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Purification of a myotoxin from the toadfish *Thalassophryne maculosa* (Günter) venom

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Abstract

Venom was milked by gently pressing the base of the opercular and dorsal fin spines. Three fractions were obtained by molecular exclusion high pressure liquid chromatography (HPLC) (Protein Pak™ 125SW, Millipore Corporation) column, but only the last one with 22.7 min retention time (rt) was biological active (TmPP-22.7). This fraction was rechromatographed on reversed phase HPLC chlorobutylsilane columns (C4, Vydac) nine fractions were obtained, but only one (TmC4-47.2) with 47.2 min rt was biologically active. MALD-TOF mass analysis was carried out on two samples of TmC-47.2 and the results were 15,161.36 and 15,154.70 a.m.u., respectively. Raw venom (1040 μg/ml) depolarised frog (*Hyla crepitans*) muscle irreversibly from −85 (−88, −81) mV ($n=20$, median and its 95% CI) to −18 (−24, −15) mV ($n=24$). The biological activity in TmPP-22.7 (38 μg/ml), which depolarised muscle fibres from −79 (−82, −76) mV ($n=20$) to −63 (−69 −57) mV ($n=24$). The depolarising fraction was TmC4-47.2 (50 μg/ml) which depolarised muscles from −87 (91, −82) mV ($n=33$) to −63 (−76 −51) mV ($n=53$); the depolarising effect at this concentration was completely reversed on washing with normal saline for 2 h. Muscles treated with 1 μM tetrodotoxin (TTX) were depolarised from −80 (−85, −72) mV ($n=49$) to −44 (−56, −31) mV ($n=44$) when 100 μg/ml TmC4-47.2 were applied with TTX; washing 130 min with 1 μM TTX repolarised to −59 (−69, −50) mV ($n=25$). We also present evidence that TmC4-47.2 induces myonecrosis in mice.

Keywords: Calcium conductance; Toadfish venom; myotoxin; Skeletal muscle; Thalassophryne

1. Introduction

The toadfish *Thalassophryne maculosa*, is a common cause of accidents in many Caribbean sea sandy beaches. The envenoming occurs when people step on a fish burrowing in the sand, or carelessly handle the fish, and are stung by the dorsal or opercular spines. In Venezuela, the envenoming appears immediately as excruciating and persisting local pain, inflammation and in some instances necrosis around the sting, secondary infection often occurs. Systemic symptoms such as dizziness and fever are common. Anaesthesia or paraesthesia in the affected limb is also observed. The symptoms depend on the amount of venom received, as well as the size, weight and immunoreactivity of the victim. The healing of the lesion may be torpid, and both the wound and the sensory alteration may persist for month. Similar effects have been reported with *Thalassophryne natteri* fish venom from the north-east of

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Brazil (Lopes-Ferreira et al., 2001; Haddad Junior et al., 2003; Lima et al., 2003). Toadfish venom has been shown to have nephrotoxic effects (Faco et al., 2003) and haemostatic and cytokine releasing effects (Lopes-Ferreira et al., 2002) in mice. Still, the studies on the toxicity of the toadfish are scarce (Nair et al., 1982) and the knowledge on both the mechanism of effect and composition of its venom, are fragmentary.

We studied the venom of *T. maculosa* from Margarita Island (Venezuela) by means of high performance liquid chromatography (HPLC) and purified a polypeptide which induces severe alteration in frog (*Hyla crepitans*) muscle fibres and nerve endings but is ineffective on squid [*Loligo* (*Dorytheutis*) *plei*] giant axons.

2. Materials and methods

2.1. Source of venom

T. maculosa specimens were captured with a scoop net from the muddy sand bottom of La Restinga lagoon [10° 58.7′ N, 64° 9.7′ W; Margarita Island, Nueva Esparta state, Venezuela], and were immediately taken to the laboratory to milk venom. Venom was obtained by gently pressing on the base of dorsal and opercular spines while holding a non heparinised microhaematocrit tube (Fisher Scientific Co., Fair Lawn, NJ, USA) at the tip of the spine. Volume was registered from the liquid column in the tubes, and venom was immediately frozen on dry ice until lyophilised and stored at −80 °C. At IVIC venom was restored to its initial volume with bi-distilled water and centrifuged for 15 min at a 27.200*g* in a refrigerated centrifuge (Sorvall Model RC28S, Dupont Medical Products Dept., Newtown, CT, USA) to eliminate particulated material which may damage HPLC columns. The supernatant was lyophilised and stored at −80 °C until used. Venom protein content was determined as 1 mg per absorbance unit/cm path length measured at 280 nm. Milking 21 specimens produced a pool of 2464 μl of raw venom with 30.5 mg/ml protein.

2.2. Chemicals and solutions

All reagents were either of analytical (Sigma Chemical Co., St Louis, MO, USA) or HPLC grade. For the bioassays in frog muscle, the toxins were added to buffer solution of the following composition (mM): Na^+, 118; K^+, 2.5; Ca^{2+}, 1.8; Cl^-, 121; *N*-2-hydroxyethylpiprazine-*N*′-2-ethanesulfonic acid (HEPES), 6; pH was adjusted to 7.5 ± 0.05 with NaOH. Zero sodium frog muscle experiments were carried out in saline with the following composition (mM): 2-amino-2-hydroxymethyl-1-3-propanediol (Tris), 117; sucrose, 14.6; K^+, 2.5; Ca^{2+}, 1.8; Cl^-, 107; pH was adjusted to 7.5 ± 0.05 with HCl. Experiments in squid giant axons were carried out in artificial sea water (ASW) of the following composition (mM): Na^+, 457; K^+, 10; Ca^{2+}, 3; Mg^{2+}, 53; HEPES, 6 and Cl^-, 576; pH was adjusted to 7.5 ± 0.05 with NaOH and osmolarity set between 1000 and 1010 mOsmol/kg.

2.3. High performance liquid chromatography methods

Soluble venom obtained as indicated above, was subject of molecular exclussion HPLC chromatography on a Protein Pak™ 125SW (7.8 × 300 mm, Millipore Corporation, Waters Chromatography Division, Milford, MA, USA) with a Shimadzu LC 6B (Shimadzu Corp., Kyoto, Japan) HPLC system. Elution was with 0.2 M $CH_3 \cdot COONH_4$ at 0.5 ml/min and room temperature (≈20 °C). The elution was monitored with a UV–Visible absorbance detector (Shimadzu SPD 6AV) at 230 nm. Several injections allowed us to fraction ≈3.0 mg of venom.

The active peak isolated by molecular exclusion was purified by rechromatographing on a reversed phase chlorobutylsilane column (C4 TP-1010, 10 × 250 mm, Vydac, Hesperia, CA, USA), with a linear gradient of 0.12% $CF_3 \cdot COOH$ (TFA, Aldrich Chemical Co., St Louis, MO, USA) in water (solvent A) to 0.10% TFA in CH_3.CN (solvent B, Mallinckrodt Baker Inc., Paris, KY, USA), during 70 min at 1 ml/min. Eluate's absorbance was monitored at 230 nm.

2.4. Mass spectrometry and protein sequencing

The HPLC purified peptide was rechromatographed twice on the C4 column and subjected to matrix-assisted laser desorption time of flight (MALD-TOF) mass spectrometry (Skoog and Leary, 1997) with a Lasserman 1000 mass spectrometer, using α-cyano-4-hydroxyconamic acid as the matrix.

2.5. Electrophysiological procedures

The electrophysiological experiments in frogs (*Hyla crepitans*) were carried out using the sartorius neuromuscular preparations. All experiments were at room temperature (20 °C).

Squid nerve experiments were carried out with freshly dissected nerves of *Loligo (Doryteuthis) plei* at the Fundaciencias Marine Biological Station (Mochima Bay, Sucre state, Venezuela). Paramedian giant axons were dissected out of the mantle in natural sea water and immediately transferred to ASW.

Membrane potential was recorded with microelectrodes pulled from glass capillaries of 1.2 mm external diameter (Type GC120F-6, Clark Electromedical Instruments, Pangbourne, Reading, UK) filled with 3 M KCl. The microelectrodes were connected to a high input impedance amplifier via a Ag-AgCl electrodes. When action potentials were recorded, the capacitance of the microelectrodes was compensated with a home made pre-amplifier with a differential input stage. The input stage consisted of two