Jack bean urease modulates neurotransmitter release at insect neuromuscular junctions

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\textbf{ABSTRACT}

\textbf{Background:} Plants have developed a vast range of mechanisms to compete with phytophagous insects, including entomotoxic proteins such as ureases. The legume Canavalia ensiformis produces several urease isoforms, of which the more abundant is called Jack Bean Urease (JBU). Previous work has demonstrated the potential insecticidal effects of JBU, by mechanisms so far not entirely elucidated. In this work, we investigated the mechanisms involved in the JBU-induced activity upon neurotransmitter release on insect neuromuscular junctions.

\textbf{Methods:} Electrophysiological recordings of nerve and muscle action potentials, and calcium imaging bioassays were employed.

\textbf{Results and conclusion:} JBU (0.28 mg/animal/day) in Locusta migratoria 2nd instar through feeding and injection did not induce lethality, although it did result in a reduction of 20% in the weight gain at the end of 168 h (n = 9, p ≤ 0.05). JBU (0.014 and 0.14 mg) injected directly into the locust hind leg induced a dose and time-dependent decrease in the amplitude of muscle action potentials, with a maximum decrease of 70% in the amplitude at the highest dose (n = 5, p ≤ 0.05). At the same doses JBU did not alter the amplitude of action potentials evoked from motor neurons. Using Drosophila 3rd instar larvae neuromuscular preparations, JBU (10⁻⁷ M) increased the occurrence of miniature Excitatory Junctional Potentials (mEJPs) in the presence of 1 mM CaCl\textsubscript{2} (n = 5, p ≤ 0.05). In low calcium (0.4 mM) assays, JBU (10⁻⁷ M) was not able to modulate the occurrence of the events. In Ca\textsuperscript{2+}-free conditions, with EGTA or CoCl\textsubscript{2}, JBU induced a significant decrease in the occurrence of mEPJs (n = 5, p ≤ 0.05). Injected into the 3rd abdominal ganglion of Nauphoeta cinerea cockroaches, JBU (1 μM) induced a significant increase in Ca\textsuperscript{2+} influx (n = 7, p ≤ 0.01), similar to that seen for high KCl (35 mM) condition. Taken together the results confirm a direct action of JBU upon insect neuromuscular junctions and possibly central synapses, probably by disrupting the calcium machinery in the pre-synaptic region of the neurons.

1. Introduction

Plants have developed a vast range of mechanisms to defend themselves against the continuous threat posed by phytophagous insects. This defense armory ranges from morphological adaptations to the production of entomotoxic compounds, such as toxic proteins and peptides [1,2]. These natural compounds affect many insect species by binding to different targets, including the nervous system. In insects, alterations in nervous system homeostasis can lead to neuroexcitation, resulting in hyperactivity, tremors, rigid paralysis and muscular fatigue [3]. On the other hand, plant-derived compounds can also induce neuroinhibition that may result in flaccid paralysis and reduced respiratory capacity that ultimately leads to the insect’s death [3, 4]. Among plant defense-related compounds, ureases have been recently recognized as natural insecticides against some insect species [5–7]. Ureases are metalloenzymes that catalyze the hydrolysis of urea into...
ammonia and carbon dioxide [8]. These enzymes are synthesized by bacteria, fungi and plants [9]. In plants, the main role of ureases is thought to be related to the use of urea as a nitrogen source [10,11]. The legume Canavalia ensiformis (jack bean) produces at least three urease isoforms, including the major isoform jack bean urease (JBU or JBURE I) [12], cananoxin (CNTX) [13,14] and JBURE II [15,16]. Ureases display biological activities that are independent of their enzymatic activity [7] such as platelet aggregation [17–19], antifungal activity [20] and entomotoxic effects [2,22–24]. The entomotoxic potential of JBU and derived peptides has been studied by our group for more than one decade [21–23,25–28]. Jack bean urease activity is variable, depending on the insect species, concentration and mode of administration. Ureases are lethal when fed to insect species that rely on cathespin-like enzymes as their main digestive enzymes, as found in Rhodnius prolixus and Callosobruchus maculatus. On the other hand, ureases are not lethal to insects relying on trypsin-like digestive enzymes present in Schistocerca americana, Aedes aegypti, D. melanogaster [22,29] or Nauphoeta cinerea [24]. Although peptides formed upon hydrolysis of plant ureases by insect cathespin-like enzymes play an important role on their entomotoxicity [21,29–31], it is now evident that the whole protein is toxic itself, with no need for cleavage, and can interfere with physiological functions of different insect’s systems [7,22,32,33].

One of the suggested mechanisms of JBU-induced toxicity is the deregulation of calcium ion mobility across the cell membrane [24,32–34]. The importance of calcium ions in JBU-induced toxicity has been evaluated using the Malpighian tubule secretion and hemocyte aggregation assays. In both preparations, JBU toxicity is reduced when Ca2+ concentration is decreased [27,32]. Moreover, although not cytolytic, JBU induces the formation of ion channels that are highly selective to cations in planar lipid bilayers and in lipid vesicles [35,36]. The neurotoxic effects of JBU in cockroaches include neuromuscular blockage, that can be seen in vivo and leads to a progressive reduction in muscle strength [24].

There are multiple biological signals regulating the neuromuscular junction in insects [3,4,37–41]. Depolarization caused by an action potential in the insect motor nerve terminal activates the calcium channels triggering an influx of calcium ions. These ions stimulate the release of the neurotransmitter L-glutamate from nerve endings [42–44]. Free L-glutamate diffuses across the synapic cleft and binds to receptor-operated ion channels on the post-synaptic cell resulting in influx of sodium and calcium ions [41,45] subsequently activating calcium channels in the sarcoplasmatic reticulum of skeletal muscle; the increased concentration of cytosolic calcium ions then leads to skeletal muscle contraction [46]. Based on our previous data regarding the interaction of ureases with insect tissues, we hypothesize that peripheral neurotransmission may be a potential target for JBU-induced entomotoxicity. Here we have gained a better understanding of the mechanism underlying the neuromuscular blockage induced by the C. ensiformis major urease (JBU) on insect neuromuscular communication.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents were of the highest purity available and were obtained from Sigma-Aldrich, Merck, Roche, Life Technologies or BioRad. Test-solutions were prepared daily by dilution in locust saline [47], Drosophila saline (HL3) [48] or cockroach saline [49] immediately before use. Highly purified crystalline urease of C. ensiformis (type C3) was obtained from Sigma-Aldrich, Canada. The protein (hexameric molecular mass 545 kDa) was dialysed against the insect solution used in each specific protocol. Stocks were kept at 4 °C and diluted in the appropriate insect saline before use.

2.2. Insects

Adult locusts (Locusta migratoria), cockroaches (Nauphoeta cinerea) and wandering third instar larvae (Drosophila melanogaster) were employed as experimental models. Drosophila flies (Oregon-R wild-type strain) were kept at room temperature (23–25 °C) in a 12:12 h light/dark cycle and fed with Bloomington standard food medium. Locusts were raised under crowded conditions with a 12:12 h light/dark cycle at 30 °C and 50% humidity and fed with fresh wheat seedling and bran. Cockroaches were raised under crowded conditions, maintained at room temperature (23–25 °C) in a 12:12 h light/dark cycle with water and feed (dog chow) ad libitum.

2.3. Lethality assay and weight gain

Second instar locusts (L. migratoria) were used to perform the lethality assay administrating JBU by feeding and injection. For the feeding protocol, animals were divided into control and JBU-treated groups and placed into containers (nine animals each, in triplicates). The animals were fed daily with lettuce disks (~30 mm diameter) with one disk for each animal in the group, twice a day. A droplet of JBU was added to the surface of the lettuce disk for the JBU-treated group, in a final volume of 5 μL, and allowed to air dry before placing the dishes inside the containers. Jack bean urease (~0.14 mg/animal/twice a day) was diluted in locust saline (in mM) (NaCl 150, CaCl2 10, KCl 4, MgCl2 2, NaHCO3 4, HEPES 5, sucrose 90 and trehalose 5) [47] was used for the JBU-treated group. For the control group, only locust saline was added to the lettuce surface. Lethality rate and weight gain were measured at 24, 48, 72, 96, 120, 144 and 168 h after the beginning of the experiment. For the injection protocol, animals were also divided into control and JBU-treated groups and placed in containers, five animals each, in triplicates. Animals were injected in the abdomen near to the hind leg using a Hamilton syringe. For the JBU-treated group, JBU (0.28 mg/animal), single dose, diluted in locust saline was injected in a final volume of 5 μL. Control group was injected with locust saline only [47]. Lethality rate was measured 12, 24, 48 and 72 h after the beginning of the experiment. Throughout the experiments animals were maintained in a 12:12 h light/dark cycle at 30 °C and 50% humidity. For the injection protocol, the animals were also provided with feed and water ad libitum.

2.4. Measurement of muscle potentials and nerve action potentials in Locusta migratoria

Muscle potentials from the tarsus depressor muscle of L. migratoria were recorded extracellularly from an isolated locust leg. The hind leg of an adult animal was removed and pinned onto a platform with attached electrodes. The stimulating electrodes were positioned in the femur and the recording electrodes were positioned in the tibia near the tarsus, with a ground electrode placed in between. Different treatments were administered, through a small window cut in the femur cuticle, using a Hamilton syringe, in a final volume of 2.5 μL. Three doses of JBU were assayed: 0.0014, 0.014 and 0.14 mg/hind leg, and controls were injected with locust saline. Muscle potentials were recorded for 15 min prior to the injection of JBU. Each group consisted of 6 animals. Electrical stimuli were applied using a stimulator (model SD98, Grass Technologies, Warwick, USA) at voltage of approx. 5 V, frequency of 0.2 PPS and a duration of 0.5 ms. The recordings were made using a differential amplifier (model 1700, A-M Systems, Sequim, WA, USA). Nerve action potentials (AP) of locust leg were measured using the same equipment as for muscle potential measurements. For the nerve AP, the position of stimulating and recording electrodes was reversed. The stimulating electrode was placed in the tibia near the tarsus and the recording electrode was placed in the femur to acquire the APs occurring in the tarsal fast depressor motoneurons (FDtia) [50]. Signal conversion was made using a PC-based data acquisition system (8
channels Powerlab, Dunedin, New Zeland), recorded with Chart software version 4 and the analysis made with Chart version 6.

2.5. Miniature excitatory junctional potentials in D. melanogaster larvae

The measurements of miniature excitatory junction potentials (mEJPs) was performed in third instar D. melanogaster wandering larvae neuromuscular junction as described by Nguyen and Stewart (2016) [51]. D. melanogaster larvae were dissected in HL3 solution, in mM: 70 NaCl, 5 KCl, 20 MgCl2, 10 NaHCO3, 5 Trehalose, 115 Sucrose, 5 HEPES and with 1 mM CaCl2 added [48]. Glass intracellular electrodes were made using a micropipette puller (model P-97, Sutter instruments, Novato, CA, USA) and filled with 3 M KCl, the electrodes had a resistance of approximately 30 MΩ. For the mEJP protocol, muscle number six from the body wall was impaled with the recording electrode and the occurrence and amplitude of the events were recorded. Only muscles with a resting membrane potential of at least −60 mV were used for the recordings. The acquisition of the events was made using an AxoClamp amplifier (model 2B, Axon Instruments, Sunnyvale, CA, USA) and the analog signal was converted to digital using an Axon Digidata (model 1322A, Axon Instruments, Sunnyvale, CA, USA). The recording of the events was carried out using pClamp 8.2 software and the analyses were performed using Clampfit 10 software. For the treated conditions, 10−5 M JBU was added in the bath solution along with different concentrations of CaCl2. JBU was also assayed with calcium-free HL3 solution, containing the calcium chelating agent EGTA (ethylene glycol tetraacetic acid), and with CaCl2 (Ca2+ is a well-known calcium channel blocker). The preparations were pre-incubated with JBU for 1 min before the beginning of the recordings. Controls were recorded in the same preparations before the addition of JBU into the bath, the recordings had a duration of 2 min.

2.6. Calcium imaging

All imaging experiments were performed on a digital epifluorescence imaging system (WinFluor, J. Dempster, University of Strathclyde, Glasgow, Scotland) mounted on an Olympus IX71 microscope (Olympus America, Center Valley, PA, USA) using a 20× objective. Calcium imaging was performed using ventral nerve cords (VNC) from cockroaches (N. cinerea). After dissection, the VNCs were loaded with Fluo-4 AM (5 μM, 45–60 min, at 32 °C in an incubator) prior to the assay. Experiments were performed with VNCs perfused (1–2 mL min−1) with cockroach saline solution with the following composition (in mM): NaCl 140, KCl 5, MgCl2 2, CaCl2 2, HEPES 10, D-glucose 10, pH 7.4, 310 mOsm, at room temperature. All compounds investigated were added to the perfusion solution. Data were calculated as changes in fluorescence ratio and expressed as ΔF/F0.

2.7. Statistical analysis

The results were expressed as mean ± S.E.M. Each experiment was repeated at least three times. For comparison between means of two different experimental groups the Student “t” test was employed. ANOVA was employed to analyze data from more than two experimental groups followed by Bonferroni multiple comparison as the post hoc test. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The values were considered significantly different when p ≤ 0.05.

3. Results

3.1. Lethality assay and weight gain

JBU was administrated to L. migratoria second instar locusts by feeding or injection. JBU administered orally at a daily dose of 0.28 mg/insect significantly reduced the insects’ weight gain. At the end of day 7 (168 h) the insects weighed approx. 20% less than controls (Fig. 1), p ≤ 0.05 Student “t” test, (n = 9). No lethality was observed by feeding or injection of JBU (0.28 mg/insect) (data not shown).

3.2. Muscle potentials and nerve action potentials

JBU (0.0014, 0.014 and 0.14 mg/hind leg) decreased the amplitude of locust tarsal depressor muscle potentials. Thus, in this set of protocols, only the highest dose assayed (0.14 mg/hind leg) significantly reduced the amplitude of the muscle potentials by 50%, in approximately 30 min and by 70% after 60 min, when compared to controls (Fig. 2A, 3A and B) p ≤ 0.0001 two-way ANOVA followed by Bonferroni’s multiple comparison test (n = 5). Although JBU was able to decrease the amplitude of muscle potentials, there was no change in the nerve compound action potentials (n = 6) (Fig. 2B, 3A and B).

3.3. Miniature excitatory junctional potentials in D. melanogaster larvae

The effects of JBU on spontaneous transmitter release were evaluated in the neuromuscular junction of D. melanogaster larvae under...
different calcium conditions.

In the presence of 1 mM CaCl₂, JBU (10⁻⁷ M) induced an increase in the occurrence of mEJPs events in all amplitude ranges (Figs. 3C and D; 4A and B). When the concentration of CaCl₂ in the medium was reduced to 0.4 mM, JBU (10⁻⁷ M) there was a slight decrease in the occurrence of mEJPs events, although not significative (Fig. 4C and D).

In the complete absence of Ca²⁺, achieved with zero CaCl₂ and 10 mM EGTA, JBU (10⁻⁷ M) did not alter the amplitude of mEJPs but did reduce the number of events (Fig. 4E and F). A trend to a decrease in the number of events was observed when the preparation was treated with CoCl₂ (Fig. 4G and H).

3.4. Increase in calcium influx

By using calcium imaging to monitor intracellular calcium levels ([Ca²⁺]), it was found that JBU (1 μM) induced a pronounced rise in ganglionic [Ca²⁺] (350 ± 50% peak increase (n = 7), p < .01, Fig. 5B, D and E). Interestingly, depolarizing the neurons by applying high potassium (35 mM) after JBU infusion caused a further increase in the fluorescence revealing that JBU treatment did not deplete stored calcium in the tissue (Fig. 5C, D and E).

4. Discussion and conclusions

This study aimed to improve our understanding of the mechanisms
by which *C. ensiformis* urease (JBU) interferes with insect nerve-muscle communication.

We started by investigating the survival rate and toxic effects on adult locusts by injecting and feeding the animals with JBU. No lethality was observed either by injection or feeding; however the animals showed a significant decrease in weight gain when fed with JBU. It is possible that JBU itself may be an antifeedant compound influencing the sense organs associated with the mouthparts. Furthermore, as referenced in Chapman and De Boer (1995) [52] there are multiple pathways that can feedback and control feeding behaviour. For example, in *L. migratoria* the ganglia of the stomatogastric nervous system (STNS) (frontal ganglion, hypocerebral ganglion, and ventricular ganglia) control movement of the foregut, and by interfering with this pathway the feeding activity is decreased [53]. Thus, in our experimental conditions it is possible that a JBU-induced activity in a number of neural pathways could result in a reduced weight gain. The lack of lethal effect of JBU on *L. migratoria* was anticipated. Previously we have reported that an isoform of JBU (canatoxin) is not lethal and did not alter the weight gain when fed to another orthopteran, *Schistocerca americana* and this lack of toxicity was associated to its trypsin-like digestive enzymes [29]. *L. migratoria* also uses mainly trypsin-like enzymes as digestive enzymes [54,55], thus this fact could account for the lack of lethality through the feeding pathway [29]. On the other hand, there may be differences between the ureases isoforms as well as between digestive enzymes of the insects to justify the distinct effects on weight gain, which require further investigation.

In previous work we have reported that the central and peripheral neurotoxicity induced by JBU in the cockroach, *N. cinerea*, associated to...
a dose- and time-dependent neuromuscular blockage [24]. Based on that data, we hypothesized that JBU’s neurotoxicity could involve alterations in the release of neurotransmitters at the insect neuromuscular junction. To test this hypothesis, here we have examined if the main locus of JBU activity is postsynaptic (on the muscle) or presynaptic.

The effect of JBU on nerve-muscle communication was assayed using extracellular electrophysiological recordings from the tarsal muscle of *T. Carrazoni et al.* Pesticide Biochemistry and Physiology 146 (2018) 63–70
of Ca²⁺ channels as some snake toxins or directly through a pore-forming activity still remains to be answered.

Conflict of interest

The authors declare no conflict of interest regarding this study.

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