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Involvement of vanilloid receptors and purinoceptors in the *Phoneutria nigriventer* spider venom-induced plasma extravasation in rat skin

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Abstract

Phoneutria nigriventer venom causes stimulation of capsaicin-sensitive primary afferent neurons in the rat dorsal skin, leading to neurogenic plasma protein extravasation due to the release of tachykinin NK_1 receptor agonist. In this study we further investigated the mechanisms involved in the venom-induced activation of capsaicin-sensitive primary afferent neurons. The plasma extravasation in response to venom intradermally injected was measured in Wistar rats as the local accumulation of i.v. injected ^{125}I-labelled human serum albumin into skin sites. The tachykinin NK_1 receptor agonist, D-Ala-[L-Pro^9,Me-Leu^8]substance P-(7–11) (GR73632; 10–100 pmol/site), induced a significant plasma leakage that was abolished by the selective tachykinin NK_1 receptor antagonist, (*S*)-1-[2-[3-(3,4-dichlorphenyl)-1 (3-isopropoxyphenylacetyl) piperidin-3-yl] ethyl]-4-phenyl-1 azaniabicyclo [2.2.2]octane chloride (SR140333; 1 nmol/site), whereas the leakage after venom (1–10 μg/site) was significantly inhibited (but not abolished) by SR140333. The calcitonin gene-related peptide (CGRP) receptor antagonist, CGRP-(8–37), failed to further reduce the residual plasma extravasation induced by venom plus SR140333. The μ-opioid receptor agonist, [D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin (DAMGO), and the local anaesthetic, lignocaine, had no effect on the venom-induced plasma extravasation. Similarly, the L-, N- and P/Q-type voltage-sensitive Ca^{2+} channel blockers (verapamil, ω-conotoxin MVIIA and MVIIC, respectively) as well as the Na^+ channel blockers, tetrodotoxin and carbamazepine, had no effect on the venom-induced effect. Neither the systemic treatment nor the local injection of ruthenium red prevented the venom-induced plasma extravasation. However, the vanilloid receptor antagonist, *N*-[2-(4-chlorophenyl) ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide (capsazepine; 120 μmol/kg, i.v.), reduced by 48% ($P < 0.05$) the venom (10 μg/site)-induced plasma extravasation. A significant inhibitory effect was also observed with the P_2 purinoceptor agonists, adenosine 5′-triphosphate (ATP; 10 and 30 nmol/site) and adenosine 5′-diphosphate (ADP; 10 nmol/site). The involvement of histamine and/or 5-hydroxytryptamine (5-HT) in the venom-induced plasma extravasation was ruled out since neither histamine and 5-HT receptor antagonists nor depletion of mast cells by compound 48/80 affected the venom response. This was further supported by the failure of venom to degranulate in vitro peritoneal mast cells. In conclusion, only vanilloid receptors and P_2 prejunctional purinoceptors had an inhibitory effect on the neurogenic plasma extravasation evoked by *P. nigriventer* venom in rat dorsal skin.

Keywords: *Phoneutria nigriventer* venom; Sensory nerve; Neurogenic plasma extravasation; Tachykinin NK_1 receptor agonist; Vanilloid receptor; Purinoceptor

1. Introduction

It is well known that neurogenic inflammation may be modulated by inhibiting neuropeptide release from peripheral endings of capsaicin-sensitive primary afferent neurons at a prejunctional level (Maggi, 1993). This can be achieved with a range of agents that include a local anaesthetic (lignocaine), a μ-opioid receptor agonist, [D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin (DAMGO; Escott et al., 1995), the vanilloid receptor antagonist, *N*-[2-(4-chlorophenyl) ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide (capsazepine; Bevan et al., 1992) and the inorganic dye, ruthenium red (Amann et al., 1990; Buckley et al., 1990), as well as other pharmacological agents including transmitters such as P_2 purinoceptor agonists, adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP; Burnstock and Wood, 1996). Postjunctional mechanisms may include neuropeptide receptor

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antagonists such as calcitonin gene-related peptide (CGRP) receptor antagonist (CGRP-(8–37); Hughes and Brain, 1991; Escott and Brain, 1993) and the tachykinin NK_1 receptor antagonist (*S*)-1-[2-[3-(3,4-dichlorphenyl)-1 (3-isopropoxyphenylacetyl)piperidin-3-yl] ethyl]-4-phenyl-1 azaniabicyclo [2.2.2]octane chloride (SR140333; Emonds-Alt et al., 1993). Furthermore, mast cell contents can modify the vascular actions of the peptides released from capsaicin-sensitive primary afferent neuron endings (Dimitriadou et al., 1997). Peripheral endings of sensory neurons also express various ligand-gated cation channels and distinct subunits that have been studied in detail (Fox et al., 1987; Maggi, 1993; Akopian et al., 1996). This is supported by findings that the application of a high-K^+ depolarizing medium produces a Ca^{2+}-dependent release of tachykinins and CGRP from a variety of peripheral organs of different species, which can be blocked by Ca^{2+} channel blockers (Maggi et al., 1988; Hata et al., 1992).

Previous studies have shown that *Phoneutria nigriventer* spider venom acts in a potent manner to induce plasma protein extravasation when intradermally (i.d.) injected in the rabbit and rat dorsal skin (Antunes et al., 1992, 1993; Marangoni et al., 1993). Recently, we demonstrated that the venom-induced plasma protein extravasation in rat skin is markedly reduced by both the tachykinin NK_1 receptor antagonist, SR140333, (Palframan et al., 1996) and neonatal capsaicin treatment to deplete sensory neuropeptides (Costa et al., 1997). These findings suggest the involvement of capsaicin-sensitive primary afferent neuron-mediated mechanisms in *P. nigriventer*-evoked plasma extravasation. In this study we have extended our original findings in order to learn more about mechanisms involved in the *P. nigriventer* venom-induced activation of sensory nerves and about mechanisms by which the exudative response may be attenuated.

2. Materials and methods

2.1. Rats

Experiments were carried out on both male and female Wistar rats (230–300 g) purchased from Central Animal House (CEMIB-UNICAMP) or Tucks (Essex, UK) under standard conditions and allowed access to normal diet and water. All experiments were carried out in accordance with the State University of Campinas (UNICAMP) guidelines for animal care and with the UK Animal (Scientific Procedures) Act 1986.

2.2. Measurement of plasma protein extravasation

Rats were anaesthetised with sodium pentobarbitone (Sagatal 40 mg/kg, intraperitoneally). A mixture of ^{125}I-human serum albumin (2.5 μCi/rat) with Evans blue dye (1 ml of a 2.5% solution) was injected in the tail vein. Plasma protein extravasation was evaluated in the shaved dorsal skin in response to i.d. injected *P. nigriventer* venom (1–10 μg/site) and test agents (0.1 ml/site made up in Tyrode solution) injected according to a balanced site design (Brain and Williams, 1985). Plasma extravasation was also assessed in the depilated rat hind paw skin in response to topical administration of capsaicin (8-methyl *N*-vanillyl-6-nonenamide) solution. Capsaicin (5% in 1:1 of dimethyl sulfoxide (DMSO):methanol v/v; 100 μl/paw) or vehicle (100 μl/paw of 1:1 DMSO:methanol v/v) was applied to the dorsal surface of the right and left hind paws. After 30 min, blood samples (5 ml) were taken by cardiac puncture, plasma was obtained and the rats were killed with an anaesthetic overdose. The dorsal skin injection sites and treated skin area of both paws (treated and sham) were removed and counted with plasma samples in a γ-counter. The amount of plasma extravasated was expressed as the volume (microliters) of plasma accumulated/dorsal skin site and per 100 mg of paw skin in comparison with the level of ^{125}I-human serum albumin present in 1 ml of plasma.

2.3. Mast cell isolation and incubation with P. nigriventer venom

Mast cells were obtained from male Wistar rats (200–300 g) by injecting 10 ml of Krebs–Ringer phosphate solution (KRP; pH 7.3) into the peritoneal cavity. The abdomen was then carefully massaged and the fluid was withdrawn and placed in polypropylene tubes prior to centrifugation at 300 g for 5 min at room temperature. The cell suspension was washed twice in KRP and resuspended in 6.5 ml of KRP. Aliquots of the peritoneal cell suspension (0.5 ml) were warmed at 37°C for 20 min prior to stimulation (final volume of 1.0 ml) with *P. nigriventer* venom (0.01–1 mg/ml) and the positive control, compound 48/80 (500 ng/ml). After 20 min at 37°C, the cells were centrifuged ($300 \times g$, 10 min), the supernatant was removed to glass tubes and added to HCl solution (200 μl of 1.0 N). HCl solution (1.0 ml of 0.1 N) was added to the cell pellet and the mixture was also transferred to the glass tubes. Both the supernatant and the cell pellet samples were heated to 100°C for 10 min and further centrifuged at $300 \times g$ for 5 min. The cell suspension from supernatant and cell pellet tubes was resuspended and stored at −4°C until assayed. The amounts of histamine and 5-hydroxytryptamine (5-HT) released were analysed by tandem mass spectrometry, using a Hewlett-Packard 100 liquid chromatograph coupled to a Micromass Quatro II mass spectrometer. All values were corrected for the spontaneous release occurring in the absence of stimulus.

2.4. Drug treatments

All drugs and doses used in our study were based on previous reports and their modes of action and references are described in Table 1.

Table 1
Multiple drug treatments used against *P. nigriventer* venom-induced plasma protein extravasation in rat skin in vivo

Drugs	Mode of action	Refs.
ω-Conotoxin MVIIA	N-type Ca^{2+} channel blocker	Fox et al., 1987; Maggi et al., 1988
ω-Conotoxin MVIIC	P/Q-type Ca^{2+} channel blocker	Maggi et al., 1988; Uchitel and Protti, 1994
Capsazepine	Vanilloid receptor antagonist	Perkins and Campbell, 1992
Carbamazepine	Tetrodotoxin-resistant Na^+ channel blocker	Arbuckle and Docherty, 1995; Akopian et al., 1996
DAMGO	μ-opioid receptor agonist	Barber, 1993; Escott et al., 1995
Lignocaine	Sensory nerve conduction blocker	Escott et al., 1995
Ruthenium red	Cationic channel blocker	Buckley et al., 1990; Newbold and Brain, 1995
Tetrodotoxin	Na^+ channel blocker	Maggi et al., 1988; Akopian et al., 1996
Verapamil	Selective L-type Ca^{2+} channel blocker	Fox et al., 1987; Costa et al., 1996

To ascertain the indirect involvement of mast cells in the venom-induced plasma extravasation, rats were pretreated with three consecutive daily s.c. injections of compound 48/80 (5 mg/kg) that produces a marked reduction in the responses to agents acting through mast cell activation (Ahluwalia et al., 1998). Skin vascular permeability increases were assayed on the third day.

2.5. Venom and drugs

P. nigriventer venom was obtained from the Arthropods Section of the Instituto Butantan (SP), Brazil. The venom was dialysed before use, as previously described (Antunes et al., 1992). Bradykinin, Bay K8644, CGRP, CGRP-(8–37), capsaicin, carbamazepine, compound 48/80, DAMGO, histamine, 5-HT, lignocaine, ω-conotoxin MVIIA, ω-conotoxin MVIIC, ruthenium red, tetrodotoxin, verapamil and dialysis tubing were purchased from Sigma (St. Louis, MO, USA). ^{125}I-Human serum albumin was either purchased from Amersham International (UK) or labeled by Dr. Tereza C.P. Rebella (IPEN/CNEN-São Paulo, Brazil). Mepyramine and methysergide were obtained from Sterilin (England) and Sandoz (Bale, Swiss). Sodium pentobarbitone was obtained from Rhone Merieux (Dublin, Eire). GR73632 and SR140333 were gifts from Dr D. Beattie, Glaxo Group Research (Ware, UK) and Dr Emonds-Alt at Sanofi (Montpellier, France), respectively.

P. nigriventer venom and test agents were stored at −20°C and diluted with a modified Tyrode solution (composition in mM: NaCl 137, KCl 2.7, $MgCl_2$ 0.5, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.6) immediately before use.

2.6. Statistical analysis

The data are presented as mean values ± S.E.M. for *n* experiments, for measurements of both dorsal and paw plasma protein extravasation. For statistical comparison of plasma extravasation in different group of animals, one of the following tests was used where appropriate: Student's or Bonferroni's modified *t*-test, Fisher's exact probability test and two-way analysis of variance (ANOVA). A level of $P < 0.05$ was taken as significant.

3. Results

3.1. Role of neuropeptides in venom-induced plasma protein extravasation

The tachykinin NK_1 receptor agonist, D-Ala-[L-Pro^9,Me-Leu^8]substance P-(7–11) (GR73632; 10–100 pmol/site), induced a dose-dependent plasma extravasation in rat skin that was abolished by the concomitant administration of the tachykinin NK_1 receptor antagonist, SR140333 (1 nmol/site; Fig. 1). The *P. nigriventer* venom (1–10 μg/site)-induced plasma extravasation in rat skin was significantly reduced, but not abolished, by the same treatment with SR140333 (Fig. 1).

To assess the role of CGRP in the venom-induced residual plasma extravasation, rats were treated with CGRP receptor antagonist, CGRP-(8–37) (400 nmol/kg; i.v.) or its respective vehicle (200 μl/rat of 0.1% bovine serum albumin in saline) 15 min before venom injection. The CGRP-(8–37) treatment failed to reduce further the venom-induced plasma extravasation that was partly reduced by SR140333 (1 nmol/site; Fig. 2). As expected, the plasma extravasation induced by GR73632 (30 pmol/site; 32 ± 2.2 μl/site) was potentiated by co-injection with CGRP (10 pmol/site; 63 ± 6.0 μl/site) and this response was significantly ($P < 0.05$) reduced in those rats treated with CGRP antagonist (43 ± 5.0 μl/site; $n = 5$). CGRP (10 pmol/site) when injected alone did not induce plasma extravasation as compared to the effect of Tyrode solution (12 ± 2.6 and 11 ± 0.6 μl/site for CGRP and Tyrode, respectively).

3.2. Involvement of mast cells

The co-injection of venom (10 μg/site) with antagonists of histamine H_1 (mepyramine; 2.5 nmol/site) and 5-HT receptors (methysergide; 2.5 nmol/site) together with SR140333 (1 nmol/site) further slightly reduced the dialysed venom-induced residual plasma extravasation (59

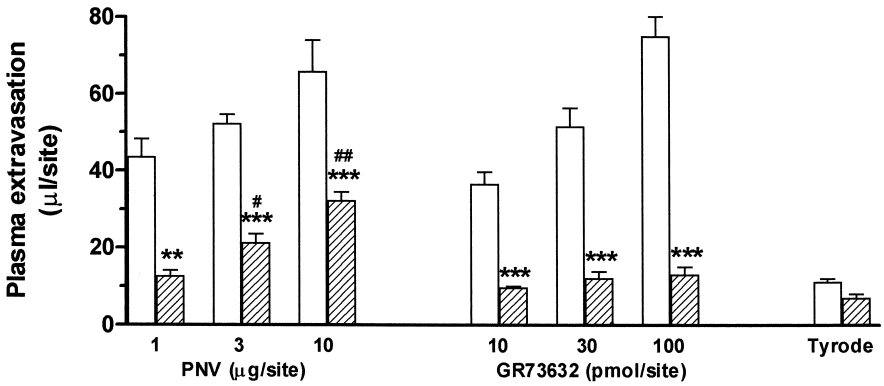

Fig. 1. Dose–response relationship for plasma protein extravasation in rat dorsal skin induced by both *P. nigriventer* venom and the tachykinin NK_1 receptor agonist, GR73632, alone (open bars) and co-injected with the tachykinin NK_1 receptor antagonist, SR140333 (striped bars). Each bar represents the mean ± S.E.M. for nine rats. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to their respective controls and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to Tyrode plus SR140333 (1 nmol/site).

± 10, 32 ± 4.0 and 25 ± 2.0 μl/site for venom alone, co-injected with SR140333 and co-injected with SR140333, mepyramine or methysergide, respectively; $n = 6$). The doses of mepyramine and methysergide used were effective because they markedly inhibited the plasma extravasation caused by both histamine (30 nmol/site; 54 ± 7.6 and 22 ± 3.4 μl/site alone and co-injected with mepyramine, respectively) and 5-HT (1 nmol/site; 66 ± 11 and 19 ± 1.5 μl/site alone and co-injected with methysergide, respectively; $n = 6$).

The plasma leakage produced by venom (1–10 μg/site) in both vehicle (saline, 0.2 ml/rat; $n = 7$)- and compound-48/80-pretreated sets of animals (5 mg/kg; $n = 6$) is shown in Fig. 3. The compound 48/80-pretreated animals showed a significant reduction ($P < 0.01$) in plasma extravasation in response to i.d. injection of compound 48/80 (500 ng/site) as compared to vehicle-pretreated rats. In contrast, i.d. injection of venom (1–10 μg/site) as well as GR73632 (30 pmol/site) produced a similar plasma extravasation in both vehicle and treated groups (Fig. 3).

3.3. Modulation of P. nigriventer venom-induced plasma protein extravasation by different agents

In order to learn more about the neurogenic plasma extravasation mechanisms induced by *P. nigriventer* venom, a range of agents known to inhibit neurogenic inflammation (Table 1) was used as described below.

Table 2 shows that prior (20 min) systemic treatment of rats with the prejunctionally acting μ-opioid receptor agonist, DAMGO (0.3 mg/kg, i.v.), had no effect on venom (10 μg/site)-induced plasma extravasation. DAMGO elicited an immediate and short-lasting decrease (3 min) in arterial blood pressure (93 ± 5, 69 ± 3 and 105 ± 8 mmHg before and 1 and 5 min post-DAMGO injection, respectively; $n = 7$) which was inhibited by previous treatment with the opioid receptor antagonist, naloxone (1 mg/kg; 99 ± 3 and 103 ± 5 mmHg before and 1 min post-DAMGO, respectively; $n = 5$). Naloxone alone had no effect on arterial blood pressure and venom-induced plasma extravasation. DAMGO-induced haemodynamic changes

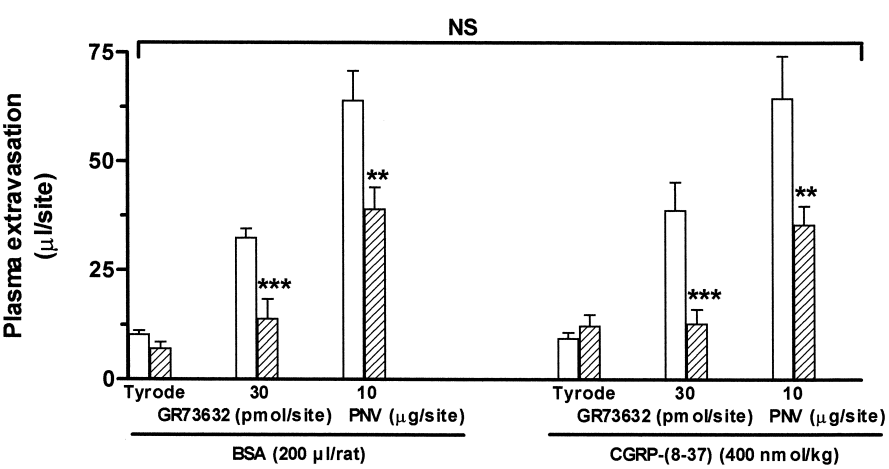

Fig. 2. Effect of CGRP receptor antagonist, CGRP-(8–37), on plasma protein extravasation induced by venom and GR73632 alone (open bars) and in the presence of SR140333 (1 nmol/site; striped bars) in vehicle- and CGRP-(8–37)-pretreated rats. Values shown are means ± S.E.M. for 10 rats. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to their respective control. NS = nonsignificant.

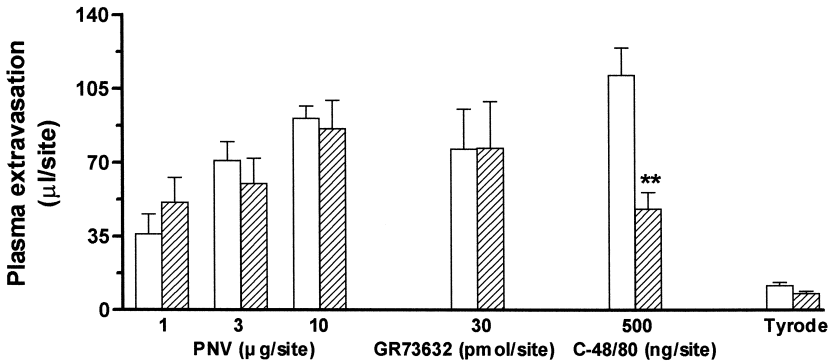

Fig. 3. Lack of effect of compound 48/80 pretreatment on plasma extravasation induced by venom. Responses to venom, GR73632 and compound 48/80 are shown by open (vehicle-pretreated group; $n = 8$) and striped bars (C-48/80-pretreated group; $n = 6$), respectively. Values are means ± S.E.M. for six to eight rats. $^{**}P < 0.01$ compared to vehicle-pretreated group.

also did not interfere with the response evoked by GR73632 (30 pmol/site; 39 ± 5.0 and 36 ± 5.0 µl/site for control and DAMGO-treated rats, respectively) and Tyrode (12.6 ± 2.5 and 12 ± 3.0 µl/site for control and DAMGO-treated rats, respectively; $n = 13$). In a separate group of rats, DAMGO (0.3 mg/kg; $n = 7$) was injected intravenously immediately before venom. With this treatment schedule, DAMGO again failed to affect the venom-induced plasma extravasation (47 ± 6 and 40 ± 1.2 µl/site for control and DAMGO-treated rats, respectively). In addition, the co-injection of DAMGO (30, 100 and 300 pmol/site) had no significant effect on the venom (3 µg/site)-induced plasma extravasation (38 ± 2.9, 40 ± 0.9, 42 ± 1.5 and 47 ± 1.3 µl/site for control and 30, 100 and 300 pmol/site of DAMGO, respectively; $n = 6$). At these doses, DAMGO did not produce significant plasma extravasation in the rat skin (not shown).

The possibility that Na^+ channels modulate the plasma protein extravasation induced by *P. nigriventer* venom was investigated by systemic treatment of the rats with both a Na^+ channel blocker, tetrodotoxin (1 nmol/rat, s.c.), and the tetrodotoxin-insensitive Na^+ channel blocker, carbamazepine (25 mg/kg, i.v.). Neither tetrodotoxin nor carbamazepine had a significant effect on the venom (10 µg/site)-induced plasma extravasation (Table 2). In the tetrodotoxin-pretreated group, the plasma extravasation induced by topical application of capsaicin solution (5%) onto the dorsal rat hind paw was markedly reduced (3.6 ± 0.7 µl/100 mg paw skin; $n = 5$) as compared to that in the control group (17 ± 4 µl/100 mg paw skin; $n = 4$) and did not significantly differ from that of the contralateral paw treated with vehicle (3.4 ± 1.1 and 2.1 ± 0.4 µl/100 mg paw skin for tetrodotoxin and control group, respectively).

Similarly, the co-injection of the local anaesthetic, lignocaine (2%), had no effect on the local plasma extravasation induced by this venom (10 µg/site; Table 2). When injected alone in the rat skin, lignocaine (2 %, i.d.) did not produce significant changes (14 ± 2.0 and 12.6 ± 0.7 µl/site for lignocaine and Tyrode, respectively; $n = 6$).

It has been shown that ruthenium red prevents sensory nerve activation by capsaicin and other ligands of the vanilloid receptors (Amann et al., 1990). The plasma protein extravasation induced by topical administration of capsaicin (5%) in the dorsal hind paw was significantly reduced in rats treated systemically with ruthenium red (2 mg/kg; 10 ± 1.3 and 4.7 ± 0.7 µl/100 mg paw skin for vehicle and ruthenium-red-treated group, respectively; $n = 7$), whereas this same treatment had no effect on venom-induced skin plasma extravasation (77 ± 9.0 and 73 ± 9.9 µl/site for vehicle- and ruthenium-red-pretreated rats; respectively; $n = 6$–9). Ruthenium red (0.3–3.0 nmol/site) co-injected with venom (10 µg/site) also had no significant effect on the plasma extravasation induced by venom (only the 3-nmol dose of ruthenium red is shown in Table 2). When injected alone, ruthenium red did not induce significant plasma extravasation (7.8 ± 0.7, 12 ± 2.0 and 20 ± 2.4 µl/site for 0.3, 1.0 and 3.0 nmol/site of ruthenium red, respectively) compared to Tyrode (14.4 ± 2.7 µl/site; $n = 6$).

The effects of L-, N-, P- and Q-type Ca^{2+} currents in the venom-induced plasma extravasation were evaluated by treatment with various Ca^{2+} channel blockers. Neither systemic treatment with the selective L-type Ca^{2+} channel

Table 2
Amount of plasma protein extravasation induced by *P. nigriventer* venom in the dorsal skin of control and drug-treated rats/sites. Values are means ± S.E.M for *n* rats

P. nigriventer venom (10 µg/site)			
Groups	*n*	Plasma extravasation (µl/site)	
		Control	Drug-treated
DAMGO	13	56 ± 7.0	43 ± 5.0
Carbamazepine	8	58 ± 11	70 ± 5.7
Tetrodotoxin	9	66 ± 4.6	68 ± 5.9
Lignocaine	6	54 ± 5.0	57 ± 2.0
Ruthenium red	6	91 ± 9.5	104 ± 10
Verapamil	11	59 ± 6.0	69 ± 5.0
ω-Conotoxin MVIIA	9	46 ± 8.7	47 ± 6.2
ω-Conotoxin MVIIC	4	63 ± 15	60 ± 14

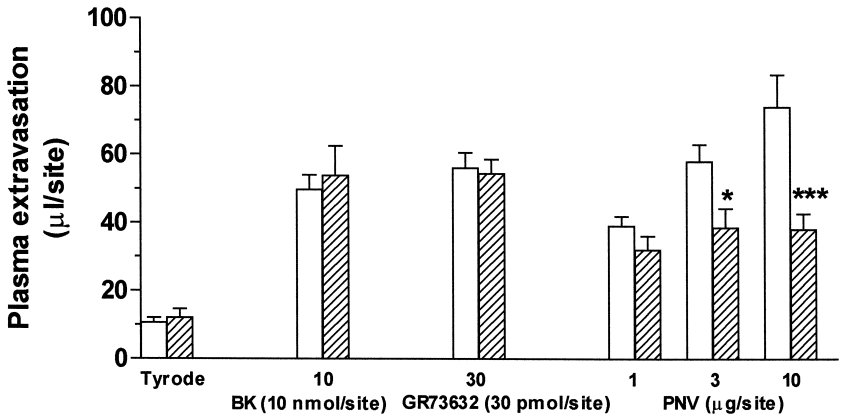

Fig. 4. Partial antagonism of venom-induced plasma extravasation in rat skin by capsaicin receptor antagonist, capsazepine. The responses to venom, GR73632, bradykinin (BK) and Tyrode are shown for vehicle- (open bars) and in capsazepine-pretreated rats (striped bars; 120 μmol/kg, 15 min before). Each bar represents the mean ± S.E.M for four to six rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to vehicle-pretreated rats.

blocker, verapamil (60 μg/kg, i.v., 15 min before, $n = 6$), nor co-injection of the nonselective P- and Q-type Ca^{2+} blocker, ω-conotoxin MVIIC (100 pmol/site; $n = 4$), significantly attenuated the plasma extravasation evoked by venom (10 μg/site; Table 2). The venom-induced plasma extravasation seen in the N-type Ca^{2+} channel blocker, ω-conotoxin MVIIA (3.2 μg/kg, i.v., $n = 9$)-pretreated group was not significantly different from that achieved above and did not differ from the control group (Table 2). At the range of doses used above, both verapamil and ω-conotoxin MVIIA significantly ($P < 0.05$) reduced the Ca^{2+} channel opener, Bay K8644 (1 μg/kg, i.v.)-induced hypertension (84 ± 7.0 and 46 ± 6.7 mmHg for control and verapamil-pretreated group or 86 ± 8.0 and 52 ± 4.6 mmHg for control and ω-conotoxin MVIIA-pretreated group). However, no significant change was noticed for the basal mean arterial blood pressure after verapamil or ω-conotoxin MVIIA (not shown).

3.4. Modification by vanilloid receptor antagonist

Systemic treatment of rats with the vanilloid receptor antagonist, capsazepine (120 μmol/kg, 15 min before; $n = 6$), produced a significant reduction in the plasma extravasation evoked by the highest doses (3 and 10 μg/site) of venom as compared to that in the group treated with vehicle (200 μl/rat; 1:1:1:7 of DMSO:Tween 80:ethanol in 0.9% w/v NaCl solution; Fig. 4). The responses elicited by GR73632 (30 pmol/site), bradykinin (10 nmol/site) and Tyrode solution were similar in both capsazepine- and vehicle-pretreated rats ($n = 5$; Fig. 4). Capsazepine (120 μmol/kg; i.v.) did not significantly affect the plasma extravasation evoked by capsaicin (5%) applied locally to the rat paw (14.4 ± 0.9 and 13.4 ± 0.7 μl/100 mg paw tissue for vehicle- and capsazepine-pretreated rats, respectively). As expected, the response evoked by capsaicin vehicle (100 μl/paw of 1:1 DMSO:methanol v/v) in the contralateral hind paw of pretreated rats was significantly smaller (3.4 ± 3.1 μl/100 mg paw skin; $n = 4$) and did not differ from that evoked by vehicle in the contralateral hind paw of vehicle-pretreated rats (4.4 ± 3.4 μl/100 mg paw skin; $n = 5$).

3.5. Partial antagonism of venom-induced plasma extravasation by nucleotides

The co-injection of increasing doses of ATP (1, 10 and 30 nmol/site) caused a significant dose-dependent reduction in the venom (10 μg/site)-induced plasma extravasation (7.5 ± 2.8, 38 ± 6.2 and 56 ± 5.0 % of reduction for 1, 10 and 30 nmol/site of ATP, respectively) as compared to that with venom alone (69 ± 2.3 μl/site; see also Fig. 5). The response to GR73632 (30 pmol/site) was not markedly changed by co-injection with ATP at a dose of 10 nmol/site (not shown). Furthermore, a significant reduction in the venom (10 μg/site)-induced plasma extravasation was observed with ADP at 10 nmol/site but not at 1 and 3 nmol/site (Fig. 6). At the lowest doses, the

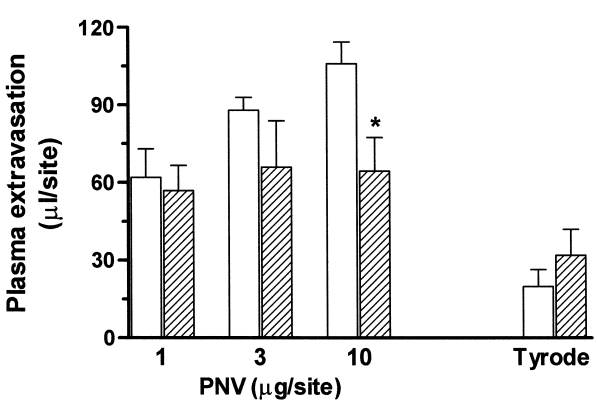

Fig. 5. Reduction of venom-induced plasma protein extravasation by co-injection with ATP (10 nmol/site; striped bars). Values shown are means ± S.E.M. for seven rats. $^{*}P < 0.05$ compared to control sites (open bars).

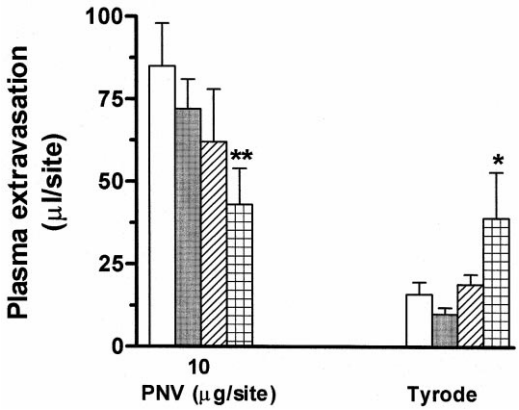

Fig. 6. Rat skin plasma extravasation in response to i.d. injection of venom alone (open bars) and co-injected with different doses of ADP (1 nmol/site; dotted bars), (3 nmol/site; striped bars) and (10 nmol/site; crossed bars). Values shown are means ± S.E.M. for four rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to their respective control sites (open bars).

plasma extravasation induced by ATP (1 and 10 nmol/site; not shown) and ADP (1 and 3 nmol/site; Fig. 6) alone was not statistically different from that with Tyrode solution whereas at the highest doses (10 and 30 nmol/site for ADP and ATP, respectively), they significantly increased permeability per se.

3.6. Histamine and 5-HT analysis in vitro

The measurement of histamine and 5-HT released by the peritoneal mast cell in response to different agents revealed that neither *P. nigriventer* venom (0.01 and 1.0 mg/ml; $n = 4$) nor GR73632 (300 pmol/ml; $n = 4$) induced degranulation of mast cells, since no significant release of histamine or 5-HT was detected. As expected, compound 48/80 (5 μg/ml; $n = 7$) caused a marked release of histamine (58 ± 3.3 %) and 5-HT (60 ± 4.6 %) from rat peritoneal mast cells.

4. Discussion

P. nigriventer is an aggressive spider found in the Southeast of Brazil where it is responsible for most of the spider accidents in humans. The intense local pain and cutaneous inflammation induced by this venom are major problems following human accidents (Lucas, 1988; Bucaretchi, 1992). Our results showed that *P. nigriventer* venom injected in the rat skin caused significant plasma extravasation, confirming previous studies (Antunes et al., 1992; Palframan et al., 1996; Costa et al., 1997). Although we noticed some variation in the magnitude of venom-induced plasma extravasation, this variability did not interfere with the results since control experiments were carried out for each treated group. This variation of the venom-induced response may reflect the use of different batches of venom and ^{125}I-human serum albumin.

P. nigriventer venom is a potent stimulant of capsaicin-sensitive primary afferent neurons when intradermally injected in the rat dorsal skin (Costa et al., 1997). We have pursued these investigations on the venom-induced plasma extravasation and tested different classes of drugs that act either via presynaptic receptors or mechanisms located on sensory nerves, or on postsynaptic receptors. This has enabled us to learn more about how this venom activates capsaicin-sensitive primary afferent neurons to release a neuropeptide that acts as tachykinin NK_1 receptor agonist (Palframan et al., 1996; Costa et al., 1997). The ability of the non peptide-selective tachykinin NK_1 receptor antagonist, SR140333, to inhibit *P. nigriventer* venom-induced plasma extravasation is clear from both our previous and present studies. It is generally assumed that the release of multiple peptides from sensory nerves provides the potential for synergistic interactions (Holzer, 1998). For example, the vasodilator action of CGRP may potentiate plasma exudation induced by tachykinin NK_1 receptor agonists in the skin, presumably by increasing blood flow at the site of leakage (Brain and Williams, 1985, 1989). However, a lack of effect of the CGRP receptor antagonist, CGRP-(8–37), on venom-induced plasma extravasation was shown. This treatment with CGRP-(8–37) (400 nmol/kg; i.v.) has been shown to inhibit cutaneous vasodilatation induced by CGRP released from stimulated sensory nerves (Escott and Brain, 1993; Siney and Brain, 1996). Furthermore, this antagonist is the only one available and the peptide ligand is likely to be limited in its application. The results suggest that either CGRP plays a minor role in the response or that the antagonist is not sufficiently effective.

We have investigated the role of mast cells in the venom-induced neurogenic plasma extravasation using histamine and 5-HT receptor antagonists as well as pretreatment of rats with compound 48/80. It is well established that *P. nigriventer* venom contains small amounts of histamine and 5-HT (Schenberg and Pereira-Lima, 1971), which play a large role in the plasma protein extravasation in rodent skin. In the present study, dialysis of the venom was performed in order to remove these components and thus eliminate possible interference of these substances in the skin assay. However, the ability of dialysed venom to produce plasma extravasation was still observed even in the presence of SR140333, suggesting that other mechanisms, independent of histamine and 5-HT content and tachykinin NK_1 receptors, may be involved in the venom-induced plasma extravasation. It has been hypothesized that substance P released from sensory nerves stimulates mast cells to release histamine (Arvier et al., 1977; Lembeck and Holzer, 1979). It would therefore be expected that, if venom-induced plasma protein extravasation is markedly reduced by the tachykinin NK_1 receptor antagonist (Palframan et al., 1996) and capsaicin treatment (Costa et al., 1997), the plasma extravasation would also be reduced in animals pretreated with compound 48/80 to

deplete mast cells. Although rats subjected to previous treatment with compound 48/80 showed a marked reduction in the response to intradermal injection of compound 48/80, inhibition of *P. nigriventer* venom-induced plasma extravasation in those treated animals was not observed. As expected, the leakage responses to GR73632, which has been proposed to be a peptidase resistant receptor agonist as well as to act solely via tachykinin NK_1 receptors was not affected by compound 48/80 pretreatment. In addition, the co-injection of histamine (mepyramine) and 5-HT (methysergide) receptor antagonists caused only a slight reduction in the remaining plasma extravasation induced by dialysed venom co-injected with SR140333. These results with compound 48/80 and mast cells amine antagonists reinforce the suggestion that *P. nigriventer* venom does not cause the release of histamine and 5-HT, and indicate that the venom-induced neurogenic plasma extravasation is not dependent in vivo on the direct activation of mast cells. This suggestion is further supported by the failure of venom to directly activate in vitro peritoneal rat mast cells. Our results agree with previous observations that mast cell activation is not essential for the initial, vascular effects of neurogenic inflammation in the rat skin (Kowalski et al., 1990; Tausk and Undem, 1995).

In further studies we investigated a range of agents that are known to influence the passage of ions into nerves. The voltage-sensitive Ca^{2+} channels (VSCC) such as the L, P and Q type have been identified in a number of peripheral nerves in several species and are involved in the release of sensory neuropeptides such as tachykinin and CGRP (Fox et al., 1987; Maggi et al., 1988; Maggi, 1991). A range of animal toxins act on different types of Ca^{2+} channels and most of these toxins have been extracted from spider venom (*Agelenopsis aperta*; ω-agatoxins) and marine snail genus *Conus* (ω-conotoxins). Curiously, *P. nigriventer* venom has been reported to contain neurotoxic peptides with different molecular weights and similarities with some Ca^{2+} channel blockers (Souccar et al., 1995; Prado et al., 1996; Guatimosim et al., 1997; Cassola et al., 1998). Although our previous report shows that *P. nigriventer* venom-induced cardiac disturbances are markedly reduced by the L-type Ca^{2+} channel blockers (Costa et al., 1996), our present findings with L (verapamil)-, N (ω-conotoxin MVIIA)- and P/Q (ω-conotoxin MVIIC)-type Ca^{2+} channel blockers were negative and thus failed to demonstrate an involvement of Ca^{2+} channels in the excitatory action of *P. nigriventer* venom on sensory neurons.

Vasodilator and exudative responses to antidromic nerve stimulation can be inhibited by opioid receptor agonists, presumably by activation of prejunctional receptors located on sensory neurons (Maggi, 1991). Our present observations that both local and systemic treatment of the animals with the μ-opioid receptor agonist, DAMGO, failed to reduce the venom-induced plasma extravasation seem surprising. However, the release of sensory neuropeptides through voltage-sensitive Ca^{2+} channels is subject to prejunctional inhibitory modulation by a number of transmitters (i.e., opioids) whilst Ca^{2+} channel-resistant release seems to be largely resistant to this inhibitory feedback (Maggi, 1991). Taken together, our results with Ca^{2+} channel blockers and DAMGO support previous findings and indicate that this venom acts independently of voltage-sensitive Ca^{2+} channels. Several other substances such as local anaesthetics and Na^+ channel blockers have recently been found to inhibit the release of sensory neuropeptides by acting elsewhere on other systems including sensory nerve conduction (Maggi, 1991). In our study lignocaine had no effect on plasma extravasation induced by venom. This is in contrast to previous studies where lignocaine significantly inhibited the neurogenic vasodilatation evoked in rat facial skin by trigeminal ganglion stimulation (Escott et al., 1995). Evidence shows that a high proportion of capsaicin-sensitive dorsal root ganglion neurons express two classes of Na^+ channels that may be distinguished by their sensitivity to tetrodotoxin (Arbuckle and Docherty, 1995; Akopian et al., 1996). We investigated the role of Na^+ channels in venom-evoked plasma extravasation, using both Na^+ channel blockers, tetrodotoxin, and the tetrodotoxin-resistant Na^+ channel blocker, carbamazepine. The use of tetrodotoxin and carbamazepine, which have also been proposed to modulate the neurogenic inflammation by acting on sensory nerve conduction, failed to elucidate the mechanisms by which *P. nigriventer* venom activates sensory nerves. The results thus excluded a role for Na^+ in the activation of sensory nerves by venom.

Our interests in mechanisms relevant to the activation of sensory nerves have also focussed on the effect of the vanilloid receptor antagonist, capsazepine (Bevan et al., 1992). We have previously demonstrated that depletion of sensory nerves by a neonatal capsaicin depletion regime leads to inhibition of venom-induced plasma extravasation (Costa et al., 1997). Our results showed that the capsaicin receptor antagonist, capsazepine, inhibited venom-induced plasma extravasation in the rat dorsal skin but failed to reduce capsaicin-evoked plasma extravasation in the rat hind paw. Although capsazepine has been shown to inhibit capsaicin-evoked bronchoconstriction and local neuropeptide release in the guinea-pig lung (Lou and Lündberg, 1992) as well as CGRP release from in vitro models such as superfused rat paw skin (Kilo et al., 1997) and rat soleus muscle (Santicioli et al., 1993), the failure of capsazepine to inhibit the capsaicin-induced responses in our study is still unclear. The lack of effect of capsazepine on the capsaicin-induced plasma protein extravasation in the lower airways corroborates our finding (Auberson and Lündberg, 1993) and may suggest that differences exist regarding capsazepine as a vanilloid receptor antagonist. Whether this reflects variation between vanilloid receptor subclasses (C- and R-type) in tissues of different species and preparations (Bevan et al., 1992; Caterina et al., 1997)

is yet to be elucidated. In this respect, a recent study showed the existence of capsazepine-insensitive vanilloid receptors on rat trigeminal ganglion neurons (Liu et al., 1998). As recently pointed out by Szallasi and Blumberg (1999), the results with capsazepine should be interpreted with caution since positive effects are not necessarily mediated by vanilloid receptors, nor do negative data rule out the involvement of these receptors. Although capsazepine has been shown to have nonspecific actions at high concentrations (Santicioli et al., 1993), it is unlikely that reduction of venom-induced plasma extravasation by this compound reflects nonspecific actions, since it had no effect on the plasma extravasation evoked by GR73632 and bradykinin.

One might also expect that ruthenium red, a compound known to inhibit the plasma extravasation induced by capsaicin application (Newbold and Brain, 1995), would block venom-induced plasma extravasation. Ruthenium red selectively modulates capsaicin-induced responses via a direct blocking effect on cation channels opened by capsaicin (Maggi, 1995). Our findings that ruthenium red inhibited capsaicin-induced plasma extravasation in the rat hind paw, but had no effect on venom-induced plasma extravasation in the rat dorsal skin indicates that venom does not act on these latter cationic channels. The results from the use of agents that are known to interact directly with the capsaicin receptor are suggestive that the in vivo pathway of inflammation in response to activation of vanilloid receptors needs reevaluation.

In the final series of experiments we investigated the modulatory effect of the purines, ADP and ATP. Purines are known to be released from vascular cells including endothelial cells in response to injury (Lundberg, 1996), and also to influence sensory nerve activation (Sawynok and Reid, 1997; Boarder and Hourani, 1998). The finding of subtypes of adenosine A receptor and P_2 purinoceptors (activated by ATP and ADP) expressed in sensory neurons has led to the exploration of the sources of ATP and their metabolites acting to modulate neurogenic mechanisms (Burnstock and Wood, 1996). Although previous studies have suggested that purines are implicated in the neurogenic inflammatory and vasodilatation response (Holton, 1959; Chahl, 1976, 1977; Burnstock and Wood, 1996), their role in the modulation of vascular permeability is still unclear. For instance, bradykinin-induced plasma extravasation in rat joints was significantly increased by concomitant injection of ATP (and adenosine A_2 receptor agonists), while in experimental arthritis, ATP (and adenosine) inhibited the inflammatory responses (Green et al., 1991). We found that the co-administration of either ATP or ADP partially reduced the venom-induced plasma extravasation. Interestingly, at higher doses, ATP and ADP significantly reduced venom-induced permeability but increased permeability when injected alone in rat dorsal skin. These results suggest that the activation of capsaicin-sensitive primary afferent neurons in the dorsal rat skin by *P. nigriventer* venom is partially modulated in an inhibitory manner via P_2 purinoceptors subunits.

In conclusion, the present results are of interest in that they suggest that this pro-inflammatory pain-producing venom may contain a factor that acts via the vanilloid receptor in capsaicin-sensitive primary afferent neurons and that P_2 purinoceptors may also play a modulatory role.

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