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Effect of the urease-derived peptide Jaburetox on the central nervous system of *Triatoma infestans* (Insecta: Heteroptera)

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ABSTRACT

Background: Triatoma infestans is the main vector of Chagas'disease in Southern Cone countries. In triatomines, symptoms suggesting neurotoxicity were observed after treatment with Jaburetox (Jbtx), the entomotoxic peptide obtained from jackbean urease. Here, we study its effect in the central nervous system (CNS) of this species.

Methods: Immunohistochemistry, Western blots, immunoprecipitation, two-dimensional electrophoresis, tandem mass spectrometry and enzymatic assays were performed.

Results: Anti-Jbtx antibody labeled somata of the antennal lobe only in Jbtx-treated insects. Western blot assays of nervous tissue using the same antibody reacted with a 61 kDa protein band only in peptide-injected insects. Combination of immunoprecipitation, two-dimensional electrophoresis and tandem mass spectrometry identified UDP-*N*-acetylglucosamine pyrophosphorylase (UDP-GlcNAcP) as a molecular target for Jbtx. The activity of UDP-GlcNAcP increased significantly in the CNS of Jbtx-treated insects. The effect of Jbtx on the activity of nitric oxide synthase (NOS) and NO production was investigated as NO is a recognized messenger molecule in the CNS of *T. infestans*. NOS activity and NO levels decreased significantly in CNS homogenates of Jbtx-treated insects.

Conclusions: UDP-GlcNAcP is a molecular target of Jbtx. Jbtx impaired the activity of *T. infestans* nitrergic system, which may be related with early behavioral effects.

General Significance: We report that the CNS of *Triatoma infestans* is a target for the entomotoxic peptide and propose that a specific area of the brain is involved. Besides potentially providing tools for control strategies of Chagas' disease vectors our data may be relevant in various fields of research as insect physiology, neurobiology and protein function.

1. Introduction

Isoforms of urease from *Canavalia ensiformis* seeds possess fungitoxic and insecticidal properties [3,7,9,35]. This entomotoxicityis mostly due to the release of an internal peptide by the hydrolytic activity of insect cathepsin-like enzymes [8,12,25,27]. *Rhodnius prolixus* and *Triatoma infestans*, two of the triatomine vectors of Chagas' disease, belong to the group of insects in which digestion is based on cathepsin-like enzymes. An entomotoxic peptide derived from jackbean urease (JBU) with 93 amino acids was obtained by heterologous expression in *Escherichia coli* and named Jaburetox [39,22,26]. Two recombinant His-tagged versions of Jaburetox (Jbtx) were developed, Jbtx-2Ec [22] and Jbtx [26], differing from each other only by the presence of a V5

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viral epitope in the first one, both displaying potent and equivalent insecticidal activity [20].

A combination of molecular modeling, leakage experiments in large unilamelar vesicles and electrophysiological studies using planar lipid bilayers revealed that both, JBU and Jbtx, disrupt acidic lipid bilayers producing membrane permeabilization and display ion channel activity [2,20,24]. This mechanism may be relevant for the in vivo action of these molecules as entomotoxic agents [6].

The effects of JBU and Jbtx were tested in several insect species and their mechanism of action has been characterized in Malpighian tubules [36] and in the crop [37] of *R. prolixus*. In triatomines, several symptoms suggestive of neurotoxicity were observed after a meal of urease or Jbtx, which may indicate that the central nervous system (CNS) of the insect is a target of the entomotoxic molecules. Furthermore, RNAi experiments revealed that the urease toxicity is linked to a phospholipase A_2 XII gene, which is abundantly expressed in the *R. prolixus* CNS [10].

T. infestans, a blood feeding heteropteran, is the main vector of Chagas' disease in Argentina and neighboring countries. Morphological, biochemical and molecular biology approaches have been employed to study the CNS of this triatomine [30,31], making this species a suitable model for the investigation of neurotoxicity of entomotoxic molecules [29]. In this study, we investigated the effect of Jbtx in the CNS of *T. infestans*. The impairment of the nitrergic system of the insect and the role of UDP-*N*-acetylglucosamine pyrophosphorylase (UDP-GlcNAcP), a putative Jbtx binding protein in *T. infestans* CNS, are also discussed.

2. Materials and methods

2.1. Chemicals

ABC reagent (Vector Laboratories Inc., Burlingame, CA, USA), rabbit polyclonal universal nitric oxide synthase (uNOS) antibody (Thermo Scientific - Pierce, IL, USA) and biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Inc) were purchased from indicated commercial sources. ECL Advance Western Blotting Detection Kit; horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies; Hybond-P PVDF membranes; protein A-Sepharose and IPG strips were acquired from GE Healthcare (Buckinghamshire, UK). Bacitracin; 3,3′-diaminobenzidine tetrahydrochloride; Permount medium; protease inhibitor cocktail; vanadium chloride; mouse monoclonal anti-β-tubulin antibody and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Insects

Male adults of *T. infestans* were used in this study. The insects, free of *Trypanosoma cruzi* and *Blastocrithidia triatomae*, were supplied by the National Center for the Control of Chagas' disease (Santa María de Punilla, Córdoba, Argentina). They were housed under controlled conditions of light (L:D = 12:12, lights on at 6.00 a.m.), temperature (27 ± 1 °C) and relative humidity (70–80%). Fifth-instar insects of the phytophagous hemipteran *Dysdercus peruvianus* (cotton-stainer bug) from a colony established in our laboratory were used in this study. The colony was kept under controlled temperature (24 ± 2 °C) and humidity (70 ± 5%) and in a 14/10 h photoperiod (light/dark). The insects were fed on cotton (*Gossypium hirsutum*) seeds and had free access to water.

2.3. Jaburetox

The recombinant peptide used in this study, previously named Jbtx-2Ec [22], contained a 93 amino acid sequence derived from jackbean urease plus a V5 viral epitope and was produced according to Mulinari et al. [22]. We have shown that the presence of the V5 antigen did not contribute nor interfere with the insecticidal activity of the urease-derived peptide [20]. Thus here, with the purpose of simplification, we called Jbtx-2Ec simply Jbtx. The rabbit polyclonal anti-Jbtx antibody employed in the experimental approaches was obtained as reported previously [22].

2.4. Injections

The insects were weighed and placed ventral side up under a dissecting microscope. Injections of Jbtx (dose of 0.1 μg/mg body weight) in 20 mM sodium phosphate buffer (PB, pH 7.1) were performed with a Hamilton syringe attached to a stereotaxic instrument (David Kopf Instruments, CA, USA). Control insects were injected with an equivalent volume of PB. Jbtx or vehicle was injected between the third tergite and the first sternite, at the midline [39]. After the injection, control and treated insects were placed in individual containers where they were allowed to recover. All the experiments were performed 3 h after injections.

2.5. Immunohistochemistry

Jbtx- and vehicle-injected insects were processed for immunohistochemistry. They were placed under a dissecting microscope and the dorsal cuticle of the head and thorax was quickly removed. The head and thorax of 5 insects for each treatment were flushed with ice-cold fixative (4% paraformaldehyde and 0.4% picric acid in 0.16 M PB, pH 6.9) [32]. Dissection of the brain and ganglia proceeded with the tissues bathed in the cold fixative. Samples were pooled separately and remained in the same fixative overnight, at 4 °C. After that, they were rinsed several times in phosphate buffered saline (PBS, 6.6 mM Na2HPO4/KH2PO4, 150 mM NaCl, pH 7.4), passed through increasing concentrations (5%–30%) of sucrose in PBS plus 0.02% bacitracin and 0.01% sodium azide for at least 48 h at 4 °C until processed for immunohistochemistry [33]. The pools of brains and CNS ganglia from Jbtx- and vehicle-injected insect groups were serially sectioned with a cryostat (Microm, Waldorf, Germany) and mounted onto gelatin coated glass slides. After that, sections were processed according to the avidin–biotin immunoperoxidase protocol [16]. The sections (16 μm) were incubated overnight with the rabbit anti-Jbtx antibody (1:5000) [22]. After that, the slides were rinsed in PBS, incubated at room temperature for 30 min in biotinylated goat anti-rabbit secondary antibodies (1:100), rinsed again in PBS and further incubated for 1 h in ABC reagent. Peroxidase activity was revealed by reaction with 3,3′-diaminobenzidine tetrahydrochloride using hydrogen peroxide and nickel salts for enhancement of the reaction product [34]. The sections were dehydrated in graded ethanol series of 20, 40, 60, 90% (v/v) to absolute ethanol and mounted with Permount medium. Photographs were taken with a Nikon E800 microscope equipped with a Nikon digital sight DS-5Mc camera. Images were modified only to enhance contrast (Adobe Photoshop, Adobe Systems Inc). The experiment was repeated three times.

The specificity of the immunohistochemistry protocol was tested by overnight pre-incubation of the anti-Jbtx antibody diluted 1:5000 with 5 μM Jbtx. This pre-absorbed antibody was used for incubating sections which were further processed following the ABC method as stated above. Other controls were performed by incubating sections without either the first or the second antibodies

2.6. Western blot

For western blot assays, three groups containing 10 brains with the attached CNS ganglia from Jbtx-injected or control insects were processed. Tissue samples were obtained by dissecting the insects with the head and thorax constantly bathed in ice-cold sterile saline solution. Samples of each group were stored at −70 °C in 0.01 M PBS plus a protease inhibitor cocktail until assayed.

To confirm that the injected Jbtx had reached the CNS, tissue samples were homogenized, centrifuged at 10,000 *g* (10 min at 4 °C)