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The analgesic effect of crotoxin on neuropathic pain is mediated by central muscarinic receptors and 5-lipoxygenase-derived mediators

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ABSTRACT

Crotoxin (CTX), a neurotoxin isolated from the venom of the South American rattlesnake *Crotalus durissus terrificus*, induces analgesia. In this study, we evaluated the antinociceptive effect of CTX in a model of neuropathic pain induced by rat sciatic nerve transection. Hyperalgesia was detected 2 h after nerve transection and persisted for 64 days. Immersion of proximal and distal nerve stumps in CTX solution (0.01 mM for 10 s), immediately after nerve transection, blocked hyperalgesia. The antinociceptive effect of CTX was long-lasting, since it was detected 2 h after treatment and persisted for 64 days. CTX also delayed, but did not block, neurectomy-induced neuroma formation. The effect of CTX was blocked by zileuton (100 mg/kg, p.o.) and atropine (10 mg/kg, i.p.), and reduced by yohimbine (2 mg/kg, i.p.) and methysergide (5 mg/kg, i.p.). On the other hand, indomethacin (4 mg/kg, i.v.), naloxone (1 mg/kg, i.p.), and *N*-methyl atropine (30 mg/kg, i.p.) did not interfere with the effect of CTX. These results indicate that CTX induces a long-lasting antinociceptive effect in neuropathic pain, which is mediated by activation of central muscarinic receptors and partially, by activation of α-adrenoceptors and 5-HT receptors. Eicosanoids derived from the lipoxygenase pathway modulate the action of crotoxin.

1. Introduction

Neuroma formation is always incited by nerve injury and it is the most common complication reported in the literature (Adams et al., 1974; Evans et al., 1970; Schebitz and Wilkens, 1967). Transection of a nerve, named neurectomy, induces the development of neuroma at the site of injury. Neuromas are known to be a source of ectopic sensory input, being painful and causing major disability (Mathews and Osterholm, 1972; Meyer et al., 1985; Zeltser et al., 2000). It is well demonstrated that prolonged ectopic sensory input contributes to spontaneous and evoked neuropathic pain (Zeltser et al., 2000). Recordings from single nerve fibers arising from experimental neuromas in the sciatic nerve of rat, mouse, and cat have demonstrated that certain regenerating fibers display spontaneous action potential activity (Govrin-Lippmann and Devor, 1978; Scadding et al., 1981), whereas others may be activated by mechanical distortion of the neuromas (Scadding et al., 1981). The pharmacotherapy for neuropathic pain has had limited success (Przewlocki and Przewlocka, 2001; Woolf and Mannion, 1999), resulting in major disability and leading to the need of developing new treatments for neuroma prevention. A variety of non-surgical approaches have been tried, including retrograde axoplasmic transport of distinct substances for destruction of ganglion cells, however these approaches have presented limited efficacy (Brandner et al., 1989).

Several lines of evidence have suggested that crotoxin (CTX), the main neurotoxic component of the South American rattlesnake *Crotalus durissus terrificus*, composed of a weakly toxic basic phospholipase A_2 and of an acidic non-toxic and non-enzymatic polypeptide named crotapotin (Bon, 1989; Slotta, 1938), induces antinociceptive effect in experimental models of acute pain, mediated by an action on the central nervous system (Zhang et al., 2006b). Recent data from our group have demonstrated that CTX inhibits tumor growth and induces antinociception in an experimental model of cancer pain (Brigatte, P. and Cury, Y., unpublished data). Data from the literature have also demonstrated that the toxin inhibits tumoral cell proliferation (Corin et al., 1993; Newman, 1993; Rudd et al., 1994). In addition, a phase I clinical trial study showed that patients with advanced cancer treated with CTX presented analgesia and reduction of tumor mass (Cura et al., 2002).

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Despite these observations, the effect of crotoxin on neuropathic pain remains to be determined. The present study was undertaken to evaluate the antinociceptive effect of crotoxin in a model of neuropathic pain induced by neurectomy. The possible mechanisms involved in the antinociceptive effect of the venom were also evaluated.

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing between 160 and 180 g, were used throughout this study. The rats were housed under controlled humidity, at a temperature of 22 °C±1 subjected to a 12 h light–dark cycle and in a sound-attenuated room. Food and water were available *ad libitum* until 2 h before sciatic nerve transection and crotoxin administration. All the tests were performed between 9:00 and 16:00 h. All experiments were in accordance with the guidelines for the ethical use of conscious animals in pain research, published by the International Association for the Study of Pain (Zimmermann, 1983). The procedures were approved by the Institutional Animal Care Committee at Butantan Institute (CEUAIB, protocol number 201/05).

2.2. Crotoxin (CTX)

Crotoxin was purified from *C. d. terrificus* venom by anion-exchange chromatography as previously described by Faure et al. (1994) using a Mono-Q HR 5/5 column in a FPLC system (Pharmacia, Uppsala, Sweden). Fractions (1 ml/min) were eluted by a linear gradient of NaCl (0–1 M in 50 mM Tris–HCl; pH 7.0). Three pools (pool I, pool II and pool III) were obtained during the crotoxin purification process, being pool II the correspondent pure crotoxin fraction. Fractions containing crotoxin (pool II, total volume collected = 10 ml) were checked for homogeneity by non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%), and phospholipase A_2 activity was assessed by a colorimetric assay using a synthetic chromogeneic substrate. One-hundred microliters of phosphate buffer saline (PBS, pH 7.4) containing 12.5 or 25 μg of crotoxin were added to 1.0 ml of reaction medium (10 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 100 mM NaCl) and incubated for 20 min at 37 °C, in the presence of 100 μl of 3 mM chromogeneic substrate 4-nitro-3-(octanoyloxy) benzoic acid (Sigma Chem. Co, USA), dissolved in 5 mM acetonitrile. The reaction was stopped by placing the tubes on ice and the quick addition of 100 μl Triton X-100 (2.5%). Tubes were held at room temperature for 5–10 min and absorbance determined at 425 nm. All assays were run in duplicate.

2.3. Testing

2.3.1. Induction of neuropathic pain (neurectomy)

Neurectomy was performed based on the method described by Kingery and Vallin (1989). Rats were anesthetized with halothane, and a small incision was made in the skin and femoral biceps. The sciatic nerve was exposed unilaterally by careful dissection, transected in two locations at the mid-thigh level and 0.5 cm of the nerve was removed to ensure adequate separation between the proximal and distal stumps. Sham controls were subjected to the same surgical procedure, without manipulation of the nerve.

2.3.2. Evaluation of mechanical hyperalgesia (Randall–Selitto test)

The rat paw pressure test (Randall and Selitto, 1957) was used for determination of hyperalgesia. An Ugo-Basile pressure apparatus was used to assess pressure pain thresholds prior to sciatic nerve transection and again at different times after crotoxin or saline treatment. Testing was blind in regard to group designation. Briefly, increasing force (in g, 16 g/s) was applied to the right hind paw. The force needed to induce paw withdrawal was recorded as the pain threshold. To reduce stress, rats were habituated to the testing procedure the day before the experiment. To minimize damage to the paw, a maximum force of 160 g (i.e. cut-off) was established for the test applied after the pharmacological treatments.

2.3.3. Evaluation of general motor activity (open-field test)

Possible changes in locomotor activity induced by crotoxin were investigated in the open field test. The open field consisted of a white circular arena (97 cm in diameter surrounded by a 32.5 cm high wall). The floor of that arena is divided into three concentric circles, which in turn, are subdivided by straight segments into nineteen roughly equal areas. Hand-operated counters were used to score ambulation (locomotion) (number of floor units entered) and rearing (number of times the animal stood on hind legs) frequencies. Each animal was individually placed in the centre of the open field and behavioral parameters were recorded for 3 min. The open field was washed with water–alcohol (5%) before the animals were placed in it to avoid possible biasing effects of odor clues left by previous subjects. The test was applied 2 h after crotoxin treatment in animals submitted to sciatic nerve transection.

2.4. Drugs

Atropine sulfate, atropine methyl nitrate, yohimbine, methysergide, naloxone hydrochloride, atenolol and indomethacin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Zileuton was purchased from Abbott Laboratories (North Chicago, USA).

Atropine sulfate, *N*-methyl atropine, yohimbine, atenolol, naloxone, methysergide were mixed in 0.85% sterile saline solution. Zileuton was mixed in 10% hydro-alcoholic solution. Indomethacin was mixed in Tris 1 M, pH 8.0. Drugs were mixed fresh daily.

2.5. Pharmacological treatments

2.5.1. Crotoxin (CTX)

Crotoxin was dissolved in sterile physiological saline (0.01 mM in 0.95% sterile saline) and applied directly to the nerve stumps immediately after nerve transection. For the treatment, the nerve stumps were laid on the inside portion of the tip of 1.5 ml plastic centrifuge tube and CTX was delivered (50 μl) over the proximal and distal nerve stumps. The stumps were maintained for 10 s in crotoxin solution. Sterile saline applied directly to nerve stumps, was used as control.

In another set of experiments, CTX or saline was administered by s.c. route (18 μg/rat), immediately before nerve transection or on day 14 after surgery. This dose of crotoxin was based on previous study of our group (Sampaio et al., 2003; Zambelli et al., 2008) and did not cause clinical signs of crotalid envenomation.

2.5.2. Receptor antagonists and inhibitors

In order to characterize the involvement of 5-HT receptors in the anti-hyperalgesic effect of crotoxin, methysergide (5 mg/kg, i.p.) was administered 30 min before the toxin. To evaluate the involvement of central and peripheral muscarinic receptors, atropine sulfate (10 mg/kg, i.p.) or methyl atropine nitrate (30 mg/kg, i.p.), was administered 30 and 20 min before the toxin, respectively. Yohimbine (2 mg/kg, i.p.), an α-adrenergic receptor antagonist, was administered 15 min before surgery. Atenolol (1 mg/kg, i.p.), a β-adrenergic receptor antagonist, was administered 30 min before crotoxin. To characterize the involvement of opioid receptors in the anti-hyperalgesic effect of crotoxin, naloxone hydrochloride (1 mg/kg, i.p.), a non-selective opioid receptor antagonist, was administered 105 min after crotoxin. To evaluate the involvement of eicosanoids, indomethacian (4 mg/kg, i.v.), a cyclooxygenase inhibitor or zileuton (100 mg/kg, p.o.), a 5-lipoxygenase inhibitor, was administered 60 min before the toxin.

The doses of the drugs used were based on data from the literature or on previous study of our group (Campos et al., 2006; Chacur et al.,

2003; Coimbra et al., 2001; Horizoe et al., 1998; Jancar et al., 1989; Picolo et al., 2000; Sampaio et al., 2006; Tavares-de-Lima et al., 1989; Zarrindast et al., 2000; Zhang et al., 2006a; Zweifel et al., 2008). The 3-fold increase in the dose of *N*-methyl atropine relative to that of atropine was adopted based on the difference in antagonistic potency these drugs display against contractions of the rat isolated vas deferens induced by the muscarinic receptor agonist carbachol.

In these assays, the paw pressure test was applied 2 h after crotoxin administration. In all assays, testing was blind in regard to group designation.

2.6. Creatine phosphokinase (CK) dosage

The possible rat muscle injury induced by crotoxin was evaluated by serum CK activity (Azevedo-Marques et al., 1985; de Sousa-e-Silva et al., 2003; Furtado et al., 2003). Animals were anaesthetized with halothane and all blood samples (2 ml) were collected by abdominal aorta 4 and 8 h after local crotoxin or saline administration during the surgery process (Furtado et al., 2003). Blood was allowed to clot, centrifuged at 4 °C and 3000 rpm, and serum creatine kinase (CK) activity was determined by colorimetric method based on creatine formation through ADP/phosphocreatine reaction (CK activated with Nac; Merck-1-Test, Merck, Rio de Janeiro, Brazil). CK value was expressed in international units per liter (U/L) of serum.

2.7. Statistical analysis

Results are presented as mean ± S.E.M. Statistical evaluation of data was carried out by analysis of variance (ANOVA) and sequential differences among means were compared according to Tukey contrast analysis at $p<0.05$.

3. Results

3.1. Evaluation of hyperalgesia induced by sciatic nerve transection

The sciatic nerve transection caused a significant decrease in pain threshold detected 2 h after surgery, as measured by the Randall and Selitto test. This phenomenon was detected up to 64 days after surgery (Fig. 1). Sham-operated (control) animals did not present alteration in pain threshold as compared to basal values obtained immediately before the surgical procedures (Fig. 1).

3.2. Antinociceptive effect of crotoxin

Crotoxin 0.01 mM (13 μg/50 μl saline/rat), applied directly to the nerve stumps immediately after nerve transection, blocked hyperalgesia induced by neurectomy. The antinociceptive effect of CTX was detected 2 h after treatment and persisted for at least 64 days (Fig. 2A). It is interesting to note that in addition to the anti-hyperalgesic effect, CTX also increased the pain threshold of the animals, as compared to basal values obtained before surgery (Fig. 2A).

In another set of experiments, CTX was administered by s.c. route, immediately before or 14 days after sciatic nerve transection. The rat paw pressure test was applied 2 or 24 h after treatment. Results presented in Fig. 2B and C showed that CTX administered by s.c. route also blocked hyperalgesia induced by nerve transection and increased the pain threshold of the animals, as compared to basal values obtained before surgery; however the antinociceptive effect of the toxin was detected only 2 h after treatment, disappearing 24 h thereafter.

Based on the results showing that the local treatment with crotoxin-induced a more long-lasting antinociceptive effect than the systemic (s.c.) treatment, the studies on the characterization of the mediators involved in the effect of the toxin were performed using the former treatment.

3.3. Effect of crotoxin on spontaneous motor activity and on creatine kinase serum levels

Crotoxin presents neurotoxic and myotoxic activities (Arruda et al., 2002; Bon, 1989; Mebs et al., 1983), which could alter the locomotor activity of the animals. Since motor activity inhibition could interfere with the behavior of the rats in the nociceptive test, the possible influence of crotoxin on spontaneous motor activity was investigated. Local administration of the CTX did not alter locomotion and rearing frequencies (60 and 18, respectively) in the open field, as compared to control animals treated with saline (56 and 15, respectively).

CK serum levels of rats treated with crotoxin (4 h = 1547 U/L; 8 h = 996 U/L) were not significantly different of that observed in animals treated with saline (4 h = 1633 U/L; 8 h = 803 U/L).

3.4. Effects of naloxone on the antinociceptive effect of CTX

Pre-treatment with naloxone did not interfere with the antinociceptive effect of CTX. Naloxone, per se, also did not modify the hyperalgesic effect induced by sciatic nerve transection (Fig. 3A).

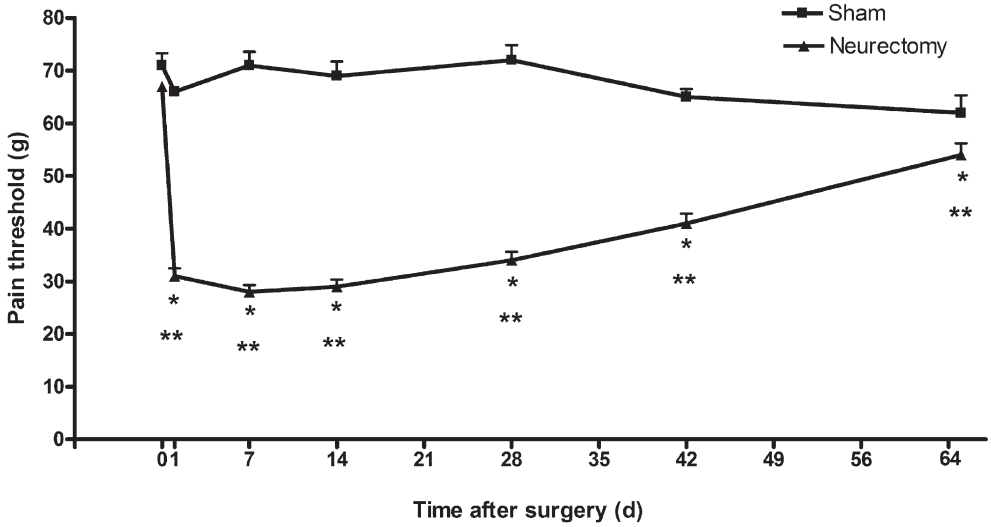

Fig. 1. Evaluation of hyperalgesia induced by rat sciatic nerve transection (neurectomy). Pain threshold was estimated in the rat paw pressure test applied before (time 0), and 2 h or on days (d) 1, 7, 14, 28, 42 and 64 after surgery. Sham-operated (Sham) rats were subjected to the same surgical procedure, without manipulation of the nerve. Time 0 corresponds to pain threshold measure before surgery. Data represent mean values ± S.E.M. for 10 rats per group. *Significantly different from mean values obtained before surgery (time 0). **Significantly different from mean values of Sham group ($p<0.05$).

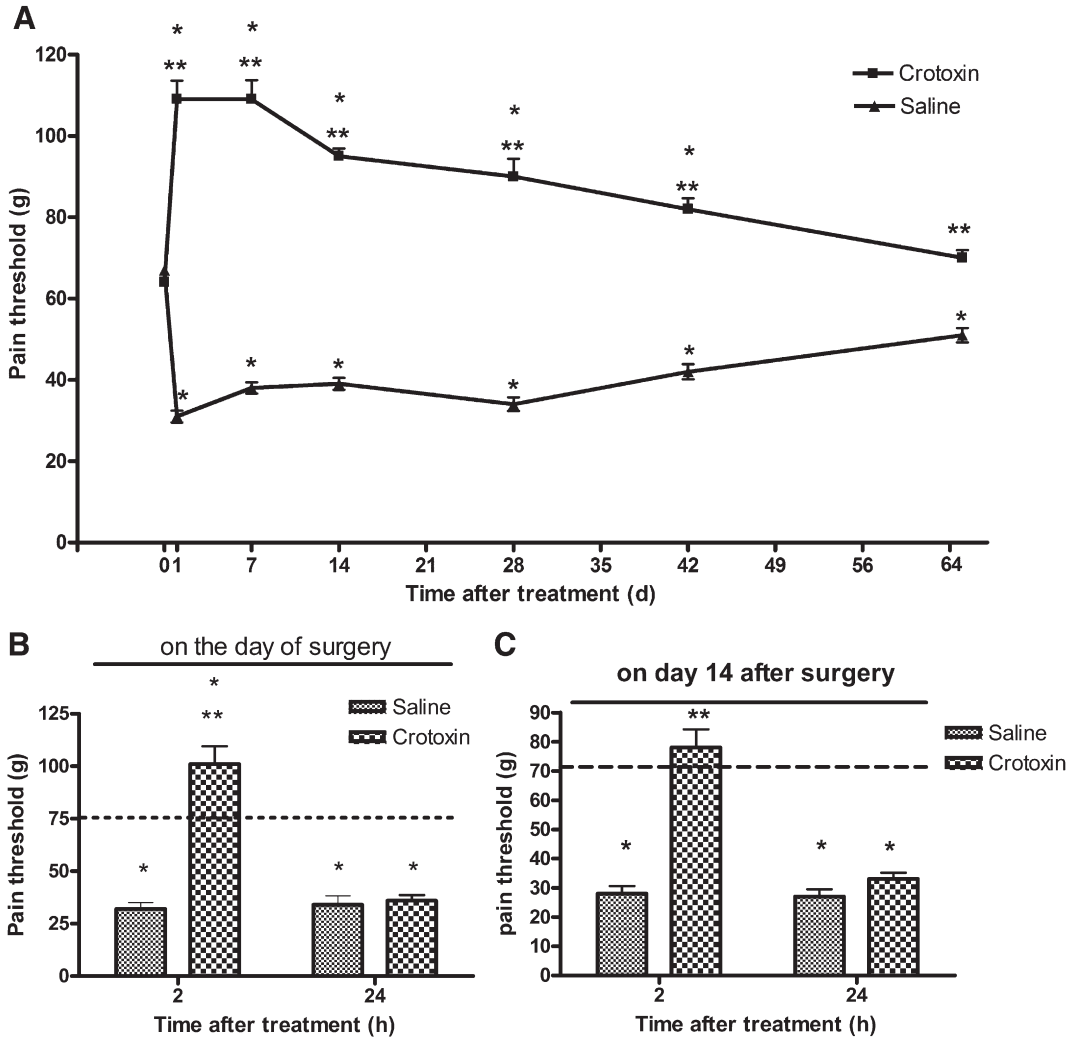

Fig. 2. Antinociceptive effect of crotoxin. In Panel A, the toxin (0.01 mM) or saline (control) was applied to the proximal and distal nerve stumps, immediately after nerve transection. In Panels B and C, crotoxin (18 μg/rat) or saline (control) was administered by s.c. route, immediately before nerve transection (Panel B) or on day 14 after surgery (Panel C). In Panel A, the pain threshold was estimated in the rat paw pressure test applied before (time 0) and 2 h or on days (d) 1, 7, 14, 28, 42 and 64 after treatments. In Panels B and C, the pain threshold was estimated in the rat paw pressure test applied 2 and 24 h after treatments. The dash line represents the values of pain threshold obtained before surgery. Data represent mean values ± S.E.M. for 10 rats per group. *Significantly different from mean values obtained before surgery (dash line). **Significantly different from mean values of saline (control group) ($p<0.05$).

3.5. Involvement of 5-HT and adrenergic receptors in the antinociceptive effect of crotoxin

Pre-treatment with methysergide (Fig. 3B) or yohimbine (Fig. 3C) partially inhibited (46% and 51%, respectively) the antinociceptive effect of crotoxin. On the other hand, atenolol did not interfere withy the antinociceptive effect of CTX (Fig. 3D). The antagonists per se, did not modify the hyperalgesic effect induced by sciatic nerve transection.

3.6. Involvement of muscarinic receptors in antinociception induced by crotoxin

Pre-treatment with atropine blocked the antinociceptive effect of CTX (Fig. 3E). In contrast, pre-treatment with *N*-methyl atropine did not interfere with the antinociceptive effect of crotoxin (Fig. 3F).

The antagonists per se, did not modify the hyperalgesic effect induced by sciatic nerve transection.

3.7. Involvement of eicosanoids in the antinociceptive effect of crotoxin

Indomethacin did not modify the antinociceptive effect of crotoxin (Fig. 3G). On the other hand, pre-treatment with zileuton reverted crotoxin-induced antinociception (Fig. 3H). The inhibitors per se, did not modify the hyperalgesic effect induced by sciatic nerve transection.

4. Discussion

Peripheral nerve injury is often accompanied by persistent functional loss and chronic neuropathic pain. Pathophysiological nociceptive pain observed after nerve injury, usually occurs soon after the injury and it is resistant to most therapeutic strategies (Zimmermann, 2001). The sciatic nerve transection is considered a good model to study neuropathic pain and pain related behavior observed after nerve transection has been well studied (Kingery and Vallin, 1989; Vallin and Kingery, 1991).

Results herein presented demonstrate that transection of rat sciatic nerve induces the development of hyperalgesia. This phenomenon was detected 2 h after surgery and persisted at least for 64 days. These results are in agreement with data from Dowdall et al. (2005) showing a rapid drop in mechanical threshold (hyperalgesia) after nerve transection that remains for 56 days after surgery.

Peripheral nerve section causes the development of mechanical hyperalgesia in the adjacent innervated distal extremity (Kingery and

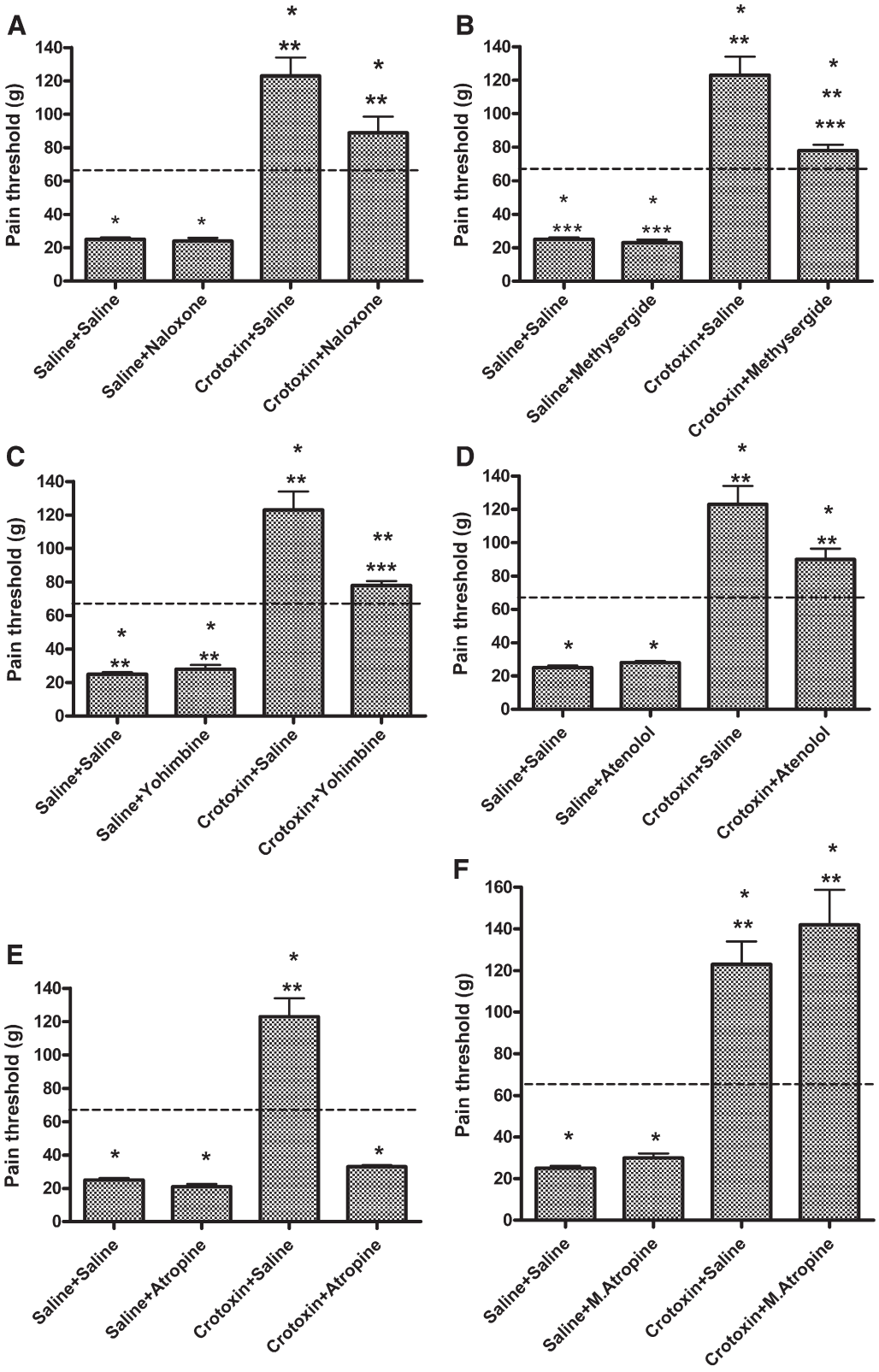

Fig. 3. Effect of distinct pharmacological treatments on antinociception induced by crotoxin. Pain threshold was estimated in the rat paw pressure test applied before (dash line) and 2 h after rat sciatic nerve transection. Crotoxin (0.01 mM) or saline was applied to the proximal and distal nerve stumps, immediately after nerve transection. Naloxone (Panel A, 1 mg/kg, i.p.) was administered 105 min after surgery. Methysergide (Panel B, 5 mg/kg, i.p.) and yohimbine (Panel C, 2 mg/kg, i.p.) were injected 30 or 15 min before surgery, respectively. Atenolol (Panel D, 1 mg/kg, i.p.) and atropine (Panel E, 10 mg/kg, i.p.) were administered 30 min before surgery. *N*-methyl atropine (Panel F, M.Atropine, 30 mg/kg, i.p.) was injected 20 min before surgery. Indomethacin (Panel G, 4 mg/kg, i.v.) and zileuton (Panel H, 100 mg/kg, p.o.) were administered 60 min before surgery. Animals treated with the same vehicle used for drug dilution, according to the same experimental protocol, were used as controls. Data represent mean values ± S.E.M. for ten rats per group. *Significantly different from mean values obtained before surgery (dash line). **Significantly different from mean values for the saline + saline group ($p < 0.05$). *** Significantly different from mean values for the crotoxin + saline group ($p < 0.05$).

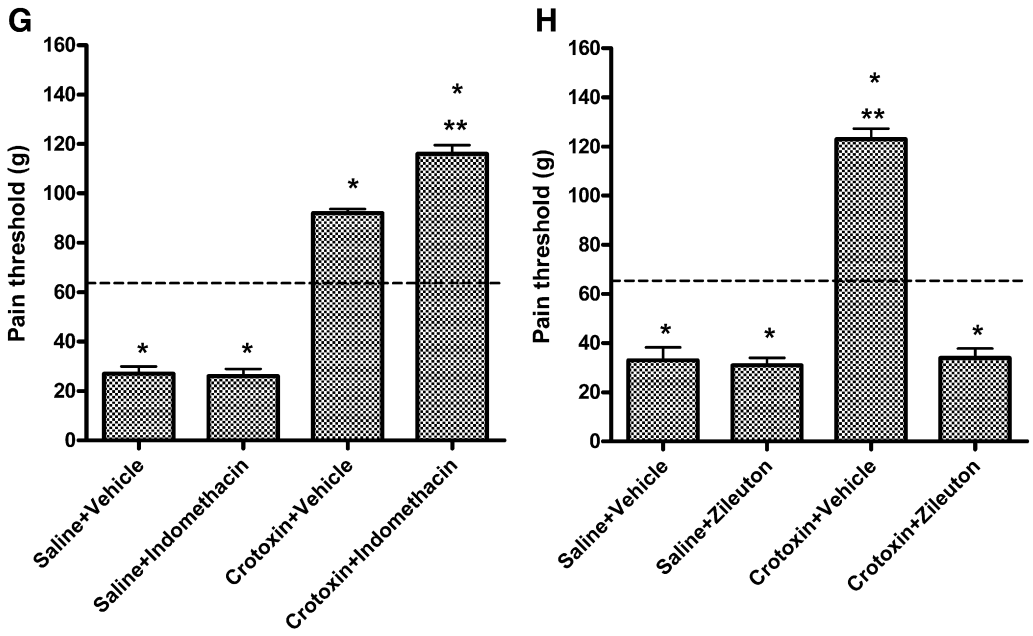

Fig. 3 (*continued*).

Vallin, 1989). Data from the literature have indicated that distinct mechanisms may contribute to the development of such mechanical hyperalgesia. These mechanisms include the development of collateral sprouts from high-threshold mechanoreceptors (Devor et al., 1979); an increase in nociceptor density following nerve sprouting (Devor et al., 1979; Markus et al., 1984); changes in dorsal horn modulation of afferent input, characterized by disinhibition of nociceptive afferents with subsequent increased excitability of central neurons (Finnerup et al., 2007; Kingery and Vallin, 1989).

Crotoxin, administered directly to nerve stumps, immediately after nerve transection, blocked hyperalgesia induced by nerve lesion. This effect was long-lasting, since it was detected 2 h after surgery and persisted up to 64 days. It is important to stress that the effect of different concentrations (3.2 nM–0.01 mM) of crotoxin were evaluated in this model of neuropathic pain, and the concentration of 0.01 mM was the lowest one that inhibited the nociceptive phenomenon induced by nerve transection (data not shown). Therefore, this concentration of CTX was chosen for the subsequent characterization of the antinociceptive activity of the toxin. To our knowledge, this is the first demonstration that crotoxin inhibits chronic (neuropathic) pain and that this effect is long-lasting. Recent data from the literature has evidenced that crotoxin induces antinociceptive effect in experimental models of acute pain, by an action in the central nervous system (Zhang et al., 2006a).

The mechanisms involved in the long-lasting antinociceptive activity of crotoxin, observed after local application of the toxin, were not presently investigated. Interestingly, Luvisetto et al. (2007) observed that the *Botulinum* neurotoxin induces a long-lasting (at least 21 days) local antinociceptive effect in an experimental model of neuropathic pain induced by chronic constriction injury of sciatic nerve. Taken together, these data suggest that such long-lasting antinociceptive effect may be peculiar to neurotoxins exerting effects at synaptic junctions.

In another set of experiments crotoxin was administered by s.c. route immediately before surgery or on day 14 after nerve transection. The results showed that the toxin is effective in inhibiting neuropathic pain when administered by s.c. route, however the antinociceptive effect was evidenced only 2 h after toxin administration, disappearing 24 h thereafter. The results indicate that crotoxin is also effective in inhibiting neuropathic pain when administered by a systemic route, even at the peak of the hyperalgesic response caused by nerve transection (14 days), however, the s.c. administration of the toxin, in contrast to the toxin applied directly to the nerve stumps, induces a short-lasting antinociceptive effect.

As pointed out earlier, a number of peripheral mechanisms are involved in neuropathic pain, including the development of neuromas (Bennett, 1993; Seltzer et al., 1991; Wang and Wang, 2003). Preliminary histopathological studies have indicated that crotoxin, applied directly to the nerve stumps of the animals, delays the development of neuromas, since in animals pre-treated with saline, histopathological alterations compatible with neuroma formation were detected on day 7 after surgery, whereas in the CTX-treated group, neuroma formation was observed 14 days after neurectomy. The delay in neuroma formation could contribute to the long-lasting antinociceptive effect induced by crotoxin and to the effectiveness of the toxin when applied directly to nerve stumps. However, at the end of the experimental period (64 days), no significant differences in the incidence of neuromas were observed between the control (80%) and CTX (77%) group (Nogueira-Neto, F. and Cury, Y., unpublished data). Ectopic excitability manifested at the neuroma, is an important feature that contributes to neuropathic pain, and upregulation of voltage-gated sodium channels is considered one of the factors responsible for this phenomenon (Woolf et al., 2004). Despite the observation that CTX delays neuroma formation, a possible effect of the toxin on sensory neurons excitability, which might also contribute to the long-lasting action of the toxin, was not investigated in this study.

Several lines of evidence have indicated that crotoxin displays immunomodulatory and anti-inflammatory activities, affecting the cellular and vascular components of the inflammatory and immune responses (Cardoso and Mota, 1997; Sampaio et al., 2003; Zambelli et al., submitted for publication). There is an increasing evidence that inflammatory and immune mechanisms play an important role in the generation of neuropathic pain and several inflammatory and immune-like glial cells have been implicated in its pathogenesis (review in Moalem and Tracey, 2006). Whether the anti-inflammatory and immunomodulatory actions of crotoxin contribute to the antinociceptive effect of CTX remains to be determined.

Peripheral nerve injury is usually followed by autotomy (Inbal et al., 1980; Kingery and Vallin, 1989; Wall et al., 1979). In our experimental conditions, autotomy behavior began in the first postoperative

week, increasing during the second and third weeks. The treatment with crotoxin, applied directly to the nerve stumps, caused a 25% decrease in the number of animals presenting autotomy (data not shown).

Crotoxin presents neurotoxic and myotoxic activities (Arruda et al., 2002; Bon, 1989; Mebs et al., 1983; Radvanyi and Bon, 1984), which could interfere with the motor response of the animals to the nociceptive stimulus. However, the antinociceptive effect of the toxin is not due to alterations in motor activity of the animals or to a toxic effect in muscle cells, since locomotor activity evaluated in the open field, and the levels of serum CK were not different from that observed in control animals. In our study, the CK levels were determined 4 and 8 h after crotoxin administration. Furtado et al. (2003) showed, that, for the crude crotalid venom injected into the thigh muscle of the right hind leg of mice, the peak of CK release is detected 4 h after venom injection, decreasing thereafter. Therefore, in our study, the lack of alterations in CK levels at 4 and 8 h after CTX administration is indicative that the toxin, in the concentration used, did not induce myotoxic effect. It is interesting to note that 8 h after crotoxin or saline treatments there was a decrease in the levels of CK as compared to the values obtained 4 h after treatments. The higher CK levels observed in the earlier periods of time may result from muscle injury caused by the surgical procedure, since the CK levels were determined in blood collected from animals submitted to nerve transection and treated with crotoxin or saline.

In this study, we also analyzed the possible mechanisms involved in the antinociceptive effect of crotoxin. Based on the results showing that the local treatment with crotoxin induced a more long-lasting antinociceptive effect than the systemic (s.c.) treatment, the studies on the characterization of the mediators involved in the effect of the toxin were performed using the former treatment.

Previous studies of our group have shown that *C. d. terrificus* venom induces antinociceptive effect in experimental models of acute and chronic pain, mediated by activation of central and peripheral opioid receptors (Brigatte et al., 2001; Giorgi et al., 1993; Picolo and Cury, 2004, unpublished data). However, opioid receptors are not involved in the antinociceptive effect of crotoxin in this model of neuropathic pain, since naloxone, a non-specific opioid receptor antagonist, did not interfere with this effect. These data are in accordance with Zhang et al. (2006a,b) and Zhu et al. (2008) that did not observe the involvement of opioid receptors in the analgesic action of crotoxin in experimental models of acute pain.

Experimental data have indicated that neurotoxins isolated from Elapid venoms induce antinociception when evaluated in phasic and tonic rodent pain models. This antinociceptive effect is attributed to activation of muscarinic receptors, by an action of the toxins on central cholinergic neurons (Chen and Liu, 2006; Chen and Huang, 1992; Chen and Robinson, 1990; Zhang et al., 2006a). Based on these data, we have now investigated the involvement of muscarinic receptors in the effect of crotoxin. Atropine blocked whereas methyl-atropine did not modify the antinociceptive effect of the toxin, indicating that central muscarinic receptors mediates the effect of the toxin in this model of neuropathic pain. The mechanisms involved in central cholinergic system activation by the toxin have not been determined yet. It is important to stress that Zhang et al. (2006a,b) did not detect the involvement of cholinergic system in the antinociceptive action of crotoxin in experimental models of acute pain. The reason for this discrepancy is not apparent. However, differences in the experimental model used for pain evaluation and/or in the doses and route of administration of crotoxin could contribute to these distinct results. An increase in central muscarinic acetylcholine receptors (mAChRs) in neuropathic pain states has been postulated (Chen and Luo, 2003), which might contribute to the detection of a cholinergic activity for crotoxin. Experimental data have shown that muscarinic agonists are effective in the control of neuropathic pain (Clayton et al., 2007; Sullivan et al., 2007). In addition, these studies have indicated that some muscarinic receptor agonists that were not active in models of acute pain (Duttaroy et al., 2002; Starling and Wei, 1994), can induce antinociceptive effect in neuropathic pain models (Sullivan et al., 2007).

The antinociceptive effect observed following activation of mAChRs are thought to be mediated, in part, through an increase in the activity of the central descending inhibitory pathways and release of inhibitory transmitters and also to a decrease in the release of excitatory transmitters (review in Millan, 2002). The neurotransmitter 5-hydroxytryptamine (5-HT) is an important participant in the brain and spinal inhibition of nociceptive transmission (Baba et al., 2001; Honda et al., 2003; Kawamata et al., 2003; Ma and Han, 1992; Zhang et al., 1987) and activation of central serotonergic system has been implicated in the inhibitory effect of crotoxin on the pain-evoked discharge of neurons in rats (Zhu et al., 2008). Results herein presented indicate that 5-HT receptors are at least partially involved in the antinociceptive effect of crotoxin in neuropathic pain, since methysergide reduced the anti-hyperalgesic effect of the toxin.

The noradrenergic neurons of the descending pain inhibitory systems inhibit pain transmission from primary afferent neurons and the descending noradrenergic inhibitory system may be recruited after nerve injury, limiting hyperalgesia observed in such conditions (Jasmin et al., 2003). In our study, yohimbine, but not atenolol, partially inhibited antinociception induced by crotoxin, suggesting the involvement of α-adrenoceptors in this effect.

Taken together, our results indicate that the central cholinergic, serotonergic, and noradrenergic systems are involved in the antinociceptive effect of crotoxin in neuropathic pain. An interplay between these systems has been proposed (Millan, 2002), and might be considered for the antinociceptive action of crotoxin.

The crotoxin molecule is composed of an acidic non-toxic and non-enzymatic polypeptide named crotapotin and of a weakly toxic phospholipase A_2 (PLA_2) (Bon, 1989; Slotta, 1938). Recent study of our group demonstrated that the PLA_2 subunit is responsible for the inhibitory action of crotoxin on inflammatory cells (Sampaio et al., 2005; Zambelli et al., submitted for publication). Type II secreted phospholipase A_2, which hydrolyses the sn-2 ester bound of membrane phospholipids, release arachidonate. Free arachidonic acid can then be converted into eicosanoids (Needleman et al., 1986; Smith, 1989). Prostanoids and leukotrienes have a potent pro-inflammatory activity (Ford-Hutchinson and Chan, 1985). However, increasing evidence indicates that arachidonate-derived mediators, such as prostaglandins and lipoxins, also exert anti-inflammatory function, being involved in inflammatory resolution (Gilroy et al., 2003; Levy et al., 2001). Sampaio et al. (2006) and Zambelli et al. (2008) showed that the inhibitory action of crotoxin and its PLA_2 subunit on macrophages and lymphocytes is mediated by eicosanoids derived from the lipoxygenase pathway. Therefore, in order to evaluate the involvement of eicosanoids in the antinociceptive effect of crotoxin, the rats were pre-treated with indomethacin or zileuton. The results demonstrated that eicosanoids derived from the lipoxygenase pathway mediates the effect of the toxin and indicate that a phospholipase A_2 activity is important for crotoxin-induced antinociception. We did not determined the type of eicosanoid responsible for the antinociceptive effect of crotoxin in this model of neuropathic pain, but Sampaio et al. (2006) demonstrated that macrophages incubated with the toxin secrete high levels of lipoxin A_4. In addition, preliminary results of our group indicate that this mediator is responsible for the inhibitory effects of crotoxin on inflammatory cells (unpublished data). The anti-inflammatory activity of lipoxin A_4 is well documented (Serhan, 2005). More recently, Svensson et al. (2007) demonstrated that lipoxins attenuate nociception not only at the site of inflammation but also by interfering with pain processing in the spinal cord. In the periphery, lipoxins could interfere with nociception by regulating the communication between the immune and sensory nervous systems. Therefore, in addition to a possible central mechanism involved in

the antinociceptive effect of CTX, a peripheral action of toxin, via the release of lipidic (lipoxin) mediators that interfere with the inflammatory response evoked by nerve transection, might be also considered.

It is important to mention that, based on the results showing the involvement of lipoxygenase-derived mediators and muscarinic receptors in the antinociceptive effect of crotoxin applied directly to nerve stumps, we also investigated the possible role of these mediators and receptors in the effect of crotoxin administered by s. c. route on day 14 after nerve transection. Pre-treatment with zileuton or atropine also blocked the antinociceptive effect of CTX, indicating that, independent of the route of administration, the toxin promotes antinociception by the release of lipidic mediators and activation of muscarinic receptors.

In conclusion, the results herein described indicate that crotoxin induces a long-lasting antinociceptive effect in neuropathic pain induced by transection of rat sciatic nerve. The antinociceptive effect of the toxin is mediated by activation of central muscarinic receptors and partially by α-adrenoceptors and serotonergic receptors. 5-lipoxygenase-derived lipidic mediators are involved in the modulation of this effect.

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