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δ-Opioid receptors and nitric oxide mediate the analgesic effect of *Crotalus durissus terrificus* snake venom

Gisele Picolo, Renata Giorgi, Yara Cury *

Laboratory of Pathophysiology, Butantan Institute, Av. Vital Brazil, 1500, 05503-900, São Paulo, SP, Brazil

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Abstract

The antinociceptive effect of *Crotalus durissus terrificus* venom was investigated in a model of inflammatory hyperalgesia induced by carrageenin. The rat paw pressure test was applied before and 3 h after the intraplantar (i.pl.) injection of carrageenin. The venom administered per os before and 1 or 2 h after carrageenin blocked hyperalgesia. When carrageenin was injected in both hind paws and naloxone into one hind paw, antinociception was abolished only in the paw injected with naloxone. D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP) and nor-binaltorphimine, antagonists of μ- and κ-opioid receptors, respectively, did not alter the effect of the venom. *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI 174,864), an antagonist of δ-opioid receptors, antagonised this effect. Prolonged administration of the venom did not induce tolerance to this antinociceptive effect. N^G-methyl-L-arginine (L-NMMA) and methylene blue, inhibitors of nitric oxide synthase and soluble guanylate cyclase, respectively, injected i.pl., antagonised antinociception. These data indicate that both δ-opioid receptors and nitric oxide participate in the mediation of the peripheral antinociceptive effect of *C. durissus terrificus* venom.

Keywords: *Crotalus durissus terrificus* venom; Analgesia; Opioid receptor; Nitric oxide (NO)

1. Introduction

Neuropharmacological studies have demonstrated the analgesic activity of snake venoms or substances isolated from them (Chen and Robinson, 1990; Dutta and Chaudhuri, 1991; Xiong et al., 1992a,b; Pu et al., 1995). *Crotalus durissus terrificus* venom causes antinociception in mice. There is evidence that this effect is mainly due to a supraspinally integrated response since it is evident in the hot-plate but not in the tail-flick test (Giorgi et al., 1993; Picolo et al., 1998). Opioid receptors are involved in this antinociceptive effect (Giorgi et al., 1993) and, in addition to this central effect, the venom causes antinociception in the acetic acid-induced writhing test, a model of inflammatory hyperalgesia (Giorgi et al., 1993).

Hyperalgesia, which results from the sensitization of nociceptors to mechanical, thermal or chemical stimulation, is a fundamental event in the manifestation of inflammatory pain (Ferreira, 1980). Analgesic drugs with peripheral activity that interfere with inflammatory hyperalgesia may act by (a) preventing the sensitization of pain receptors (Ferreira, 1972; Ferreira et al., 1973), e.g., nonsteroidal anti-inflammatory drugs effect, or (b) directly abolishing the ongoing sensitization of pain receptors (Ferreira, 1990; Ferreira and Nakamura, 1979a,b; Ferreira et al., 1984; Lorenzetti and Ferreira, 1982, 1985), e.g., dipyrone and peripheral opioids effects. The mechanism by which opioids induce peripheral analgesia involves the participation of μ-, κ- and δ-opioid receptors (Ferreira and Nakamura, 1979a,b; Stein et al., 1988, 1989, 1990; Kayser et al., 1991). On the other hand, the L-arginine/nitric oxide/cyclic GMP pathway also participates in the antinociceptive activity of some drugs with opioid activity. This conclusion is based on the fact that inhibitors of nitric oxide generation from L-arginine or from guanylate cyclase activation abolish the analgesic effect of these drugs (Ferreira et al., 1991a,b, 1995; Granados-Soto et al., 1997; Nozaki-Taguchi and Yamamoto, 1998).

Considering that *C. durissus terrificus* venom induces antinociception in the acetic-acid-induced writhing test, the present study was designed to investigate (a) the possible antinociceptive effect of the venom on inflammatory hy-

* Corresponding author. Tel.: +55-11-813-7222; fax: +55-11-815-1505.

E-mail address: yarac@attglobal.net (Y. Cury).

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peralgesia induced by carrageenin, (b) the participation of peripheral opioid receptors in this effect, (c) the type of opioid receptor involved and (d) the participation of L-arginine/nitric oxide/cGMP pathway in this phenomenon.

2. Materials and methods

2.1. Venom

Lyophilised venom of *C. durissus terrificus* was obtained from the Laboratory of Herpetology, Butantan Institute, São Paulo, Brazil, and stored at −20°C. The venom was dissolved in sterile physiological saline (0.85% w/v NaCl solution) at the moment of use and administered by the oral (p.o.) route. The dose and time of venom administration are indicated in Results.

2.2. Animals

Male Wistar rats, weighing between 170 and 190 g, were used throughout this study.

2.3. Evaluation of hyperalgesia

Hyperalgesia was produced by the intraplantar (i.pl.) administration of 0.1 ml of sterile saline solution containing carrageenin (Marine Colloids; 200 μg/paw) into one of the hind paws. The pain threshold was measured before carrageenin injection and 3 h thereafter, using an Ugo Basile® pressure apparatus, essentially as described by Randall and Selitto (1957). Briefly, a force with increasing magnitude (16 g/s) was applied to the paw. When the animals reacted by withdrawing the paw, the force (in grams) needed to induce this response represented the pain threshold.

2.4. Evaluation of oedema

Oedema was induced by the injection by the i.pl. route of 0.1 ml of sterile saline solution containing carrageenin (200 μg/paw) into one of the hind paws. The contralateral paw received the same volume of sterile saline solution (control paw). The volume increase (oedema) of paws up to the tibio-tarsal articulation was measured plethysmographically before the injection of the irritant and at selected time intervals thereafter, according to the method of Van Arman et al. (1965). Results were calculated as the difference between the values obtained in both paws as percent increase in paw volume.

2.5. Evaluation of general motor activity

Possible changes in activity provoked by the venom were investigated in an open-field arena (Broadhurst, 1960). Hand-operated counters were used to score ambulation (locomotion) frequency (number of floor units entered) and rearing frequency (number of times the animal stood on hind legs). Each animal was individually placed in the centre of the open field and behavioural 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 odour clues left by previous subjects.

2.6. Pharmacological treatments

2.6.1. Opioid antagonists

In order to establish the involvement of peripheral opioid receptors on the antinociceptive effect of the venom, carrageenin was injected simultaneously into both hind paws of the rats and naloxone (Rhodia do Brasil, 1 μg/paw), a nonspecific opioid receptor antagonist, into one hind paw, concomitant with carrageenin. To characterise the type of opioid receptor involved in the antinociceptive effect of the venom, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP, RBI, USA, 20 μg/paw), norbinaltorphimine (RBI, 50 μg/paw) or *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI 164,864, RBI, 10 μg/paw), μ-, κ- and δ-opioid receptor antagonists, respectively, were injected by the i.pl. route simultaneously with carrageenin.

2.6.2. Methylene blue and L-NMMA

To investigate the participation of the L-arginine/nitric oxide/cGMP pathway on the antinociceptive effect of the venom, N^G-methyl-L-arginine (L-NMMA, Sigma, USA, 50 μg/paw), an inhibitor of nitric oxide (NO) synthase, or methylene blue (Sigma, 500 μg/paw), an inhibitor of the activation of guanylate cyclase, was injected, i.pl., 60 min before carrageenin.

2.7. Presentation of data and analysis

Results are presented as the mean ± S.E.M. Statistical evaluation of data was carried out by analysis of variance and sequential differences among means according to Tukey contrast analysis at $P < 0.05$ (Sokal and Rohlf, 1981).

3. Results

3.1. Effect of oral administration of C. durissus terrificus venom on hyperalgesia and oedema induced by i.pl. injection of carrageenin

The i.pl. injection of carrageenin caused a significant decrease in pain threshold. The venom (25–800 μg/kg) administered immediately before the i.pl. injection of the

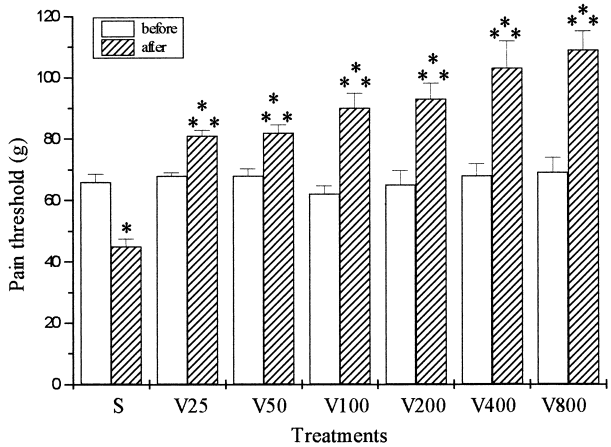

Fig. 1. Effect of *C. durissus terrificus* venom on the hyperalgesia induced by carrageenin. Pain threshold was estimated in the rat paw pressure test applied before and 3 h after i.pl. injection of carrageenin (200 μg/paw). The venom (25–800 μg/kg), V, or saline (control group), S, was administered p.o. immediately before the injection of carrageenin. Data represent mean values ± S.E.M. for eight rats per group. * Significantly different from mean values before carrageenin injection. ** Significantly different from mean values of control group ($P < 0.05$).

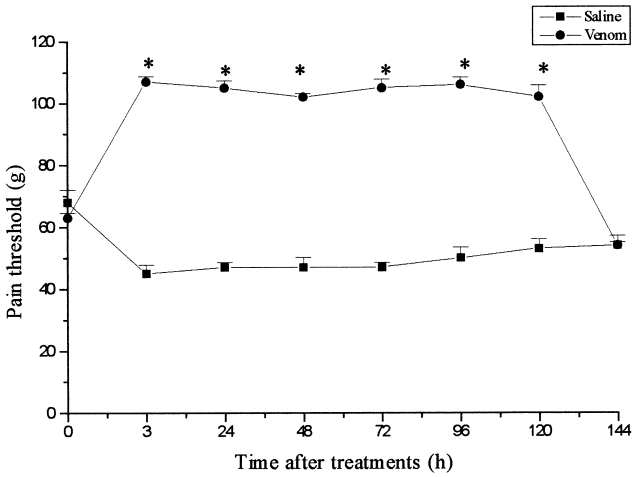

Fig. 3. Duration of antinociception induced by *C. durissus terrificus* venom. Pain threshold was estimated in the rat paw pressure test applied before (time 0) and at different times after oral administration of venom (200 μg/kg) or saline (control group). Carrageenin (200 μg/kg) was injected by the i.pl. route, 3 h before each pain threshold evaluation, except at time 0. Data represent mean values ± S.E.M. for eight rats per group. * Significantly different from mean values of control group ($P < 0.05$).

irritant induced antinociception (Fig. 1). Similar results were observed when the venom (200 μg/kg) was administered 1 or 2 h after carrageenin (Fig. 2).

As expected, carrageenin also induced an oedematogenic response. The peak of oedema was observed 3 h after injection of the irritant (54% increase in paw volume) and thereafter the oedema gradually decreased. The administration of the venom (200–800 μg/kg) immediately before carrageenin injection did not alter the intensity or the time course of this oedematogenic response (data not shown).

3.2. Duration of antinociception following oral administration of the venom

Inflammatory hyperalgesia was induced at different times after a single administration of the venom (200

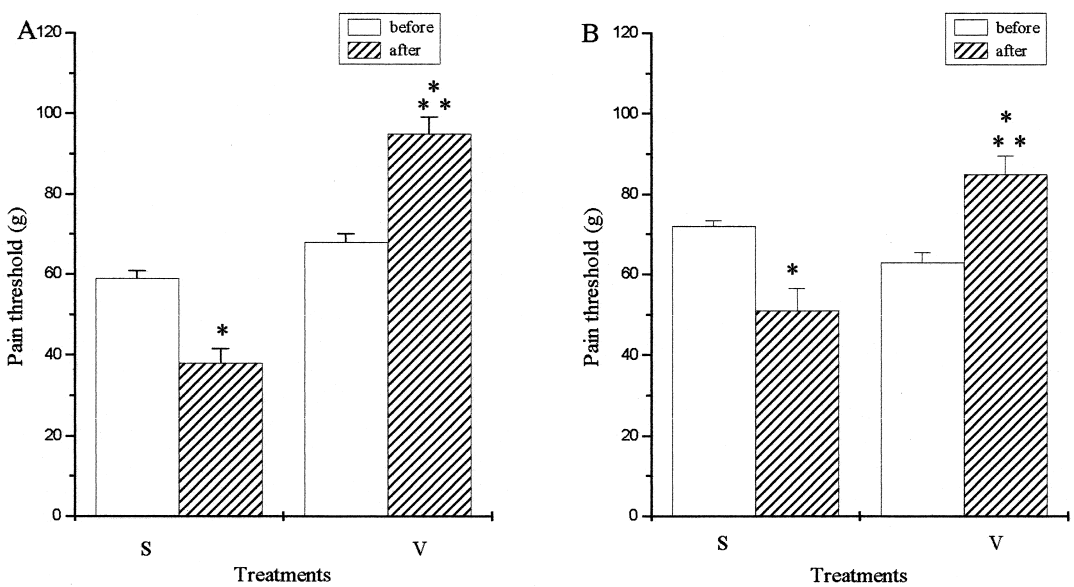

Fig. 2. Antinociceptive effect of *C. durissus terrificus* venom administered 1 or 2 h after carrageenin. Pain threshold was estimated in the rat paw pressure test applied before and 3 h after i.pl. injection of carrageenin (200 μg/paw). The venom (200 μg/kg), V, or saline (control group), S, was administered p.o. 1 h (A) or 2 h (B) after carrageenin. Data represent mean values ± S.E.M. for six rats per group. * Significantly different from mean values before carrageenin injection. ** Significantly different from mean values of control group ($P < 0.05$).

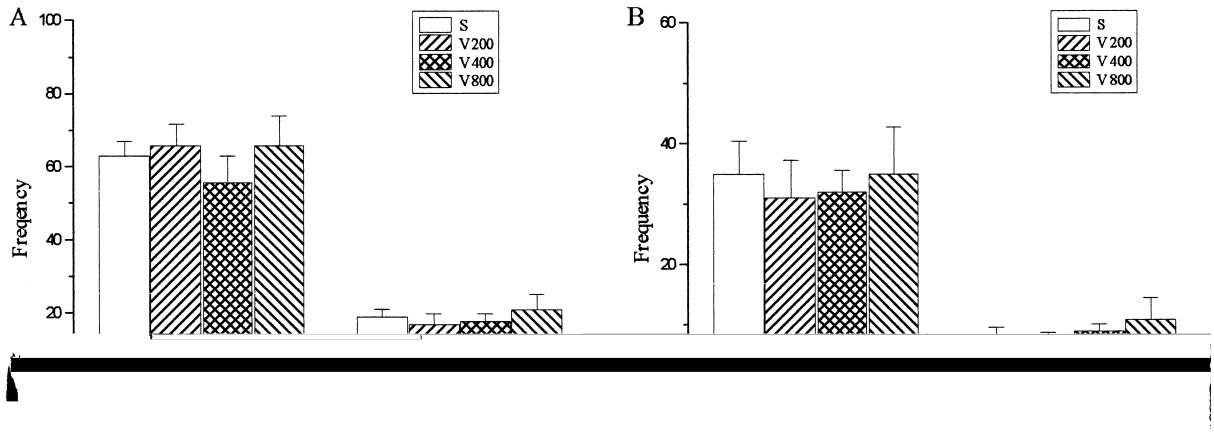

Fig. 4. Effect of *C. durissus terrificus* venom on open-field behaviour. Locomotion (LO) and rearing (RE) frequencies were determined 1 h (A) or 3 h (B) after oral administration of venom (200–800 μg/kg), V, or saline, S, (control group). Data represent mean values ± S.E.M. for eight rats per group.

μg/kg) by the oral route. Different groups of rats were used for each measurement of hyperalgesia. The antinociceptive effect was detected up to 120 h after venom administration (Fig. 3).

3.3. Effect of oral administration of C. durissus terrificus venom on spontaneous motor activity

As motor activity inhibition could interfere with the behaviour of the rats in the nociceptive test, the possible influence of the venom on spontaneous motor activity was investigated. As shown in Fig. 4, oral administration of the venom (200–800 μg/kg) had no effect on locomotion and rearing, as compared to those of control animals.

3.4. Effect of opioid receptors antagonists on the antinociceptive action of the venom

When carrageenin was injected in both hind paws and naloxone into one hind paw, the antinociceptive activity of

Fig. 5. Effect of naloxone on the antinociception induced by *C. durissus terrificus* venom. Pain threshold was estimated in the rat paw pressure test applied before and 3 h after intraplantar injection of carrageenin (200 μg/paw) in both hind paws (A and B). The venom (200 μg/kg), V, or saline (control group), S, was administered p.o. immediately before the injection of carrageenin. Naloxone (1 μg/paw), N, was injected into one hind paw, concomitantly with carrageenin (A). Data represent mean values ± S.E.M. for six rats per group. * Significantly different from mean values before carrageenin injection. ** Significantly different from mean values for the S + C + S group ($P < 0.05$).

the venom (200 μg/kg) was abolished in the naloxone injected paw (Fig. 5).

Fig. 6 shows the effect of i.pl. injection of CTOP, nor-binaltorphimine and ICI 174,864 on the antinociceptive action of the venom. The administration of μ- (Fig. 6A) and κ- (Fig. 6B) opioid receptor antagonists did not alter the action of the venom. The δ-opioid receptor antagonist, however, abolished the antinociception induced by the venom (Fig. 6C). To confirm the participation of peripheral δ-opioid receptors on venom-induced antinociception, carrageenin was injected in both hind paws and the δ-opioid receptor antagonist into one of the hind paws. Venom antinociceptive activity was abolished only in the paw injected with the antagonist (data not shown).

The i.pl. injection of opioid antagonists did not affect carrageenin hyperalgesia (Figs. 5 and 6).

3.5. Effect of prolonged treatment with C. durissus terrificus venom on hyperalgesia induced by i.pl. injection of carrageenin

Animals were daily treated with the venom (200 μg/kg) for 14 days. Carrageenin was injected on days 1, 7 and 14, immediately after venom administration. Hyperalgesia

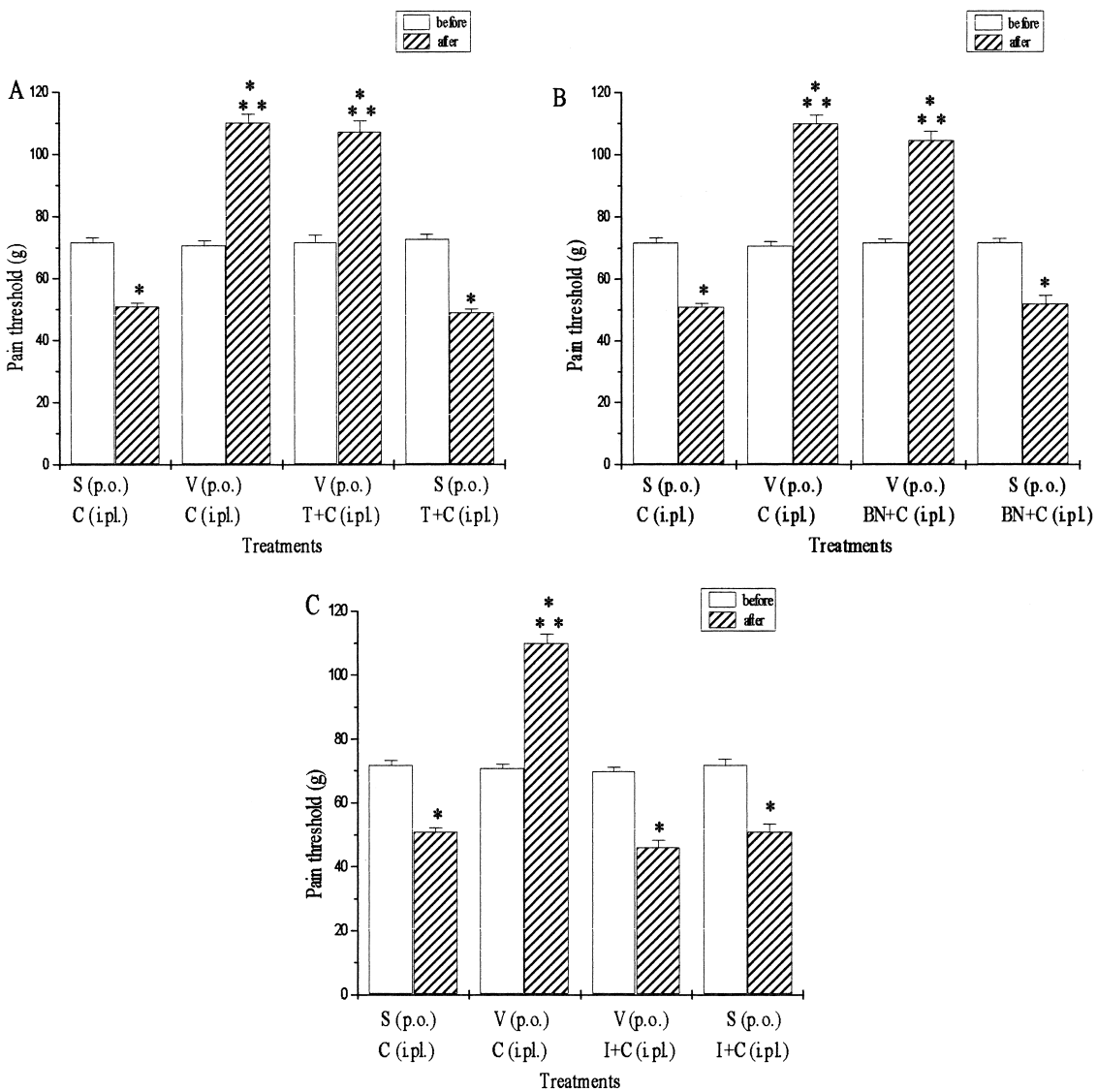

Fig. 6. Effect of μ-, κ- or δ-opioid receptor antagonists on the antinociception induced by *C. durissus terrificus* venom. Pain threshold was estimated in the rat paw pressure test applied before and 3 h after intraplantar injection of carrageenin (200 μg/paw), C. The venom (200 μg/kg), V, or saline (control group), S, was administered p.o. immediately before the injection of carrageenin. The antagonists were injected by the intraplantar route, concomitantly with carrageenin. (A) CTOP (20 μg/paw), T; (B) nor-binaltorphimine (50 μg/paw), BN; (C) ICI 174,864 (10 μg/paw), I. Data represent mean values ± S.E.M. for six rats per group. * Significantly different from mean values before carrageenin injection. ** Significantly different from mean values of S + C group ($P < 0.05$).

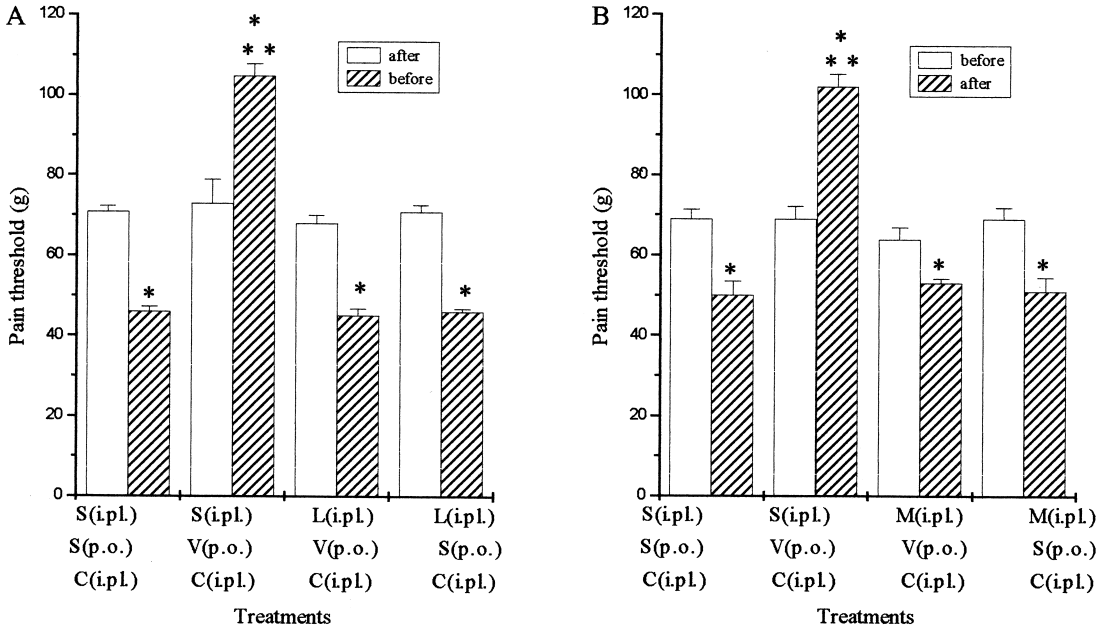

Fig. 7. Effect of L-NMMA or methylene blue on the antinociception induced by *C. durissus terrificus* venom. Pain threshold was estimated by the rat paw pressure test applied before and 3 h after intraplantar injection of carrageenin (200 μg/paw), C. The venom (200 μg/kg), V, or saline (control group), S, was administered p.o. immediately before the injection of carrageenin. (A) L-NMMA (50 μg/paw), L, or (B) methylene blue (500 μg/paw), M, was injected by i.pl. route 1 h before carrageenin. Data represent mean values ± S.E.M. for six rats per group. *Significantly different from mean values before carrageenin injection. **Significantly different from mean values for the S + S + C group ($P < 0.05$).

was measured 3 h after carrageenin injection. The antinociceptive effect of the venom was not modified by this prolonged treatment The pain threshold observed in venom-treated animals was: day 1, 106 ± 1.47 g; day 7, 105 ± 0.94 g; and day 14, 108 ± 2.1 g and in control (saline) rats: day 1, 43 ± 2.30 g; day 7, 45 ± 1.89 g; and day 14, 44 ± 3.69 g.

3.6. L-NMMA and methylene blue inhibit C. durissus terrificus venom-induced antinociception

L-NMMA injected i.pl. did not interfere with the hyperalgesia induced by carrageenin but abolished the antinociceptive effect of the venom (200 μg/kg) (Fig. 7A). A similar response was observed in animals pretreated with methylene blue (Fig. 7B).

4. Discussion

Data presented herein demonstrate that *C. durissus terrificus* snake venom produces antinociception of the inflammatory hyperalgesia induced in the rat paw by carrageenin. This effect was long lasting since it persisted for 120 h after venom administration. This finding is in agreement with previous observation showing that in the hot-plate test venom-induced antinociception was also detected after this period of time (Giorgi et al., 1998). Furthermore, the inflammatory hyperalgesia was also inhibited when the venom was administered 1 or 2 h after the i.pl. injection of carrageenin. This result suggests that the venom is able to abolish the ongoing sensitization of pain receptors. This action is characteristic of analgesics such as peripheral opioids and dypirone, which directly antagonise ongoing hyperalgesia (Ferreira and Nakamura, 1979a,b; Ferreira et al., 1984; Lorenzetti and Ferreira, 1982, 1985).

C. durissus terrificus snake venom has toxic substances that interfere with neuromuscular transmission and induce myotoxic effects (Vital-Brazil, 1980). These actions of the venom could influence the performance of the animals in the nociceptive test used. Nevertheless, the antinociceptive effect of the venom is not due to alterations in the general activity of the animals since the frequency of locomotion and rearing, evaluated in the open field, was not affected by the venom. Further, it is important to point out that, in the present study, the venom was always administered by the oral route. Finally, it has been known for a long time that gastric or rectal administration of venoms of *Crotalineas* does not alter the physiological state of dogs and rabbits and does not induce antibody production in horses (Brazil and Pestana, 1909).

The antinociceptive effect of the venom on carrageenin-induced hyperalgesia could be due to anti-inflammatory (anti-oedematogenic) activity. Our data disprove this hypothesis because the venom did not interfere with the oedematogenic response induced by the phlogistic agent. However, it has been demonstrated that *C. durissus terrificus* venom (or fractions) interferes with the cellular and vascular components of the inflammatory response. Leukocyte migration in response to an inflammatory stim-

uli and macrophage activation are inhibited by this venom (Sousa e Silva et al., 1996). Moreover, crotapotin, a nontoxic nonenzymatic chaperon protein which is normally complexed to a phospholipase A_2 in the venom blocked the carrageenin-induced paw oedema in rats (Landucci et al., 1995). The utilisation of crude venom and the oral route for venom administration may have contributed to the lack of an anti-oedematogenic effect observed in our experiments.

The mechanism of action of the venom in the model of inflammatory hyperalgesia seems to involve a peripheral opioid effect since its antinociceptive effect was antagonised by i.pl. injection of naloxone, a nonspecific opioid antagonist. Opioids can induce peripheral analgesia through an interaction with peripheral μ-, κ- and δ-opioid receptors present on primary afferent neurones (Ferreira and Nakamura, 1979a,b; Stein et al., 1988, 1989, 1990; Kayser et al., 1991; Nozaki-Taguchi and Yamamoto, 1998). In the present work, when specific receptor opioid antagonists were used, instead of naloxone, only ICI 164,864 was able to interfere with the action of the venom. This result indicates that δ-opioid receptors are involved in the peripheral action of the venom. Although the mechanism of opioid activity of the venom was not fully investigated, opioid-like substances secreted by adrenal glands are not involved in the effect of the venom since adrenalectomy did not interfere with its antinociceptive activity (data not shown).

The development of tolerance to the analgesic effect of opioids can be observed after repeated administration (Koob and Bloom, 1988). Our results demonstrated that animals treated for 14 consecutive days with the venom did not show this phenomenon. These data do not agree with a previous observation in the hot-plate test in which tolerance was observed after prolonged treatment with the venom (Hoffmann et al., 1994; Brigatte et al., 1998). Differences between both nociceptive tests used may explain these distinct results. The hot-plate paw-licking test has been used for studying the neuronal mechanism of opioid analgesia and other central analgesic-acting substances (Gebhart et al., 1971; Jacob and Ramabadran, 1978). Models of inflammatory hyperalgesia are usually used for the screening of analgesics with peripheral activity. Recently, it was demonstrated for morphine that peripheral analgesia is resistant to the development of tolerance even when its central effect is reduced (Tokuyama et al., 1998).

Morphine and other opioid-like substances inhibit hyperalgesia by stimulating the L-arginine/nitric oxide/cyclic GMP pathway (Ferreira et al., 1991a,b, 1995; Granados-Soto et al., 1997; Nozaki-Taguchi and Yamamoto, 1998). Accordingly with this evidence, we have shown that L-NMMA, an NO synthase inhibitor, and methylene blue, an inhibitor of the activation of guanylate cyclase, administered by the i.pl. route, abolished the antinociceptive activity of the venom. These results are corroborated by recent data showing that the analgesic effect of peripherally applied [D-Pen]enkephalin (DPDPE), a δ-opioid receptor agonist, during inflammation induced by formalin injection in the rat is, at least partially, mediated by the NO-cGMP pathway (Nozaki-Taguchi and Yamamoto, 1998). Finally, further corroborating our data, Pu et al. (1995) showed the participation of NO as a mediator of analgesia induced by hannalgesin, a substance isolated from the venom of king cobra (*Ophiophagus hannah*).

Despite the data here presented about the antinociceptive effect of the venom and its mechanism of action, the rat paw pressure test used to evaluate inflammatory hyperalgesia provides information about the pain threshold. The use of other specific methods will allow the evaluation of the effect of the venom on the increased magnitude of suprathreshold responses during hyperalgesia.

In conclusion, peripheral δ-opioid receptors and the stimulation of NO-cGMP pathway are involved in the *C. durissus terrificus* snake-venom-induced antinociception of inflammatory hyperalgesia.

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