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Activation by *Phoneutria nigriventer* (armed spider) venom of tissue kallikrein-kininogen-kinin system in rabbit skin *in vivo*

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1 The purpose of the present study was to investigate the mechanisms by which venom from *Phoneutria nigriventer* spider induces increases in vascular permeability in rabbit skin.

2 Local oedema formation, in response to intradermally-injected agents, was measured in male New Zealand white rabbits as the local accumulation of i.v. injected ^{125}I-labelled human serum albumin into skin sites.

3 *Phoneutria nigriventer* venom (10–30 μg/site) increased vascular permeability, which was inhibited by trasylol (10 μg/site) and the bradykinin B_2 receptor antagonists D-Arg,[Hyp^3,Thi^{5,8},D-Phe^7]-BK (3 nmol/site) and Hoe 140 (0.3 nmol/site). In addition, the oedema induced by the venom was potentiated by the kinase II inhibitor, captopril (1 nmol/site). The lipoxygenase inhibitor, BWA4C (10 nmol/site) and the PAF antagonist, WEB 2086 (100 nmol/site) had no effect on the venom-induced increase in vascular permeability.

4 Incubation of rabbit plasma with *Phoneutria nigriventer* venom *in vitro* did not cause bradykinin formation. Further, the plasma kallikrein inhibitor, soybean trypsin inhibitor (10 μg/site), had no effect on the venom-induced increase in vascular permeability in rabbit skin.

5 These results indicate that the oedema produced by *Phoneutria nigriventer* venom is dependent on the activation of the tissue kallikrein-kinin system.

Keywords: Trasylol; bradykinin; kallidin; soybean trypsin inhibitor; captopril; oedema; *Phoneutria nigriventer*

Introduction

Phoneutria nigriventer is a spider responsible for most human accidents involving spider bites in South America, mainly in Brazil (Lucas, 1988). The human victim presents several signs and symptoms including intense and radiating local pain, neurogenic shock, cardiac disturbances, pulmonary oedema and priapism (Schenberg & Pereira-Lima, 1971). *Phoneutria nigriventer* venom contains neurotoxin(s) which activates sodium channels leading to either neuromuscular blockade on the phrenic nerve-diaphragm muscle preparation (Fontana & Vital-Brazil, 1985) or release of acetylcholine and noradrenaline by the autonomic nerve endings in the guinea-pig auricles (Vital-Brazil *et al.*, 1988). This venom also contains substance(s), probably peptides, which induce rabbit vascular smooth muscle contractions (Antunes *et al.*, 1990; 1993; Marangoni *et al.*, 1993) and local oedema formation in rat and rabbit skin (Antunes *et al.*, 1992). The oedema formation observed in animal skin is mainly due to an increase in microvascular permeability and not to an increased microvascular blood flow, as it is markedly potentiated by the co-injection of the prostanoid vasodilator, prostaglandin E_1, or the peptide vasodilator, calcitonin gene-related peptide (Antunes *et al.*, 1992). The venom from *Phoneutria nigriventer* contains histamine and 5-hydroxytryptamine (5-HT) (Schenberg & Pereira-Lima, 1971) but oedema formation is still clearly observed in rabbit skin after removal of these amines (Antunes *et al.*, 1992). In this study we describe the mechanisms by which this venom increases microvascular permeability in rabbit skin.

Methods

Measurement of local oedema formation in rabbit skin

Local oedema formation was measured in male New Zealand White rabbits (1.5–2.5 kg; provided by Cemib-UNICAMP, Brazil) as the local accumulation of i.v. injected ^{125}I-labelled human serum albumin into skin sites as described previously (Williams, 1979; Brain & Williams, 1985). The rabbits were anaesthetized with pentobarbitone sodium (Sagatal, 30–40 mg kg^{-1}) injected via the marginal ear vein and maintenance doses were given when required. ^{125}I-human serum albumin (2 μCi kg^{-1}) and Evans blue dye (0.5 ml kg^{-1}, 2.5% w/v) were injected by the same route. The agents under test were made up in sterile saline and injected intradermally in 100 μl volumes into the shaved dorsal skin according to a balanced site pattern with six replicates per dose. After 30 min, a 5 ml cardiac blood sample was taken into heparin and the animal killed by a Sagatal overdose. The dorsal skin was removed and the injection sites punched out (15 mm diameter) and counted for radioactivity in a gamma counter. Oedema formation at each site was expressed as plasma volume, calculated from the counts in 1 ml plasma.

Kinin-generating system in rabbit plasma in vitro

Rabbit blood was collected in siliconized tubes in presence of heparin (20 iu ml^{-1}) and centrifuged at 3000 r.p.m. for 15 min. An aliquot of plasma (0.2 ml) was added to an 0.1% acetic acid solution (1.8 ml). The mixture was boiled for 30 min and pH was adjusted to 7.4–7.8. Tris buffer solution (0.2 M, pH 7.8, 0.5 ml) and trypsin (2000 u contained in 0.1 ml) were added to the mixture and then incubated at 37°C for 30 min. The reaction was stopped by adding 0.2% acetic acid solution (0.1 ml) and the mixture was boiled (10 min) again in order to destroy trypsin (Diniz & Carvalho, 1963). Similar experiments were carried out with either saline or *Phoneutria nigriventer* venom (0.2 mg) instead of trypsin. Bradykinin formation *in vitro* was assayed by the contractions obtained in the guinea-pig isolated ileum in a selective bioassay using Krebs solution containing the histamine H_1 antagonist, mepyramine (0.35 μM), the 5-HT antagonist, methysergide (0.60 μM), the acetylcholine antagonist, atropine (0.35 μM), the β-adrenoceptor antagonist, propranolol (6.5

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μM), the α-adrenoceptor antagonist, phenoxybenzamine (0.05 μM) and the prostanoid cyclo-oxygenase inhibitor, indomethacin (5.6 μM; see Gilmore *et al.*, 1968; Eckenfels & Vane, 1972).

Dialysis of Phoneutria nigriventer *venom*

Phoneutria nigriventer venom [3–4 ml of a 2 mg ml^{-1} 0.9% (w/v) saline solution] was dialyzed for up to 48 h at 4–6°C against two litres of 0.9% saline in order to remove histamine and 5-HT. The dialyzing solution was changed four times during this period. A venom sample was withdrawn prior to dialysis for subsequent comparison with the dialyzed material. Efficient removal (>99.9%) of histamine and 5-HT was confirmed by bioassay on rabbit mesenteric artery strip. Dialyzed *Phoneutria nigriventer* venom was used throughout the study in order that we could investigate mechanisms of oedema formation induced by the venom that are independent of histamine and 5-HT.

Venom and reagents

Lyophilized *Phoneutria nigriventer* venom (PNV) was purchased from the Butantan Institute (São Paulo, Brazil). The venom was collected from the spiders by electrical stimulation.

Trasylol (aprotinin), soybean trypsin inhibitor, arachidonic acid, bradykinin, des-Arg^9-bradykinin, histamine, L-α-phosphatidylcholine β-acetyl-γ-O-alkyl (platelet-activating factor, PAF), bradykinin B_2 receptor antagonist (D-Arg,[Hyp^3,Thi^{5,8}, D-Phe^7]-BK), propranolol, atropine, indomethacin and porcine pancreas kallikrein were obtained from Sigma (St. Louis, U.S.A.). Pentobarbitone sodium (Sagatal) was obtained from May & Baker (Dagenham, Essex, U.K.). Prostaglandin E_1 was kindly provided by Dr John Pike (Upjohn Co., Kalamazoo, U.S.A.). Evans blue dye and mepyramine maleate were obtained from Merck (Darmstadt, Germany) and May & Baker (Dagenham, Essex, U.K.), respectively. BWA4C (N-(3-phenoxycinnamyl) acetohydroxamic acid) and captopril were kindly provided by Wellcome Research Laboratories (Beckenham, U.K.) and Squibb Inc. (U.S.A.), respectively. Methysergide maleate and phenoxybenzamine were a gift from Sandoz Ltd (Basel, Switzerland) and Smith, Kline & French (Stevenage, U.K.). Hoe 140 (D-Arg-[Hyp^3,Thi^5,DTic^7, Oic^8]-BK) was kindly provided by Hoechst AG (Frankfurt, Germany). ^{125}I-human serum albumin (50 μCi ml^{-1}, 20 mg albumin ml^{-1}) was bought from Amersham International (Amersham, U.K.). Dialysis tubing (mol. wt. cutoff 12000–14000) was bought from Philip Harris Scientific (London, U.K.). WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-(3,2f) (1,2,4)-triazolo-(4,3-a) (1,4)-diazepine-2-yl)-(4-morpholinyl)-1-propanone) was obtained from Boehringer-Ingelheim (Germany).

Statistical analysis

Data are presented as the mean ± s.e.mean and have been analysed by Analysis of Variance and Student's paired *t* test for within animal treatments. A *P* value of less than 0.05 was considered as significant.

Results

Figure 1 shows that tissue kallikrein (5.7 mu/site), bradykinin (0.1 nmol/site) and PNV (30 μg/site) induced local skin oedema formation which were all potentiated when injected with the vasodilator PGE_1 (0.1 nmol/site). In further experiments PGE_1 was routinely used to potentiate oedema formation. The protease inhibitor trasylol (10 μg/site), known to inhibit bradykinin synthesis, significantly inhibited both kallikrein- and PNV-induced oedema, without affecting the oedema induced by bradykinin (Figure 1).

Soybean trypsin inhibitor (SBTI, 10 μg/site), an inhibitor of plasma kallikrein, failed to affect significantly the increased vascular permeability induced by tissue kallikrein (180 ± 7.0 μl and 237 ± 24.0 μl for control and SBTI-treated sites, respectively, mean ± s.e.mean, $n = 3$), bradykinin (108 ± 17.0 μl and 132 ± 9.3 μl for control and SBTI-treated sites, respectively, mean ± s.e.mean, $n = 3$) and PNV (121 ± 9.8 μl and 136 ± 19.0 μl for control and SBTI-treated sites, respectively, mean ± s.e.mean, $n = 4$).

The bradykinin B_2 receptor antagonist D-Arg,[Hyp^3,Thi^{5,8}, D-Phe^7]-BK (3 nmol/site) significantly reduced bradykinin-

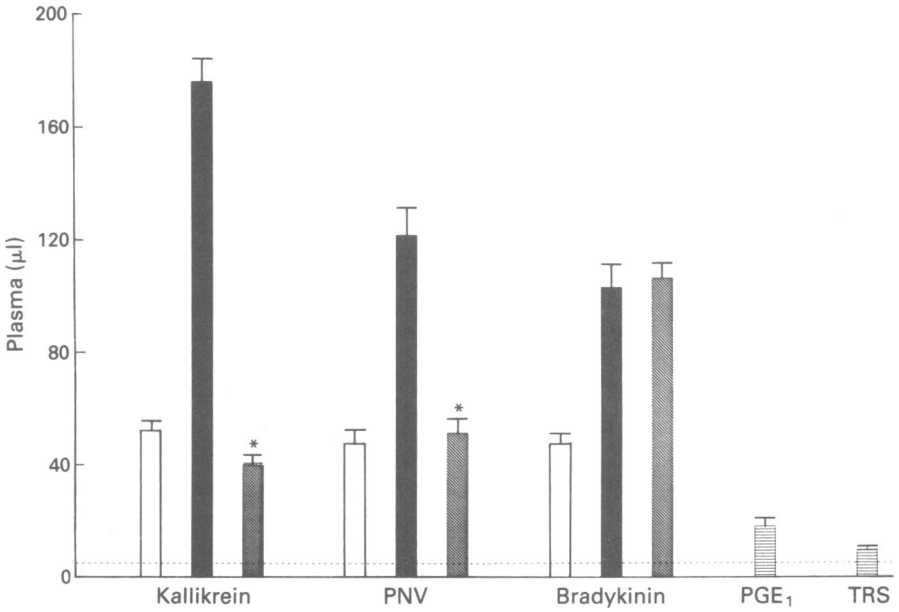

Figure 1 Increased vascular permeability induced by *Phoneutria nigriventer* venom (PNV) is inhibited by trasylol in rabbit skin. Kallikrein (5.7 mu/site), bradykinin (0.1 nmol/site) and PNV (30 μg/site) were injected alone (open columns) and co-injected with prostaglandin (PGE_1, 0.1 nmol/site; solid columns). The hatched columns represent the co-injection of the inflammatory agents (kallikrein, PNV or bradykinin) + PGE_1 + trasylol (10 μg/site). The dashed line represents sites injected with saline alone. Oedema response induced by PGE_1 and trasylol (TRS) injected alone is also shown (striped columns). The results are expressed as mean (with s.e.mean) values from 6–7 rabbits. *$P < 0.001$ when compared with oedema in the absence of trasylol.

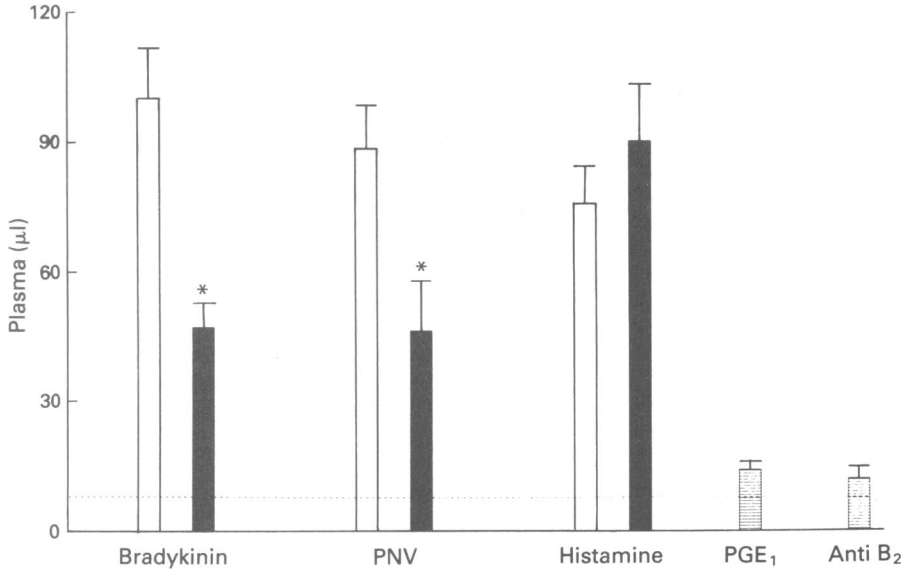

Figure 2 Bradykinin B_2 receptor antagonist reduces the increased vascular permeability induced by *Phoneutria nigriventer* venom (PNV) in rabbit skin. Bradykinin (0.1 nmol/site), PNV (30 μg/site) and histamine (3 nmol/site) were co-injected with prostaglandin E_1 (PGE_1, 0.1 nmol/site) in the absence (open columns) or presence (solid columns) of the bradykinin B_2 receptor antagonist D-Arg,[Hyp^3,Thi^{5,8},D-Phe^7]-BK (3 nmol/site). The dashed line represents sites injected with saline alone. Oedema response induced by PGE_1 and bradykinin B_2 receptor antagonist (Anti-B_2) injected alone is shown by the striped columns. Results are expressed as the mean (with s.e.mean) values from 4 rabbits. $^*P<0.01$ and $^{**}P<0.05$.

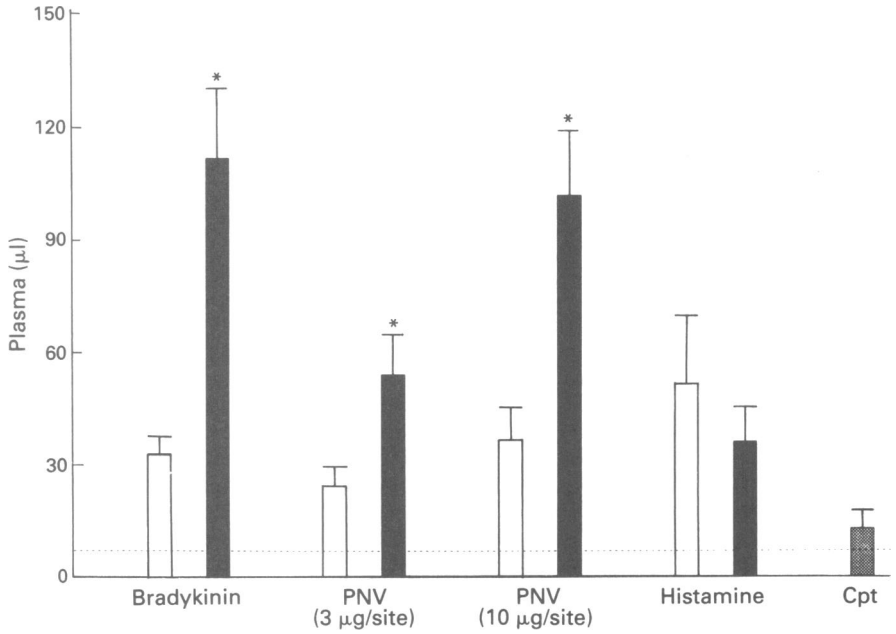

Figure 3 Captopril potentiates *Phoneutria nigriventer* venom (PNV)-induced oedema in rabbit skin. Bradykinin (0.1 nmol/site), PNV (3 and 10 μg/site) and histamine (10 nmol/site) were injected alone (open columns) and in combination with captopril (1 nmol/site; solid columns). The dashed line represents sites injected with saline alone. Oedema response induced by captopril (Cpt) alone (hatched column) is also shown. Results are expressed as the mean (with s.e.mean) values from 4 rabbits. $^*P<0.05$.

and PNV-induced oedema (when in the presence of PGE_1) but not that induced by the combination of histamine (3 nmol/site) and PGE_1 (0.1 nmol/site; Figure 2). The stable B_2 receptor antagonist, D-Arg-[Hyp^3,Thi^5,DTic^7,Oic^8]-BK (Hoe 140; 0.3 nmol/site) also significantly reduced the oedema induced by bradykinin (104 ± 19 μl and 49.8 ± 4.1 μl for control and Hoe-treated sites, respectively, mean ± s.e.mean, $n = 3$, $P<0.05$) and virtually abolished that induced by PNV (111 ± 16.2 μl and 19.3 ± 1.3 μl for control and Hoe-treated sites, respectively, mean ± s.e.mean, $n = 3$, $P<0.05$). At the dose used above, Hoe 140 did not significantly affect histamine (3 nmol/site)-induced oedema (115 ± 20 μl and 108 ± 21.7 μl for control and Hoe-treated sites, respectively, mean ± s.e.mean., $n = 3$). The selective bradykinin B_1 receptor agonist, des-Arg^9-BK (0.1 nmol/site) caused a small oedematous response when compared to an equimolar dose of BK (31 ± 5.4 μl and 92 ± 7.2 μl, respectively, $n = 3$, $P<0.05$). The increased vascular permeability induced by both bradykinin and PNV were significantly potentiated by the kininase II inhibitor, captopril (1 nmol/site, $n = 4$; Figure 3). At the dose used, captopril did not affect histamine (10 nmol/site)-induced oedema (Figure 3).

In further experiments we investigated whether other mediators of increased microvascular permeability could also be

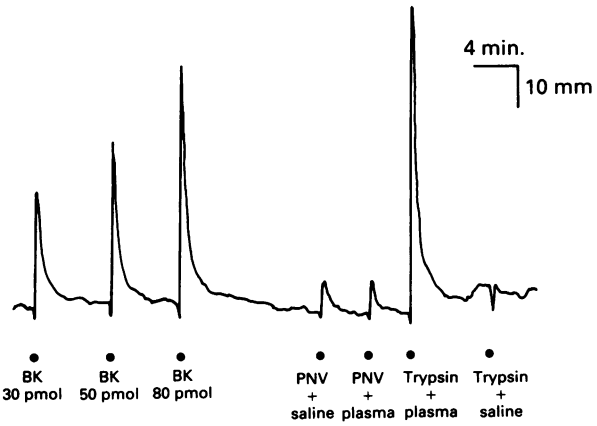

Figure 4 *Phoneutria nigriventer* venom (PNV) does not induce bradykinin formation in rabbit plasma *in vitro*. Guinea-pig isolated ileum strips were previously treated with mepyramine (0.35 μM), methysergide (0.6 μM), atropine (0.35 μM), phenoxybenzamine (0.05 μM), propranolol (6.5 μM) and indomethacin (5.6 μM). Both trypsin and PNV were incubated with either denatured rabbit plasma or saline (as described in Methods) and given as bolus injection (100 μl) in the guinea-pig ileum strips. Similar results were obtained in another four experiments.

involved in the response to intradermal PNV. The platelet-activating factor antagonist WEB 2086 (100 nmol/site) significantly reduced (44.0 ± 8.7%, $n = 4$, $P < 0.05$) the oedema caused by the co-injection of PAF (3 nmol/site) and PGE_1 (0.1 nmol/site) without affecting the oedema induced by PNV and PGE_1 (111 ± 5.9 μl and 120 ± 9.3 μl for control and WEB 2086-treated sites respectively, mean ± s.e.mean, $n = 4$). Similarly, the lipoxygenase inhibitor BWA4C (10 nmol/site) did not significantly affect the oedema formation induced by either BK (82 ± 6.9 μl and 103 ± 6.5 μl, for control and BWA4C-treated sites, respectively; mean ± s.e.mean, $n = 4$) or PNV (110 ± 10.7 μl and 120.7 ± 11.6 μl, for control and BWA4C-treated sites, respectively; mean ± s.e.mean, $n = 4$) in presence of PGE_1. BWA4C (10 nmol/site) significantly inhibited the oedema resulting from the co-injection of bradykinin (0.1 nmol/site) and arachidonic acid (3 nmol/site; 42.0 ± 9.4% of inhibition, $n = 4$, $P < 0.05$).

The bradykinin formation *in vitro* obtained by incubation of rabbit plasma with trypsin was confirmed by contractions obtained in guinea-pig ileum previously treated with a mixture of antagonists (phenoxybenzamine, propranolol, mepyramine, methysergide and atropine; Figure 4). These contractile responses were abolished when plasma was pre-incubated (10 min) with SBTI (100–500 μg) before adding trypsin (not shown). The contractions were compared to standard doses of bradykinin (1 –100 ng). However, guinea-pig ileum contractions were not observed when PNV or saline were used instead of trypsin to stimulate bradykinin formation (Figure 4).

Discussion

Phoneutria nigriventer venom causes increased vascular permeability in rabbit and rat skin independently of its 5-HT or histamine content and mast cell activation (Antunes *et al.*, 1992). Here we demonstrate that the oedema observed in the rabbit skin occurs mainly due to tissue kallikrein-kinin system activation.

Several mediators can interact to induce inflammatory oedema formation. For instance, PAF increases vascular permeability in rabbit (Wedmore & Williams, 1981), rat (Hwang *et al.*, 1985), guinea-pig (Morley *et al.*, 1983) and human (McGivern & Basran, 1984) skin. Since the PAF antagonist, WEB 2086 (Casals-Stenzel *et al.*, 1986) had no effect on PNV-induced oedema, we may assume that this mediator does not participate in the oedema observed. Similarly, the leukotrienes B_4, C_4 and D_4 induce oedema formation (Ueno *et al.*, 1981; Bray *et al.*, 1981). However, the failure of the lipoxygenase inhibitor BWA4C (Higgs *et al.*, 1988) to inhibit PNV-induced oedema indicates that leukotrienes do not appear to be involved.

Locally generated prostaglandins, such as prostaglandin E_2 and I_2, are known to increase oedema formation as a consequence of their vasodilator activity (Williams & Morley, 1973; Williams & Peck, 1977; Williams, 1979). Interestingly, the *Phoneutria nigriventer* bite is characterized by intense local pain (Schenberg & Pereira-Lima, 1971) and prostaglandins have a potent hyperalgesic effect on peripheral nerve terminals (Ferreira, 1972; Ferreira *et al.*, 1978). Thus, it is possible that prostaglandins could be involved in both potentiating the oedema formation and sensitizing the nerve terminals. However in the rabbit skin it is necessary to inject vasodilator doses of PGE_1 to allow full observation of oedema potential. This suggests that either pain producing amounts of prostaglandins are below the threshold for increasing blood flow, or that *Phoneutria nigriventer* venom does not release prostaglandins in the rabbit skin, as happens in guinea-pig isolated lungs (Antunes *et al.*, 1993).

Another important mediator implicated in the modulation of vascular permeability and pain is bradykinin (Spector & Willoughby, 1968; Bathon & Proud, 1991). Kinins can be formed by kallikreins acting on kininogens of either low or high molecular weight, the latter are considered to occur mainly in plasma. Interestingly, the kallikrein inhibitor, trasylol (Vogel, 1979) and the bradykinin B_2 receptor antagonists (Vavrek & Stewart, 1985; Wirth *et al.*, 1991) caused a significant reduction on the venom-induced oedema. Furthermore, captopril which inhibits the breakdown of bradykinin, potentiated PNV- but not histamine-induced increased vascular permeability, indicating that this effect was not linked to its vasodilator activity. These results indicate that the oedema induced by PNV in rabbit skin is dependent mostly on kallikrein activation and consequent kinin formation. Although we did not observe kinin formation in rabbit plasma when it was incubated with venom, this apparent discrepancy could be explained by considering that the venom may be activating tissue and not plasma kallikrein. In this aspect, it is interesting to note that the plasma kallikrein inhibitor SBTI (Spector & Willoughby, 1968) has no effect on PNV-induced increased vascular permeability.

Kallikreins derived from tissue sources are acidic glycoproteins (mol. wt. 25,000–43,000) which act locally near their site of origin and greatly differ from plasma kallikreins (Fukushima *et al.*, 1985; Evans *et al.*, 1988; Proud & Kaplan, 1988; Margolius, 1989). A fragment of 24 amino acids is removed from human pancreatic kallikrein to form the activate enzyme (Fukushima *et al.*, 1985), but the endogenous protease responsible for this reaction has not been identified. Tissue kallikreins act specifically on low molecular weight kininogen causing local formation of the decapeptide kallidin (Lys-bradykinin) which acts at bradykinin B_2 receptors (de Nucci *et al.*, 1988; Regoli *et al.*, 1990). The physiological role of kallidin has not yet been established. However, in traumas such as spider bites, it is likely that extravascularly generated kinins such as kallidin may be more important modulators of vascular permeability and pain than intravascular ones, such as bradykinin. It would be worth investigating whether clinical use of trasylol or the B_2 antagonists under present development could alleviate the local symptoms as well as perhaps some of the systemic ones (e.g. pulmonary oedema) induced by *Phoneutria* bites.

Wasp and ant venoms are known to contain kinins (Piek, 1991); however, no tissue kallikrein activator has been described in venoms so far. The biochemical identification of the peptide(s) responsible for this activity in *Phoneutria* venom might provide a useful tool to understand further the role of the tissue kallikrein-kinin system.

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