Available online at www.sciencedirect.com
SCIENCE DIRECT
PEPTIDES
Peptides 26 (2005) 2412–2426
www.elsevier.com/locate/peptides

Discovery of an MIT-like atracotoxin family: Spider venom peptides that share sequence homology but not pharmacological properties with AVIT family proteins

Suping Wen [a], David T.R. Wilson [b], Sanjaya Kuruppu [c], Michael L.J. Korsinczky [b], Joseph Hedrick [d], Ling Pang [d], Tim Szeto [e,1], Wayne C. Hodgson [c], Paul F. Alewood [b], Graham M. Nicholson [a,*]

[a] *Neurotoxin Research Group, Department of Health Sciences, University of Technology, Sydney, P.O. Box 123, Broadway, NSW 2007, Australia*
[b] *Institute for Molecular Biosciences, University of Queensland, St. Lucia, Qld 4072, Australia*
[c] *Monash Venom Group, Department of Pharmacology, Monash University, Clayton, Vict. 3800, Australia*
[d] *Schering-Plough Corporation, 2000 Galloping Hill Road, Kenilworth, NJ 07033-1329, USA*
[e] *Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, CT 06032, USA*

Received 13 April 2005; received in revised form 6 May 2005; accepted 6 May 2005
Available online 24 June 2005

Abstract

This project identified a novel family of six 66–68 residue peptides from the venom of two Australian funnel-web spiders, *Hadronyche* sp. 20 and *H. infensa*: Orchid Beach (Hexathelidae: Atracinae), that appear to undergo N- and/or C-terminal post-translational modifications and conform to an ancestral protein fold. These peptides all show significant amino acid sequence homology to atracotoxin-Hvf17 (ACTX–Hvf17), a non-toxic peptide isolated from the venom of *H. versuta*, and a variety of AVIT family proteins including mamba intestinal toxin 1 (MIT1) and its mammalian and piscine orthologs prokineticin 1 (PK1) and prokineticin 2 (PK2). These AVIT family proteins target prokineticin receptors involved in the sensitization of nociceptors and gastrointestinal smooth muscle activation. Given their sequence homology to MIT1, we have named these spider venom peptides the MIT-like atracotoxin (ACTX) family. Using isolated rat stomach fundus or guinea-pig ileum organ bath preparations we have shown that the prototypical ACTX–Hvf17, at concentrations up to 1 μM, did not stimulate smooth muscle contractility, nor did it inhibit contractions induced by human PK1 (hPK1). The peptide also lacked activity on other isolated smooth muscle preparations including rat aorta. Furthermore, a FLIPR Ca^{2+} flux assay using HEK293 cells expressing prokineticin receptors showed that ACTX–Hvf17 fails to activate or block hPK1 or hPK2 receptors. Therefore, while the MIT-like ACTX family appears to adopt the ancestral disulfide-directed β-hairpin protein fold of MIT1, a motif believed to be shared by other AVIT family peptides, variations in the amino acid sequence and surface charge result in a loss of activity on prokineticin receptors.

Keywords: ACTX–Hvf17; Mamba intestinal toxin 1; Bv8; Prokineticin; Funnel-web spider

Abbreviations: ACh, acetylcholine; ACTX, atracotoxin; BCA, bicinchoninic acid; Bv8, 8 kDa protein from the skin secretions of toads (*Bombina variegata*); CHO, Chinese hamster ovary; DDH, disulfide-directed β-hairpin; EST, expressed sequence tag; FLIPR, fluorometric imaging plate reader; HEK, human embryonic kidney; ICK, inhibitory cystine-knot; MIT1, mamba intestinal toxin 1 from the venom of the black mamba snake *Dendroaspis p. polylepis*; PK, prokineticin (also known as endocrine-gland vascular endothelial growth factor or EG-VEGF); PKR, prokineticin receptor; RACE, rapid amplification of cDNA ends; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid

* Corresponding author. Tel.: +61 2 9514 2230; fax: +61 2 9514 2228.
E-mail address: Graham.Nicholson@uts.edu.au (G.M. Nicholson).
[1] Present address: Ludwig Institute for Cancer Research, Courtauld Building, 91 Riding House Street, London W1W 7BS, UK.

0196-9781/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2005.05.012

1. Introduction

Recently a novel non-toxic peptide was isolated from the venom of one of the world's most lethal group of arachnids, the Australian funnel-web spiders (Araneae: Hexathelidae: Atracinae) [42]. This 68-residue protein, ACTX–Hvf17, was isolated from the venom of the Blue Mountains funnel-web spider *H. versuta*. Interestingly, it does not function like classical funnel-web spider atracotoxins to modulate mammalian or insect voltage-gated ion channel function (for reviews see [35,43]) since it lacks insecticidal activity and fails to affect vas deferens smooth muscle or skeletal muscle contractility. Despite some sequence homology with a variety of colipases it also lacks colipase activity. Accordingly we have been interested in identifying the target of ACTX–Hvf17.

We have recently determined that ACTX–Hvf17 shows significant sequence homology with a variety of novel peptides from fish, frog, snake and several mammalian species belonging to the AVIT family [19] as shown in Fig. 3. This includes MIT1 from the venom of the black mamba snake *Dendroaspis p. polylepis* [2,39], Bv8 (*Bombina variegata* 8 kDa protein; [32]) and its orthologs Bm8a–f (*B. maxima* 8 kDa protein; [4,21]) from the skin secretions of toads from *Bombina* spp., and the recently identified prokineticin 1 (PK1; also known as endocrine-gland vascular endothelial growth factor or EG-VEGF) and prokineticin 2 (PK2) peptides [27]. These are all 77–94 residue peptides, containing 10 cysteines with conserved spacing, whose N-terminal four residues, 'AVIT', are all identical (Fig. 3). All these peptides, including ACTX–Hvf17, display some limited sequence homology with a variety of colipases but lack colipase activity, are highly resistant to classical specific proteolytic enzymes, and have no overt toxic effects in vertebrates or insects [19,42]. ACTX–Hvf17 shares 32% sequence identity with Bv8, including the 10 conserved cysteine residues, and is 44% homologous if conservative substitutions are included (Fig. 3). The 3D fold of ACTX–Hvf17 remains undetermined as it is intractable to NMR analysis due to aggregation under a wide variety of conditions. Nevertheless, it is believed that ACTX–Hvf17 adopts the MIT1/colipase fold [42]. Recently two non-AVIT family peptides PRTx16C0 and PRTx16C1 with the highest reported homology to ACTX–Hvf17 have also been isolated from the venom of the Brazilian armed spider *Phoneutria reidyi* (SWISS-PROT data bank accession numbers P83893 and P83997) (Fig. 3). Unfortunately, like ACTX–Hvf17, no target has yet been identified for these non-toxic arachnid peptides. This is not unique with only a few conotoxins within the venom of marine cone snails (*Conus* spp.) causing death or overt toxicity, requiring the target of the remaining peptides to be carefully determined.

AVIT family proteins are distributed widely in mammalian tissues and have been shown to cause a variety of actions. These include intestinal contraction [39], hyperalgesia [32,34], spermatogenesis [48], protection of neuronal cells from apoptosis [31], control of behavioral circadian rhythms [5] and stimulation of endocrine gland angiogenesis [23–26]. In particular, MIT1, Bv8 and the prokineticins have all been shown to potently stimulate contraction of guinea-pig ileum smooth muscle with EC_{50} values in the low to subnanomolar range [27,32,39]. However, they do not affect a range of other smooth muscle preparations including vas deferens, trachea, aorta, uterus, and gallbladder. Recent studies have shown that PK1 and PK2 are cognate ligands for two G-protein-coupled receptors designated ZAQ (PK1 receptor, PKR1) and I5E (PK2 receptor, PKR2), respectively [28,29]. PK1 and PK2 induce a transient increase in intracellular calcium ($[Ca^{2+}]_i$) with nanomolar potency in Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK293) cells expressing PKR1 and PKR2 receptors [28,29]. They bind to these receptors with high affinity but PK2 has a higher affinity for both receptors than PK1.

In the present study, we have identified six novel MIT-like ACTX orthologs using a combination of HPLC and molecular biology techniques. Given the homology to the AVIT protein family, we hypothesized that ACTX–Hvf17 and these MIT-like spider venom peptides stimulate nociceptors and gastrointestinal motility. Thus, these peptides may be useful compounds in the development of prokinetic agents in the treatment of conditions involving poor gastrointestinal motility, or novel analgesics. We show that while ACTX–Hvf17 shares sequence homology to AVIT family proteins and conforms to a similar ancestral protein fold, known as the disulfide-directed β-hairpin, it lacks the ability to stimulate vertebrate gastrointestinal smooth muscle contractility. It also fails to bind to prokineticin receptors that mediate hyperalgesia and the prokinetic actions on smooth muscle. Modeling of the 3D structure of the MIT-like ACTX family and AVIT family proteins, based on the known structure of MIT1, revealed that the surface charges were also significantly different between these two families of peptides suggesting that the two families may target different receptors and are likely to be functionally distinct.

2. Materials and methods

2.1. Peptide purification

Venom was collected from both male and female specimens of the Australian funnel-web spider species *H.* sp. 20 ("Illawarra") and *H. versuta*, and female specimens of the Orchid Beach variant of *H. infensa* (*H. infensa*: Orchid Beach) by aspirating venom from the fang-tips (chelicerae) of aggravated spiders into polyethylene pipette tips. Collected venom was dissolved in 0.1% TFA (v/v) for sample transfer to polyethylene tubes and dried down in a Savant SpeedVac for storage at −20 °C.

ACTX–Hvf17 was isolated from *H. versuta* venom as described previously [42]. Lyophilized crude *H.* sp. 20 and *H. infensa*: Orchid Beach venoms were dissolved in 0.1% TFA (v/v) and components purified using an analytical C18