Pharmacological characterization of rabbit corpus cavernosum relaxation mediated by the tissue kallikrein-kinin system

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1 The roles of the tissue kallikrein-kinin system and nitric oxide (NO) release in *Phoneutria nigriventer* venom-induced relaxations of rabbit corpus cavernosum (RbCC) smooth muscle have been investigated by use of a bioassay cascade.

2 *Phoneutria nigriventer* venom (10–30 µg), porcine pancreatic kallikrein (100 mu), rabbit urinary kallikrein (10 mu), bradykinin (BK, 0.3–3 nmol), acetylcholine (ACh, 0.3–30 nmol) and glyceryl trinitrate (GTN, 0.5–10 nmol) caused relaxations of the RbCC strips. Captopril (1 µM) substantially potentiated *Phoneutria nigriventer* venom- and BK-induced RbCC relaxations without affecting those elicited by GTN.

3 The bradykinin B₂ receptor antagonist, Hoe 140 (D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK, 50 nM), aprotinin (10 µg ml⁻¹) and the tissue kallikrein inhibitor, Pro-Phe-Aph-Ser-Val-Gln-NH₂ (KIZD-06, 1.3 µM) significantly inhibited *Phoneutria nigriventer* venom-induced RbCC relaxations, without affecting those provoked by GTN and ACh. The B₁ receptor antagonist, [Leu⁹]des Arg¹⁰BK (0.5 µM) and soybean trypsin inhibitor (SBTI, 10 µg ml⁻¹) had no effect on *Phoneutria nigriventer* venom-induced RbCC relaxations.

4 The relaxations induced by *Phoneutria nigriventer* venom, porcine pancreas kallikrein, BK and ACh were significantly inhibited by N^ω-nitro-L-arginine methyl ester (L-NAME, 10 µM) but not by D-NAME (10 µM). L-NAME did not affect GTN-induced relaxations. L-Arginine (300 µM), but not D-arginine (300 µM), significantly reversed the inhibitory effect of L-NAME.

5 Our results indicate that *Phoneutria nigriventer* venom activates the tissue kallikrein-kininogen-kinin system in RbCC strips leading to NO release and suggest a functional role for this system in penile erection.

Keywords: Kinin receptors; penile erection; priapism; Hoe 140; aprotinin; prokallikreins; KIZD-06; *Phoneutria nigriventer* venom

Introduction

Tissue kallikreins are a subgroup of serine protease glycoproteins (mol. wt. 24–45 kD) encoded by a multigene family and are found in a wide variety of tissues (for reviews see Margolius, 1989; and Bhoola *et al.*, 1992) including the pancreas, salivary glands (Proud *et al.*, 1977), kidneys (Proud & Kaplan, 1988), gut (Schachter *et al.*, 1983), prostate (Lazure *et al.*, 1984) and pituitary gland (Powers & Nasjletti, 1982; 1983; Fuller *et al.*, 1985). In all tissues examined to date, these kallikreins are synthesized in the form of a preproenzyme, termed prokallikrein. Tissue prokallikreins have been characterized from both human (Corthon *et al.*, 1979; Spragg, 1983; Takada *et al.*, 1985) and rat (Takaoka *et al.*, 1984) urine. Although trypsin, thermolysin (Takada *et al.*, 1985), rat submandibular gland arginine esterase (Kamada *et al.*, 1990), plasmin and plasma kallikrein can activate prokallikrein, the mechanism of endogenous tissue kallikrein activation is unknown.

The venom of the armed spider *Phoneutria nigriventer* causes local oedema formation in both rat and rabbit skin *in vivo* (Antunes *et al.*, 1992) by activation of the tissue kallikrein-kinin system leading subsequently to local kinin formation (Marangoni *et al.*, 1993). Severe *Phoneutria nigriventer* envenomation in man causes intense and radiating local pain, cardiac disturbances, and in children is often accompanied by priapism, a disorder characterized by a prolonged and painful penile erection. Subcutaneous injection of *Phoneutria nigriventer* venom in mice and dogs also causes

intermittent and long-lasting priapism accompanied by local oedema formation in the distal portion of the penis (Schenberg & Pereira-Lima, 1978). Since relaxation of the corpus cavernosum plays an essential role in penile erection, we have investigated the involvement of the tissue kallikrein-kinin system in this response.

Methods

Rabbit corpus cavernosum (RbCC) preparation

Male New Zealand white rabbits (1.5–2.5 kg, provided by CEMIB-UNICAMP) were anaesthetized with sodium pentobarbitone (Sagatal, 30–40 mg kg⁻¹, i.v.) and exsanguinated via the carotid artery. Following penectomy, the rabbit corpus cavernosum (RbCC) was dissected in Krebs solution and cleared of the tunica albuginea and surrounding tissues. Strips of RbCC were superfused in a cascade system (Vane, 1964) with warmed (37°C) and oxygenated (95% O₂ + 5% CO₂) Krebs solution at a flow rate of 5 ml min⁻¹. The tissue responses (tension of 1.5 g) were detected with auxotonic levers attached to Harvard heart/smooth muscle transducers and displayed on a Watanabe multichannel pen recorder (model WTR 381). After a 60–90 min period of equilibration, RbCC strips were precontracted with noradrenaline (3 µM) in order to increase the basal tone. The agonists *Phoneutria nigriventer* venom, acetylcholine (ACh), bradykinin (BK), glyceryl trinitrate (GTN), porcine pancreas kallikrein and rabbit urinary kallikrein were administered as

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single bolus injection (10–50 μ l). N^ω-nitro-L-arginine methyl ester (L-NAME), D-NAME, L-arginine, D-arginine, aprotinin (Trasylol), soybean trypsin inhibitor (SBTI), captopril, Hoe 140 (D-Arg-[Hyp³,Thi⁵,D-Tic⁷,Oic⁸]-BK), the bradykinin B₁ receptor antagonist [Leu⁹]des Arg¹⁰ BK and the tissue kallikrein inhibitor KIZD-06 (Pro-Phe-Aph-Ser-Val-Gln-NH₂) were infused (0.1 ml min⁻¹) over the tissues 15 min before and during bolus injection of the appropriate agonists.

Purification of rabbit urinary kallikrein

Rabbit urinary kallikrein was partially purified by a modified procedure (Stella *et al.*, 1989) from 650 ml of rabbit urine. The urine was dialysed against distilled water, lyophilized and the crude powder suspended in 100 ml of sodium phosphate buffer (0.05 M, pH 7.0). This material was applied to a DEAE-Sephadex A-50 resin column (100 ml) previously equilibrated with 0.05 M sodium phosphate buffer. The resin was extensively washed with the equilibration buffer and with 300 ml of this same buffer containing 0.05 M NaCl. The active material (see below) was eluted by washing the column with 100 ml of phosphate buffer containing 0.3 M NaCl. This active pool was dialysed and lyophilized. Urinary kallikrein was then submitted to gel filtration on a Superose 12 column equilibrated with 0.05 M phosphate buffer. This procedure was repeated several times. The final preparation was purified 23 fold with a 12% yield and contained 80% of the active enzyme, as determined by titration against a previously titrated aprotinin solution. Rabbit urinary kallikrein activity was followed by the hydrolysis of 0.86 mM S-2266 in 0.05 M Tris-HCl, pH 8.0 at 37°C, in a final volume of 0.5 ml. The reaction was stopped by adding 0.5 ml of 15% acetic acid and the amount of product formed was determined by measuring the absorbance at 405 nm (Oliva *et al.*, 1988).

Dialysis of *Phoneutria nigriventer* venom

Since *Phoneutria nigriventer* venom contains small amounts of histamine (0.06–1.0%) and 5-hydroxytryptamine (5-HT, 0.03–0.25%; Schenberg & Pereira-Lima, 1978), we have routinely used dialysed *Phoneutria nigriventer* venom throughout this study in order to avoid the interference of these substances in the bioassay. *Phoneutria nigriventer* venom [5 ml of a 2 mg ml⁻¹ 0.9% (w/v) saline solution] was dialysed for up to 48 h at 4–6°C against 2 l of 0.9% saline. The dialysing solution was changed four times during this period (Antunes *et al.*, 1992).

Venom and reagents

Phoneutria nigriventer venom was obtained by electrical stimulation of spiders maintained by the Arthropods Section, Instituto Butantan, São Paulo (Brazil) and desiccated in a vacuum desiccator containing sodium hydroxide pellets at room temperature.

Bradykinin (BK), [Leu⁹]des Arg¹⁰-BK, acetylcholine (ACh), noradrenaline, porcine pancreas kallikrein, aprotinin, soybean trypsin inhibitor (SBTI), N^ω-nitro-L-arginine methyl ester (L-NAME), L-arginine and D-arginine were purchased from Sigma Co (St. Louis, U.S.A.). Glyceryl trinitrate (GTN, ampoules containing 1 mg ml⁻¹ in isotonic saline), captopril and D-NAME were obtained from Lipha Pharmaceuticals (London, U.K.), Squibb Inc. (U.S.A.) and Bachem (London, U.K.), respectively. Pentobarbitone sodium (Sagatal) was purchased from May & Baker (Dagenham, Essex, U.K.). Hoe 140 (D-Arg-[Hyp³,Thi⁵,D-Tic⁷,Oic⁸]-BK) was obtained from Hoechst AG (Frankfurt, Germany). KIZD-06 (Pro-Phe-Aph-Ser-Val-Gln-NH₂) was synthesized by Dr J. Burton (Boston University, U.S.A.). Dialysis tubing (mol. wt. cutoff 12,000–14,000) was bought from Sigma Chem. Co., Inc. (St Louis, MO, U.S.A.).

The composition of the Krebs solution was (mM): NaCl

118, NaHCO₃ 25, glucose 5.6, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.17 and CaCl₂·6H₂O 2.5.

Statistical analysis

The relaxations induced by *Phoneutria nigriventer* venom, ACh, BK, porcine pancreas kallikrein and rabbit urinary kallikrein were expressed relative to the sub-maximal relaxation induced by GTN which was taken to be 100%. The results are shown as the mean $\pm$ s.e.mean of *n* experiments. Analysis of variance and Student's unpaired *t* test were employed to evaluate the data. A *P* value < 0.05 was considered to be significant.

Results

Involvement of the tissue kallikrein-kinin system in *Phoneutria nigriventer* venom-induced RbCC relaxations

Single bolus injections of *Phoneutria nigriventer* venom (1–30 μ g) caused dose-dependent relaxations of the RbCC strips (not shown). An infusion of captopril (1 μ M) significantly (*P* < 0.01) potentiated the relaxations induced by both BK (42 $\pm$ 8% relaxation before vs. 66 $\pm$ 9% relaxation during infusion of captopril, *n* = 8) and *Phoneutria nigriventer* venom (40 $\pm$ 4% relaxation before vs. 62 $\pm$ 7% relaxation during infusion of captopril, *n* = 9) but had no effect on those elicited by GTN (Figure 1, *n* = 7).

The stable bradykinin B₂ receptor antagonist, Hoe-140 (50 nM), abolished the relaxations induced by BK and *Phoneutria nigriventer* venom without affecting those caused by ACh (76 $\pm$ 6% relaxation before vs. 74 $\pm$ 6% relaxation during infusion of Hoe-140, *n* = 6) and GTN (Figure 2, *n* = 6). In contrast, the bradykinin B₁ receptor antagonist, [Leu⁹]des Arg¹⁰ BK (0.5 μ M), did not affect either BK- or *Phoneutria nigriventer* venom-induced relaxations (45 $\pm$ 12% and 55 $\pm$ 16% relaxation before vs. 49 $\pm$ 9% and 59 $\pm$ 20% relaxation during infusion of this antagonist in the presence of BK and *Phoneutria nigriventer* venom, respectively, *n* = 6).

The infusion of the protease inhibitor, aprotinin

Figure 1 Captopril (1 μ M) significantly potentiates the rabbit corpus cavernosum (RbCC) relaxation induced by bradykinin (BK, 1 nmol) and *Phoneutria nigriventer* venom (PNV, 30 μ g) without affecting that induced by glyceryl trinitrate (GTN, 1 nmol). RbCC strips were superfused in cascade and previously contracted with noradrenaline as described in the Methods. This is a representative tracing of seven experiments (auxotonic lever).

($10 \mu\text{g ml}^{-1}$) virtually abolished the RbCC relaxations induced by both porcine pancreas kallikrein (100 mu, $n = 4$) and *Phoneutria nigriventer* venom (3 μg , $n = 6$; Figure 3). At this concentration, aprotinin did not affect relaxations induced by BK ($47 \pm 7\%$ relaxation before vs. $49 \pm 9\%$ relaxation during infusion of aprotinin, $n = 5$) and GTN (Figure 3). Infusion of the tissue kallikrein inhibitor KIZD-06 ($1.3 \mu\text{M}$) abolished the relaxations induced by rabbit urinary kallikrein and *Phoneutria nigriventer* venom (Figure

4) without affecting those induced by ACh ($90 \pm 9\%$ relaxation before vs. $86\% \pm 2\%$ relaxation during KIZD-06 infusion, $n = 3$) and GTN (Figure 4, $n = 3$). The plasma kallikrein inhibitor SBTI ($10 \mu\text{g ml}^{-1}$) failed to affect significantly *Phoneutria nigriventer* venom-, BK-, ACh- and porcine pancreas kallikrein-induced relaxations (55 ± 12 , 54 ± 17 , 85 ± 26 and $35 \pm 6\%$ relaxation before vs. 67 ± 20 , 54 ± 8 , 98 ± 8 and $33 \pm 8\%$ relaxation during SBTI infusion, respectively, $n = 4$).

Figure 2 The bradykinin (BK) B_2 antagonist Hoe-140 (50 nm) abolished the rabbit corpus cavernosum (RbCC) relaxation induced by BK (3 nmol) and *Phoneutria nigriventer* venom (PNV, 10 μg). The relaxations induced by both glyceryl trinitrate (GTN, 9 nmol) and acetylcholine (ACh, 30 nmol) were not significantly affected by the infusion of Hoe-140. This is a representative tracing of six experiments (auxotonic lever).

Figure 3 Infusion of aprotinin ($10 \mu\text{g ml}^{-1}$) abolished the rabbit corpus cavernosum (RbCC) relaxations induced by *Phoneutria nigriventer* venom (PNV, 3 μg) and by porcine pancreas kallikrein (PPK, 100 mu). The relaxations induced by glyceryl trinitrate (GTN, 2.2 nmol) and bradykinin (BK, 0.3 nmol) were not significantly affected by the infusion of aprotinin. This is a representative tracing of four experiments (auxotonic lever).

Figure 4 The specific tissue kallikrein inhibitor Pro-Phe-Aph-Ser-Val-Gln-NH₂ (KIZD-06, 1.3 μ M) abolished the rabbit corpus cavernosum (RbCC) relaxations induced by both *Phoneutria nigriventer* venom (PNV, 10 μ g) and rabbit urinary kallikrein (RbUK, 10 μ u) without significantly affecting those evoked by glyceryl trinitrate (GTN, 4.5 nmol) and acetylcholine (ACh, 300 pmol). This is a representative tracing of three experiments (auxotonic lever).

Figure 5 Effects of N^o-nitro-L-arginine methyl ester (L-NAME, 10 μ M), D-NAME (10 μ M), L-arginine (L-Arg, 300 μ M) and D-Arg (300 μ M) on rabbit corpus cavernosum (RbCC) strips. The infusion of L-NAME (but not D-NAME) further increased the RbCC tone and abolished the relaxation induced by both acetylcholine (ACh, 0.3 nmol) and *Phoneutria nigriventer* venom (PNV, 10 μ g). The relaxations induced by glyceryl trinitrate (GTN, 0.5 nmol) were not significantly affected by L-NAME infusion. Subsequent infusion of L-Arg (but not D-Arg) partially reversed the increased RbCC tone and also partially restored the relaxations induced by ACh and PNV. This is a representative tracing of six experiments (auxotonic lever).

Involvement of NO in the RbCC relaxations induced by Phoneutria nigriventer venom

Figure 5 shows that GTN (0.5 nmol), ACh (0.3 nmol) and *Phoneutria nigriventer* venom (10 μ g) relaxed RbCC strips ($n = 6$). The infusion of nitro-D-arginine methyl ester (D-NAME, 10 μ M) did not significantly affect either the basal tone of the RbCC strips or the relaxations induced by the above substances. In contrast, an infusion of L-NAME (10 μ M) increased the tone of the tissues by $59 \pm 9\%$ ($P < 0.05$) and abolished the *Phoneutria nigriventer* venom- and ACh-induced relaxations without affecting those evoked by GTN. An infusion of L-arginine (300 μ M), but not of D-arginine (300 μ M), significantly reversed the increased tone

and restored the relaxations induced by *Phoneutria nigriventer* venom and ACh (Figure 5).

Discussion

Our results show that tissue kallikrein causes RbCC relaxation by a mechanism dependent on the release of NO since the relaxations were significantly inhibited by the NO synthase inhibitor L-NAME (Moore *et al.*, 1989) but not by its inactive enantiomer D-NAME. The reversal of the inhibitory effect of L-NAME by L-arginine (but not by D-arginine) further confirms the involvement of NO in this response. In erectile tissues, NO has been implicated recently as an essen-

tial mediator involved in the nonadrenergic, noncholinergic neurotransmission that induces the relaxation of the corpus cavernosum and triggers penile erection (Ignarro *et al.*, 1990; Burnett *et al.*, 1992; Anderson, 1993).

Activation of the tissue kallikrein-kinin system leads to kallidin release (for review see Bhoola *et al.*, 1992) which can in turn act on bradykinin B₂ receptors (Regoli & Barabé, 1980) to release NO from endothelial cells (De Nucci *et al.*, 1988; D'Orleans-Juste *et al.*, 1989). Since corpus cavernosum tissues are characterized by a rich network of sinusoidal capillaries containing endothelial cells (De Groot & Steers, 1988), it is possible that a similar mechanism for NO release may be also operative in this tissue.

The involvement of the tissue kallikrein-kinin system in the RbCC relaxations induced by *Phoneutria nigriventer* venom is supported by several observations. First, the protease inhibitor aprotinin (Vogel & Werle, 1970) and the tissue kallikrein inhibitor KIZD-06, selectively inhibited both the tissue kallikrein- and *Phoneutria nigriventer* venom-induced RbCC relaxations. As expected, the plasma kallikrein inhibitor SBTI (Vogel & Werle, 1970) had no effect on either the porcine pancreas kallikrein- or the *Phoneutria nigriventer* venom-induced relaxations indicating that plasma kallikrein activation is not involved. Second, the kinase II inhibitor, captopril, significantly potentiated the RbCC relaxations induced by BK and *Phoneutria nigriventer* venom. Third, a specific bradykinin B₂ receptor antagonist (Hoe-140; Wirth *et al.*, 1991) selectively abolished the relaxing effects of BK and *Phoneutria nigriventer* venom. The failure of the bradykinin B₁ receptor antagonist, [Leu⁹]des Arg¹⁰ BK, to block these relaxations eliminated the involvement of B₁ receptors.

The observation that porcine pancreas kallikrein and rabbit urinary kallikrein relaxed the RbCC strips by a kinin-dependent mechanism as discussed above indicates that RbCC contains low molecular weight kininogen which is the substrate for tissue kallikreins. Although high molecular weight kininogen is restricted to plasma, kininogens have also been detected in some organs such as brain (Shikimi *et*

al., 1973), kidney (Figueroa *et al.*, 1988), uterus (Figueiredo *et al.*, 1990), sweat and sexual glands and psoriatic skin (Poblete *et al.*, 1991).

While there are no reports on the existence of tissue kallikrein in corpus cavernosum smooth muscle, these enzymes have been detected in vascular smooth muscle tissues including rat aorta (Oza *et al.*, 1990), rat mesenteric artery (Nolly & Lama, 1982) and rat tail vein and artery (Nolly *et al.*, 1985). It seems plausible to suggest, therefore, that *Phoneutria nigriventer* venom causes RbCC relaxation by activating and/or stimulating the secretion of tissue kallikrein localized in the RbCC thereby resulting in the release of kallidin. Alternatively, *Phoneutria nigriventer* venom could function as an activator of tissue prokallikreins. An additional possibility is that *Phoneutria nigriventer* venom may itself contain tissue kallikrein. Although *Phoneutria nigriventer* venom contains histamine, 5-HT and some enzymes (Schenberg & Pereira-Lima, 1978), there are no reports on the existence of kallikreins in spider venoms. The active fraction of *Phoneutria nigriventer* venom responsible for activation of the tissue kallikrein-kinin system in rabbit skin has been partially purified (Antunes *et al.*, 1993) but further biochemical studies are necessary to elucidate the mechanisms involved.

It is interesting to note that tissue kallikreins are believed to play a functional role in spermatogenesis since they are reported to increase sperm motility, to cause sperm maturation and to increase blood flow in the testis (Saitoh & Kumamoto, 1988; Clements *et al.*, 1988). In addition, testosterone enhances tissue kallikrein gene expression in sex glands (Clements *et al.*, 1988). While our results have indicated that tissue kallikrein activation may be important in penile erection, the precise role of the tissue kallikrein-kinin system in this phenomenon remains to be established.

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