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Purification and characterization of a cysteine-rich secretory protein from *Philodryas patagoniensis* snake venom

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ABSTRACT

Cysteine-rich secretory proteins (CRiSPs) are widespread in reptile venoms, but most have functions that remain unknown. In the present study we describe the purification and characterization of a CRiSP (patagonin) from the venom of the rear-fanged snake *Philodryas patagoniensis*, and demonstrate its biological activity. Patagonin is a single-chain protein, exhibiting a molecular mass of 24,858.6 Da, whose NH_2-terminal and MS/MS-derived sequences are nearly identical to other snake venom CRiSPs. The purified protein hydrolyzed neither azocasein nor fibrinogen, and it could induce no edema, hemorrhage or inhibition of platelet adhesion and aggregation. In addition, patagonin did not inhibit contractions of rat aortic smooth muscle induced by high K^+. However, it caused muscular damage to murine gastrocnemius muscle, an action that has not been previously described for any snake venom CRiSPs. Thus, patagonin will be important for studies of the structure-function and evolutionary relationships of this family of proteins that are widely distributed among snake venoms.

1. Introduction

Cysteine-rich secretory proteins (CRiSPs) encompass a growing family of proteins commonly found in venoms of lizards and snakes (Mackessy, 2002; Yamazaki and Morita, 2004), and they are related to proteins present in the mammalian male reproductive tract. They are secreted single-chain proteins with molecular masses of about 20–30 kDa, and exhibit homologous amino acid sequences containing 16 highly conserved cysteine residues, which form 8 disulfide bonds (Guo et al., 2005). Remarkably, 10 of these cysteines are clustered in the C-terminal third of the polypeptide chain, the cysteine-rich domain and the "hinge" region. However, the NH_2-terminus sequence is more highly conserved as compared to other regions of these proteins (Yamazaki and Morita, 2004; Osipov et al., 2005).

The first discovered CRiSP was the acidic epididymal glycoprotein in rats in 1981 (Kierszenbaum et al., 1981), and since then many CRiSPs have been sequenced and characterized. Recent studies have revealed that CRiSPs are widespread in snake venoms from all five continents, including those of front-fanged and rear-fanged snake families (Yamazaki et al., 2003; Vidal et al., 2007), suggesting that their inclusion into the venom proteome occurred early in snake evolution (Fry et al., 2003; Fry and Wuster, 2004). However, biological activities have not yet been demonstrated for most of these proteins.

Several snake venom CRiSPs – such as ablomin from *Gloydius blomhoffi* (Viperidae), triflin from *Protobothrops flavoviridis* (Viperidae), and latisemin from *Laticauda semifasciata* (Elapidae) – inhibit depolarization-induced contraction of rat tail arterial smooth muscle, similar to L-type Ca^{2+} channel blockers (Yamazaki et al., 2002b). Others, such as pseudechetoxin and pseudecin (from the venoms of the elapid snakes *Pseudechis australis* and *P. porphyriacus*, respectively), are cyclic nucleotide-gated ion channel-blocking toxins (Brown et al., 1999; Yamazaki et al., 2002a). In addition, piscivorin, ophanin and catrin-2, from the venoms of *Agkistrodon piscivorus piscivorus* (Viperidae), *Ophiophagus hannah* (Elapidae) and *Crotalus atrox* (Viperidae), respectively, have also been reported to present a small but significant inhibition of smooth muscle contraction evoked by high K^+ (Yamazaki et al., 2003). Recently, natrin from *Naja atra* (Elapidae) venom has been demonstrated to block ion channel currents of the high-conductance calcium-activated and voltage-gated potassium channels (BK_{Ca} and Kv1.3) (Wang et al., 2005; Wang et al., 2006). However, the functions and even the molecular targets of most snake venom CRiSPs, particularly those from rear-fanged snakes, remain to be determined. Tigrin, from the venom of *Rhabdophis tigrinus tigrinus*, is the only CRiSP isolated in native form from a rear-fanged snake venom (Yamazaki et al., 2002b), but its biological activity is still unknown. In the present study we describe the purification, biochemical characterization and biological activity on

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mammalian skeletal muscle of a CRiSP from the venom of *Philodryas patagoniensis* (patagonin), an opisthoglyphous snake widespread in South America.

2. Materials and methods

2.1. Materials

Philodryas patagoniensis venom solution was prepared as previously described (Peichoto et al., 2007). BCA (bicinchoninic acid) protein assay kit, BSA (bovine serum albumin), collagen, bovine thrombin, ADP (adenosine 5'-diphosphate), ristocetin, divalent cation ionophore A23187, azocasein, azocoll and α-cyano-4-hydroxycinnamic acid were obtained from Sigma-Aldrich Chemical. Molecular mass markers and sequencing grade side-chain protected porcine trypsin were obtained from Bio-Rad and Promega, respectively. All other chemicals were of analytical reagent grade or better.

2.2. Animals

In order to determine hemorrhagic and edematogenic activities of patagonin, male Swiss mice (*Mus musculus*) (18–22 g) were used. To determine the effect of patagonin on smooth muscle contractions induced by high K^+, male Wistar rats (200–250 g) were used. These animals were supplied by the Animal House of Butantan Institute, Brazil. To determine local and systemic damage induced by patagonin, male CF-1 mice (18–20 g), supplied by the Animal House of Faculty of Veterinary Sciences, Northeastern National University, Argentina, were used. All experiments followed the ethical standards for animal experiments in toxinological research recommended by the International Society of Toxinology (Meier et al., 1993), and they were approved by the Ethical Committee for the Use of Animals at Butantan Institute (process 530/08).

2.3. Purification of patagonin

Venom was fractionated on a Mono-Q column as described previously (Peichoto et al., 2007). Basic fractions were pooled, dialyzed, and brought to 1 M $(NH_4)_2SO_4$ prior to being applied to an HiTrap Phenyl HP column (1 mL) (GE Healthcare, Sweden) equilibrated with 50 mM Tris–HCl, pH 7.4, 1 M $(NH_4)_2SO_4$, using an FPLC system. After washing the column, bound proteins were eluted with a decreasing linear gradient of 1 to 0 M $(NH_4)_2SO_4$. Protein concentration was monitored by measuring the absorbance at 280 nm. One mL fractions were collected, and SDS-PAGE was performed on selected fractions.

2.4. Protein quantification

Protein concentration of samples was determined by BCA protein assay, using BSA as standard (Smith et al., 1985).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were electrophoresed on 12% polyacrylamide slab gels following the method of Laemmli (1970) and then silver stained (Blum et al., 1987).

2.6. Mass spectrometry

In order to determine the molecular mass of patagonin, approximately 0.5 μg protein in 50% acetonitrile was spotted onto sinapinic acid matrix and allowed to dry. Mass spectra were obtained using an Applied Biosystems Voyager DE Pro MALDI-TOF mass spectrometer, operating in delayed extraction and linear mode (Mackessy et al., 2006).

For protein identification, protein bands of interest were excised from a silver-stained SDS-PAGE gel, destained and subjected to reduction with DTT, alkylation with iodoacetamide, and then in-gel digestion with sequencing grade side-chain protected porcine trypsin using a ProGest digestor (Genomic Solutions), following the manufacturer's instructions. The tryptic peptide mixtures were dried in a Speed-Vac and redissolved in 5 μL of 70% acetonitrile and 0.1% TFA (trifluoroacetic acid). Digests (0.65 μL) were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA, dried, and analyzed with a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer. Singly-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were sequenced by collision-induced dissociation tandem mass spectrometry. Spectra were interpreted using the on-line form of the MASCOT program at http://www.matrixscience.com.

2.7. NH2-terminal sequence analysis

The NH_2-terminal amino acid sequence of patagonin was determined by automated Edman degradation using an Applied BioSystems Procise sequencer at the Protein Structure Core Facility, University of Nebraska Omaha Medical Center (USA).

Homologous protein sequences were searched using the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al., 1997). Multiple sequence alignment was carried out using the ExPASy proteomics tools (http://www.expasy.org/tools/), in FASTA format (Pearson and Lipman, 1988). Amino acid sequences were aligned using the T-Coffee software (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi) (Notredame et al., 2000).

2.8. Protease activities

Potential cleavage of bovine fibrinogen (Biggs, 1976) by patagonin was determined by SDS-PAGE using 12% polyacrylamide gels (Peichoto et al., 2007). Azocasein (Wang and Huang, 2002) and azocoll (Váchová and Moravcová, 1993) assays were also used to determine proteolytic activity of patagonin.

2.9. Biological activities

The hemorrhagic (Peichoto et al., 2007) and edema-inducing (Lomonte et al., 1993) activities of patagonin were evaluated as described elsewhere. The effect of patagonin on the aggregation of washed human platelets or PRP (platelet-rich plasma) was tested as described previously (Santoro et al., 1999), using a Chrono-log lumiaggregometer (model 560VS). Platelet adhesion-inhibiting activity was measured by a microtiter-plate assay (Bellavite et al., 1994). The local and systemic effects induced by patagonin in mice were determined according to a method previously reported (Peichoto et al., 2007). Rat aortic smooth muscle contraction experiments were performed using a modification of a previously described method (Hooker et al., 1977). Briefly, animals were killed by decapitation. Thoracic aorta was rapidly dissected and placed in Krebs-bicarbonate solution (129.87 mM NaCl, 4.69 mM KCl, 1.56 mM $CaCl_2$, 1.17 mM KH_2PO_4, 1.17 mM $MgSO_4$, 14.87 mM $NaHCO_3$, 5.5 mM glucose, pH 7.4). Adhering perivascular tissue was carefully removed, a 4 mm ring was cut and the endothelium was removed with a wooden stick. The vessel was mounted onto two thin stainless steel holders in a 5 mL organ bath containing Krebs-bicarbonate solution, at 37 °C, continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2. A resting tension of 2 g was applied on the ring. A depolarizing solution, made by the replacement of NaCl with equimolar KCl (60 mM, final concentration, high K^+ solution), was used to induce smooth muscle contraction. The isometric tension was recorded with