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Jack bean urease modulates neurotransmitter release at insect neuromuscular junctions

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ABSTRACT

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). Previews 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.

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

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 ($n = 9$, $p \leq 0.05$). JBU (0.014 and 0.14 mg) injected direct 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 ($n = 5$, $p \leq 0.05$). 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 $CaCl_2$ ($n = 5$, $p \leq 0.05$). In low calcium (0.4 mM) assays, JBU (10^{-7} M) was not able to modulate the occurrence of the events. In Ca^{2+}-free conditions, with EGTA or $CoCl_2$, JBU induced a significant decrease in the occurrence of mEPJs ($n = 5$, $p \leq 0.05$). Injected into the 3rd abdominal ganglion of *Nauphoeta cinerea* cockroaches, JBU (1 μM) induced a significant increase in Ca^{2+} influx ($n = 7$, $p \leq 0.01$), 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

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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], canatoxin (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 toxicity is variable, depending on the insect species, concentration and mode of administration. Ureases are lethal when fed to insect species that rely on cathepsin-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 cathepsin-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 through 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 Ca^{2+} 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 synaptic 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 sarcoplasmic 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 entomotoxicty. 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 ^{O}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 disks inside the containers. Jack bean urease (~0.14 mg/animal/twice a day) diluted in locust saline (in mM) (NaCl 150, $CaCl_2$ 10, KCl 4, $MgCl_2$ 2, $NaHCO_3$ 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 SD9B, 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 motorneurons (FDTa) [50]. Signal conversion was made using a PC-based data acquisition system (8