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# A novel sea anemone peptide that inhibits acid-sensing ion channels

Armando Alexei Rodríguez[a,*], Emilio Salceda[b], Anoland Georgina Garateix[a], André Junqueira Zaharenko[c], Steve Peigneur[d], Omar López[b], Tirso Pons[e], Michael Richardson[f], Maylín Díaz[a], Yasnay Hernández[a], Ludger Ständker[g], Jan Tytgat[d], Enrique Soto[b]

[a] Centro de Bioproductos Marinos (CEBIMAR), Loma y 37, Alturas del Vedado, CP 10400 Habana, Cuba
[b] Instituto de Fisiología, Universidad Autónoma de Puebla, 14 sur 6301, CU, San Manuel, Puebla, Puebla CP 72750, Mexico
[c] Laboratory of Genetics, Butantan Institute, São Paulo 05503-900, Brazil
[d] Laboratory of Toxicology, University of Leuven (KU Leuven), Campus Gasthuisberg O&N2, Herestraat 49, P.O. Box 922, 3000 Leuven, Belgium
[e] Centro Nacional de Investigaciones Oncológicas (CNIO), C/ Melchor Fernández Almagro 3, 28029 Madrid, Spain
[f] Fundação Ezequiel Dias-FUNED, Rua Conde Pereira Carneiro 80, CEP 30510-010 Belo Horizonte, MG, Brazil
[g] Kompetenzzentrum Ulm Peptide Pharmaceuticals (U-PEP), Universität Ulm (West), Albert-Einstein Allee 47, 89081 Ulm, Germany

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ABSTRACT

Sea anemones produce ion channels peptide toxins of pharmacological and biomedical interest. However, peptides acting on ligand-gated ion channels, including acid-sensing ion channel (ASIC) toxins, remain poorly explored. PhcrTx1 is the first compound characterized from the sea anemone *Phymanthus crucifer*, and it constitutes a novel ASIC inhibitor. This peptide was purified by gel filtration, ion-exchange and reversed-phase chromatography followed by biological evaluation on ion channels of isolated rat dorsal root ganglia (DRG) neurons using patch clamp techniques. PhcrTx1 partially inhibited ASIC currents ($IC_{50} \sim 100$ nM), and also voltage-gated $K^+$ currents but the effects on the peak and on the steady state currents were lower than 20% in DRG neurons, at concentrations in the micromolar range. No significant effect was observed on $Na^+$ voltage-gated currents in DRG neurons. The N-terminal sequencing yielded 32 amino acid residues, with a molecular mass of 3477 Da by mass spectrometry. No sequence identity to other sea anemone peptides was found. Interestingly, the bioinformatic analysis of Cys-pattern and secondary structure arrangement suggested that this peptide presents an Inhibitor Cystine Knot (ICK) scaffold, which has been found in other venomous organisms such as spider, scorpions and cone snails. Our results show that PhcrTx1 represents the first member of a new structural group of sea anemones toxins acting on ASIC and, with much lower potency, on $K_v$ channels. Moreover, this is the first report of an ICK peptide in cnidarians, suggesting that the occurrence of this motif in venomous animals is more ancient than expected.



## 1. Introduction

Sea anemones are venomous animals that produce a great number of bioactive peptides and proteins, comprising cytolysins, phospholipases, ion channel toxins and protease inhibitors [44]. These proteinaceous molecules are used as weapons for preying on small crustaceans and fishes, and for defense against predators. The most commonly known bioactive proteins are the 20 kDa actinoporins [4], whereas more than a hundred peptide toxins, mainly comprising voltage-gated ion ($Na^+$ and $K^+$) channel toxins [44], have been isolated and characterized from these organisms during the last four decades. Nevertheless, recent transcriptomic and peptidomic studies have shown that the diversity of peptide toxins produced by sea anemones is more complex than previously estimated [36,51,52,66], thus opening up new possibilities in the search for novel structures and biological activities from these organisms, including new peptide toxins acting on diverse receptors, and voltage-gated and ligand-gated ion channels.

Sea anemone peptide toxins acting on ligand-gated ion channels, such as acid-sensing ion channel (ASICs), are barely known in contrast with the known large number of voltage-gated ion channel toxins. ASICs are $H^+$-gated $Na^+$ channels that belong to the ENaC/degenerin superfamily of sodium channels [48]. ASICs are

* Corresponding author. Tel.: +53 7 8811298; fax: +53 7 8811298.
*E-mail addresses:* armando@cebimar.cu, aara259@gmail.com (A.A. Rodríguez), emilio.salceda@gmail.com (E. Salceda), agarateix@gmail.com (A.G. Garateix), a.j.zaharenko@ig.com.br (A.J. Zaharenko), Steve.Peigneur@pharm.kuleuven.be (S. Peigneur), omar.lpz.r@gmail.com (O. López), tirsopons@gmail.com (T. Pons), mmikeana@yahoo.com.br (M. Richardson), maylin.diaz60@yahoo.com (M. Díaz), yasnay@cebimar.cu (Y. Hernández), l.standker@gmx.de (L. Ständker), Jan.Tytgat@pharm.kuleuven.be (J. Tytgat), esoto24@gmail.com (E. Soto).

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involved in sensory perception, synaptic plasticity, learning, memory formation, cell migration and proliferation, nociception, and neurodegenerative disorders, among other processes [48]; therefore those molecules that specifically target these channels are of growing pharmacological and biomedical interest. Few proteinaceous ASIC toxins have been isolated and characterized from venomous animals. Up to date, APETx2 is the only well-known ASIC toxin from a sea anemone species (*Anthopleura elegantissima*), which selectively inhibits the ASIC3 subunit in *Xenopus laevis* oocytes ($IC_{50}$ = 63 nM) [20] as well as the ASIC3-like current of DRG neurons with an $IC_{50}$ of 216 nM [20], although it also inhibits the tetrodotoxin (TTX)-resistant $Na_v$ 1.8 currents of DRG neurones ($IC_{50}$ = 2.6 μM) [9], and channel subtypes $Na_v1.2$ and $Na_v1.8$ in *X. laevis* oocytes [45]. Recently, another peptide toxin (π-AnmTX Hcr 1b-1) acting on ASIC3 in *X. laevis* oocytes was characterized from a sea anemone [37], however its potency ($IC_{50}$ = 5.5 μM) is much lower than the one exhibited by APETx2. Some other proteinaceous compounds acting on ASICs have been isolated from tarantula [25] and snake species [10,21]. Therefore the finding of ASIC modulators from animal venoms remains limited considering the large number of biologically active peptide and proteins produced by these organisms.

In the present work we isolated and characterized a new ASIC inhibitor from the aqueous extract of the sea anemone *P. crucifer*. This peptide toxin, named PhcrTx1, inhibited the ASIC currents in rat sensory neurons and produced a smaller but significant inhibitory effect on voltage-gated $K^+$ currents, with no action on voltage-gated $Na^+$ currents. PhcrTx1 is the first toxin isolated and characterized from *P. crucifer* and it has no significant sequence similarity to any known sea anemone peptide. Our analysis indicated that this peptide presents the Inhibitor Cystine Knot (ICK) motif, which has not been previously identified in cnidarians. These results show that PhcrTx1 represents the first isolated and characterized member of a new structural group of sea anemone toxins.

## 2. Materials and methods

### 2.1. Sample preparation and chromatographic separation of the bioactive peptide

Fifteen specimens of the sea anemone *P. crucifer* (Le Sueur, 1817) were collected at the north coast of Havana. A voucher sample (No. ANC 03.3.2.001) was deposited at the Cuban National Aquarium. In the laboratory the specimens were immediately separated from stones, and then homogenized together with the secreted mucus using a blender. The homogenate was lyophilized and the dried material (27 g) stored at −20 °C.

An amount of 5 g of the dry homogenate was mixed with 100 ml of 0.1 M ammonium acetate (p.a, Merck, Germany), stirred during 30 min and centrifuged at 4000 × *g* during 1 h at 4 °C. The supernatant (350 mg/90 ml) was applied onto a Sephadex G-50 M (Pharmacia, Sweden) column (5 cm × 93 cm) previously calibrated [52] and the separation was done at a flow rate of 2 ml/min using 0.1 M ammonium acetate as eluent. Fractions of 20 ml each were collected and manually read at 280 nm on a UV-1201 spectrophotometer (Shimadzu, Japan).

The biologically active samples from gel filtration were applied to a Fractogel EMD $SO_3^-$ 650 M (Merck, Germany) cation-exchange column of dimensions 1.8 cm × 5 cm. The ion-exchange step was performed under the following conditions: a 400 ml (31 column volumes) gradient of ammonium acetate was run at a flow rate of 1 ml/min, from 0.01 to 1 M, using a gradient mixer GM-1 (Pharmacia, Sweden). Eighty fractions of 5 ml each were collected and manually read at 280 nm.

The biologically active samples from ion-exchange chromatography were submitted to reversed-phase chromatography in a Hypersil H5 ODS column (Unicam, UK) of dimensions 4.6 mm × 250 mm, previously equilibrated in solvent A, 0.1% trifluoroacetic acid (HPLC grade, AppliChem, Germany). Elution was carried out at a flow rate of 0.8 ml/min using stepwise elution at 100% A during 10 min, followed by an ascending linear gradient of solvent B, 0.05% TFA in acetonitrile (0–80% B in 80 min). The peptide was re-purified in a Discovery RPC18, 4.6 mm × 250 mm (Supelco, USA) HPLC column, using the gradient 10–20%B in 5 min, and then 20–30% in 50 min, at 1 ml/min. Eluting compounds were detected at 214 nm.

The protein content of the aqueous extract and chromatographic fractions were estimated with a BCA protein assay kit (AppliChem, Germany) [58].

### 2.2. Patch clamp experiments on isolated neurons

The biological activity screening of chromatographic fractions was performed on ASIC currents using the whole-cell patch clamp technique in primary cultured rat dorsal root ganglion (DRG) neurons. The pure compound was characterized on the ASIC currents and also on the voltage-gated ($Na^+$ and $K^+$) currents in DRG neurons. Animal care and procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering. The number of animals used for this work was kept to the minimum necessary for a meaningful interpretation of the data.

The DRG neurons were isolated from Wistar rats at postnatal ages P5 to P9 without sex distinction, and cultured according to a previously reported procedure [53]. Briefly, for the cell culture the rats were anesthetized and killed with an overdose of sevofluorane. The dorsal root ganglia were isolated from the vertebral column and incubated (30 min at 37 °C) in Leibovitz L15 medium (L15) (Invitrogen, USA) containing 1.25 mg/ml trypsin and 1.25 mg/ml collagenase (Sigma–Aldrich, USA). After enzymatic treatment, the ganglia were washed three times with sterile L15. Cells were mechanically dissociated using a Pasteur pipette and then plated on 12-mm × 10-mm glass coverslips (Corning, USA) pre-treated with poly-D-lysine (Sigma–Aldrich, USA) and placed onto 35-mm culture dishes (Corning, USA). Neurons were incubated 4–8 h in a humidified atmosphere (95% air, 5% $CO_2$, at 37 °C) using a $CO_2$ water-jacketed incubator (Nuaire, USA) to allow the isolated cells to settle and adhere to the coverslips. The plating medium contained L15, with added 15.7 mM $NaHCO_3$ (Merck, Mexico), 10% fetal bovine serum, 2.5 μg/ml fungizone (both from Invitrogen), 100 U/ml penicillin (Lakeside, Mexico), and 15.8 mM HEPES (Sigma–Aldrich, USA).

Whole-cell recording was carried out using an Axopatch-1D amplifier (Axon Instruments, USA). The pulse generation and the data sampling were controlled by Pclamp 9.2 software (Axon Instruments, USA) using a 16-bit data-acquisition system, Digidata 1320A (Axon Instruments, USA). All experiments were performed at room temperature (23–25 °C). The patch electrodes were pulled from borosilicate glass and had a resistance in the range of 1.5–2.5 MΩ when filled with the intracellular solution (see Table 1). The capacitance and series resistance (80%) were electronically compensated. Experiments were rejected when the voltage error exceeded 5 mV after compensation for series resistance at the maximum peak current, but no corrections were made for smaller values.

ASIC currents were elicited by a fast (about 40 ms) pH change from 7.4 to 6.1 for 5 s, by shifting one of the three outlets of a fast change perfusion system (SF-77B, Warner Inst., USA) while keeping the cell at a holding potential ($V_h$) of −60 mV. The time interval between the pH change steps was 1 min to guarantee that



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