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Peptides from puff adder *Bitis arietans* venom, novel inhibitors of nicotinic acetylcholine receptors

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ABSTRACT

Phospholipase A_2 (named bitanarin) possessing capability to block nicotinic acetylcholine receptors (nAChRs) was isolated earlier (Vulfius et al., 2011) from puff adder *Bitis arietans* venom. Further studies indicated that low molecular weight fractions of puff adder venom inhibit nAChRs as well. In this paper, we report on isolation from this venom and characterization of three novel peptides called baptides 1, 2 and 3 that reversibly block nAChRs. To isolate the peptides, the venom of *B. arietans* was fractionated by gel-filtration and reversed phase chromatography. The amino acid sequences of peptides were established by *de novo* sequencing using MALDI mass spectrometry. Baptide 1 comprised 7, baptides 2 and 3 −10 amino acid residues, the latter being acetylated at the N-terminus. This is the first indication for the presence of such post-translational modification in snake venom proteins. None of the peptides contain cysteine residues. For biological activity studies the peptides were prepared by solid phase peptide synthesis. Baptide 3 and 2 blocked acetylcholine-elicited currents in isolated *Lymnaea stagnalis* neurons with IC_{50} of about 50 μM and 250 μM, respectively. In addition baptide 2 blocked acetylcholine-induced currents in muscle nAChR heterologously expressed in *Xenopus* oocytes with IC_{50} of about 3 μM. The peptides did not compete with radioactive α-bungarotoxin for binding to *Torpedo* and α7 nAChRs at concentration up to 200 μM that suggests non-competitive mode of inhibition. Calcium imaging studies on α7 and muscle nAChRs heterologously expressed in mouse neuroblastoma Neuro2a cells showed that on α7 receptor baptide 2 inhibited acetylcholine-induced increasing intracellular calcium concentration with IC_{50} of 20.6 ± 3.93 μM. On both α7 and muscle nAChRs the suppression of maximal response to acetylcholine by about 50% was observed at baptide 2 concentration of 25 μM, the value being close to IC_{50} on α7 nAChR. These data are in accord with non-competitive inhibition as follows from α-bungarotoxin binding experiments. The described peptides are the shortest peptides without disulfide bridges isolated from animal venom and capable to inhibit nAChR by non-competitive way.

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1. Introduction

Snake venoms affect various targets in the prey organism including cardiovascular, immune and nervous systems (Warrell, 2010). To accomplish this task, venoms comprise a large number of different compounds interacting selectively with biological targets. Therefore, venoms can be used as a rich source of tools for studying the structure and functioning principles of the vital systems of animals at the molecular level. Good examples of such tools are α-neurotoxins of three-finger toxin family from *Elapidae* snakes, which allowed obtaining comprehensive information on the

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structure and mechanism of action of nicotinic acetylcholine receptors (nAChRs). These receptors are pentameric transmembrane proteins from the superfamily of ligand gated ion channels; they are widely distributed in the organisms of both vertebrates and invertebrates (Corringer et al., 2000; Karlin, 2002; Millar, 2003; Papke, 2014; Fasoli and Gotti, 2015). nAChRs perform fast transmission of excitation from the motor nerve to the skeletal muscle, between the neurons of the central and peripheral nervous system, modulate the release of neurotransmitters from the presynaptic terminal, and play an important role in synaptic plasticity (Wonnacott et al., 2006; Dani and Bertrand, 2007). Disturbances in the nAChR expression or function lead to serious neurodegenerative diseases (Dani and Bertrand, 2007; Dineley et al., 2015). There are 17 different nAChR subunits which, assembling as pentamers, form a number of subtypes that are distinguished in biophysical characteristics as well as in sensitivity to various agonists or antagonists (Vulfius, 2006; Gotti et al., 2010). These differences are used to identify the nAChR subtypes in tissues or cells (Albuquerque et al., 1997; Kehoe and McIntosh, 1998; Nicke et al., 2004; Vulfius et al., 2005).

The toxins of snakes and marine mollusks are often used for this purpose (Tsetlin and Hucho, 2004; Kasheverov et al., 2009a; Tsetlin et al., 2009). α-Conotoxins, peptide toxins from sea snails of *Conus* genus, are from 12 to 30 amino acid residues long and exhibit high selectivity to certain nAChR subtypes (Nicke et al., 2004; Kasheverov et al., 2009a). Due to the small size, α-conotoxins and their analogues can be prepared by peptide synthesis, but the presence of multiple disulphide bonds significantly hinder the preparation due to a possibility of improper closure of disulphide bonds. Peptide toxins waglerins with only one disulphide bridge were isolated (McArdle et al., 1999) previously from the venom of the temple viper *Tropidolaemus wagleri*; these toxins block muscle type nAChRs. Recently from the venom of Fea's viper *Azemiops feae* we have isolated (Utkin et al., 2012) peptide azemiopsin which contains 21 amino acid residues and no disulphide bond, but still is an nAChR blocker.

We were looking for polypeptide compounds capable of interacting with nAChRs in the venoms of *Viperidae* snakes and found that a low molecular weight fraction obtained after gel filtration of venom from African puff adder *Bitis arietans* has the ability to inhibit the current induced by acetylcholine in neurons of a pond snail *Lymnaea stagnalis* (Vulfius et al., 2009). In this report we present the data about sequence and biological activity for three isolated peptides which were named baptides 1, 2 and 3. Baptide 1 contained 7 amino acid residues while baptides 2 and 3—10. No cysteine residues were present in all three peptides. Baptide 2 blocked acetylcholine-induced currents in isolated *L. stagnalis* neurons with IC_{50} of about 250 μM and in mouse muscle nAChR heterologously expressed in *Xenopus* oocytes with IC_{50} of about 3 μM. A lack of competition with α-bungarotoxin suggests a non-competitive nAChR inhibition by baptide 2. Fluorescence calcium imaging assays also showed that baptide 2 acts as a non-competitive inhibitor at both muscle and α7 nAChRs. Baptide 3 suppressed cytisine- or acetylcholine-elicited current in *Lymnaea* neurons with IC_{50} of about 50 μM, but was inactive on muscle and α7 type nAChRs. Baptide 1 was a very weak antagonist on *L. stagnalis* neurons.

2. Material and methods

2.1. Materials

The *Bitis arietans* puff adder venom was obtained as earlier described (Vulfius et al., 2011). Trizma-HCl, EGTA, Pronase E, acetylcholine iodide, PNU-120596 and HEPES were purchased from Sigma (USA). Trypsin was from Calbiochem (La Jolla, CA), 4-vinylpyridine was from Aldrich, dithiothreitol was from Bio-Rad, acetonitrile was from Kriochrom (Russia), and trifluoroacetic acid was from Merck. nAChR-enriched membranes from the electric organs of *Torpedo californica* were kindly provided by Prof. F. Hucho (Free University of Berlin). GH4C1 cells transfected with human α7 nAChR were a gift from Eli-Lilly (England). Mono-iodinated (3-[^{125}I] iodotyrosyl^{54})-α-Bgt (~2000 Ci/mmol) was from Amersham Biosciences. Other reagents were of the highest quality commercially available.

2.2. Purification of peptides

Crude *B. arietans* venom (600 mg) was dissolved in 2 ml of 0.1 M ammonium acetate buffer (pH 6.2) containing 0.01% sodium azide. It was applied onto a Sephadex G50 sf (GE Healthcare, U.S.A.) column (4.5 × 150 cm) that had been equilibrated with the same buffer and was then eluted at a flow rate of 60 ml/h. The eluted proteins were detected by absorbance at 230 nm. The fractions were pooled (Fig. 1) and analyzed for capability to suppress acetylcholine-induced current in *L. stagnalis* neurons. The active fraction VII (Fig. 1) was separated on a Jupiter C18 (4.6 × 250 mm, 5u, 300 A, Phenomenex, USA) column in a gradient of 2%—30% acetonitrile in 84 min in the presence of 0.1% trifluoroacetic acid, at a flow rate of 1.0 ml/min.

2.3. MALDI mass spectrometry

MALDI-TOF MS analysis was performed on UltrafleXetreme MALDI-TOF-TOF mass spectrometer (Bruker Daltonik, Germany) equipped with Nd laser. Aliquots (0.5 μl) of the sample were mixed on a steel target with an equal volume of 2,5-dihydroxybenzoic acid (Aldrich, Milwaukee, WI) solution (20 mg/ml in 30% acetonitrile/0.5% trifluoroacetic acid), and the droplet was left to dry at room temperature. Every mass spectrum was obtained as a sum of minimum 500 laser shots. The MH+ molecular ions were measured in reflector mode; the accuracy of mass peak measurement was 0.005%. Spectra of fragmentation were obtained in LIFT mode, the accuracy of daughter ions measurement was 1 Da. Peptide mass fingerprinting was performed as described (Serebryakova et al., 2006).

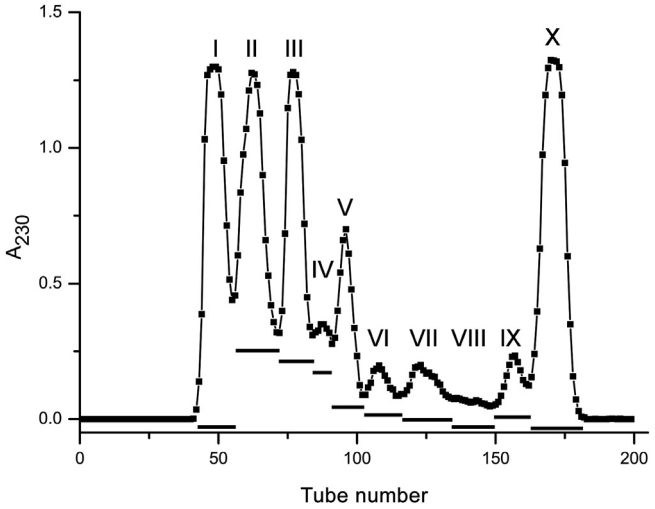

Fig. 1. Separation of puff adder *Bitis arietans* venom by gel-filtration on a Sephadex G50 sf (GE Healthcare, U.S.A.) column (4.5 × 150 cm) equilibrated with 0.1 M ammonium acetate buffer (pH 6.2), flow rate - 60 ml/h.

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