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Characterization of the mechanisms underlying the inflammatory response to *Polistes lanio lanio* (paper wasp) venom in mouse dorsal skin

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ABSTRACT

Stings by *Polistes* wasps can cause life-threatening allergic reactions, pain and inflammation. We examined the changes in microvascular permeability and neutrophil influx caused by the venom of *Polistes lanio* a paper wasp found in southeastern Brazil. The intradermal injection of wasp venom caused long-lasting paw oedema and dose-dependently increased microvascular permeability in mouse dorsal skin. SR140333, an NK_1 receptor antagonist, markedly inhibited the response, but the NK_2 receptor antagonist SR48968 was ineffective. The oedema was reduced in capsaicin-treated rats, indicating a direct activation of sensory fibres. Dialysis of the venom partially reduced the oedema and the remaining response was further inhibited by SR140333. Mass spectrometric analysis of the venom revealed two peptides (QPPTPPEHRFPGLM and ASEPTALGLPRIFPGLM) with sequence similarities to the C-terminal region of tachykinin-like peptides found in *Phoneutria nigriventer* spider venom and vertebrates. Wasp venom failed to release histamine from mast cells *in vitro* and spectrofluorometric assay of the venom revealed a negligible content of histamine in the usual dose of *P. l. lanio* venom (1 nmol of histamine/7 μg of venom) that was removed by dialysis. The histamine H_1 receptor antagonist pyrilamine, but not bradykinin B_1 or B_2 receptor antagonists, inhibited venom-induced oedema. In conclusion, *P. l. lanio* venom induces potent oedema and increases vascular permeability in mice, primarily through activation of tachykinin NK_1 receptors by substance P released from sensory C fibres, which in turn releases histamine from dermal mast cells. This is the first description of a neurovascular mechanism for *P. l. lanio* venom-mediated inflammation. The extent to which the two tachykinin-like peptides identified here contribute to this neurogenic inflammatory response remains to be elucidated.

1. Introduction

Stings by wasps and bees, including Brazilian species, pose a public health problem because of the widespread occurrence of these insects and the immediate, life-threatening systemic allergic reactions that they can cause, in addition to pain, oedema, erythema and ocular lesions (Nakajima et al., 1986; Castro et al., 1994; Fran et al., 1994; Arcieri et al., 2002; Beleboni et al., 2004; Bilo et al., 2005; Severino et al., 2006). Although rare, wasp stings can also cause cardiovascular disturbances and death (Nakajima et al., 1986), possibly as a result of severe anaphylaxis (Fitzgerald and Flood, 2006).

The venoms of social paper wasps (*Polistes* spp.; Hymenoptera: Vespidae) are a complex mixture of enzymatic and non-enzymatic proteins (principally allergen 5, hyaluronidase and phospholipases) (Habermann, 1972;

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Ring et al., 1978; Bernheimer et al., 1982; Schmidt et al., 1986; Watala and Kowalczyk, 1990; Hoffman, 1993; King et al., 1996; Moawad et al., 2005), as well as peptides such as antimicrobial peptides, kinins, mast cell degranulating peptides and mastoparans (Arbuzova and Schwarz, 1999; Zhang et al., 2003; Wang et al., 2006), low molecular mass components such as amines, and a variety of volatile substances (Bruschini et al., 2006). Some of these substances cause mast cell degranulation (Findlay et al., 1977), neutrophil migration and the production of pro-inflammatory mediators such as cytokines and nitric oxide (Petricevich, 2004; Souza et al., 2005; de Paula et al., 2006). The pain and inflammatory responses caused by wasp venoms may involve components (kinins, neuropeptides and phospholipases) and mechanisms, e.g., ion channel activation, similar to those described for other arthropod venoms (Costa et al., 2000, 2003; Zanchet and Cury, 2003).

The paper wasp *Polistes lanio lanio* is a common species in southeastern Brazil and lives in small colonies of several individuals around buildings and human dwellings. Despite the common occurrence of this species, the pharmacology and biological activities of its venom are virtually unknown. In this work, we examined the changes in dorsal skin vascular permeability (based on the extravascular accumulation of intravenously injected ^{125}I-albumin), the increase in myeloperoxidase activity (a well-standardized marker of neutrophil recruitment in tissue) and the paw oedema caused by *P. l. lanio* venom. We also identified two tachykinin-like peptides by mass spectrometry.

2. Material and methods

2.1. Drugs and chemicals

Bovine serum albumin (BSA), bradykinin, capsaicin, carrageenan, compound 48/80, des-Arg^9-[Leu^8]-bradykinin, *o*-dianisidine dihydrochloride, hexadecyl trimethylammonium bromide (HTAB), histamine, hydrogen peroxide, icatibant (Hoe 140), neurokinin A (NKA), *o*-phthaldialdehyde, Percoll, substance P (SP) and urethane were from Sigma Chemical Co. (St. Louis, MO, USA). The bradykinin B_1 receptor antagonist des-Arg^9-[Leu^8]-bradykinin was provided by Dr. João Bosco Pesquero (UNIFESP, São Paulo, SP, Brazil) and the tachykinin receptor antagonists (*S*)1-(2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl) piperidin-3-yl]ethyl) -4-phenyl-1-azoniabicyclo[2.2.2]octane chloride (SR140333) and (*S*)-*N*-methyl-*N*-[4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide (SR48968) were kindly donated by Dr. Estelle Weinling (Sanofi-Aventis, Chilly-Mazarin, France). [^{125}I]-Bovine serum albumin (specific activity = 0.037 MBq/μg) was supplied by the Instituto de Pesquisas Nucleares (IPEN, São Paulo, SP, Brazil). Lidocaine gel (2%) was purchased from Cristália Produtos Químicos Ltda (Itapira, São Paulo, Brazil).

2.2. Animals

Isogenic male and female C57BL/6 mice (8–12 weeks old, 23–32 g) were obtained from the Animal House of the Department of Immunology (ICB/USP, São Paulo, SP, Brazil) and male and female Wistar rats (200–250 g) were obtained from the Multidisciplinary Center for Biological Investigation at the State University of Campinas (CEMIB/UNICAMP, Campinas, SP, Brazil). The mice and rats were housed at 22–23 °C with free access to rodent chow and water. The experiments described here were approved by the Animal Care and Use Committee of the University of São Paulo (protocol no. 127) and were performed according to guidelines of the Brazilian College for Animal Experimentation (COBEA). Animals of both sexes were used because there are no significant differences in the inflammatory responses of dorsal skin in male and female mice or rats (Cao et al., 1999; Costa et al., 1997).

2.3. Wasp venom extraction

Colonies of *P. l. lanio* (Giannotti and Machado, 1994) were collected locally on the university campus at UNICAMP, killed with CO_2 gas and stored at −20 °C. When required, the wasps were thawed on ice and the venom sacs were removed by gently pulling on the sting with forceps so as to withdraw the venom apparatus. The venom sacs were subsequently separated from the stings and venom was obtained by gently compressing the venom sacs to expel the contents into a beaker, after which the venom sacs were washed by gently stirring in distilled water (approximately 200 sacs/20–30 ml) for 2 h at 4 °C to ensure maximum venom recovery. The suspension was centrifuged and the supernatant was lyophilized and stored at −20 °C.

2.4. Venom dialysis

When required, low molecular mass components such as catecholamines, free amino acids, histamine, serotonin and small peptides were removed from the venom by dialysis for up to 24 h at 4 °C against 2 l of distilled water using dialysis tubing with a nominal molecular mass cut-off of 2 kDa (Costa et al., 1997).

2.5. Microvascular permeability in rodent dorsal skin

Mice were anaesthetised with urethane (25% w/v, 100 μl/10 g, i.p.) and the dorsal skin was shaved prior to the intradermal (i.d.) injection (maximum of six sites/mouse) of the test substances using a randomized scheme. ^{125}I-Bovine serum albumin (^{125}I-BSA, 0.042 MBq/mouse) was injected intravenously (i.v.) via the tail vein. Five minutes later, *P. l. lanio* venom (0.3–30 μg/site), agonists (bradykinin, histamine or SP and antagonists, or vehicle (Tyrode) solution, was injected into the dorsal skin in a fixed volume of 50 μl. Thirty minutes after the i.d. injection of test agents, a blood sample was obtained via cardiac puncture (1 ml) and centrifuged at 6000 g for 4 min to obtain plasma. The mice were killed via an overdose of urethane and cervical dislocation. The dorsal skin was removed and the injected sites punched out and weighed. The radioactivity in the plasma and skin samples was counted in a γ-counter and the amount of plasma extravasation in the skin was determined by comparing the amount of radioactivity in each skin site with that present in plasma (1 ml) from the same mouse. The plasma extravasation was expressed as μl/g of wet tissue (Costa et al.,

2006a). In experiments in which rats were used instead of mice, the rats were anaesthetised with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) and the changes in microvascular permeability were quantified as described above, except that the plasma extravasation was expressed as μl/skin site (Costa et al., 2000).

2.6. Mouse paw oedema

To examine the time of onset and duration of venom-induced plasma extravasation, paw oedema was monitored for 4 h after a subplantar injection of venom (7 μg/paw, $n = 6$) and venom plus the tachykinin NK_1 receptor antagonist (SR140333; 1 nmol/paw, $n = 4$). In a parallel experiment, carrageenan (0.3 mg/paw, $n = 6$) was used as a positive control. The basal paw volume was assessed by hydroplethysmometry (model 7120 plethysmometer, Ugo Basile, Italy) immediately before subplantar injection and 30, 60, 120, 180 and 240 min thereafter. Paw volume was always assessed in a double blind manner and by the same investigator. The increase in paw volume (oedema, expressed in ml) in response to the test agents was calculated by subtracting the initial paw volume (basal) from the paw volume measured at each time point. The anti-inflammatory effect of SR140333 was evaluated by the trapezoidal method based on the area under the curve (AUC) of the hindpaw volume–time profiles. The mice were subsequently killed with an overdose of anaesthesia followed by cervical dislocation. The injected paw skin was removed, weighed and stored at −80 °C until analyzed for myeloperoxidase activity.

2.7. Myeloperoxidase (MPO) activity

The MPO activity in paw skin injected with venom and test agents was measured with minor modifications as described elsewhere (Bradley et al., 1982; Costa et al., 2006b). Each paw skin sample was weighed and placed in potassium phosphate buffer solution (KPB, 50 mM, pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (1 ml per 50 mg of wet tissue). Samples were snap frozen in liquid nitrogen, homogenised (45 s/cycle) and the homogenates were then centrifuged (10,000*g*, 15 min, 4 °C) after which 10 μl aliquots of the supernatants were transferred to a 96-well plate (in duplicate). Immediately thereafter, 200 μl of *o*-dianisidine solution (0.167 mg of *o*-dianisidine dihydrochloride/ml in 0.0005% hydrogen peroxide) was added to the wells. The absorbance at 460 nm was measured every 30 s over a period of 5 min using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The results were expressed as MPO units (U) per g of skin (1 U of MPO was defined as the amount of enzyme responsible for the degradation of 1 μmol of hydrogen peroxide/min at 25 °C).

2.8. Mast cell isolation and degranulation assay

Rats were exsanguinated under halothane anaesthesia and 10 ml of Krebs–Ringer phosphate solution (KRP; pH 7.4; composition, in mM: NaCl 150, KCl 6.1, Na_2HPO_4 10, $MgSO_4$ 1.5, $CaCl_2$ 42.9 and glucose 5.6.) was injected into the peritoneal cavity. The abdomen was carefully massaged and the injected fluid was removed along with the peritoneal exudates, placed in polypropylene tubes and centrifuged (300 *g*, 5 min, 4 °C). Mast cells from the peritoneal cavity of 10 rats were purified on a Percoll gradient as described previously (Enerback and Svenson, 1980). The resulting cell pellet (of which mast cells comprised ~10%) was gently resuspended in KRP solution (1 ml), layered over the isotonic Percoll gradient and kept at room temperature for 10 min prior to centrifugation (150 *g*, 25 min, 4 °C). The gradient zone containing mast cells was removed and washed twice in KRP solution. The purity of the mast cell suspensions (95%) was determined based on the routine microscopic analysis of Cytospin® preparations stained with May–Grünwald Giemsa. The mast cell viability (98%) was assessed based on the exclusion of trypan blue dye (0.1%).

Aliquots (0.5 ml) of mast cell suspension containing 4×10^5 cells/ml were warmed to 37 °C for 10 min and *P. l. lanio* venom (3–100 μg/ml) or compound 48/80 (1 μg/ml) was added to the suspension to give a final volume of 1.0 ml. Following a 15 min incubation, the reaction was stopped by immersing the tubes in ice cold-water. The cells were then centrifuged (300 *g*, 10 min, 4 °C) and the supernatant was removed for histamine quantification. The pellet was resuspended in KRP solution (1.0 ml) and boiled at 100 °C for 10 min in order to release the residual histamine. The histamine concentration was determined spectrofluorometrically (see next section) and the results expressed as a percentage of the total cellular content of this amine.

2.9. Spectrofluorometric quantification of venom histamine content

The histamine content of *P. l. lanio* venom was determined spectrofluorometrically by the method of Shore et al. (1959). The method is based on the formation of a fluorescent product following the reaction of histamine with *o*-phthaldialdehyde. Briefly, 1 ml aliquots from serial dilutions of crude or dialysed venom (final concentrations of 5–100 μg/ml) in KRP solution were mixed with 300 μl of NaOH solution (1 mol/l) containing *o*-phthaldialdehyde (1%; 80 μl). After 4 min, the reaction was stopped by adding 150 μl of HCl (3 mol/l) and the solution was mixed vigorously. The histamine concentration of the venom samples was determined spectrofluorometrically (excitation: 360 nm and emission: 450 nm;) by comparing the reaction rate (fluorescence) of the samples with that of a standard curve of histamine (5–150 μg/ml). The calibration curve was linear ($r^2 = 0.998$) over the range of 5–100 μg of venom/ml. The results were expressed as percentage of the dry weight of venom.

2.10. Denervation by treatment with capsaicin

To assess the direct involvement of capsaicin-sensitive neurons in the venom-induced plasma extravasation, capsaicin denervation was done in neonatal rats on the second day of life (body weight: 7–8 g), as previously described (Jancsó et al., 1977; Costa et al., 1997), with minor changes. Briefly, the rostral part of the back of neonatal rats

was cleaned with 70% ethanol and ~50 mg of topical anaesthetic (2% lidocaine gel) was applied. After 10 min, the rats received a single subcutaneous (s.c.) injection of capsaicin (50 mg/kg) or corresponding volume (100 μl) of vehicle (1:1:8; ethanol:Tween 80:0.9% NaCl solution, v/v) and were used 60–70 days later.

2.11. Mass spectrometric analysis of venom peptides

The venom peptide content was analyzed by bidimensional CapLC-nano-electrospray ionization mass spectrometry (nanoESI-MS/MS) using a quadrupole time-of-flight hybrid mass spectrometer (Q–Tof Waters, Micromass, Manchester, UK) equipped with a nano Z-spray source operating in positive ion mode coupled to a capillary liquid chromatographic system (CapLC, Waters, Manchester, UK).

Initially, lyophilized venom was dissolved in distilled water (0.5 mg/ml) and centrifuged (4000 *g*, 1 h, 4 °C) prior to filtration through an Amicon Ultra-4 centrifugal filter (5 kDa cut-off; Millipore Corporation, Belford, MA, USA). The resulting peptide-rich filtrate was lyophilized and stored at −20 °C. The venom sample was resuspended (final concentration, 1 mg/ml) in 5% acetonitrile in distilled water containing 0.1% formic acid. An aliquot of this solution (3 μl) was injected into the system through the CapLC autosampler coupled to a Waters Opti-Pak C_{18} trap column followed by a NanoEase capillary column (i.d. 75 μm) packed with C_{18} silica (Waters, Manchester, UK). Peptides were eluted with a water:acetonitrile gradient (flow rate: 200 nl/min) over 60 min, starting with an aqueous solution containing 5% acetonitrile that increased linearly up to 50% acetonitrile; after pumping with this concentration of acetonitrile for 5 min, the linear gradient was continued up to 85% acetonitrile (all of these solutions contained 0.1% formic acid throughout the chromatographic run).

The ionization conditions for the mass spectrometer included a capillary voltage of 3 kV, a cone voltage of 50 V, an extractor voltage of 4 V, a low mass resolution (LM) of 5 V and a high mass resolution (HM) of 15 V, with different collision energies, depending on the mass and charge state (m/z) of the precursor ions. The source temperature was 80 °C and the desolvation gas was N_2 at a flow rate of 80 l/h. Argon was used for ion fragmentation in the collision cell and collisional cooling. Calibration was done with 0.1% phosphoric acid in acetonitrile (1:1, v/v) over a mass range of 50–2500 m/z.

The spectra were acquired with the TOF analyzer in "V-mode" (Tof kV = 7.2) and the MCP voltage set at 2100 V. Acquisition was assessed in data-dependent acquisition (DDA) mode, and double and triple charged ions were analyzed by MS/MS. The product-ion MS/MS spectra were deconvoluted using MaxEnt3 and sequenced manually with the PepSeq application included in MassLynx (Waters, Manchester, UK).

2.12. Peptide sequence alignment and phylogenetic analysis of tachykinin-like peptides

The peptide sequences detected in the venom were used to search the SWISSPROT and UNIPROT-TREMBL databanks (http://br.expasy.org/sprot/) for similar sequences. The amino acid sequences retrieved were aligned using CLUSTAL W 1.83v and a phylogenetic tree was constructed and displayed with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) using the output file dendogram "dnd" from CLUSTAL W (data not shown; Cogo et al., 2006).

2.13. Statistical analysis

The results were expressed as the mean + SEM of n experiments and statistical comparisons were done by using Student's unpaired t-test or ANOVA followed by Bonferroni's modified t-test. A value of $P < 0.05$ indicated significance. All statistical calculations were done with Prism 4.0 software (GraphPad Inc., San Diego, CA, USA).

3. Results

3.1. Venom-induced increase in vascular permeability

The i.d. injection of non-dialysed *P. l. lanio* venom (1–30 μg/site, $n = 3$–8) produced a dose-dependent increase in microvascular permeability (plasma extravasation) compared to the vehicle (Tyrode)-injected sites in mouse dorsal skin 30 min after injection (Fig. 1). Dialysed *P. l. lanio* venom (3–30 μg/site, $n = 4$) also produced potent plasma extravasation, although this was generally less marked than that with non-dialysed venom (Fig. 1). In subsequent protocols, doses of 7–10 μg were used, depending on the activity of the venom batches.

3.2. Time course of venom-induced plasma extravasation in mouse hindpaw and MPO activity

The subplantar injection of *P. l. lanio* venom (7 μg; $n = 6$) caused substantial paw oedema with a bell-shaped time curve that was maximal after 120 min (Fig. 2A). Based on the area under the curve from zero to 4 h post-venom injection ($AUC_{[0-4h]}$), the tachykinin NK_1 receptor antagonist, SR140333 (1 nmol/site; $n = 4$) significantly attenuated venom-induced oedema formation ($P < 0.001$, Fig. 2B). The subplantar injection of carrageenan (0.3 mg/

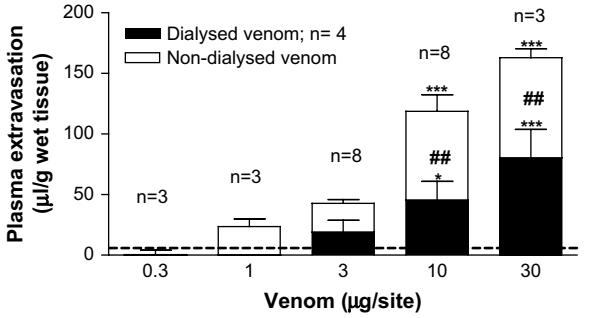

Fig. 1. Increase in vascular permeability caused by non-dialysed and dialysed *P. l. lanio* venom in mouse dorsal skin. The results are expressed as microlitres of plasma extravasated/g of wet tissue. The columns represent the mean ± SEM of 3–8 mice. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared to Tyrode solution alone (control, dashed line). Data were analyzed by ANOVA followed by Bonferroni's modified t-test. $\#\#P < 0.001$ compared to non-dialysed venom (Student's unpaired t-test).

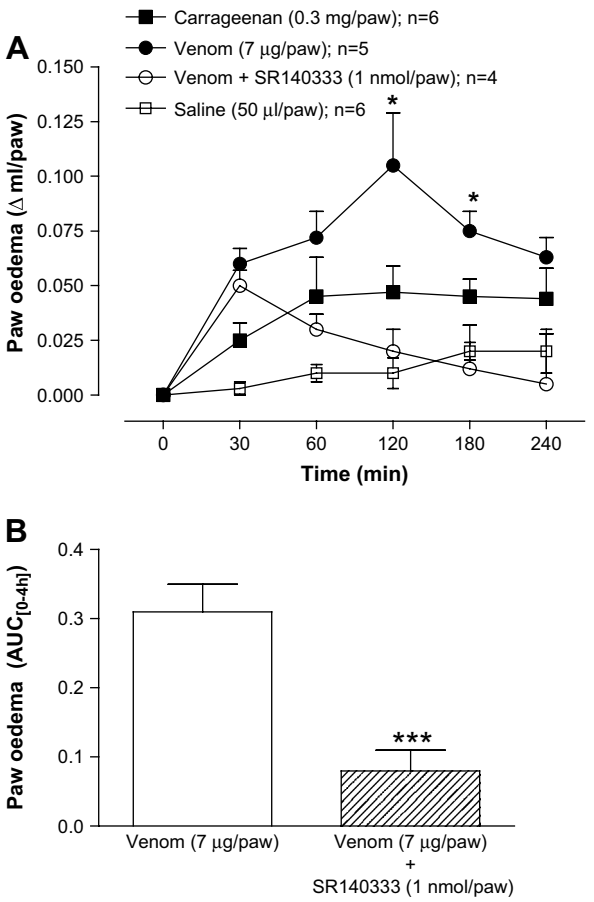

Fig. 2. A. Time course of mouse hindpaw oedema induced by *P. l. lanio* venom and carrageenan. The oedema was expressed as the change in paw volume and the points represent the mean ± SEM of 4–6 mice for carrageenan (■; 0.3 mg/paw) and for venom (7 μg/site) in the absence (●) and presence (○) of SR140333 (1 nmol/paw). The control group (vehicle saline) is represented by (□). $^{*}P < 0.05$ compared to venom + SR140333. Data were analyzed by ANOVA followed by Bonferroni's modified *t*-test. B. Area under the curve from zero hour to 4 h post-injection ($AUC_{[0-4h]}$) for venom-injected paws in the absence and presence of SR140333. The AUC values were calculated from the time curves shown in panel A. The columns represent the mean ± SEM of 4–6 mice. $^{***}P < 0.001$ compared to venom alone. Data were analyzed by Student's unpaired *t*-test.

paw; $AUC_{[0-4h]}$: 0.14 ± 0.03 ml h; $n = 6$), a well-known pharmacological tool used as a comparative control here, produced only half of the oedematogenic response seen with venom, but was still significantly different ($P < 0.05$) from the response caused by vehicle alone ($AUC_{[0-4h]}$: 0.05 ± 0.02 ml h).

To determine whether there was any neutrophil infiltration, we examined the increase in tissue MPO activity 4 h after the subplantar injection of *P. l. lanio* venom (7 μg/paw, $n = 4$). The injection of venom enhanced MPO activity in the ipsilateral paws as compared to the contralateral paws ($P < 0.05$, Fig. 3). The subplantar injection of carrageenan (0.3 mg/paw, $n = 5$) also markedly increased MPO activity in ipsilateral versus contralateral paws ($P < 0.05$, Fig. 3; $n = 6$).

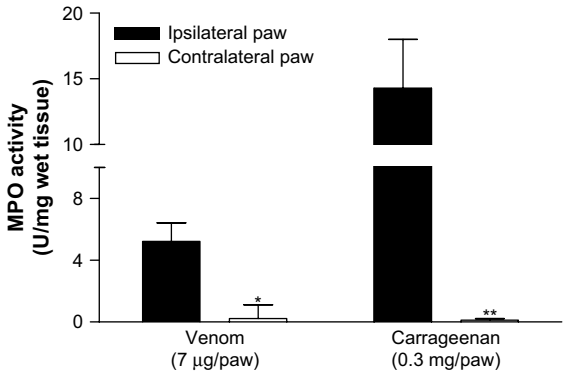

Fig. 3. Myeloperoxidase (MPO) activity in mouse hindpaws injected with *P. l. lanio* venom. The columns represent the mean ± SEM of $n = 4$ mice for venom (7 μg/site) and $n = 6$ mice for carrageenan (0.3 mg/paw) and their respective contralateral paws. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to ipsilateral paws. Data were analyzed by Student's unpaired *t*-test.

3.3. Lack of participation of kinins in venom-induced plasma extravasation

Bradykinin produced a dose-dependent increase in microvascular permeability in C57BL/6 mice (6.4 ± 2.3, 45.2 ± 11* and 87.6 ± 15* μl/g, for bradykinin doses of 1, 3, and 10 nmol/site, respectively; $n = 6$ each; $^{*}P < 0.05$ compared to its vehicle, Tyrode: 4 ± 9.2 μl/g). Pretreatment with icatibant (Hoe 140, 0.8 nmol/kg, i.v.), a bradykinin B_2 receptor antagonist, 5 min before bradykinin (3 nmol/site) significantly inhibited ($P < 0.05$) the plasma protein extravasation produced by this peptide. In contrast, plasma extravasation induced by *P. l. lanio* venom (7 μg/site) was not significantly affected by Hoe 140 (Fig. 4A).

The plasma protein extravasation caused by *P. l. lanio* venom and bradykinin was not mediated by the activation of bradykinin B_1 receptors since the B_1 receptor antagonist des-Arg^9-Leu^8-bradykinin (3 μmol/kg, i.v.; Cao et al., 2000) did not significantly change the responses evoked by these agents (Fig. 4B). As a negative control, the plasma protein extravasation mediated by histamine (30 nmol/site) was not affected by either Hoe 140 (83 ± 25 μl/g and 103 ± 24 μl/g for histamine in the absence and presence of antagonist, respectively; $n = 4$ each) or des-Arg^9-[Leu^8]-bradykinin (3 μmol/kg, i.v., given 5 min before; 88 ± 39.8 μl/g and 128 ± 13 μl/g for histamine in the absence and presence of antagonist, respectively; $n = 4$ each).

3.4. Involvement of tachykinin NK_1 and NK_2 receptors in venom-induced plasma extravasation

SP is one of the most important tachykinins involved in neurogenic inflammation, and its action is mediated mainly by NK_1 receptors. The local treatment of dorsal skin with SR140333 (Costa et al., 1997), an NK_1 receptor antagonist, blocked SP (300 pmol/site)-induced plasma extravasation and significantly attenuated the increase in plasma extravasation caused by non-dialysed *P. l. lanio* venom (Fig. 5A).

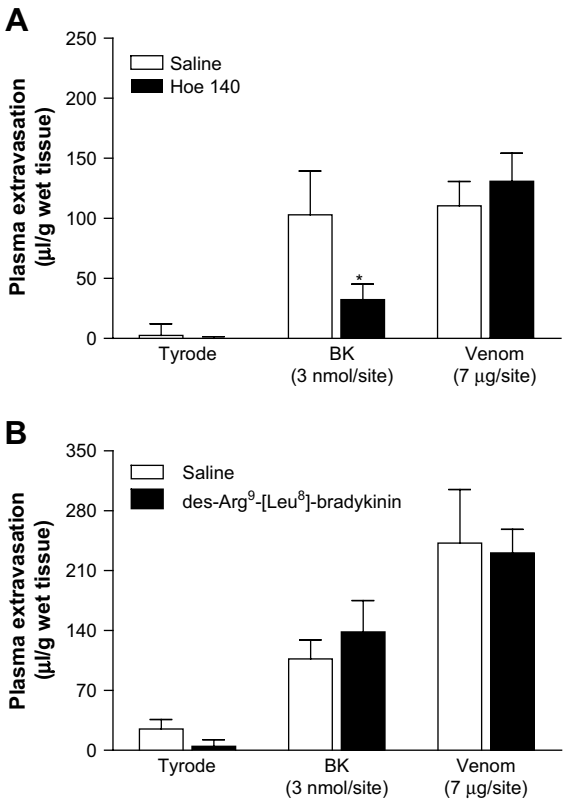

Fig. 4. Lack of effect of bradykinin (BK) B_1 and B_2 receptor antagonists on *P. l. lanio* venom (7 µg/site)-induced plasma extravasation in mouse dorsal skin. Panel A shows the effect of the B_2 receptor antagonist Hoe 140 (0.8 nmol/kg, i.v., given 5 min before; $n = 9$) and panel B shows the response to the B_1 receptor antagonist des-Arg^9-[Leu^8]-bradykinin (3 µmol/kg, i.v., given 5 min before; $n = 8$) on bradykinin- and venom-induced plasma extravasation. Control mice received only the vehicle used to dissolve the antagonists (0.9% saline, 0.05 ml/mouse, i.v.; $n = 6$ for panel A and $n = 8$ for panel B). The white columns represent the responses to bradykinin and venom alone (controls) and the black columns represent mice treated with antagonists. The columns represent the mean ± SEM $^*P < 0.05$ compared to bradykinin alone. Data were analyzed by ANOVA followed by Bonferroni's modified *t*-test.

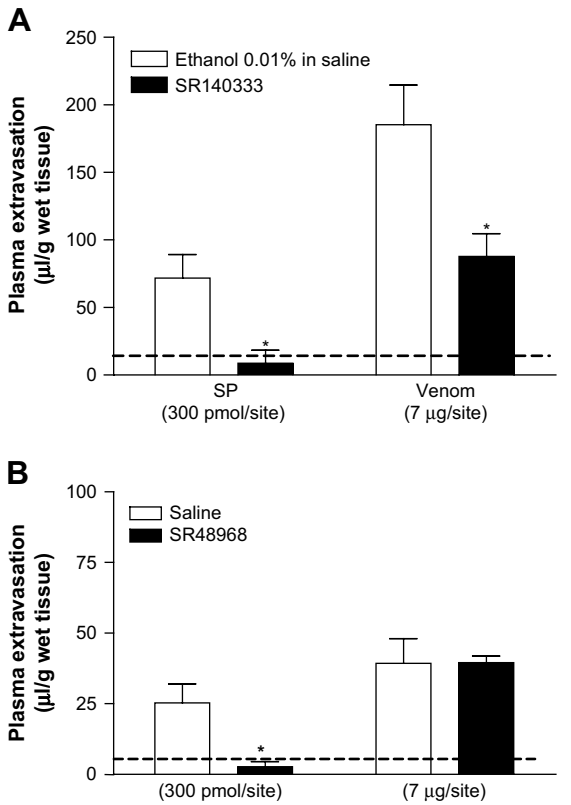

Fig. 5. Effect of tachykinin receptor antagonists on *P. l. lanio* venom-induced plasma extravasation. A. Inhibition of plasma extravasation by the NK_1 receptor antagonist SR140333 (1 nmol/site) co-injected with venom or substance P (SP). B. Lack of effect of the NK_2 receptor antagonist SR48968 (3 mg/kg, i.v.) on the response to venom. Note that the neurokinin A (NKA) response (positive control) was inhibited by SR48968. The white and black columns represent the responses in the absence (control) and presence of antagonist, respectively. The dashed line indicates the response to Tyrode, and the columns represent the mean ± SEM of *n* animals ($n = 5$ for panel A and $n = 4$ for panel B). $^*P < 0.05$ compared to the respective non-treated control. Data were analyzed by ANOVA followed by Bonferroni's modified *t*-test.

The remaining oedema evoked by dialysed *P. l. lanio* venom (30 µg/site), as seen in Fig. 1, was further markedly reduced by co-treatment with SR140333 (84.3 ± 18.4 and $37.2 \pm 7^*$ µl/g, for dialysed *P. l. lanio* venom alone and in the presence of SR140333, respectively; $n = 5$; $^*P < 0.05$ compared to dialysed *P. l. lanio* venom alone).

The involvement of tachykinin NK_2 receptors in *P. l. lanio* venom-induced oedema was investigated using SR48968, a selective antagonist of this receptor (Lam and Wong, 1996). Neurokinin A (NKA; 300 pmol/site), a preferential NK_2 receptor agonist, caused discrete plasma extravasation in mouse dorsal skin. In mice pretreated with SR48968 (3 mg/kg, i.v., given 5 min before), there was significant inhibition of NKA-induced plasma extravasation without affecting the venom-induced response (Fig. 5B).

3.5. Effect of C-fibre depletion on venom-induced plasma extravasation

The i.d. injection of *P. l. lanio* venom (7 µg) in rat dorsal skin resulted in plasma protein extravasation that was less pronounced than that seen in mouse skin (Table 1; compare with Fig. 4). In rats treated with capsaicin (50 mg/kg; s.c.) when neonates, there was partial but significant inhibition of *P. l. lanio* venom-induced plasma extravasation when compared to rats not treated with capsaicin (Table 1). The co-injection of SR140333 (1 nmol/site) inhibited the venom-induced response in rats not treated with capsaicin and further reduced the residual venom-induced oedema in rats treated with capsaicin (Table 1).

3.6. Involvement of histamine in venom-induced plasma extravasation and spectrofluorometric determination of histamine in P. l. lanio venom

The co-injection of pyrilamine (0.1 µg/site), an H_1 receptor antagonist, markedly inhibited the plasma extravasation caused by histamine (10 nmol/site; $n = 4$) and *P. l. lanio* venom (7 µg/site). The response to pyrilamine alone did not differ significantly from that produced by Tyrode solution (Fig. 6). In contrast to this involvement of

Table 1
Plasma extravasation caused by *P. l. lanio* venom in capsaicin-treated rats. The results are expressed as microlitres of plasma extravasation/g of wet tissue and are the mean ± SEM of 4–10 rats. $^{*}P < 0.05$ compared to the control group (capsaicin-vehicle). Data were analyzed by ANOVA followed by Bonferroni's modified *t*-test.

Agents	Vehicle-treated	Capsaicin treated	Change in plasma extravasation (%)
Venom (7 μg/site)	18.12 ± 8.20 ($n = 7$)	7.44 ± 1.43* ($n = 10$)	−58.9
Venom + SR140333 (1 nmol/site)	3.63 ± 4.86 ($n = 4$)	0.02 ± 0.81 ($n = 4$)	−99.5
Tyrode solution	2.41 ± 0.98 ($n = 7$)	2.44 ± 1.10 ($n = 10$)	+ 1.24

histamine in venom-induced plasma extravasation, *P. l. lanio* venom (3–100 μg/ml) did not stimulate the release of histamine from rat peritoneal mast cells when compared with the spontaneous release from mast cells incubated with KRP alone. On the other hand, incubation with compound 48/80 (positive control; 1 μg/ml) resulted in a marked release of histamine ($P < 0.001$; Table 2).

Spectrofluorometric analysis showed that *P. l. lanio* venom contained a small amount of histamine (1 nmol of histamine/7 mg of venom), whereas no histamine was detected in dialysed venom.

3.7. *Mass spectrometric analysis of* P. l. lanio *venom peptides*

The analysis of a peptide-enriched filtrate of *P. l. lanio* venom by bidimensional LC-nanoESI-MS/MS revealed the presence of 20 peptides with molecular masses ranging from 492.4 to 2606.7. Screening of these peptides to detect the presence of the immonium ion *m/z* 104 of methionine (an amino acid present in many tachykinin-like peptides) resulted in the identification of two peptides, PllTkP-I (1602.80 Da, theoretical p*I* = 6.75; UniProt Knowledgebase – UniProtKB accession number P85879) and PllTkP-II (1768.95 Da, theoretical p*I* = 6.05; UniProtKB accession number P85880), that shared homology with the C-terminal sequence FXGLM-NH_2 present in tachykinin-like peptides from several invertebrates and vertebrates, including peptides found in *P. nigriventer* spider venom (Pimenta et al., 2005) which causes neurogenic inflammation in rodents (Palframan et al., 1996; Costa et al., 1997, 2000; Zanchet and Cury, 2003). Mass spectra for these two peptides (*Polistes l. lanio* tachykinin-like peptides **I** and **II**) are provided in the Supplementary material. Table 3 compares the sequence homology of PllTkP-I with several tachykinins and tachykinin-like peptides from various sources. Both of the wasp peptides had amidated C-terminals, in common with the other peptides shown in Table 3.

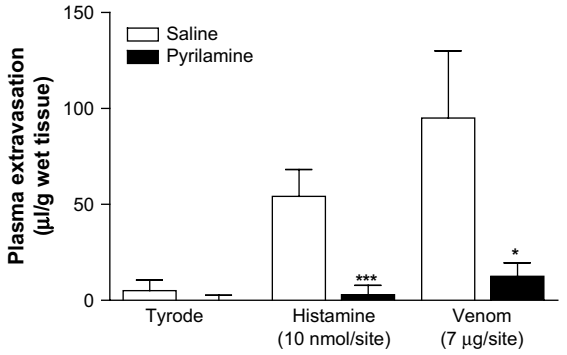

Fig. 6. Inhibition of *P. l. lanio* venom-induced plasma extravasation by the histamine antagonist pyrilamine. The white columns represent the responses to histamine and venom alone and the black columns show the effect in the presence of pyrilamine (0.8 μg/site). The columns represent the mean ± SEM of 5 mice. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to the respective non-treated control. Data were analyzed by ANOVA followed by Bonferroni's modified *t*-test.

As suggested by the sequence alignments in Table 3, phylogenetic analysis of the PllTkP-I and PllTkP-II sequences showed that these peptides were related to tachykinin-like peptides from spider venom (*P. nigriventer*) and to a variety of frog peptides (data not shown). However, excluding the C-terminal pentapeptide, the *P. l. lanio* venom peptides shared only limited homology with the *P. nigriventer* venom peptides. Whilst the latter peptides were rich in lysine and also contained arginine, the wasp peptides contained no lysine and only one arginine residue each; consequently, these peptides had a slightly acidic p*I* (~ 6.1–6.8) compared to the basic p*I* (~ 8.5–9.5) of the spider peptides; this acidic p*I* of the wasp peptides was however similar to that of eledoisin, a variety of frog peptides, and neuromedins K and L. In contrast to the spider venom peptides which contained no proline (except for PhTkP-XV that had one proline residue), the wasp peptides contained two or more prolines that accounted for ~36% of the amino acids in PllTkP-I; this high proline content is unusual among the tachykinin-like peptides shown in Table 3, including the frog peptides which contained 1–3 prolines (8.3–27% of amino acids); note that the presence of pyroglutamic acid (pGlu or Z) at the N-terminal of several of the frog peptides resulted in a conservative substitution for proline since pGlu no longer had the negative charge of Glu and was now a cyclic hydrophobic residue (Takai et al., 2006).

4. Discussion

The intradermal injection of *P. l. lanio* venom caused significant plasma protein extravasation in mouse and rat

Table 2
Polistes l. lanio venom does not degranulate rat peritoneal mast cells. The histamine release was expressed as a percentage of the total histamine content of the cells. The results are the mean ± S.E.M. a pool of cells from 10 rats. $^{***}P < 0.001$ compared to the basal (spontaneous) release. Data were analyzed by ANOVA followed by Bonferroni's modified *t*-test.

Stimulus	Concentration (μg/ml)	Histamine release (%)
Basal (KRP)	–	16.0 ± 3.1
Compound 48/80	1	89.1 ± 4.6***
P. l. lanio venom	3	21.2 ± 1.4
	10	12.1 ± 0.7
	30	10.6 ± 1.0
	100	18.7 ± 0.5

KRP – Krebs–Ringer phosphate solution.

Table 3
Sequence alignment of PllTkP-I from *P. l. lanio* venom with other tachykinin-like peptides from selected invertebrates (octopus) and vertebrates. The sequences were aligned as described in Section 2. Peptide identifications: eledoisin is from octopus (*Eledone aldovrandi*) salivary gland, frog skin peptides include hylambatin from *Kassina* (*Hylambates*) *maculatus*, kassinin from *Kassina senegalensis*, phyllomedusin from *Phyllomedusa bicolor*, physalaemin from *Physalaemus biligonigerus* (*fuscumaculatus*) and uperolein from *Uperuleia marmorata*. The ranatachykinins (RTK-A, B, C, D) are frog (*Rana catesbeiana*) brain/gut peptides. Neuromedins K and L (originally identified in porcine spinal cord) and substance P are tachykinins of mammalian origin. See Severini et al. (2002) and Satake and Kawada (2006) for references to the original reports describing the purification and identification of these peptides, and for the sequences of additional tachykinin-like peptides. PnTkP (I-XV) and PhM1 are tachykinin-like peptides from *Phoneutria nigriventer* spider venom (Pimenta et al., 2005). Grey shading = conserved (identical) amino acid residues, black shading = conservative substitutions, Z = pyroglutamic acid (pGlu).

Peptide	1 . . . . . 10	% Identity	% Homology	pI	MW (Da)
PllTkP-I	Q P P T P P E H R F P - G L M	100	100	6.75	1602.80
RTK-A	- K P S - P D - R F Y - G L M	50	64	8.59	1309.65
Hylambatin	D P P D - P D - R F Y - G M M	50	57	3.93	1439.58
Eledoisin	- Z P S - K D - A F I - G L M	36	57	5.84	1205.61
Phyllomedusin	- Z - N - P N- R F I - G L M	43	50	9.75	1188.61
Uperolein	- Z P D - P N- A F Y - G L M	43	50	3.80	1251.56
Physalaemin	- Z A D - P N- K F Y - G L M	36	50	5.83	1282.60
PnTkP-IX	E K K D - K D - R F Y - G L M	36	50	8.53	1528.77
PnTkP-XI	E K N D K K D - R F Y - G L M	36	50	8.53	1642.81
PnTkP-XIII	E K K D K K D - R F Y - G L M	36	50	9.41	1656.87
PnTkP-XV	E K K D K K D - R F P N G L V	36	50	9.53	1672.93
PnTkP-VI	E - - - K K D - R F L - G L M	36	50	8.69	1235.67
PnTkP-X	E K K D K K D - K F Y - G L M	29	50	9.31	1628.86
RTK-D	- K P N - P E - R F Y - AP M	43	43	8.59	1348.66
Substance P	- R P K - P N- N F F - G L M	43	43	11.00	1319.68
Kassinin	D V P K - S D - N F V - G L M	36	43	8.59	1348.66
PnTkP-XIV	E K K D K K D - R F Y - G L F	29	43	9.41	1672.89
PnTkP-VII	E - - - K K D - R F L - G L F	29	43	8.69	1251.70
PnTkP-XII	E K K D K K D - K F Y - G L F	21	43	9.31	1644.89
RTK-C	- - H N - P A- S F I - G L M	36	36	6.74	1085.53
RTK-B	- - Y K - S D - S F Y- G L M	29	36	5.83	1209.54
Neuromedin K	- - D M - H D - F F V - G L M	29	36	4.20	1210.52
Neuromedin L	- - H K - T D - S F V - G L M	29	36	6.74	1133.55
PhM1	- - K D K K D - C F Y - G L M	29	36	8.16	1346.64
PnTkP-V	E K K D K K D - R F - - - - -	14	29	9.53	1192.66
PnTkP-VIII	E K K D K K D - R F Y - - - -	14	29	9.41	1355.72
PnTkP-IV	E K K D K K D - K F - - - - -	7	29	9.41	1164.65
PnTkP-III	E K K D K K D - R - - - - - -	7	21	9.53	1045.59
PnTkP-II	E K K D K K D - K - - - - - -	0	21	9.41	1017.58
PnTkP-I	E K K D K K D - - - - - - - -	0	14	8.53	889.49

dorsal skin, with the oedematogenic response being more pronounced in mice. The subplantar injection of venom also produced marked hindpaw oedema in mice, with a bell-shaped time–response curve that was maximal after 2 h. In addition, there was a significant increase in the MPO activity of mouse hindpaw homogenates 4 h after venom administration. Together, these data indicate that *P. l. lanio* venom potently increases microvascular permeability (oedema) and causes neutrophil accumulation in mouse skin.

Our data are partially consistent with those reported by Eno (1997), who demonstrated that the venom of the African paper wasp, *Polistes fuscatus*, also caused dose- and time-dependent rat hindpaw oedema, with a maximum response after 5 h. More recent work has shown that the venom of a Brazilian paper wasp, *Polybia paulista*, stimulates a potent inflammatory response, including leukocyte infiltration and plasma protein extravasation, in the rat peritoneal cavity (de Paula et al., 2006). The general profile of these venoms is similar to the well-characterized inflammatory activity of spider (Costa et al., 1997; Ospedal et al., 2002) and snake (Farsky et al., 1997; Camargo et al., 2005; Fernandes et al., 2006) venoms in the cutaneous microvasculature of rats or mice.

The dialysis of *P. l. lanio* venom partially attenuated the increase in vascular permeability in mouse dorsal skin, suggesting that dialyzable, low molecular mass components (e.g. histamine) or biologically active tachykinin-like peptides contributed to the venom-mediated inflammatory response. On the other hand, the spectrofluorometric analysis showed a negligible content of histamine in the dose of *P. l. lanio* venom commonly used here; dialysis of the venom effectively eliminated this amine, as also reported for other *Polistes* spp. venoms (Lahiri and Sarangi, 1979; Eno, 1997). These results indicate that *P. l. lanio* venom contain a significant amount of histamine that could contribute to the immediate local oedema seen following venom injection. In contrast to this, histamine and/or serotonin have been implicated in the rodent acute hindpaw

oedema caused by the venoms of wasps such as *P. fuscatus* (Eno, 1997), *Vespula vulgaris* (Griesbacher et al., 1998), the black-bellied hornet (*Vespa basalis*) (Ho and Hwang, 1991) and *Polybia paulista* (de Paula et al., 2006), as well as bees (*Apis mellifera*) (Calixto et al., 2003).

Although the venom was devoid of significant quantities of histamine capable of producing oedema in the mouse dorsal skin, our findings show that the histamine H_1 receptor antagonist pyrilamine inhibited the increase in vascular permeability caused by i.d. injection of *P. l. lanio* venom, thus suggesting an indirect role for histamine in this response. In fact, histamine is the principal mediator released in skin during immediate allergic responses to bee and wasp venoms (Findlay et al., 1977; Koller et al., 1992; Bilo et al., 2005). Exogenous histamine or histamine released from mast cells induces oedema in humans and several animal species, including rodents, via the stimulation of histamine H_1 receptors (Inoue et al., 1997; Teixeira et al., 2006). Besides its effect in increasing microvascular permeability, the massive release of histamine by mast cells can also result in erythema, vasodilatation, hyperthermia, pruritus, and a hypersensitivity reaction characteristic of individuals sensitive to hymenopteran venoms (Calixto et al., 2003; Tavares, 2005; Fitzgerald and Flood, 2006). Thus, These findings suggest that histamine released from mast cells by endogenous SP could be a secondary mechanism involved in the *P. l. lanio* venom-induced plasma extravasation.

The endogenous tachykinins SP, NKA and NKB bind to tachykinin NK receptors by their NH_2-terminal portion. SP is the preferred ligand for NK_1 receptors, which are coupled to G-proteins (for review, see Satake and Kawada, 2006). In agreement with this mode of action, we and others have used NK_1 receptor knockout mice and/or selective NK_1 receptor antagonists to show that SP and non-peptide NK_1 receptor agonists produce cutaneous oedema mainly via these receptors (Palframan et al., 1996; Cao et al., 1999). The *P. l. lanio* venom-induced inflammatory response was significantly inhibited by the co-injection of SR140333, an NK_1 receptor antagonist, and by capsaicin, a well-known depletor of neuropeptides (Jancsó et al., 1977; Costa et al., 1997). Moreover, the residual oedema seen after dialysis, which effectively eliminated the low content of histamine from the venom, was further reduced by SR140333 possibly through a mechanism mediated by other venom components such as peptides. Mass spectrometry showed that *P. l. lanio* venom contained a variety of peptides (molecular mass: 492–2607 Da), two of which (PllTkP-I and PllTkP-II) had C-terminal sequences similar to those of other tachykinin-like peptides (Lembeck et al., 1985; Pimenta et al., 2005; for review, see Severini et al., 2002; Rawlingson et al., 2004 and Satake and Kawada, 2006). Our findings agree with those of Pimenta et al. (2005) who detected a large series of tachykinin-like peptides in *P. nigriventer* spider venom. Experimental studies have shown that the inflammatory and nociceptive responses to *P. nigriventer* venom are partly mediated by central or peripheral NK_1 receptors (Costa et al., 1997, 2006a; Zanchet and Cury, 2003; Zanchet et al., 2004) that may be activated by the peptides described by Pimenta et al. (2005); a similar situation could apply to *P. l. lanio* venom. Experiments with synthetic analogues of these spider and wasp tachykinin-like peptides would be useful in assessing their involvement in the inflammatory and nociceptive actions of the corresponding venoms. In contrast to NK_1 receptors, NK_2 receptors were apparently not involved in the venom-induced response since SR48968, an antagonist of this receptor, failed to significantly affect the venom-induced plasma extravasation. These data, together with the results obtained following neuropeptide depletion by treatment with capsaicin, provide evidence that a sensory neurogenic component, i.e., tachykinin-like peptides, is involved in the venom-induced increase in microvascular permeability.

Many wasp venoms, including those of *Polistes* species, contain mastoparans (Yajima et al., 1980; Wang et al., 2006), tetradecapeptides that share common properties with SP at the cellular level and in their action on purified G-proteins (Mousli et al., 1989). Like SP, mastoparans can activate mast cells (Devillier et al., 1985; Mousli et al., 1989; Farquhar et al., 2002; Mendes et al., 2005). Although we have not systematically screened *P. l. lanio* venom for mastoparans, the inability to directly stimulate histamine release from mast cells *in vitro* suggests that this venom is devoid of these peptides.

Kinins such as bradykinin are among the best studied bioactive peptides in insect venoms, including wasps (Piek, 1991; Konno et al., 2002; Mendes and Palma, 2006). Bradykinin is a vasoactive, proinflammatory nonapeptide that promotes the expression of cell adhesion molecules, leukocyte sequestration, inter-endothelial gap formation, and protein extravasation in post-capillary venules in the cutaneous microvasculature of humans and animals (Marangoni et al., 1993; for review, see Regoli et al., 1998). However, as shown here, there was apparently no involvement of kinins in the *P. l. lanio* venom-induced increase in vascular permeability since bradykinin B_2 (Hoe 140) and B_1 (des-Arg^9-[Leu^8]-bradykinin) receptor antagonists had no significant effect on the venom-induced response in mouse dorsal skin. This lack of involvement of kinins contrasts with the presence of these peptides in various wasp venoms (Eno, 1997; Piek, 1991; Griesbacher et al., 1998; Konno et al., 2002; Murata et al., 2006).

Based on the results of this study, we suggest that *P. l. lanio* venom increases vascular permeability by primarily activating tachykinin NK_1 and then indirectly activate histamine H_1 receptors. NK_1 receptors in post-capillary venules may be activated directly by venom tachykinin-like peptides or by endogenous SP released from sensory fibres in response to the venom. In addition, endogenous SP may bind to mast cell NK_1 receptors to stimulate the release of histamine (Ogawa et al., 1999); venom peptides (mastoparans or tachykinin-like peptides) are unlikely to be involved in direct histamine release since the venom did not degranulate mast cells *in vitro*. Histamine released endogenously (as described above) or present in the venom can activate H_1 histamine receptors, leading to increased vascular permeability.

Of the receptor pathways involved in the enhanced vascular permeability, the most important would appear to be the release of endogenous SP since (1) removal of histamine from the venom by dialysis did not abolish the increase in permeability (the decrease in the response

following dialysis probably reflects both the removal of histamine as well as the potential loss of peptides, possibly including part of the tachykinin-like peptides, with molecular masses close to the nominal 2 kDa molecular mass cut-off of the dialysis membrane), (2) the residual activity of the venom seen after dialysis (which effectively removed histamine from the venom) was proportionally greater than that seen in mice pretreated with pyrilamine and non-dialysed venom and it was further inhibited by SR140333 (suggesting that endogenously released histamine was more important than venom histamine), (3) the quantity of histamine or histamine-like substances in the venom was too low to account for the potent increase in vascular permeability caused by the venom; thus the histaminergic activity of the venom observed in the present study was mainly due to the release of endogenous histamine, and (4) the contribution of the venom tachykinin-like peptides to this phenomenon is still unclear.

This is the first description of a neurovascular mechanism for *P. l. lanio* venom-mediated inflammation. The extent to which the two tachykinin-like peptides identified here contribute to this neurogenic inflammatory response remains to be elucidated. Our results suggest that NK_1 antagonists or capsaicin-mediated desensitization of sensory nerves (through the use of creams) could be a useful adjuvant for treating stings by this species.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.toxicon.2008.10.006

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