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Identification of a new vascular smooth muscle contracting polypeptide in *Phoneutria nigriventer* spider venom

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Abstract—The fractionation of *Phoneutria nigriventer* spider venom by gel filtration (Sephadex G-10-120) followed by ion-exchange chromatography (microgranular CM-cellulose-52) resulted in sixteen fractions (C_I to C_{XVI}) from which $C_{VII+VIII}$, C_{IX} and C_{X+XI} caused dose-dependent and short-lived contractions of both arterial and venous rabbit vessels. Fraction C_{X+XI} was further purified by a reverse phase HPLC, and a contractile polypeptide (PNV2) was isolated. The amino terminal sequence of PNV2 (LAKRADICQPGKTSQRACET) indicated that it represents a pure polypeptide consisting of a single chain. Furthermore, the amino acid analysis of PNV2 revealed the presence of four disulfide bridges, a high content in Lys (14%), Glx (11%), and the absence of His. The global amino acid composition showed that this polypeptide is composed of 102 residues (Trp not included) with a calculated molecular weight of 12,114. Whether this peptide is responsible for the vascular alterations observed in *Phoneutria* envenomation, such as lung edema and priapism, remains to be further investigated.

Phoneutria nigriventer is the species responsible for most human spider bites in the center, east and south of Brazil [1]. The bite of this spider causes intense and radiating local pain, autonomic dysfunction, and paralysis [2–5]. *Phoneutria nigriventer* venom (PNV)* contains several toxins that exert important biological effects such as voltage-dependent sodium channel activation [6, 7], local edema formation *in vivo* [8], and vascular smooth muscle contractions [9, 10]. Recently, a polypeptide responsible for the contractile activity has been identified [11], which greatly differs from other peptides isolated from PNV [12, 13]. We report here the isolation and biochemical characterization (amino acid composition, N-terminal amino acid sequence and a calculated molecular weight) of a new contractile polypeptide purified from PNV.

Materials and Methods

Venom and reagents. Phoneutria nigriventer venom was obtained by electrical stimulation of spiders maintained by the Arthropods Section, Institute Butantan, São Paulo (SP, Brazil) and desiccated using a vacuum desiccator containing NaOH tablets at room temperature. All chemicals and solvents were of HPLC grade purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) or the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Reverse phase chromatography was performed using a Waters (991-PDA) system. The composition of the Krebs solution was (mM): NaCl, 118; $NaHCO_3$, 25; glucose, 5.6; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.17; and $CaCl_2 \cdot 6H_2O$, 2.5.

Fractionation of PNV. Desiccated PNV was fractionated as described previously [11]. Briefly, PNV (900 mg; dissolved in acetic acid) was separated by gel filtration at 5° on a 2.5 cm × 190 cm column of Sephadex G-10-120, equilibrated with 2% acetic acid. The eight fractions (S_I–S_{VIII}) obtained were lyophilized and assayed on rabbit arterial and venous vascular smooth muscle. The vasoactive fraction S_I (450 mg) was dissolved in ammonium acetate buffer (3.5 mL, 0.05 M, pH 5.0) and chromatographed on a cation exchange column (microgranular CM-cellulose-52; Whatman). The column (2.5 cm × 55 cm) was equilibrated previously with this buffer at flow rate of 20 mL/hr (5°), the sample applied, and stepwise elution (0.1 to 3.0 M buffer, pH 5.0) started at an effluent volume of 150 mL. The sixteen new fractions (C_I to C_{XVI}) obtained were lyophilized, redissolved in acetic acid (2%), and desalted at 5° on a 2 cm × 200 cm column of Sephadex G-10-120, equilibrated with the same solvent, followed by lyophilization. Each fraction was assayed on rabbit arterial and venous vascular smooth muscle.

Reverse phase liquid chromatography. The smooth muscle active fraction is mainly retained in fractions $C_{VII+VIII}$, C_{IX} and C_{X+XI} [11]. Fraction C_{X+XI} obtained from ion-exchange chromatography was purified by reverse phase HPLC on a 0.39 cm × 30 cm μBondapack column (Waters System) with a linear gradient of 0–66% acetonitrile in 0.1% trifluoroacetic acid (buffer B), at a flow rate of 1 mL/min. The resulting fractions were lyophilized for bioassay and the active fraction was repurified on reverse phase HPLC using step-wise elution in the range of the first purification. Proteins were detected by their absorbance at 220 nm. The purified active fraction was named PNV2.

N-Terminal amino acid sequence of PNV2. Twenty micrograms of the isolated polypeptide (PNV2) were used to determine its N-terminal sequence by automated Edman degradation in an Applied Biosystems model 477A Sequencer. Phenylthiohydantoin amino acids were identified in a model 120-A PTH-amino acid analyzer (Applied Biosystems), according to the retention times of a 20 PTH-amino acid standard.

Amino acid composition of PNV2. Amino acid analysis was performed on a Pico-Tag amino acid analyzer (Water System) as described by Heinriksen and Meredith [14]. The purified sample (10 μg) was hydrolyzed with 6 N hydrochloric acid (Pierce—Sequenal Grade) containing 1% phenol (v/v) at 106° for 24 hr. Hydrolyzates reacted with 20 μL of fresh derivatization solution (ethanol:triethylamine:water:phenylisothiocyanate, 7:1:1:1, by vol.) for 1 hr at room temperature. The phenylthiocarbamyl (PTC) amino acids were identified by HPLC, comparing their retention times with those of a standard mixture.

Superfusion of vascular smooth muscle. Male New Zealand white rabbits (2.0 to 2.5 kg) were anesthetized with thiopental sodium (30 mg/kg, i.v.) and exsanguinated via the carotid artery. The abdominal and thoracic cavities were opened and the rabbit pulmonary artery (RbPA), mesenteric vein (RbMesV) and vena cava (RbVC) were removed and placed in Krebs solution. The vessels were cleared of adipose tissue and the endothelial layer was removed mechanically to avoid interference of endothelial-

* Abbreviations: PNV, *Phoneutria nigriventer* venom; RbPA, rabbit pulmonary artery; RbMesV, rabbit mesenteric vein; and RbVC, rabbit vena cava.

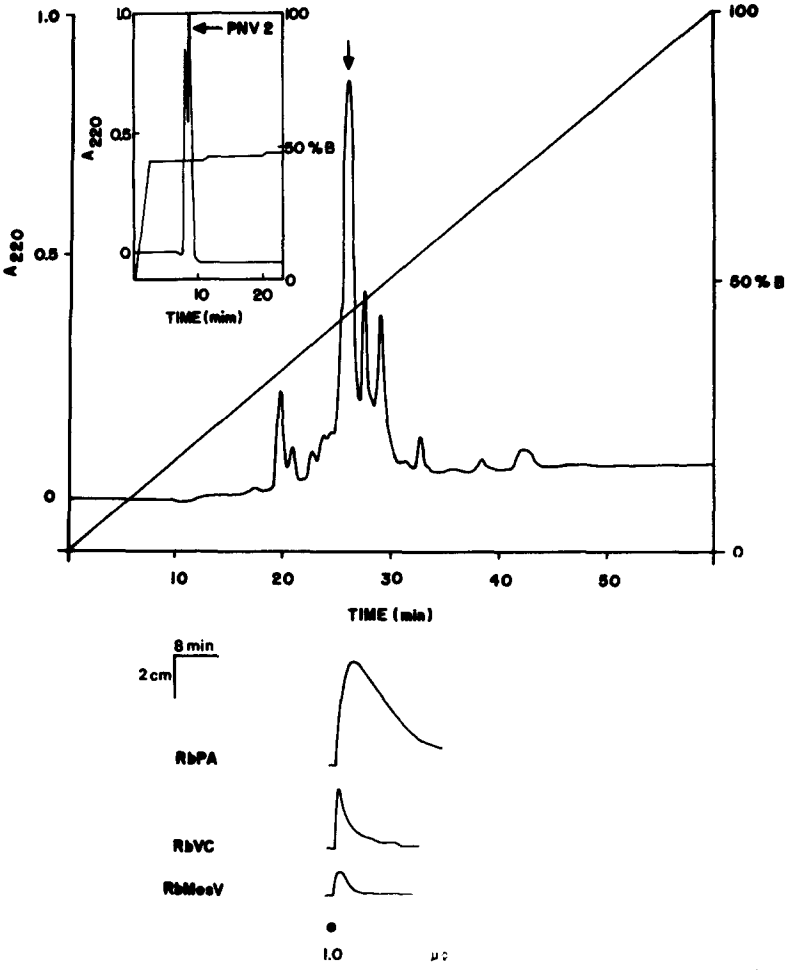

Fig. 1. Spasmogenic activity in rabbit pulmonary artery (RbPA), vena cava (RbVC) and mesenteric vein (RbMesV) of peaks obtained by reverse phase HPLC of fraction X + XI on a 0.39 cm × 30 cm μBondapack C18 analytical column. For elution, a linear gradient from 0–66% acetonitrile in 0.1% trifluoroacetic acid was used. The vascular tissues were de-endothelialized and superfused in cascade. The peak containing the spasmogenic activity (indicated by the arrow) was repurified by HPLC and revealed two other peaks (inset), the latter being responsible for the contractile activity.

derived vasoactive factors. The removal of endothelium was assessed by the lack of relaxation induced by acetylcholine (10^{-6} M) in noradrenaline-precontracted tissues. The tissues were suspended in a cascade [15] and continuously superfused with oxygenated (95% O_2 + 5% CO_2) and warmed (37°) Krebs solution at 5 mL/min. Responses of the tissues were detected with auxotonic levers [16] attached to Harvard heart/smooth muscle transducers and displayed on a Watanabe multichannel pen recorder (model WTR 381). After an equilibration period of approximately 60 min, the fractions obtained from PNV separation were injected as a single bolus.

Results and Discussion

Resolution of fraction X + XI was achieved through HPLC (Fig. 1). Spasmogenic activity in both rabbit arterial (RbPA) and venous (RbVC and RbMesV) tissues was detected in the peak indicated by the arrow (Fig. 1). Similarly to whole PNV [10], the contractile activity present in this peak was dose-dependent and of short-lived duration. No spasmogenic activity was detected in the other peaks (not shown).

Repurification of the described active peak by HPLC using discontinuous linear gradient between 40 and 42% of buffer B revealed the presence of two other peaks (inset of Fig. 1), from which only the latter (indicated as PNV2) presented spasmogenic activity on the vascular tissues (not shown).

The amino terminal sequence of this vascular smooth muscle active fraction (PNV2) and its amino acid composition are shown in Tables 1 and 2, respectively. Results from N-terminal amino acid sequence support the interpretation that PNV2 represents a pure polypeptide consisting of a single chain. Amino acid analysis indicated the presence of four disulfide bridges, a high content in Lys (14%), Glx (11%) and the absence of His. The global amino acid composition indicated the presence of 102 residues (Trp not included) and a calculated molecular weight of 12,114.

Entwistle *et al.* [12] purified a neutral polypeptide (5.5 to 5.9 kDa) with four disulfide bridges responsible for repetitive action potentials and twitching of locust skeletal muscles. Three other neuropeptides (6–9 kDa) with seven (PhTx1), nine (PhTx2) and eight (PhTx3) disulfide bridges

Table 1. Amino terminal sequence of PNV2 toxin purified from *Phoneutria nigriventer* venom: Comparison of the N-terminal sequence of the first 20 amino acid residues of PNV2 toxin with other toxin peptides isolated from the same spider venom

Toxin*	N-Terminal sequence
	1 20
PNV2	LAKRADICQPGKTSQRACET...
PNV1	EAFPGQST...
PhTx1	AELTSCFPVGHECDGDASNC...
PhTx2	ATCAGQDQTCK...
PhTx3	GCIGRNESQKKDNVYKFKE...

* PNV2: this paper; PNV1: Ref. 11; and PhTx: Ref. 13.

Table 2. Amino acid composition of PNV2 purified from *Phoneutria nigriventer* venom

Amino acid	PNV2
Asx	8.12 (8)*
Glx	10.80 (11)
Ser	7.18 (7)
Gly	10.35 (10)
His	0
Arg	3.85 (4)
Thr	7.70 (8)
Ala	11.34 (11)
Pro	2.48 (2)
Tyr	3.22 (3)
Val	2.51 (3)
Met	2.72 (3)
Cys	7.85 (8)
Ile	2.63 (3)
Leu	0.88 (1)
Phe	5.89 (6)
Lys	14.09 (14)
Trp	ND†

* Numbers in parentheses represent the nearest integer.
† ND = not determined.

were isolated from PNV and thought to be responsible for the neurotoxicity presented by the venom [13, 17]. We have recently purified a peptide (PNV1) with two disulfide bridges and a larger molecular weight (13,899) that induces short-lived contractions on rabbit vascular smooth muscle [11]. Here we report another peptide (PNV2) with a similar molecular weight (12,114) that also presents spasmogenic activity on rabbit isolated blood vessels. Our results indicate that PNV2 lacks free sulfhydryl groups even though no corrections for losses during acid hydrolysis were done concerning its half-cystine content. Considering that the five toxins described above do not present free cysteines, we assume that the cysteines in PNV2 are arranged to form four disulfide bridges.

Since the spasmogenic effect of whole PNV in rabbit vascular smooth muscle is not affected by tetrodotoxin [10], it is unlikely that sodium channel activation plays an important role in these tissues, as it does in both rat phrenic-diaphragm muscle-nerve preparation [6] and guinea pig isolated atria [7]. Furthermore, the finding that the α-adrenoceptor antagonist phenoxybenzamine does not affect PNV-induced contractions [10] excludes the possibility that PNV induces endogenous noradrenaline release from autonomic nerve endings present in the vascular walls, as occurs in guinea pig auricles [7]. *Phoneutria* envenomation is mainly characterized by severe local pain, but it may be accompanied by vascular disturbances such as lung edema and priapism [2–5]. Whether these peptides with vascular smooth muscle spasmogenic activity are responsible for the permeability alterations mentioned remains to be further investigated.

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