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The ω-atracotoxins: Selective blockers of insect M-LVA and HVA calcium channels☆

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ABSTRACT

The ω-atracotoxins (ω-ACTX) are a family of arthropod-selective peptide neurotoxins from Australian funnel-web spider venoms (Hexathelidae: Atracinae) that are candidates for development as biopesticides. We isolated a 37-residue insect-selective neurotoxin, ω-ACTX-Ar1a, from the venom of the Sydney funnel-web spider *Atrax robustus*, with high homology to several previously characterized members of the ω-ACTX-1 family. The peptide induced potent excitatory symptoms, followed by flaccid paralysis leading to death, in acute toxicity tests in house crickets. Using isolated smooth and skeletal nerve-muscle preparations, the toxin was shown to lack overt vertebrate toxicity at concentrations up to 1 μM. To further characterize the target of the ω-ACTXs, voltage-clamp analysis using the whole-cell patch-clamp technique was undertaken using cockroach dorsal unpaired median neurons. It is shown here for the first time that ω-ACTX-Ar1a, and its homolog ω-ACTX-Hv1a from *Hadronyche versuta*, reversibly block *both* mid–low- (M-LVA) and high-voltage-activated (HVA) insect calcium channel (Ca_v) currents. This block occurred in the absence of alterations in the voltage-dependence of Ca_v channel activation, and was voltage-independent, suggesting that ω-ACTX-1 family toxins are pore blockers rather than gating modifiers. At a concentration of 1 μM ω-ACTX-Ar1a failed to significantly affect global K_v channel currents. However, 1 μM ω-ACTX-Ar1a caused a modest 18% block of insect Na_v channel currents,

☆ The amino acid sequence of ω-ACTX-Ar1a reported in this paper has been deposited in the Swiss-Prot Database under accession code P83580. The DNA sequences of the ω-ACTX-Ar1 family have been deposited in GenBank under accession numbers EF523494, EF523495, EF523497, EF523498, and EF523499.

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Abbreviations: ω-ACTX, ω-atracotoxins from Australian funnel-web spiders; BK_{Ca} channel, large conductance calcium-activated potassium channel; Ca_v channel, voltage-gated calcium channel; CNS, central nervous system; DUM, dorsal unpaired median; ESI-Q-ToF, electrospray ionization quadrupole time-of-flight; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; HVA, high-voltage-activated; IC_{50}, median inhibitory concentration; ICK, inhibitory cystine-knot; KD_{50}, median knockdown dose; K_v channel, voltage-gated potassium channel; LD_{50}, median lethal dose; M-LVA, mid-low-voltage-activated; MIT, mamba intestinal toxin; Na_v channel, voltage-gated sodium channel; rpHPLC, reverse-phase high-performance liquid chromatography; TAG, terminal abdominal ganglion; TFA, trifluoroacetic acid; TEA, tetraethylammonium; (+)-TC, (+)-tubocurarine; TTX, tetrodotoxin

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similar to the minor block of Na_v channels reported for other insect Ca_v channel blockers such as ω-agatoxin IVA. These findings validate both M-LVA and HVA Ca_v channels as potential targets for insecticides.

1. Introduction

The evolution of insect resistance to one or more classes of commonly used agrochemicals has now been reported in most major insect crop pests and disease vectors [1,2]. Over the period 1996–1998, pests were estimated to destroy around 18% of the world's food supply, with the major damage being caused by arthropods [3]. In addition, arthropods are vectors for the transmission of many new and re-emerging diseases of significant medical and veterinary importance [4]. This has necessitated the development of new strategies to combat highly resistant herbivorous and hematophagous pest species. New biological approaches include the production of transgenic crops that express insecticidal toxins from the soil bacterium *Bacillus thuringiensis* [5] and the release of insect-specific recombinant baculoviruses that express a variety of insecticidal neurotoxins from animal venoms [6]. Recent studies have investigated the potential of expressing ω-atracotoxins from the venom of Australian funnel-web spiders in plants or as orally active acaricidal agents [7,8].

The ω-atracotoxin-1 (ω-ACTX-1) toxins constitute the first family of insect-specific peptide toxins isolated from the venom of Australian funnel-web spiders (Mygalomorphae: Hexathelidae: Atracinae). These toxins are reported to inhibit insect, but not mammalian, Ca_v channels [9–12]. All family members are 36–37 residues in length, and contain six cysteine residues with a strictly conserved disulfide pattern. The three-dimensional solution structure of ω-ACTX-Hv1a comprises a structurally disordered N-terminus (residues 1–3), a core region rich in β-turns and disulfides (residues 4–21), and a β-hairpin (residues 22–37) that protrudes from the disulfide-rich core [10]. The three disulfide bonds form an inhibitory cystine-knot (ICK) motif that is present in the majority of atracotoxin structures determined to date, and which is common in peptide neurotoxins targeting ion channels [13–15]. Site-directed mutagenesis [11] and synthetic truncates [9] have been used to elucidate the toxin insectophore, the key residues involved in binding to the insect target site, of the prototypic family member ω-ACTX-Hv1a. The primary insectophore residues, Pro^{10}, Asn^{27} and Arg^{35}, form a small contiguous patch of ~200 $Å^2$ on one face of the toxin surface [11,12] (Fig. 1C). Residues Gln^9 and Tyr^{13} appear to be of minor functional importance in orthopterans and dictyopterans, but not dipterans, suggesting that there might be minor species-specific variations in the toxin insectophore (Fig. 1C).

These toxins are lethal over a wide range of arthropod orders including Acarina, Coleoptera, Dictyoptera, Diptera, Hemiptera, Lepidoptera, and Orthoptera [7,8,10,11,16–18]. ω-ACTX-1 toxins cause irreversible spastic paralysis, preceeding flaccid paralysis and death, yet no toxic effects have been reported following testing on vertebrate preparations. In insect preparations, ω-ACTX-Hv1a acts directly on CNS neurons rather than interganglionic axons or the peripheral neuromuscular junction [10,17]. Electrophysiological studies have shown that the phyletic specificity of this family of toxins is believed to be derived from their action on invertebrate, but not vertebrate, voltage-gated calcium (Ca_v) channels [10,12]. In preliminary experiments in unidentified cockroach metathoracic ganglia neurons, ω-ACTX-Hv1a partially blocked Ca_v channels at concentrations up to 1 μM. Competitive binding assays using radioiodinated ω-atracotoxin-Hv1a revealed that the toxin binds to orthopteran channels at nanomolar concentrations [12], whereas it had no effect on whole-cell Ca_v channel currents in a variety of vertebrate-derived neuron preparations at concentrations as high as 1 μM [10]. Moreover, the toxin does not block rat HVA $Ca_v1.2$ (L-type), $Ca_v2.1$ (P/Q-type) or $Ca_v2.2$ (N-type) channels at concentrations up to 10 μM [12]. However, the mode of channel block and the precise insect Ca_v channel subtype targeted by the ω-ACTX-1 toxins remains to be determined, and their potential action on other voltage-gated ion channels has not been investigated in detail.

Recently, ω-ACTX-Hv1a has been trialed as a novel biopesticide for protection of plants from phytophagous pest insects following expression of the toxin transgene in tobacco plants (*Nicotiana tabacum*). Transgenic expression of ω-ACTX-Hv1a effectively protected tobacco plants from the larvae of two recalcitrant agricultural pests, *Helicoverpa armigera* and *Spodoptera littoralis*, with 100% mortality at 48 h [7]. Surprisingly, a recombinant thioredoxin–ω-ACTX-Hv1a fusion protein was lethal to *H. armigera* and *S. littoralis* caterpillars when applied topically [7]. In addition, ω-ACTX-Hv1a is orally active against ticks [8] and mosquitoes (J. Huang, G. King, S. Wikel, unpublished data). These studies indicate that at least some insecticidal peptide toxins have the potential to be developed as orally active biopesticides.

Here we describe the pharmacological characterization of a novel member of the ω-ACTX-1 family that we isolated from the venom of the Sydney funnel-web spider, *Atrax robustus*. This 37-residue peptide, ω-ACTX-Ar1a, shows selective toxicity against house crickets, but it has no affect on vertebrate nerve-muscle preparations. We show that ω-ACTX-Ar1a and ω-ACTX-Hv1a block both M-LVA and HVA Ca_v channels in cockroach neurons, with minor activity against Na_v but not K_v channels. This block did not alter the voltage-dependence of Ca_v channel activation, and it was voltage-independent, suggesting that the ω-ACTX-1 toxins are pore blockers rather than gating modifiers. As far as we are aware, the ω-ACTX-1 toxins are the first peptide toxins demonstrated to selectively block both M-LVA and HVA insect Ca_v channels.

2. Materials and methods

2.1. Toxin purification and peptide sequencing

Venom was collected from female Sydney funnel-web spiders, *A. robustus*, via direct aspiration from the fangs. Venom was