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Activation of peripheral ATP-sensitive K^+ channels mediates the antinociceptive effect of *Crotalus durissus terrificus* snake venom

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Abstract

The role of peripheral potassium channels on the antinociceptive effect of *Crotalus durissus terrificus* venom, a mixed δ- and κ-opioid receptor agonist, was investigated in hyperalgesia induced by carrageenin or prostaglandin E_2. Rat paw pressure test was applied before and 3 h after the intraplantar (i.pl.) injection of the nociceptive stimuli. Oral administration of venom 2 h after carrageenin or prostaglandin E_2 induces antinociception. Local pretreatment with 4-aminopyridine and tetraethylammonium (blockers of voltage-dependent K^+ channel) or charybdotoxin and apamin (inhibitors of large- and small-conductance Ca^{2+}-activated K^+ channel, respectively) did not modify venom effect. On the other hand, glybenclamide, an inhibitor of ATP-sensitive K^+ channel abolished antinociception induced by the venom. Glybenclamide also inhibited the antinociceptive effect of [D-Pen^{2,5}] enkephalin (DPDPE), a *delta* opioid receptor agonist, but did not modify the effect of (+)-*trans*-(1*R*,2*R*)-U-50488 (U50488), a *kappa* opioid receptor agonist. Diazoxide and pinacidil, two ATP-sensitive K^+ channel openers, injected by intraplantar route, induced a long-lasting increment of pain threshold of the animals and produced antinociception in both models of hyperalgesia. These results suggest that the antinociceptive effect of crotalid venom is mediated by activation of ATP-sensitive K^+ channels at peripheral afferent neurons.

Keywords: *Crotalus durissus terrificus* venom; Analgesia; ATP-sensitive K^+ channel; δ-Opioid receptor agonist, peripheral; κ-Opioid receptor agonist, peripheral

1. Introduction

Crotalus durissus terrificus snake venom induces antinociception mediated by opioid receptors (Giorgi et al., 1993). The participation of these receptors depends on the type and duration of the nociceptive stimulus, since in the hot plate test, this effect involves the activation of κ-opioid receptors (Brigatte et al., 2001), whereas in the carrageenin-induced hyperalgesia, antinociception involves the activity of peripheral δ-opioid receptors (Picolo et al., 2000). Furthermore, we have recently demonstrated that in the prostaglandin E_2-induced hyperalgesia, both κ- and δ-opioid receptors are involved in the antinociceptive effect of this venom (Picolo and Cury, unpublished data). In addition to the opioid mechanisms, activation of the L-arginine-nitric oxide (NO)-cGMP pathway contributes to venom-induced antinociception (Picolo et al., 2000).

Several lines of evidence have suggested that opening of ATP-sensitive K^+ channels mediates the analgesic action of NO and cGMP. Soares et al. (2000) demonstrated that the peripheral antinociceptive effect of the nitric oxide donor, sodium nitroprusside involves the opening of ATP-sensitive K^+ channels. Furthermore, Soares and Duarte (2001) showed that dibutyrylguanosine 3:5′-cyclic monophosphate (DbcGMP), a membrane permeable analogue of cyclic GMP, induces peripheral antinociception through specific opening of ATP-sensitive K^+ channels. Recent investigations have shown that nociceptor desensitization induced by various drugs capable of stimulating the L-arginine-NO-cGMP pathway, such as morphine, ketorolac or diclofenac, is mediated by activation of ATP-sensitive K^+ channels (Rodrigues and Duarte, 2000; Lázaro-Ibáñez et al., 2001; Ortiz et al., 2002). Taken together, these data suggest a link between the activation of the NO-cGMP pathway and the opening of ATP-sensitive K^+ channels.

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Since we have previously reported that *C. durissus terrificus* snake venom activates the NO-cGMP pathway in the periphery and to further characterize the cascade of molecular events involved in the antinociceptive effect of the crotalid venom, the present work was undertaken to determine the role of peripheral K^+ channels on this effect. For this purpose, we tested the action of intraplantar administration of specific and nonspecific blockers of K^+ channels on the antinociceptive effect of venom. Hyperalgesia induced in the rat paw by prostaglandin E_2 or carrageenin was used for nociceptive evaluation, since distinct opioid receptors are involved in venom-induced antinociception in both models of hyperalgesia.

2. Materials and methods

2.1. Venom

Lyophilised venom of *C. durissus terrificus* was obtained from the Laboratório de Herpetologia, Instituto Butantan, São Paulo, Brazil, and stored at −20 °C. Venom was dissolved in sterile physiological saline (0.85% NaCl solution) at the moment of use and administered by oral (p.o.) route (200 μg/kg) 2 h after intraplantar injection of prostaglandin E_2 or carrageenin.

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 (200 μg/paw) or prostaglandin E_2 (100 ng/paw) into one of the hind paws. Pain threshold was measured before and 3 h after prostaglandin E_2 or carrageenin injection, 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 g) needed to induce this response represented the pain threshold. To reduce stress, the rats were habituated to the apparatus 1 day before the experiments.

2.4. Pharmacological treatments

In order to evaluate the role of K^+ channels in the antinociceptive effect induced by venom, glybenclamide (80 μg/paw), a blocker of ATP-sensitive K^+ channel (Edwards and Weston, 1993), charybdotoxin (2 μg/paw) or apamin (10 μg/paw), selective blockers of large- and small-conductance Ca^{2+}-activated K^+ channels, respectively (Romey et al., 1984; Miller et al., 1985; Stretton et al., 1992), and 4-aminopyridine (100 μg/paw) or tetraethylammonium (640 μg/paw), blockers of voltage-dependent K^+ channel (Cook and Quast, 1990) were injected immediately after the administration of venom. The doses of blockers used in the present work are effective in inhibiting the in vivo prejunctional effect of morphine on peripheral sensory nerves (Yonehara and Takiuchi, 1997) and the antinocicep-

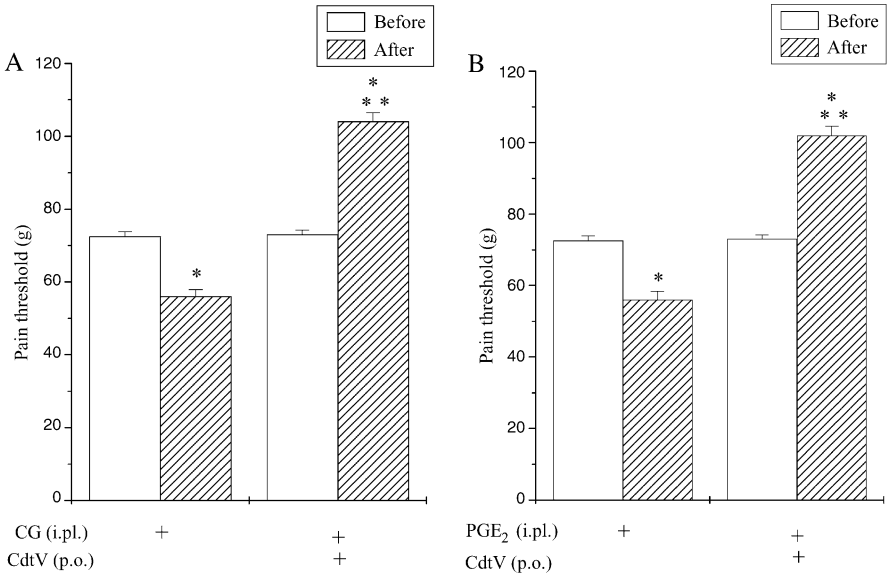

Fig. 1. Effect of *C. durissus terrificus* venom (CdtV) on hyperalgesia induced by carrageenin or prostaglandin E_2. Pain threshold was estimated in the rat paw pressure test applied before and 3 h after intraplantar injection of (A) carrageenin (CG, 200 μg/paw) or (B) prostaglandin E_2 (PGE_2, 100 ng/paw). CdtV (200 μg/kg) was administered p.o. 2 h after the injection of the hyperalgesic agents. Data represent mean values ± S.E.M. for six rats per group. *Significantly different from mean values before carrageenin or prostaglandin E_2 injection. **Significantly different from mean values of CG or PGE_2 group ($P<0.05$).

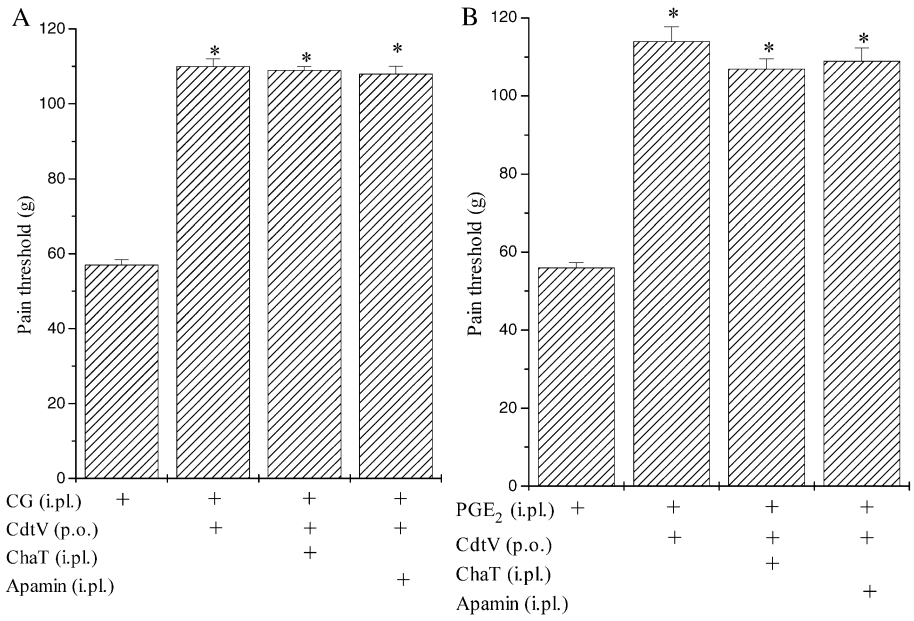

Fig. 2. Effect of blockers of Ca^{2+}-sensitive K^+ channel on the antinociception induced by *C. durissus terrificus* venom (CdtV). Pain threshold was estimated in the rat paw pressure test applied before and 3 h after intraplantar injection of (A) carrageenin (CG, 200 μg/paw) or (B) prostaglandin E_2 (PGE_2, 100 ng/paw). CdtV (200 μg/kg) was administered p.o. 2 h after the injection of the hyperalgesic agents and immediately before the i.pl. injection of charybdotoxin (ChaT, 2 μg/paw) or Apamin (10 μg/paw). Data represent mean values ± S.E.M. for six rats per group. *Significantly different from mean values for the CG or PGE_2 group ($P<0.05$).

tive effect of morphine in the formalin test (Ortiz et al., 2002). To confirm that opening of peripheral ATP-sensitive K^+ channels is able to induce antinociception, diazoxide (50–200 μg/paw) and pinacidil (50–200 μg/paw), openers of these channels (Steinberg et al., 1988; Quast and Cook, 1989) were used as positive controls. In addition, to determine if the peripheral analgesic action of δ- and κ-opioid receptors involves the opening of peripheral ATP-sensitive K^+ channels, the effect of glybenclamide on the antinociception induced by intraplantar injection of [D-Pen^{2,5}] enke-

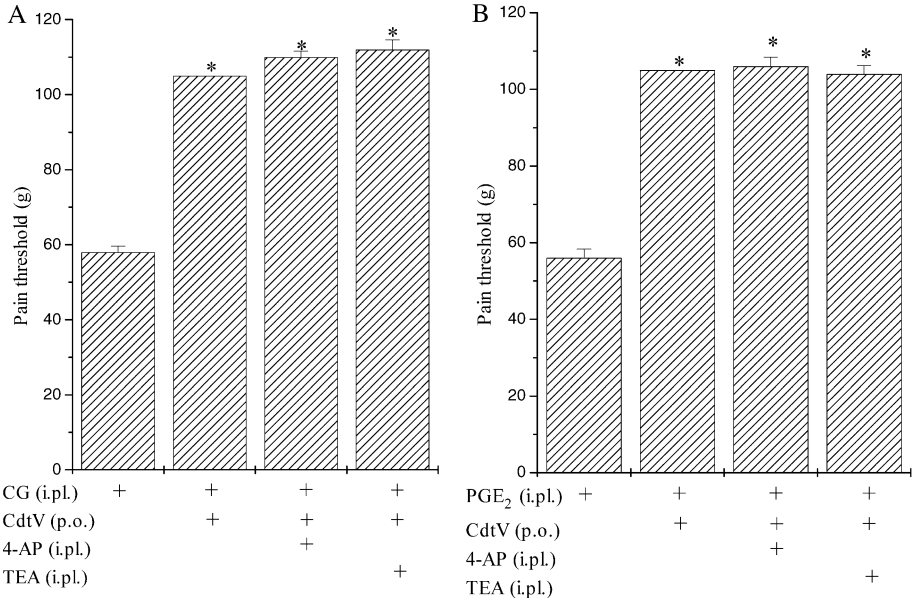

Fig. 3. Effect of blockers of voltage-dependent K^+ channel on the antinociception induced by *C. durissus terrificus* venom (CdtV). Pain threshold was estimated in the rat paw pressure test applied before and 3 h after intraplantar injection of (A) carrageenin (CG, 200 μg/paw) or (B) prostaglandin E_2 (PGE_2, 100 ng/paw). CdtV (200 μg/kg) was administered p.o. 2 h after the injection of the hyperalgesic agents and immediately before the i.pl. injection of 4-aminopyridine (4-AP, 100 μg/paw) or tetraethylammonium (TEA, 640 μg/paw). Data represent mean values ± S.E.M. for six rats per group. *Significantly different from mean values for the CG or PGE_2 group ($P<0.05$).

phalin (DPDPE, 20 μg/paw) and (+)-*trans*-(1*R*,2*R*)-U-50488 (U50488, 10 μg/paw), agonists of δ- and κ-opioid receptors, respectively, was evaluated.

2.5. Drugs used

Carrageenin was purchased from Marine Colloids (USA). 4-Aminopyridine, apamin, charybdotoxin, diazoxide, glybenclamide (glyburide), prostaglandin E_2, pinacidil, and tetraethylammonium were purchased from Sigma (USA). DPDPE and U50488 were purchased from RBI (USA). Carrageenin and U50488 were dissolved in saline. Diazoxide and pinacidil were dissolved in dimethyl sulfoxide (16%) and saline. DPDPE, 4-aminopyridine, apamin, charybdotoxin, glybenclamide, and tetraethylammonium were dissolved in distilled water. A stock solution of prostaglandin E_2 was prepared by dissolving 500 μg of prostaglandin E_2 in 1 ml of absolute ethanol. For injection into rat paw, an aliquot of this solution was diluted in sterile saline. The percentage of ethanol in the solution injected into the hind paw was less than 0.1%.

2.6. Ethics

All the experiments are 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) and the procedures were approved by the Institutional Animal Care Committee at the Butantan Institute (CEUAIB, protocol number 019/2000).

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 were tested by 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

The intraplantar injection of either prostaglandin E_2 or carrageenin caused a significant decrease in pain threshold (Fig. 1A and B, respectively). Venom (200 μg/kg) administered 2 h after the i.pl. injection of the nociceptive stimuli, induced antinociception (Fig. 1).

3.2. Effect of blockers of K^+ channels on the antinociceptive effect of *C. durissus terrificus* venom

Local pretreatment with either charybdotoxin or apamin (Fig. 2), as well as with either 4-aminopyridine or tetraethylammonium (Fig. 3) did not modify the antinociceptive effect of venom on hyperalgesia induced by carrageenin or prostaglandin E_2. On the other hand, the intraplantar administration of the blocker of ATP-sensitive K^+ channel glybenclamide abolished the effect of venom on both nociceptive tests (Fig. 4). None of the drugs interfere per

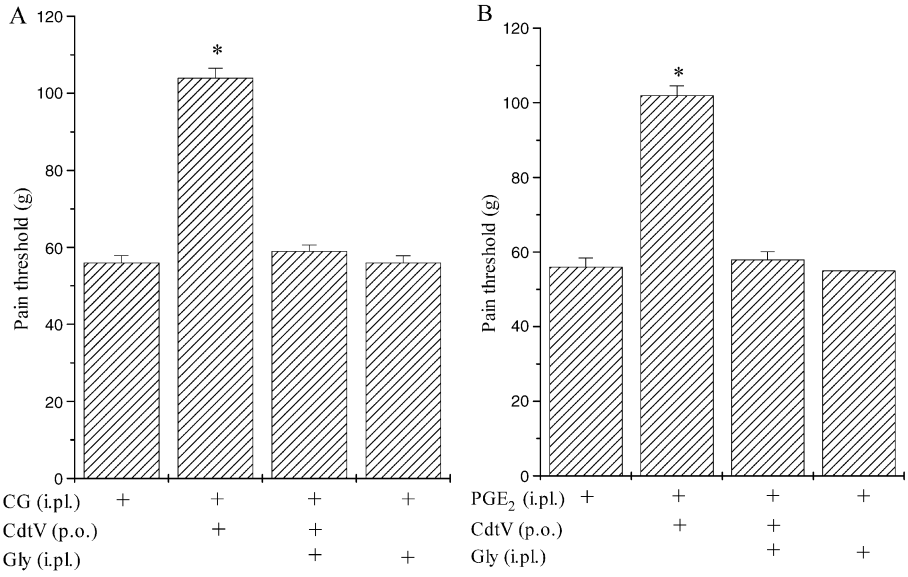

Fig. 4. Effect of blocker of ATP-sensitive K^+ channel on the antinociception induced by *C. durissus terrificus* venom (CdtV). Pain threshold was estimated in the rat paw pressure test applied before and 3 h after intraplantar injection of (A) carrageenin (CG, 200 μg/paw) or (B) prostaglandin E_2 (PGE_2, 100 ng/paw). CdtV (200 μg/kg) was administered p.o. 2 h after the injection of the hyperalgesic agents and immediately before the i.pl. injection of glybenclamide (Gly, 80 μg/paw). Data represent mean values ± S.E.M. for six rats per group. *Significantly different from mean values for CG or PGE_2 group ($P < 0.05$).

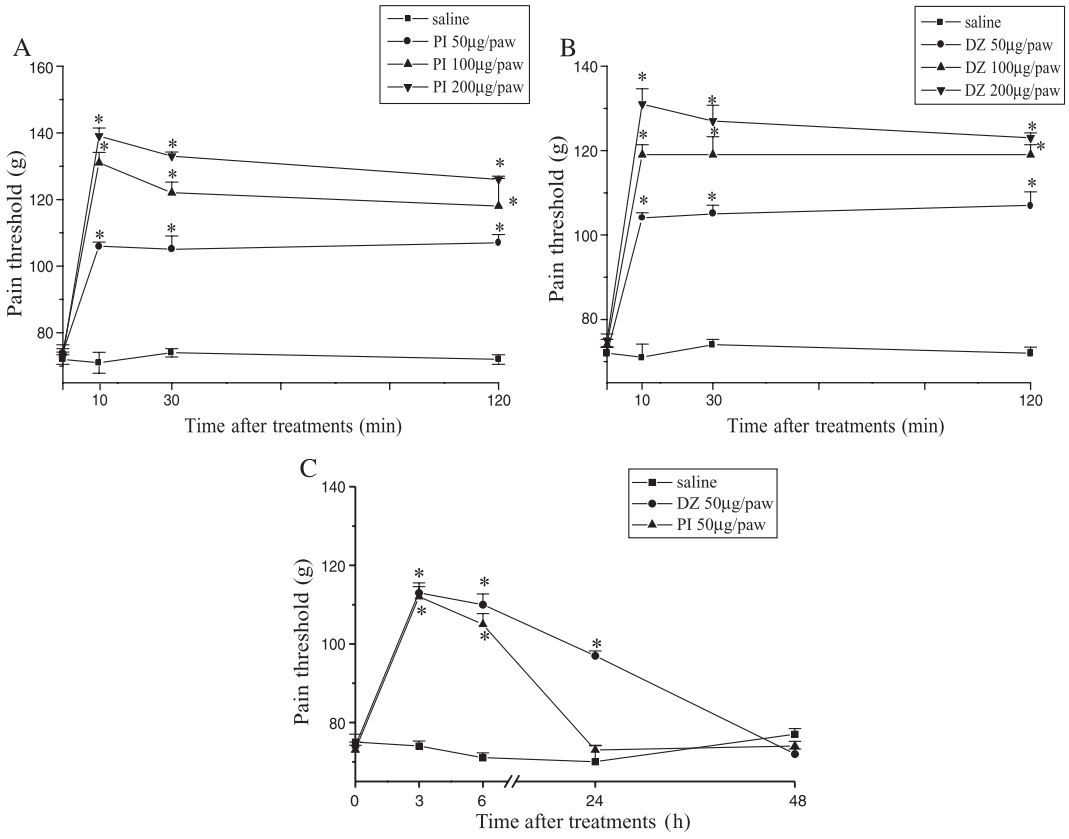

Fig. 5. Effect of ATP-sensitive K^+ channel openers on rat pain threshold. Pain threshold was estimated in the rat paw pressure test applied before and at different times after i.pl. administration of (A) pinacidil (PI; 50, 100 or 200 µg/paw) or (B) diazoxide (DZ; 50, 100 or 200 µg/paw) or saline (control group). (C) Duration of antinociception induced by PI or DZ. Data represent mean values ± S.E.M. for six rats per group. *Significantly different from mean values of control group ($P < 0.05$).

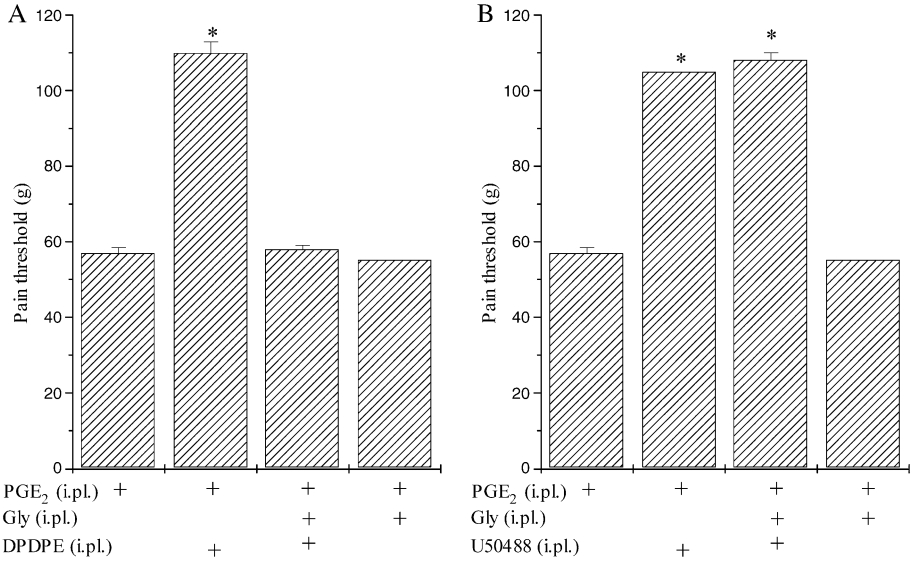

Fig. 6. Effect of blocker of ATP-sensitive K^+ channel on the antinociception induced by δ- and κ-opioid receptors agonists. Pain threshold was estimated in the rat paw pressure test applied before and 3 h after intraplantar injection of prostaglandin E_2 (PGE_2, 100 ng/paw). (A) DPDPE (20 µg/paw) or (B) U50488 (10 µg/paw) were administered 2 h after the injection of the hyperalgesic agent and 30 min after the i.pl. injection of glybenclamide (Gly, 80 µg/paw). Data represent mean values ± S.E.M. for six rats per group. *Significantly different from mean values for PGE_2 group ($P < 0.05$).

se with hyperalgesia induced by carrageenin or prostaglandin E_2, since the pain threshold observed in carrageenin- or prostaglandin E_2-treated rats was 56 ± 1.87 and 56 ± 2.39 g, respectively, whereas the pain threshold of the animals treated with the hyperalgesic stimuli plus blockers of K^+ channels ranged between 53 and 59 g.

3.3. Peripheral antinociceptive effect of openers of K^+ channels

In order to confirm the antinociceptive activity of local ATP-sensitive K^+ channels, diazoxide and pinacidil (50, 100 and 200 μg/paw), two specific openers of these channels (Steinberg et al., 1988; Quast and Cook, 1989), were administered by intraplantar route. Both drugs produced a dose-dependent (Fig. 5A and B) and long-lasting (Fig. 5C) increase in the pain threshold of animals, in the absence of hyperalgesia, and caused an antinociceptive effect on hyperalgesia induced by either carrageenin (carrageenin: 55 ± 2.08 g; carrageenin + diazoxide: 107 ± 2 g; carrageenin + pinacidil: 105 ± 4.18 g) or prostaglandin E_2 (prostaglandin E_2: 53 ± 1.66 g; prostaglandin E_2 + diazoxide: 106 ± 1.25 g; prostaglandin E_2 + pinacidil: 103 ± 3.39 g).

3.4. Effect of glybenclamide on the antinociceptive effect of δ- and κ-opioid receptors agonists

The δ-opioid receptor agonist, DPDPE, or the κ-opioid receptor agonist, U50488, administered by i.pl. route, blocked prostaglandin E_2-induced hyperalgesia (Fig. 6). Local pretreatment with glybenclamide abolished the antinociception induced by the δ-opioid receptor agonist, but had no effect on the antinociceptive effect induced by the κ-opioid receptor agonist (Fig. 6).

4. Discussion

Data presented herein evidenced that the antinociceptive effect of *C. durissus terrificus* venom on hyperalgesia involves the activation of peripheral ATP-sensitive K^+ channels, since glybenclamide, a blocker of these K^+ channels (Amoroso et al., 1990; Davies et al., 1991; Nichols and Lederer, 1991; Edwards and Weston, 1993), antagonises the venom effect. Furthermore, blockers of other types of K^+ channels, such as voltage-dependent (4-aminopyridine and tetraethylammonium) and Ca^{2+}-activated (charybdotoxin and apamin) K^+ channels, at doses reported in the literature to be effective in various models (Yonehara and Takiuchi, 1997; Ortiz et al., 2002), did not modify antinociception induced by venom. At the doses presently used, the blockers of K^+ channel did not interfere per se with hyperalgesia induced by either carrageenin or prostaglandin E_2, indicating that glybenclamide does not affect the nociceptive behaviour of the animals. To confirm that opening of ATP-sensitive K^+ channels at the peripheral terminal of afferent neurones is able to induce antinociception, diazoxide and pinacidil, two specific openers of these channels (Steinberg et al., 1988; Quast and Cook, 1989), were i.pl. administered to rats. The results demonstrate that activation of these K^+ channels increases pain threshold and antagonises hyperalgesia.

The dose of venom presently used (200 μg/paw) was based on previous work showing the effectiveness of such a dose on the hot plate test and on hyperalgesia induced by carrageenin (Giorgi et al., 1993; Picolo et al., 2000; Brigatte et al., 2001). In the present experimental conditions, this effectiveness was demonstrated for an ongoing sensitization of pain receptors since antinociception was detected when venom was administered 2 h after the intraplantar injection of carrageenin or prostaglandin E_2.

It is important to point out that the antinociceptive effect of venom in either carrageenin- or prostaglandin E_2-induced hyperalgesia is mediated by activation of peripheral δ or δ- and κ-opioid receptors, respectively (Picolo et al., 2000; Picolo and Cury, unpublished data). Several lines of evidence indicate that the peripheral antinociceptive effect of opioids, such as morphine, depends on the opening of ATP-sensitive K^+ channels (Rodrigues and Duarte, 2000), but not on the opening of voltage-dependent or Ca^{2+}-activated-K^+ channels (Ortiz et al., 2002). The demonstration that the antinociceptive effect of venom, a mixed δ- and κ-opioid receptor agonist, is mediated by the local opening of ATP-sensitive K^+ channels, suggest that in addition to μ-opioid receptors, these K^+ channels are also important for the peripheral analgesic effect of other type of opioid receptors. To confirm this indication, the effect of the blocker of ATP-sensitive K^+ channel, glybenclamide, on the antinociceptive effect of DPDPE and U50488, agonists of δ- and κ-opioid receptors, respectively, was presently evaluated. The results showed that peripheral ATP-sensitive K^+ channel is involved only in the antinociception induced by the δ-opioid receptor agonist. The lack of effect of glybenclamide on the antinociceptive effect of κ-opioid receptor agonist is in agreement with previous evidence showing that, centrally, inhibition of calcium channels, but not the opening of K^+ channels, is the main mechanism of the analgesic activity of κ-opioid receptor agonists (North, 1993; Wild et al., 1991; Schultz and Gross, 2001, for review). Presently we have no explanation for the complete reversal by glybenclamide of the antinociceptive effect of the venom in the hyperalgesia induced by prostaglandin E_2. As previously verified (Picolo and Cury, unpublished data) the antinociceptive action of the crotalid venom, in this condition, involves both δ- and κ-opioid receptors. Therefore, after glybenclamide, some degree of antinociception should remain, through κ-opioid receptors. Further investigation is in course to clarify the discrepancy.

Picolo et al. (2000) showed that stimulation of local L-arginine/NO/cGMP pathway is also involved in the antinociceptive effect of the venom. Soares et al. (2000) and

Soares and Duarte (2001) suggested that activation of peripheral ATP-sensitive K^+ channels could be the mechanism responsible for antinociception induced by nitric oxide and cGMP. Furthermore, several studies demonstrated that activation of NO/cGMP and ATP-sensitive K^+ channels are involved in analgesia induced by opioids (Ferreira et al., 1991a,b; Welch and Dunlow, 1993; Granados-Soto et al., 1997; Nozaki-Taguchi and Yamamoto, 1998; Rodrigues and Duarte, 2000; Schultz and Gross, 2001). Based on these data and the results presently obtained, we can suggest that, peripherally, the antinociceptive effect of the venom involves, at least partially, a cascade of molecular events characterized by local activation of δ- and κ-opioid receptors (Picolo et al., 2000; Picolo and Cury, unpublished data), followed by stimulation of neuronal NO and cGMP (Picolo et al., 2000), with subsequent opening of ATP-sensitive K^+ channels. However, a direct action of the venom on these channels cannot be ruled out. Patch-clamp studies with the analgesic fraction isolated from crude venom, assayed on neurons from dorsal root ganglia, are now in progress and will allow the further characterization of venom action on ion channels.

In conclusion, *C. durissus terrificus* venom induces antinociception in hyperalgesia induced by either carrageenin or prostaglandin E_2. This antinociceptive effect is antagonized by local administration of glybenclamide, but is not modified by 4-aminopyridine, tetraethylammonium, charybdotoxin and apamin. These results suggest that the antinociceptive effect of crotalic venom involves, peripherally, the activation of ATP-sensitive K^+ channels, which may induce hyperpolarization of peripheral endings of primary afferent neurons.

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