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Conversed mutagenesis of an inactive peptide to ASIC3 inhibitor for active sites determination

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ABSTRACT

Peptide Ugr9-1 from the venom of sea anemone *Urticina grebelnyi* selectively inhibits the ASIC3 channel and significantly reverses inflammatory and acid-induced pain *in vivo*. A close homolog peptide Ugr 9-2 does not have these features. To find the pharmacophore residues and explore structure–activity relationships of Ugr 9-1, we performed site-directed mutagenesis of Ugr 9-2 and replaced several positions by the corresponding residues from Ugr 9-1. Mutant peptides Ugr 9-2 T9F and Ugr 9-2 Y12H were able to inhibit currents of the ASIC3 channels 2.2 times and 1.3 times weaker than Ugr 9-1, respectively. Detailed analysis of the spatial models of Ugr 9-1, Ugr 9-2 and both mutant peptides revealed the presence of the basic-aromatic clusters on opposite sides of the molecule, each of which is responsible for the activity. Additionally, Ugr9-1 mutant with truncated *N*- and *C*-termini retained similar with the Ugr9-1 action *in vitro* and was equally potent *in vivo* model of thermal hypersensitivity. All together, these results are important for studying the structure–activity relationships of ligand-receptor interaction and for the future development of peptide drugs from animal toxins.

1. Introduction

In the last 40 years, venoms of animals like spiders, scorpions, sea anemones, snakes, and cone snails have provided a great variation of effective peptide molecules specifically those acting on a variety of ion channels, such as voltage-gated Na^+, K^+, and Ca^{2+} channels (Frazão et al., 2012; Quintero-Hernández et al., 2013), transient receptor potential channels (Andreev et al., 2013), acid-sensing ion channels (ASICs) (Osmakov et al., 2014), and others. Thereby, they are considered as useful tools for the structure–function relationship study of many channels for their mechanism of action determination and physiological role in living cells (McCleary and Kini, 2013).

Control of pH is very important for normal function of tissues; therefore, investigation of ASICs is an important part of understanding of normal and pathological processes. ASIC channels relate to the superfamily of amiloride-sensitive degenerin/epithelial Na^+-channels (DEG/ENaC) (Kellenberger and Schild, 2002). Six protein isoforms (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) form homo- or heterotrimeric transmembrane complexes. In response to acidic pH stimuli, all but ASIC2b and ASIC4 mediate a sodium-selective current (Akopian et al., 2000; Chen et al., 1998; Waldmann et al., 1997). ASIC channels were found in central and peripheral nerve cells where they participate in various physiological and pathological processes. It was shown that ASIC1a and ASIC2 are directly involved in synaptic plasticity, learning, transmission of nerve excitation (Wemmie et al., 2003, 2002), ischemia, and neuronal cell death (Friese et al., 2007; Gao et al., 2005; Xiong et al., 2004; Yagi et al., 2006). Peripheral ASIC1b and, especially, ASIC3 channels play an important role in the perception of pain and signal integration under the inflammation process (Ikeuchi et al., 2009; Dubinnyi et al., 2012; Lingueglia, 2007; Deval et al., 2008; Diochot et al., 2012).

To date, several peptide molecules targeting ASIC channels were isolated from venoms of various animals. The spider 40 amino-acid peptide psalmotoxin 1 (PcTx1) was isolated from the spider

Abbreviations: ASIC3, acid-sensing ion channel type 3; IC_{50}, half-maximal inhibitory concentration; CFA, complete Freund's adjuvant; NMR, nuclear magnetic resonance.

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Psalmopoeus cambridgei (Escoubas et al., 2000). PcTx1 has a high-affinity inhibitory effect on homomeric ASIC1a channels in various cellular expression systems with IC_{50} < 1 nM. However it has a potentiating action on ASIC1b channels (Chen et al., 2006). From the venom of the coral snake, *Micrurus tener tener*, a β-bungarotoxin related MitTx, was identified. It consists of two subunits, a 60 amino-acid Kunitz-type peptide MitTx-α subunit and a 120 amino-acid phospholipase A2-like protein MitTx-β subunit. MitTx is an agonist of ASIC1a, 1b and 3 subtypes, activating them in the nanomolar concentration at neutral pH. Also toxin has a very strong potentiating effect on ASIC2a (Bohlen et al., 2011). Two 57 amino-acid peptides, mambalgin-1 (Ma-1) and mambalgin-2 (Ma-2), have been found in the African black mamba *Dendroaspis polylepis polylepis* venom. The peptides differ by one substitution in position 4, Y in Ma-1 or F in Ma-2. Mambalgins in the nanomolar concentration reversibly inhibit ASIC1a as well as ASIC1b channels and exhibit analgesic effects *in vivo* in models of acute and inflammatory pain (Diochot et al., 2012).

Some molecules acting on ASIC were found in venomous marine animals. APETx2, a 42 amino-acid polypeptide component of the sea anemone *Anthopleura elegantissima* venom, reversibly inhibits ASIC3 and reduces mechanical hypersensitivity in a rat model of cutaneous inflammatory pain (Diochot et al., 2004; Karczewski et al., 2010). A structural analog of APETx2 – 41 amino-acid polypeptide π-AnmTx Hcr 1b-1 isolated from alcoholic extract of the sea anemone *Heteractis crispa* also reversibly inhibits the peak component of the current of human ASIC3 channels (Kozlov et al., 2012).

A structurally different 29 amino-acid polypeptide π-AnmTX Ugr 9a-1 identified in the venom of the sea anemone *Urticina grebelnyi* has an inhibiting effect on the ASIC3 channel with IC_{50} of 10 μM. The Ugr 9-1 close homolog, a 29 amino-acid polypeptide AnmTX Ugr 9a-2 (shortly Ugr 9-2) from the same venom has no effect on ASIC3 as well as other ASIC isoforms. Also, it was non-active *in vivo* in thermal hypersensitivity model in contrast to Ugr 9-1, which showed a significant analgesic effect at doses of 0.1–0.5 mg/kg (Osmakov et al., 2013). To find the pharmacophore residues, we produced several mutant analogs on the basis of inactive Ugr 9-2 to compare their biological activity with the activity of Ugr 9-1. We showed that the T9F substitution, as well as the Y12H are each individually important to ASIC3-inhibiting activity. Also, we produced the Ugr 9-1 shortened mutant Ugr22 truncated at both *N*- and *C*-termini, protruding from the central area, and showed that Ugr22 possessed similar *in vitro* activity as Ugr 9-1 and demonstrated a pronounced anti-inflammatory effect *in vivo*.

2. Materials and methods

2.1. Gene synthesis and construction of expression vectors

The DNA encoding mature Ugr 9-2 peptide was constructed from three synthetic oligonucleotides using the PCR technique. The target PCR fragment was amplified using forward primer containing a site for restriction enzyme BglII and the Met-codon for BrCN cleavage (5′ – GATTAGATCTC ATG ATT TCC ATT GAT CCG CCG TGC CGT ACC TGC TAT TAT – 3′), reverse primer 1 (5′ – CGC ATC ATA CAC GCA ATT GCC GGA GGA ATC ACG ATA ATA GCA GGT ACG GCA – 3′), reverse primer 2 (“R2”), containing a site for restriction enzyme XhoI and the stop-codon (5′ – GATTCCTCGAG CTA CGC GCC GCC GCA GCC AAA CGC ATC ATA CAC GCA ATT – 3′). PCR fragment-encoding peptide gene was gel-purified, digested by BglII/XhoI, and cloned into the expression vector pET32b$^+$ (Novagen). To introduce F9 and H12 substitutions, two pairs of primers (forward for F9: 5′ – GATTAGATCTC ATG ATT TCC ATT GAT CCG CCG TGC CGT TTT TGC TAT TAT – 3′ and reverse for F9 – “R2”; forward for H12: GATTAGATCTC ATG ATT TCC ATT GAT CCG CCG TGC CGT ACC TGC TAT CAT – 3′ and reverse for H12 – “R2”) were designed. The plasmid pET32b$^+$-Ugr 9-2 was used as a template. A pair of primers (forward: 5′ – GATTAGATCTC ATG GGC TGC CGT TTT TGC TAT CAT – 3′, and reverse: 5′ – GATTCCTCGAG CTA GCC GCA GCC ATA CGC ATC ATA – 3′) was designed to a construct of DNA encoding truncated Ugr 9-1 peptide. The plasmid pET32b$^+$-Ugr 9-1 (Osmakov et al., 2013) was used as a template. The resulting constructs were confirmed by sequencing.

2.2. Recombinant peptide production

Recombinant peptides were produced through fusion with a thioredoxin domain. *Escherichia coli* BL21(DE3) cells transformed with the expression vectors were cultured at 37 °C in LB medium containing 100 μg/ml ampicillin up to the culture density of OD_{600}~0.6–0.8. To induce expression, up to 0.2 mM isopropyl-1-thio-β-D-galactopyranoside was added. The cells were cultured at 25 °C for 18 h, harvested, resuspended in the start buffer for affinity chromatography (400 mM NaCl, 20 mM Tris–HCl buffer, pH 7.5), and ultrasonicated and centrifuged for 15 min at 14,000 rpm to remove all insoluble particles. The supernatant was applied to a TALON Superflow metal affinity resin (Clontech), and the fusion proteins were purified according to the manufacturers' instructions. Each fusion protein cleavage was performed in accordance with (Andreev et al., 2010). HCl to a final concentration of 0.2 M and CNBr with a molar ratio CNBr to each fusion protein of 600:1 were added. Target peptides were purified from the reaction mixture by HPLC on a reverse-phase column Jupiter C_5 250 × 10 mm (Phenomenex). The peptides' purity was checked by Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass-spectrometry (MS) and by *N*-terminal sequencing.

2.3. Mass spectrometry

Molecular weight measurement was carried out by MALDI-TOF mass spectrometry (Micromass, UK), and Ultraflex TOF–TOF (Bruker Daltonik, Germany) instruments. Calibration was performed using either a ProteoMass peptide and protein MALDI-MS calibration kit, with a mass range of 700 Da to 66,000 Da, or a ProteoMass peptide MALDI-MS calibration kit, with a mass range of 700 Da to 3500 Da (both from Sigma, USA). The molecular mass was determined in linear or reflector positive ion mode, using samples prepared by the dried-droplet method with 2.5-dihydroxy benzoic acid (10 mg/ml in 70% acetonitrile with 0.1% TFA) or α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitrile with 0.1% TFA) matrices.

2.4. Amino acid sequence analysis

N-terminal sequencing of alkylated peptide was carried out by automated stepwise Edman degradation, using a Procise model 492 protein sequencer (Applied Biosystems) according to the manufacturer's protocol.

2.5. Electrophysiology

Xenopus laevis oocytes were removed surgically, defolliculated, and injected with 2.5 ng–10 ng of human ASIC3 (hASIC3) cRNA (AF057711.1). cRNA transcripts were synthesized from NaeI-linearized ASIC3 cDNA template (pcDNA3.1 + human ASIC3 subcloned from clone EX-Q0260-B02 (GeneCopoeia, Inc.)), using a RiboMAXTM large-scale RNA production system T7 (Promega) according to the manufacturers' protocol for capped transcripts. After injection, oocytes were kept for 2–3 days at 19 °C and then up to 7