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New APETx-like peptides from sea anemone *Heteractis crispa* modulate ASIC1a channels

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ABSTRACT

Sea anemones are an abundant source of various biologically active peptides. The hydrophobic 20% ethanol fraction of tropical sea anemone *Heteractis crispa* was shown to contain at least 159 peptide compounds including neurotoxins, proteinase and α-amylase inhibitors, as well as modulators of acid-sensing ion channels (ASICs). The three new peptides, π-AnmTX Hcr 1b-2, -3, and -4 (41 aa) (short names Hcr 1b-2, -3, -4), identified by a combination of reversed-phase liquid chromatography and mass spectrometry were found to belong to the class 1b sea anemone neurotoxins. The amino acid sequences of these peptides were determined by Edman degradation and tandem mass spectrometry. The percent of identity of Hcr 1b-2, -3, and -4 with well-known ASIC3 inhibitors Hcr 1b-1 from *H. crispa* and APETx2 from *Anthopleura elegantissima* is 95–78% and 46–49%, respectively. Electrophysiological experiments on homomeric ASIC channels expressed in *Xenopus laevis* oocytes establish that these peptides are the first inhibitors of ASIC1a derived from sea anemone venom. The major peptide, Hcr 1b-2, inhibited both rASIC1a (IC_{50} 4.8 ± 0.3 μM; nH 0.92 ± 0.05) and rASIC3 (IC_{50} 15.9 ± 1.1 μM; nH 1.0 ± 0.05). The maximum inhibition at saturating peptide concentrations reached 64% and 81%, respectively. In the model of acid-induced muscle pain Hcr 1b-2 was also shown to exhibit an antihyperalgesic effect, significantly reducing of the pain threshold of experimental animals.

1. Introduction

The venoms of many terrestrial and marine organisms such as spiders, scorpions, snakes, gastropods, and sea anemones actively studied over the past several decades are known to represent a mixture of an enormous number of protein toxins and peptides possessing unique specificity and potency. Their biological role in biocenosis is attack of prey or competitors as well as protection of the host organism from predators. Peptide toxins mostly act on certain subtypes of the ion channels (Na_V, K_V, Ca_V, ASIC, AChR, TRP, P2X) involved in important physiological processes of a prey/predator organism.

It was found that sea anemones produced neurotoxins modulating K_V and Na_V channels (3–6.5 kDa), cytolytic α-pore-forming toxins (~20 kDa), and a multigene family of Kunitz-type serine proteinase inhibitors (6–7 kDa) also modulating TRPV1 receptor or several subtypes of K_V channels [1]. Recently, a new β-defensin family representatives (~4.7 kDa), helianthamide [2] and magnificamide [3], inhibiting porcine pancreatic α-amylase (PPA), has been derived from sea anemones *Stichodactyla helianthus* and *Heteractis magnifica*. According to Tysoe and coauthors [2], such amylase inhibitors can be a promising compounds for controlling blood glucose levels of patients with diabetes mellitus.

Several peptides acting on ASIC channels are considered to be a candidate for selective analgesic drugs development [4–7]. ASICs belong to the superfamily of amiloride-sensitive degenerin/epithelial sodium channels (DEG/ENaC) gated by the extracellular pH value decrease. It was shown that ASICs are formed by three subunits. To date, at least nine ASICs isoforms (1a – b, 2a – b, 3a – c, 4, 5) encoded by five genes (ACCN1-5) have been detected in mammals [8,9]. ASIC1a, ASIC2a, and ASIC2b are expressed widely in neurons of the central nervous system, moreover, ASIC1a is the most abundant in neurons of the brain [8]. ASICs involvement in synaptic plasticity, generation and transmission of a nerve impulse [10,11], development of neurodegenerative disorders or autoimmune neuroinflammation [9,11] is confirmed. Homo- and heteromeric ASIC3-containing channels are mainly expressed by neurons of the peripheral nervous system [9] involved in the pain perception of various etiologies [9,12,13].

To date, it has been established that peptide toxins isolated from

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spider and snake venoms: PcTx1 [14], Hi1a [15], Hm3a [16], MitTxα/β [17], Ma1–3 [7], α-DTx [18], mainly modulate ASIC1 [19]. PcTx1 from tarantula *Psalmopoeus cambridgei* is the first described ASIC1a peptide modulator. Until recently, it remained the most powerful and selective channel blocker (IC_{50} 0.9 nM) [19]. Now, PcTx1 yields the palm to Hi1a (IC_{50} 0.4 nM) discovered in the venom-gland transcriptome of the funnel-web spider *Hadronyche infensa* intensively studied as an effective long-acting neuroprotective agent [15].

In contrast to above mentioned peptides, molecules derived from sea anemones, APETx2 [6,20], Hcr 1b-1 [21], PhcrTx1 [22], and Ugr 9a-1 [23], target homomeric ASIC3 and heteromeric ASIC3-containing channels [19]. APETx2 from sea anemone *Anthopleura elegantissima* was considered as a specific inhibitor of ASIC3 and ASIC3-containing channels except ASIC2a/3 [6,20]. However, it has recently been shown that APETx2 is also capable to potentiate ASIC1b and ASIC2a current while having no effect on ASIC1a [5]. In addition, APETx2 at high concentrations inhibits $Na_V1.2$, $Na_V1.6$, and $Na_V1.8$ channels [24], as well as potassium channel of two subtypes, hERG [20] and $K_V3.4$ [6]. Hcr 1b-1 from *Heteractis crispa* inhibits ASIC3 31-fold weaker than APETx2 (IC_{50} 5.5 μM vs 0.175 μM for hASIC3) does, apparently, due to the smaller number of charged residues in its sequence [6,21]. Both of these peptides are shown to have an antihyperalgesic effect in the model of acid-induced muscle pain [13,25]. Also there are two ASIC modulators of structural classes 11a and 9a [26], PhcrTx1 toxin from *Phymanthus crucifer* [22] and Ugr 9a-1 from *Urticina grebelnyi* [23], respectively. Two mutants of the inactive molecule Ugr 9a-2 inhibiting ASIC3 were also obtained [27].

A complex transcriptomic/proteomic study of *S. helianthus* [28], *Stichodactyla haddoni* [29], *Stichodactyla duerdeni* [30], and *Bunodosoma granulifera* [28] venoms was performed. According to results of transcriptome analysis, both known and new representatives of APETx-like peptides were found only in sea anemone *B. granulifera*, a species from Actiniidae family [28].

In this article, we analyze diversity of peptides from hydrophobic 20% ethanol fraction of *H. crispa*. A mass spectrometry investigation of obtained fractions has revealed the presence of at least 159 peptide components exhibiting inhibitory activity to trypsin, PPA or ASIC current. The primary structures of three new peptides belonging to class 1b [26], the first inhibitors of ASIC1a channel from sea anemones, have been determined. The inhibitory effect of Hcr 1b-2, major peptide of hydrophobic 20% ethanol fraction, has been shown on rASIC1a and rASIC3 currents. It has also been shown that this peptide demonstrates an antihyperalgesic effect in the model of acid-induced muscle pain.

2. Materials and methods

The specimens of *H. crispa* were collected from the South China Sea, Vietnam (2013). The species of sea anemone was identified by Dr. E. Kostina (A.V. Zhirmunsky Institute of marine biology FEB RAS). Sea anemones were frozen and kept at −20 °C.

2.1. Extraction and chromatographic procedure of sea anemone peptides

Peptides were extracted from a whole body of sea anemone *H. crispa* with 70% ethanol for 24 h. After centrifugation and ethanol evaporation, peptides separation was performed by hydrophobic chromatography on Polychrome-1 column (4.8 × 95 cm) (Olaine, Latvia) using a step gradient of ethanol concentration at a flow rate of 120 ml/h and fraction size of 20 ml. All preparations were performed at +4 °C. The protein concentration was determined by Lowry method [31], bovine serum albumin was used as a standard.

Hydrophobic peptides eluted with 20% ethanol were separated on reversed-phase Luna C18 column (10 × 250 mm) equilibrated with 10% acetonitrile solution in 0.1% trifluoroacetic acid (TFA) on an Agilent 1100 chromatograph (Agilent Technologies, USA). Peptides elution was carried out using combined gradient of acetonitrile concentration (with 0.1% TFA and at a flow rate of 3 ml/min) according to scheme: 10–32% of acetonitrile for 22 min, 32% for 18 min, 32–35% for 1.5 min, 35% for 16 min, 35–40% for 2.5 min, 40% for 8 min, 40–70% for 15 min. The final separation of the active peptides obtained after RP-HPLC was made on the same column in two alternative gradients of acetonitrile concentration (with 0.1% TFA and at a flow rate of 3 ml/min): 10–35% of acetonitrile for 13 min, then 35% for 15 min or 10–40% for 60 min. Vacuum concentrator 5301 (Eppendorf, Germany) was used for acetonitrile evaporation.

2.2. Mass spectrometric analysis

A mass spectrometric analysis was carried out using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik, Germany). The samples were dissolved in acetonitrile/water solution (1:1, v/v) containing 0.1% TFA and mixed with 10 mg/ml sinapinic acid as a matrix. Protein molecular masses (5000–20000 Da) were obtained in linear or reflector mode with external calibration.

2.3. Tandem mass spectrometry (MS/MS)

The amino acid sequences were identified from the CID tandem mass spectra of peptide fragments obtained by the cyanogen bromide cleavage of the peptide molecule previously treated with 4-vinylpyridine. Collision-induced dissociation (CID) MS/MS experiments were performed on an ultra-high resolution quadrupole time-of-flight mass spectrometer MaXis impact (Bruker Daltonik, Germany) to determine the sequence of Hcr 1b-2 and -3 peptide fragments as well as on an Ultraflex TOF/TOF III mass spectrometer (Bruker Daltonik, Germany) to determine the sequence of Hcr 1b-4 peptide fragments. The latter instruments were equipped with an ESI and MALDI ionization source, respectively. A survey mass spectrum and tandem mass spectrum were recorded for each sample. During MS/MS, the fragment ions were generated from the isolated $[M + 3H]^{3+}$ and $[M+H]^{+}$ peptide precursor ion of Hcr 1b-2, -3, and Hcr 1b-4 peptide fragments, respectively. $[M+3H]^{3+}$ peptide precursor ions were fragmented by low-energy CID with collision energy of 50 eV for Hcr 1b-2 and 35 eV for Hcr 1b-3.

2.4. Reduction and alkylation of disulfide bridges

Peptides were reduced and alkylated with 4-vinylpyridine as described in [31]. Separation of the reaction mixture was made on reversed-phase Nucleosil C18 column (4.6 × 250 mm) equilibrated with 10% acetonitrile in 0.1% TFA. The elution was carried out using combined gradient of acetonitrile concentration (with 0.1% TFA and at a flow rate of 0.5 ml/min), 10% of acetonitrile for 30 min, 10–40% for 60 min, and then 40% for 10 min.

2.5. Cyanogen bromide cleavage of alkylated peptides

The reaction was carried out in 70% TFA at room temperature for 4 h in the dark. The molar ratio of cyanogen bromide:peptide was 100:1. Separation of the reaction mixture was made on reversed-phase Nucleosil C18 column (4.6 × 250 mm) equilibrated with 10% acetonitrile in 0.1% TFA. The elution was carried out using a combined gradient of acetonitrile concentration at a flow rate of 0.5 ml/min, 10% of acetonitrile for 20 min, 10–40% for 60 min, and then 40% for 10 min.

2.6. Ladder sequencing of alkylated peptides

The alkylated peptide (5 μg) was dissolved in 20 μl of 50 mM Tris-HCl buffer solution (pH 8.0). Carboxypeptidases A (5 μg) and B (5 μg) treated with diisopropyl fluorophosphate were added; the mixture was kept at 37 °C for 0.5, 1, 2, 4, and 6 h. The reaction mixture was analyzed by MALDI-MS as previously described.