allodynia and hyperalgesia in pain of different origins (Jabbari and Machado, 2011; Pavone and Luvisetto, 2010). Moreover, BTX-A was recently registered for treatment of chronic migraine (Dodick et al., 2010) and several controlled clinical studies in other painful conditions are in progress (Jabbari and Machado, 2011; Singh, 2010). Importance of BTX-A application in clinical practice results from its unique ability to reduce pain in a long lasting manner (up to 6 months in humans).

It was suggested that antinociceptive effect of BTX-A results from inhibition of neurotransmitter release from peripheral sensory nerve endings (Aoki, 2005; Cui et al., 2004), similarly as in neuromuscular junction. On the other hand, recent behavioral (Bach-Rojecky and Lacković, 2009; Bach-Rojecky et al., 2010; Favre-Guilmard et al., 2009; Filipovic et al., 2012) and immunohistochemical studies (Matak et al., 2011, 2012) indicate that the

Abbreviations: BTX-A, botulinum toxin type A; SNAP-25, Synaptosomal Associated Protein of 25 kDa; i.pl., intraplantarly; s.c., subcutaneously; i.t., intrathecally; i.p., intraperitoneally; PBS, phosphate-buffered saline; PBST, Triton X-100 in phosphate-buffered saline; NGS, normal goat serum.

* Corresponding author. Tel./fax: +385 1 45 66 843.
E-mail addresses: vdrinovac@pharma.hr (V. Drinovac), lbach@pharma.hr (L. Bach-Rojecky), ivica.matak@mef.hr (I. Matak), lac@mef.hr (Z. Lacković).

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antinociceptive action occurs primarily in the central nervous system where BTX-A is axonally transported (Antonucci et al., 2008; Matak et al., 2011, 2012). However, there are no *in vivo* data regarding central molecular mechanism, receptors and possible neurotransmitters involved. Here we report that the antinociceptive action of BTX-A is associated with central μ-opioid receptor activity.

2. Materials and methods

2.1. Animals

Male Wistar rats (University of Zagreb School of Medicine, Croatia) weighing 300–400 g, kept in temperature-regulated environment (23 °C) under 12 h light–dark cycle, with free access to food and water (except during testing), were used in all experiments. Experiments were conducted according to the European Communities Council Directive (86/609/EEC) and recommendations of the International Association for the Study of Pain (Zimmerman, 1983). All efforts were made to reduce the number of animals used and to reduce their suffering. Experiments were approved by the Ethical Committee of the University of Zagreb, School of Medicine (permit No. 07-76/2005-43).

2.2. Drugs

The following drugs and chemicals were used: BTX-A (Botox®, Allergan, Inc., Irvine, USA); non-selective opioid antagonist naltrexone (Sigma, St. Louis, MO, USA); selective μ-opioid antagonist naloxonazine (Santa Cruz Biotechnology, Inc., CA, USA); chloral-hydrate (Sigma, St. Louis, MO, USA); diethyl ether (Sigma, St. Louis, MO, USA); acetone (Sigma, St. Louis, MO, USA). To obtain the doses needed, BTX-A and opioid antagonists were dissolved in 0.9% saline. Each vial of Botox® contains 100 U (~4.8 ng) of purified *C. botulinum* type A neurotoxin complex.

For immunohistochemical experiment the following chemicals are used: paraformaldehyde (Sigma–Aldrich, St. Louis, MO, USA), Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA), normal goat serum (Vector, Inc., Burlingame, CA, USA), c-Fos rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA), goat anti-rabbit Alexa Fluor-448 (Invitrogen, Carlsbad, CA, USA), anti-fading agent (Fluorogel, Electron Microscopy Sciences, Hatfield, PA, USA).

2.3. Animal treatment

BTX-A was injected subcutaneously into the plantar surface of the hind paw (intraplantarly, i.pl.), ipsilateral to formalin injection/nerve injury, to conscious, gently restrained rats, in a volume of 20 μl with a 27½ gauge needle. To test the effect on formalin-induced pain, BTX-A was injected in a dose of 5 U/kg, while 7 U/kg BTX-A was used in the model of neuropathic pain. Doses were chosen based on previous experiments from our laboratory (Bach-Rojecky et al., 2005; Bach-Rojecky and Lacković, 2005).

Naltrexone was injected: 1. subcutaneously (s.c., 0.02 mg/kg–2 mg/kg) into the abdominal area in a volume of 250 μl; and 2. intrathecally (i.t., 0.07 μg/10 μl–350 μg/10 μl) at the lumbar L3–L4 level. Dose of s.c. injected naltrexone was chosen based on literature (Correa et al., 2010), while dose of i.t. naltrexone (100-fold lower than s.c. dose) was based on preliminary experiment. 1 ml of naloxonazine was injected intraperitoneally (i.p.) in a dose of 5 mg/kg, as used by other authors (De Freitas et al., 2011).

2.4. Nociceptive assessment

Nociceptive experiments were performed in a quiet laboratory, between 10 a.m. and 4 p.m. Animals were allowed to accommodate to the testing environment for 10 min. Evaluation of nociceptive testing was performed by observer unaware of the animal treatment. Animal treatment was known to other experimenter who treated and marked the animals. However, the main observer could recognize the injured hind-limb either in formalin-induced pain (paw edema) or nerve injury (limping and characteristic posture of injured paw).

2.4.1. Formalin test

Conscious, gently restrained rats were s.c. injected with saline-diluted 5% formalin solution (50 μl) into the plantar side of the right hind paw pad and immediately returned to the transparent cage for 1 h observation period. Pain was measured as the number of nocifensive behaviors (licking, flinching and shaking of the injected paw). Recording time was divided in two phases: acute phase I (0–15 min) response caused by direct stimulation of peripheral sensory nerve endings with formalin, and inflammatory phase II (15–60 min) characterized by peripheral sensitization (Tjølsen et al., 1992). Each experimental group contained 5–6 animals.

BTX-A (5 U/kg i.pl.) was injected 5 days before the formalin testing, while naltrexone (0.02 mg/kg–2 mg/kg s.c.; 0.07 μg/μl–350 μg/μl i.t.) and naloxonazine (5 mg/kg i.p.) were injected 40 min and 24 h prior to the formalin test, respectively. Control animals received 0.9% saline in the appropriate volumes. For intrathecal application of naltrexone, animals were briefly anesthetized with diethyl ether until no reflexive response to paw pinch was elicited. Animal's hair was shaved at the lumbar L3–L4 level. Small skin incision (1 cm) was performed. Naltrexone or saline were injected between the vertebrae. Animals recovered from diethyl ether anesthesia in approx. 10 min. Shortly acting diethyl ether was used to achieve fast recovery prior to nociceptive testing (40 min following i.t. injection).

To investigate whether the effect of naltrexone on BTX-A-induced antinociception is short-lasting or long-lasting, we performed an additional experiment where naltrexone was administered 24 h prior to formalin testing. 24 h point was chosen based on the time required for complete elimination of naltrexone from the organism (Verebey et al., 1976.).

2.4.2. Peripheral neuropathy

A total number of 38 rats underwent sciatic nerve partial transection, as previously described (Bach-Rojecky et al., 2005; Lindenlaub and Sommer, 2000). In brief, right sciatic nerve was exposed in rats under general anesthesia (chloral-hydrate 300 mg/kg) and the middle of the nerve trunk was pierced using a thin surgical needle. Half of the nerve diameter was transected by the scalpel in the needle direction. Six rats were subjected to sham procedure; sciatic nerve was exposed, but not transected. Six naive rats served as control.

Two weeks following the peripheral nerve injury, animals which developed mechanical sensitivity to pressure/von Frey filaments and cold allodynia (at least 20% changes from the mean of the sham-operated group) were included into the further experiment. Animals were divided in four groups (5–6 animals per group) as follows: (1) 0.9% saline (i.pl.), (2) BTX-A (7 U/kg; i.pl.), (3) naltrexone (2 mg/kg, s.c.), (4) BTX-A + naltrexone.

Nociceptive measurements were performed 5 days following BTX-A i.pl. injection, and 40 min following naltrexone s.c. injection. The assessment of each animal started with mechanical sensitivity to pressure, followed by mechanical sensitivity to von Frey filaments and cold allodynia measurements, with 30 min period between each type of measurement.

2.4.2.1. Mechanical sensitivity to paw pressure. Mechanical sensitivity was measured by the modified paw pressure test, originally described by Randall and Selitto (1957), on both hind paws. Average mechanical nociceptive threshold expressed in grams was calculated from 3 measurements. Measurements were repeated in 10 min intervals by applying increased pressure to the dorsal surface until paw withdrawal or struggling of the animal occurred (Bach-Rojecky et al., 2010).

2.4.2.2. Mechanical sensitivity to von Frey filaments. Paw withdrawal threshold in response to a mechanical stimulus was determined using a series of von Frey filaments (Stoelting Co, Wood Dale, IL, USA) ranging from 0.6 g to 26 g. Animals were placed in a plastic cage with a metal mesh floor 10 min prior to testing. Von Frey filaments were applied to the mid-plantar surface of the hind paw through the mesh floor. Each filament was applied 3 times, kept in bent position on the rat's hind paw for 4 s. Filaments were applied in ascending order, and the lowest filament that elicited a foot withdrawal response was considered the threshold stimulus (Wei et al., 1998).

2.4.2.3. Cold allodynia. Cold allodynia was measured as the number of foot withdrawal responses after an application of cold stimuli (a drop of 100% acetone) to the plantar surface of the hind paw. Testing was repeated five times with an interval of approximately 5 min between each test. Response frequency to acetone was expressed as a percent withdrawal frequency [(number of paw withdrawals/number of trials) × 100] (Park et al., 2006).

2.5. Immunohistochemistry

Immunohistochemical analysis was performed on samples collected from the formalin test experiment with s.c. applied naltrexone.

Two hours following the i.pl. formalin injection rats were deeply anesthetized using chloral-hydrate (300 mg/kg) and transcardially perfused with 250 ml of 0.9% saline, followed by 250 ml of fixative (4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.4). Spinal cord was removed and cryoprotected at 4 °C overnight in 15% sucrose-fixative solution, followed by 30% sucrose in PBS the next day, until the tissue sank. Lumbar spinal cord (L4/L5 segment) sections of 4 rats belonging to each experimental group were processed for immunohistochemical analysis. Frozen sections (30 μm), cut on cryostat (Leica, Germany), were taken for free floating in wells with PBS. Sections were washed 3 × 5 min in PBST (PBS + 0.25% Triton X-100) blocked in 10% normal goat serum (NGS) for 1 h and incubated overnight at room temperature with rabbit anti-c-Fos polyclonal antibody diluted in 1% NGS. Sections were washed in PBST and incubated for 2 h at room temperature with 1:400 goat anti-rabbit Alexa Fluor-448 fluorescent secondary antibody in the dark, diluted in 1% NGS. Sections were washed and mounted on glass slides with anti-fading agent.

Sections were visualized with fluorescent microscope (Olympus BX51, Olympus, Tokyo, Japan) connected to digital camera (Olympus DP-70, Olympus, Tokyo, Japan)