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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 °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

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 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 115 Sucrose, 5 HEPES and with 1 mM $CaCl_2$ 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 analogic 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^{-7} M JBU was added in the bath solution along with different concentrations of $CaCl_2$. JBU was also assayed with calcium-free HL3 solution, containing the calcium chelating agent EGTA (ethylene glycol tetraacetic acid), and with $CoCl_2$ (Co^{2+} 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 epi-fluorescence 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, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, D-glucose10, 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 $\Delta F/F_0$.

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 $p \leq 0.05$.

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).

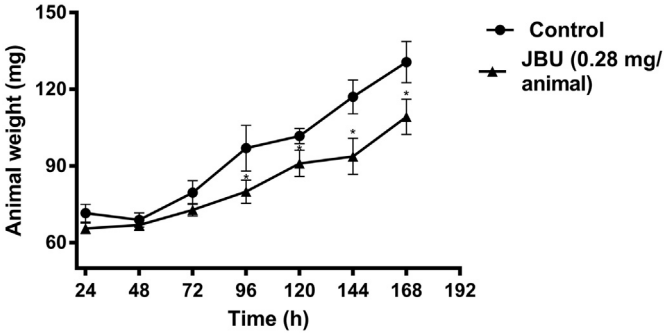

Fig. 1. Effect of JBU feeding on *Locusta migratoria* weight gain. Second instar *L. migratoria* were fed with JBU (0.28 mg/animal) added daily to lettuce disks over seven days. Symbols are mean ± S.E.M. *$p \leq 0.05$ Student "*t*-test", ($n = 9$).

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) $p \leq 0.0001$ 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

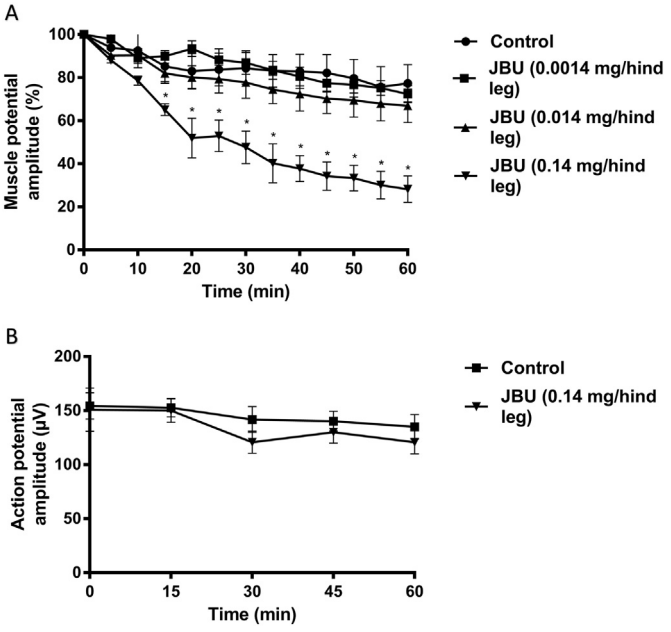

Fig. 2. Effect of JBU on amplitude of L. *migratoria* tarsal depressor muscle potentials and nerve action potentials. (A) The amplitude of the tarsal depressor muscle potentials was measured using extracellular recordings. Symbols are means ± S.E.M. *$p \leq 0.0001$ two-way ANOVA followed by Bonferroni's multiple comparison test ($n = 5$). (B) The amplitude of the leg nerve compound action potential was measured using extracellular recordings. Symbols are means ± S.E.M. $p \geq 0.05$ Student "*t*-test" ($n = 6$).

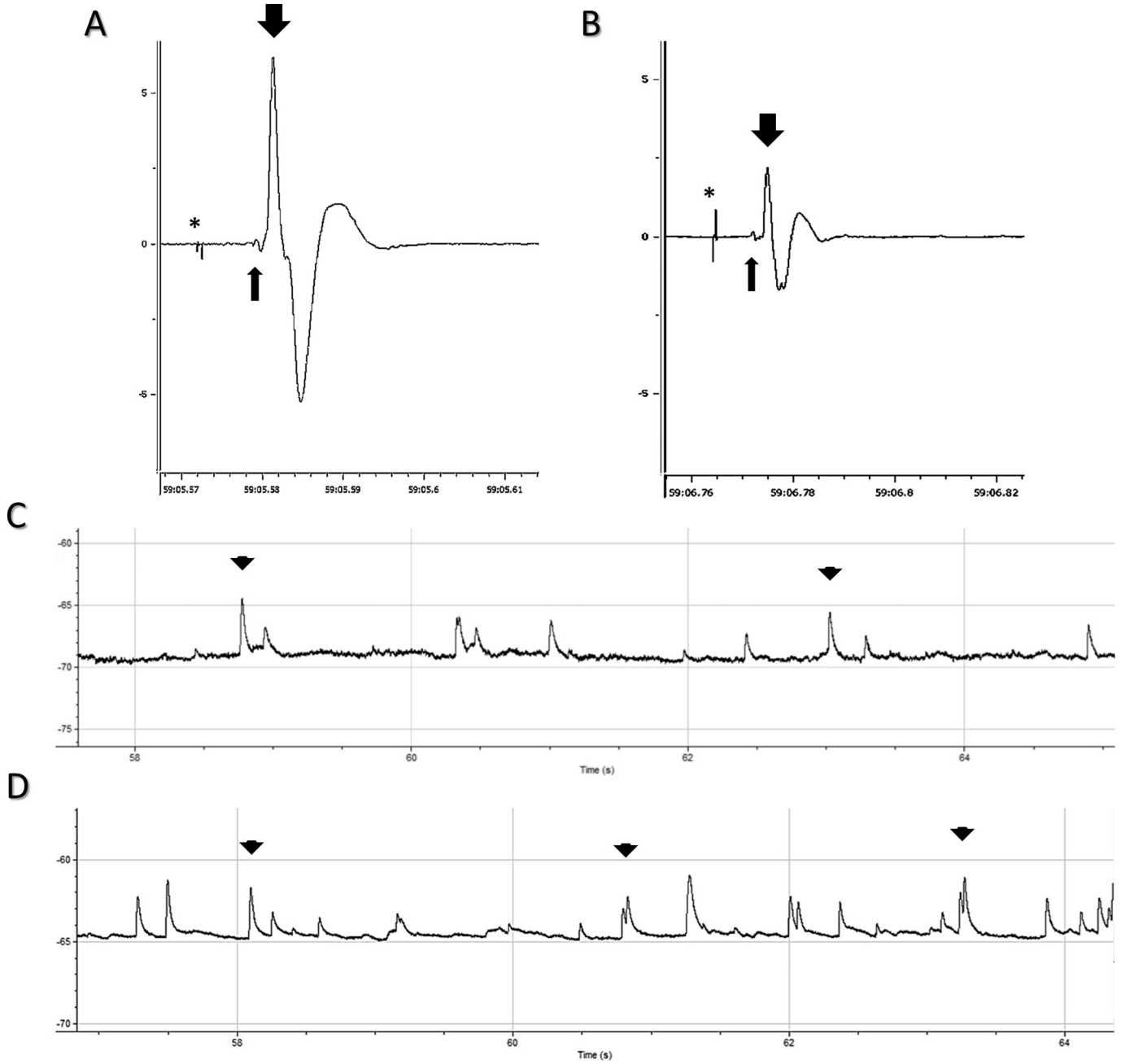

Fig. 3. Representative recordings of muscle potential and miniature Excitatory Junctional Potentials (mEJPs). (A) Representative traces of a muscle potential in control condition and (B) treated with JBU. Panel A and B show muscle potentials (thick arrows), nerve action potentials (thin arrows), and the stimulus artifact (asterisk), which indicate that an electrical stimulation is being applied. Axes are: time (min) (X axis) and amplitude (V) (Y axis), amplification ×1000. (C) Representative traces of mEJP in control condition and (D) treated with JBU, both in the presence of 1 mM $CaCl_2$. mEJPs events are indicated by arrow heads. Axes are: time (s) (X axis) and amplitude (mV) (Y axis).

different calcium conditions.

In the presence of 1 mM $CaCl_2$, 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 $CaCl_2$ 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 Ca^{2+}, achieved with zero $CaCl_2$ 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 $CoCl_2$, (Fig. 4G and H).

3.4. Increase in calcium influx

By using calcium imaging to monitor intracellular calcium levels ($[Ca^{2+}]i$), it was found that JBU (1 μM) induced a pronounced rise in ganglionic $[Ca^{2+}]i$ (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

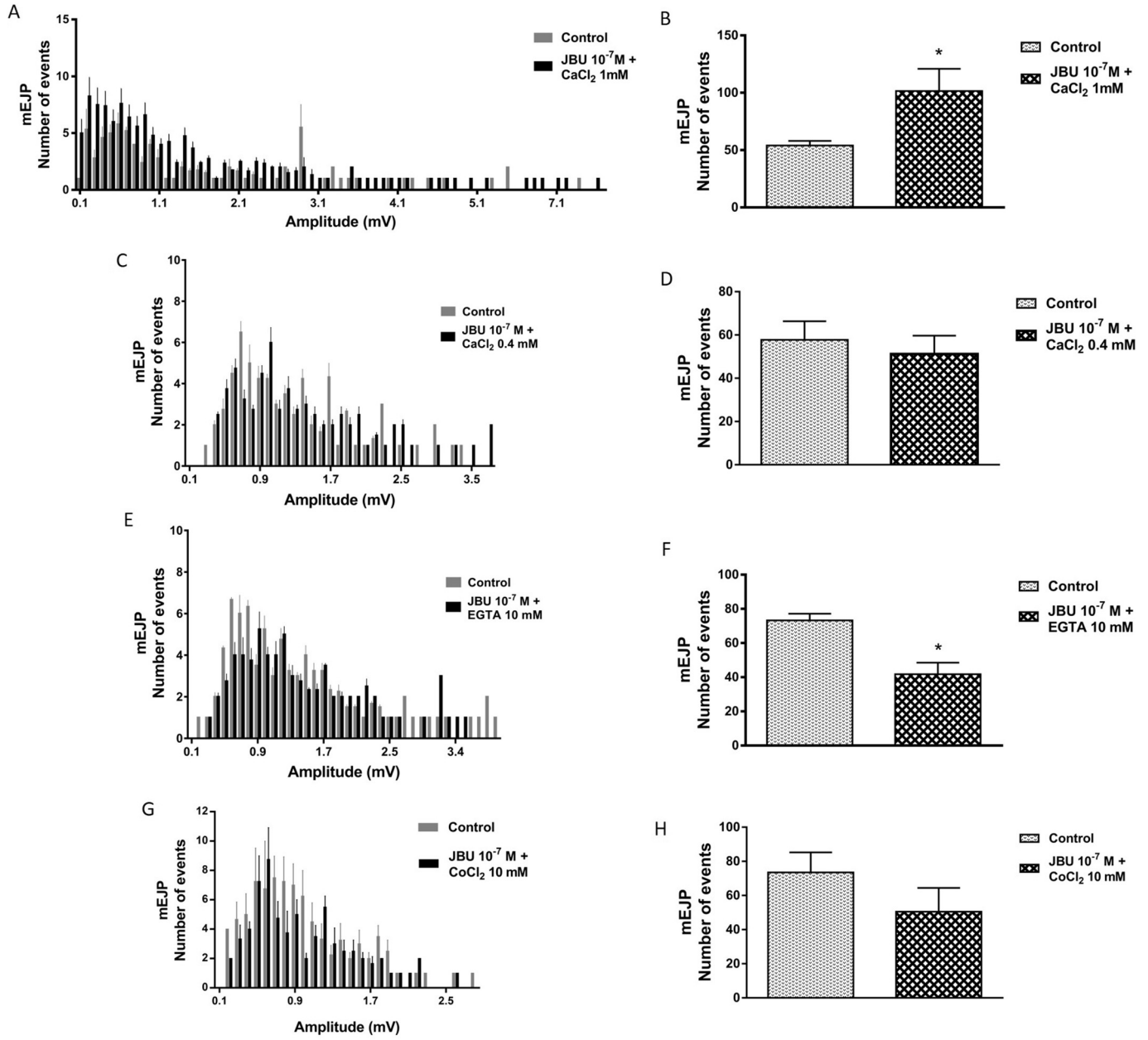

Fig. 4. Effect of JBU on the occurrence of miniature Excitatory Junctional Potentials (mEJPs) in *D. melanogaster* larval neuromuscular junction. The occurrence of spontaneous miniature Excitatory Junctional Potentials (mEJPs) was analysed by intracellular electrophysiological recordings of the 6th body wall muscle in *D. melanogaster* larvae. Panels (A, B) show an experiment conducted in the presence of 1 mM $CaCl_2$. (C, D) Experiment conducted in the presence of 0.4 mM of $CaCl_2$. (E, F) Experiment conducted in calcium-free saline. (G, H) Experiment conducted in calcium-free saline containing 10 mM $CoCl_2$. Panels (A, C, E, G) show the occurrence of events distributed by the range of the peaks. (B, D, F, H) Absolute number of events under different conditions, mean ± S.E.M. All experiments were performed at room temperature. *$p \leq 0.05$ Student "*t*-test" (n = 5).

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-liky 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 digestives 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

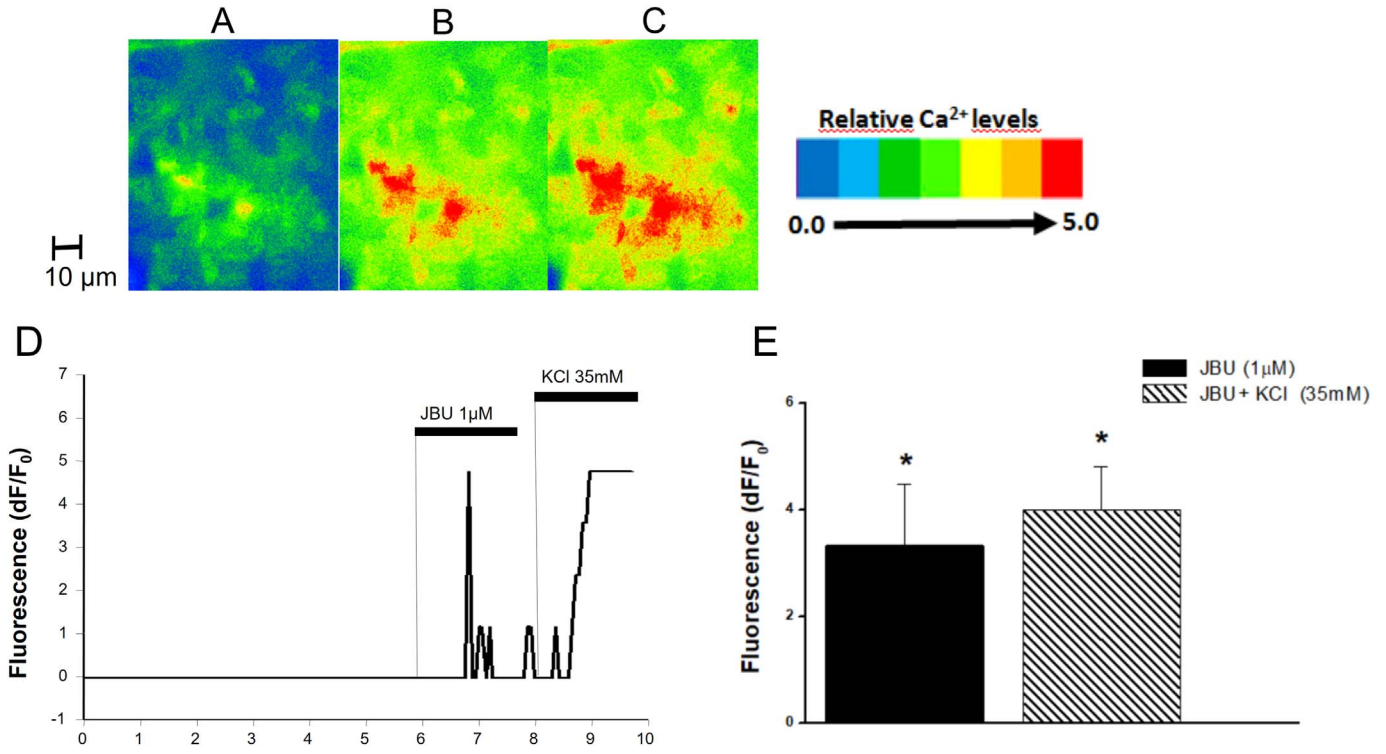

Fig. 5. Increase in Ca^{2+} influx ($[Ca^{2+}]_i$) induced by Jack Bean Urease (JBU) in the third metathoracic ganglion of *Nauphoeta cinerea* cockroaches. Panels (A, B, C) show representative images of the metathoracic ganglion loaded with Fluo-4/AM during baseline (A), 2 min exposition to 1 µM JBU (B) +35 mM KCl perfusion (C). (D) representative graph showing the kinetics of fluorescence emission ($\Delta F/F_0$) induced by 1 µM JBU and the subsequent addition of 35 mM KCl. (E) means ± S.E.M. of the maximum fluorescence emission during the perfusion of the ganglion with JBU (1 µM) + KCl (35 mM) ($n = 7$). *$p < 0.01$ compared to baseline (n = 7).

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 *L. migratoria* and intracellular electrophysiological recordings of body wall muscle of *D. melanogaster* larvae. In the locust preparation the action potentials generated through electrical stimulation are conducted along two motor neurons which innervate the tarsus depressor muscle [56]. When injected into the locust's leg, JBU produced a dose- and time-dependent decrease in the amplitude of the muscle potentials, which matches the neuromuscular depression observed *in vivo* on *N. cinerea* nerve-muscle preparation [24]. When JBU was assayed on the nerve action potential, no significant decrease in amplitude was seen. The hind leg of a locust is innervated by the thoracic nerves (3N3, 3N4, 3N5), originating in the metathoracic ganglion [57]. Extracellular recordings from these nerves in the leg showed no alteration in the APs with JBU. The absence of inhibition on nerve APs and decrease in amplitude of the muscle potential suggested an effect of JBU on neuromuscular transmission.

In order to better understand this aspect of JBU's neurotoxicity, we performed electrophysiological recordings of the *D. melanogaster* neuromuscular junction. JBU induced an increase in the occurrence of spontaneous mEJPs in the presence of 1 mM of $CaCl_2$. When the $CaCl_2$ concentration in the bath was lowered to 0.4 mM, JBU was unable to alter mEJP events. When using calcium-free saline along with a calcium chelator, the effects of JBU were abolished, indicating that calcium fluxes at the presynaptic terminal plays a central role in JBU-induced effects. The increase in occurrence of mEJPs indicates that more vesicles of neurotransmitter are being released from the pre-synaptic terminal [40]. An excess of neurotransmitter release, as frequently observed during the activity of certain neurotoxins, such as snakes toxins [58] and scorpions toxins [59,60], can cause hyperexcitation leading to neurotransmitter run out [4]. Similar effects have been seen for spider toxins on the neuromuscular junction of crayfish and insects [39,61–63] and by inducing a prolonged depolarization of the post-synaptic terminal [57,64]. These phenomenon ultimately results in neuromuscular failure, which can also explain the neuromuscular blockage produced by JBU [24]. Such physiological events are commonly preceded by an increase in the influx of Ca^{2+} in nerve terminals. Indeed, in our experimental conditions JBU increased the influx of Ca^{2+} in the cockroach metathoracic ganglion. This observation gives support to the hypothesis that JBU activity leads to exhaustion of the pre-synaptic terminal which further develops into neuromuscular failure. In this context, it is worth mentioning other biological effects of ureases (including canatoxin, an isoform of JBU) for which interference in calcium mobility across membranes has been implicated, such as platelet aggregation and induction of exocytosis [7,18,19,65]. The antidiuretic effect of JBU in *Rhodnius prolixus* was studied using a Malpighian tubule secretion assay, and revealed disruption of calcium flux across membranes as part of the inhibitory mechanism [32]. Apart from a direct effect on voltage-gated Ca^{2+} channels, JBU could also induce its effect on others nearby ion channels, such as K^+ channels, similar to the effect observed by Dal Belo and cols. working with a snake toxin [58]. In this case, the neurotoxin could block K^+ channels currents, which in turn would modulate Ca^{2+} channels opening in the nerve terminal, allowing this channel to remain open for a longer period, thereby increasing intracellular Ca^{2+} concentration.

Finally, the ability of JBU to insert itself into lipid membranes [36] and to form cation-selective ion channels in planar lipid bilayers [35] may underlie a depolarizing activity of the protein, leading to increase in quantal release at nerve terminals, similar to that produced by the pore-forming toxin pardaxin of the flatfish *Pardachirus marmoratus* [66]. Whether the neuromuscular blockage induced by JBU occurs through a depolarizing activity in the pre-synaptic terminals that involves the activation of voltage-gated Ca^{2+} channels, through indirect modulation

of Ca^{2+} 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.

Acknowledgements

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References

[1] G. Vandenborre, G. Smagghe, E.J.M. Van Damme, Plant lectins as defense proteins against phytophagous insects, Phytochemistry 72 (2011) 1538–1550, http://dx.doi.org/10.1016/j.phytochem.2011.02.024.
[2] M.F. Grossi-de-Sá, P.B. Pelegrini, I.M. Vasconcelos, C.R. Carlini, M.S. Silva, Entomotoxic plant proteins: Potential molecules to develop genetically modified plants resistant to insect-pests, Plant Toxins 10 (2017) 415–448, http://dx.doi.org/10.1007/978-94-007-6728-7_13-1.
[3] R.S. Rattan, Mechanism of action of insecticidal secondary metabolites of plant origin, Crop Prot. 29 (2010) 913–920, http://dx.doi.org/10.1016/j.cropro.2010.05.008.
[4] J.E. Casida, K.A. Durkin, Neuroactive insecticides: targets, selectivity, resistance, and secondary effects, Annu. Rev. Entomol. 117 (2013) 58–99, http://dx.doi.org/10.1146/annurev-ento-120811-153645.
[5] C.R. Carlini, M.F. Grossi-De-Sá, Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides, Toxicon 40 (2002) 1515–1539, http://dx.doi.org/10.1016/S0041-0101(02)00240-4.
[6] C.R. Carlini, J.C. Polacco, Toxic properties of ureases, Crop Sci. 48 (2008) 1665–1672.
[7] C.R. Carlini, R. Ligabue-Braun, Ureases as multifunctional toxic proteins: a review, Toxicon (Oxford) 110 (2016) 90–109, http://dx.doi.org/10.1016/j.toxicon.2015.11.020.
[8] N.E. Dixon, C. Gazzola, J.J. Watters, Jack Bean Urease (EC 3.5.1.5)—Metalloenzyme—Simple biological role for nickel, J. Am. Chem. Soc. 1 (1975) 4131–4133.
[9] C. Follmer, Insights into the role and structure of plant ureases, Phytochemistry 69 (2008) 18–28, http://dx.doi.org/10.1016/j.phytochem.2007.06.034.
[10] D.L. Eskew, R.M. Welch, W.A. Norvell, Nickel in higher plants: further evidence for an essential role, Plant Physiol. 76 (1984) 691–693, http://dx.doi.org/10.1104/pp.76.3.691.
[11] J.C. Polacco, P. Mazzafera, T. Tezotto, Opinion – nickel and urease in plants: still many knowledge gaps, Plant Sci. 199–200 (2013) 79–90, http://dx.doi.org/10.1016/j.plantsci.2012.10.010.
[12] J.B. Sumner, The isolation and crystallization of the enzyme urease, J. Bio. Chem. 69 (1926) 435–442.
[13] C.R. Carlini, J.A. Guimaraes, Isolation and characterization of a toxic protein from *Canavalia ensiformis* (Jack Bean) seeds, distinct from concanavalin A, Toxicon 19 (1981) 667–675, http://dx.doi.org/10.1016/0041-0101(81)90104-5.
[14] C. Follmer, G.B.S. Barcellos, R.B. Zingali, O.L.T. Machado, E.W. Alves, C. Barja-Fidalgo, J.A. Guimarães, C.R. Carlini, Canatoxin, a toxic protein from Jack Beans (*Canavalia ensiformis*), is a variant form of urease (EC 3.5.1.5): biological effects of urease independent of its ureolytic activity, Biochem. J. 224 (2001) 217–224, http://dx.doi.org/10.1042/bj3600217.
[15] M. Pires-Alves, M.F. Grossi-de-Sá, G.B.S. Barcellos, C.R. Carlini, M.G. Moraes, Characterization and expression of a novel member (JBURE-II) of the urease gene family from Jackbean [*Canavalia ensiformis* (L.) DC], Plant Cell Physiol. 44 (2003) 139–145, http://dx.doi.org/10.1093/pcp/pcg018.
[16] F. Mulinari, A.B. Becker-Ritt, D.R. Demartini, R. Ligabue-Braun, F. Stanisçuaski, H. Verli, R.R. Fragoso, E.K. Schroeder, C.R. Carlini, M.F. Grossi-de-Sá, Characterization of JBURE-IIb isoform of *Canavalia ensiformis* (L.) DC urease, Biochim. Biophys. Acta 2011 (1814) 1758–1768, http://dx.doi.org/10.1016/j.bbapap.2011.07.022.
[17] C. Follmer, R. Real-Guerra, G.E. Wassermann, D. Olivera-Severo, C.R. Carlini, Jackbean, soybean and *Bacillus pasteurii* ureases: biological effects unrelated to ureolytic activity, Eur. J. Biochem. 271 (2004) 1357–1363, http://dx.doi.org/10.1111/j.1432-1033.2004.04046.x.
[18] D. Olivera-Severo, G.E. Wassermann, C.R. Carlini, Ureases display biological effects independent of enzymatic activity. Is there a connection to diseases caused by urease-producing bacteria? Braz. J. Med. Biol. Res. 39 (2006) 851–861, http://dx.doi.org/10.1590/S0100-879X2006000700002.
[19] G.E. Wassermann, D. Olivera-Severo, A.F. Uberti, C.R. Carlini, *Helicobacter pylori* urease activates blood platelets through a lipoxygenase-mediated pathway, J. Cell. Mol. Med. 14 (2010) 2025–2034, http://dx.doi.org/10.1111/j.1582-4934.2009.00901.x.
[20] M. Postal, A.H.S. Martinelli, A.B. Becker-Ritt, R. Ligabue-Braun, D.R. Demartini, S.F.F. Ribeiro, G. Pasquali, V.M. Gomes, C.R. Carlini, Antifungal properties of *Canavalia ensiformis* urease and derived peptides, Peptides 38 (2012) 22–32, http://dx.doi.org/10.1016/j.peptides.2012.08.010.
[21] M.S. Defferrari, D.R. Demartini, T.B. Marcelino, P.M. Pinto, C.R. Carlini, Insecticidal effect of *Canavalia ensiformis* major urease on nymphs of the milkweed bug *Oncopeltus fasciatus* and characterization of digestive peptidases, Insect Biochem. Mol. Biol. 41 (2011) 388–399, http://dx.doi.org/10.1016/j.ibmb.2011.02.008.
[22] F. Stanisçuaski, C.R. Carlini, Plant ureases and related peptides: understanding their entomotoxic properties, Toxins 4 (2012) 55–67, http://dx.doi.org/10.3390/toxins4020055.
[23] M.S. Defferrari, H.D. Lee, C.L. Fernandes, I. Orchard, C.R. Carlini, A phospholipase A2 gene is linked to Jack Bean Urease toxicity in the Chagas' disease vector *Rhodnius prolixus*, Biochim. Biophys. Acta 1840 (2014) 396–405, http://dx.doi.org/10.1016/j.bbagen.2013.09.016.
[24] T. Carrazoni, M.A. Heberle, A.P.A. Perin, A.P. Zanatta, P.V. Rodrigues, F.D.M. Santos, C.G.M. Almeida, R.V. Breda, D.S. Santos, P.M. Pinto, J.C. Costa, C.R. Carlini, C.A. Dal Belo, Central and peripheral neurotoxicity induced by the Jack Bean Urease (JBU) in *Nauphoeta cinerea* cockroaches, Toxicology 368–369 (2016) 162–171, http://dx.doi.org/10.1016/j.tox.2016.09.007.
[25] F. Mulinari, F. Stanisçuaski, C.R. Carlini, Jaburetox-2Ec: an insecticidal peptide derived from an isoform of urease from the plant *Canavalia ensiformis*, Peptides 28 (2007) 2042–2050, http://dx.doi.org/10.1016/j.peptides.2007.08.009.
[26] P.R. Barros, H. Stassen, M.S. Freitas, C.R. Carlini, M.A.C. Nascimento, C. Follmer, Membrane-disruptive properties of the bioinsecticide Jaburetox-2Ec: implications to the mechanism of the action of insecticidal peptides derived from ureases, Biochim. Biophys. Acta 1794 (2009) 1848–1854, http://dx.doi.org/10.1016/j.bbapap.2009.09.001.
[27] M.S. Defferrari, R. Silva, I. Orchard, C.R. Carlini, Jack Bean (*Canavalia ensiformis*) Urease induces eicosanoid-modulated hemocyte aggregation in the Chagas' Disease Vector *Rhodnius prolixus*, Toxicon 82 (2014) 18–25, http://dx.doi.org/10.1016/j.toxicon.2014.02.006.
[28] L.L. Fruttero, N.R. Moyetta, A.F. Uberti, M.V.C. Grahl, F.C. Lopes, V. Broll, D. Feder, C.R. Carlini, Humoral and cellular immune responses induced by the urease-derived peptide Jaburetox in the model organism *Rhodnius prolixus*, Parasit. Vectors 9 (2016) 412, http://dx.doi.org/10.1186/s13071-016-1710-3.
[29] C.R. Carlini, A.E.A. Oliveira, P. Azambuja, J. Xavier-Filho, M.A. Wells, Biological effects of canatoxin in different insect models: evidence for a proteolytic activation of the toxin by insect cathepsinlike enzymes, J. Econ. Entomol. 90 (1997) 340–348, http://dx.doi.org/10.1093/jee/90.2.340.
[30] A.R. Piovesan, F. Stanisçuaski, J. Marco-Salvadori, R. Real-Guerra, M.S. Defferrari, C.R. Carlini, Stage-specific gut proteinases of the cotton stainer bug *Dysdercus peruvianus*: role in the release of entomotoxic peptides from *Canavalia ensiformis* urease, Insect Biochem. Mol. Biol. 38 (2008) 1023–1032, http://dx.doi.org/10.1016/j.ibmb.2008.09.004.
[31] R. Real-Guerra, C.R. Carlini, F. Stanisçuaski, Role of lysine and acidic amino acid residues on the insecticidal activity of Jackbean urease, Toxicon 71 (2013) 76–83, http://dx.doi.org/10.1016/j.toxicon.2013.05.008.
[32] F. Stanisçuaski, V. Te Brugge, C.R. Carlini, I. Orchard, In vitro effect of *Canavalia ensiformis* urease and the derived peptide Jaburetox-2Ec on *Rhodnius prolixus* Malpighian tubules, J. Insect Physiol. 55 (2009) 255–263, http://dx.doi.org/10.1016/j.jinsphys.2008.12.002.
[33] F. Stanisçuaski, V. Te Brugge, C.R. Carlini, I. Orchard, Jack bean urease alters serotonin-induced effects on *Rhodnius prolixus* anterior midgut, J. Insect Physiol. 56 (2010) 1078–1086, http://dx.doi.org/10.1016/j.jinsphys.2010.03.002.
[34] A.H.S. Martinelli, K. Kappaun, R. Ligabue-Braun, M.S. Defferrari, A.R. Piovesan, F. Stanisçuaski, D.R. Demartini, C.A. Dal Belo, C.G.M. Almeida, C. Follmer, H. Verli, C.R. Carlini, G. Pasquali, Function studies on Jaburetox, a recombinant insecticidal peptide derived from Jack Bean (*Canavalia ensiformis*) urease, Biochim. Biophys. Acta 1840 (2014) 935–944, http://dx.doi.org/10.1016/j.bbagen.2013.11.010.
[35] A.R. Piovesan, A.H.S. Martinelli, R. Ligabue-Braun, J.L. Schwartz, C.R. Carlini, *Canavalia ensiformis* urease, Jaburetox and derived peptides form ion channels in planar lipid bilayers, Arch. Biochem. Biophys. 547 (2014) 6–17, http://dx.doi.org/10.1016/j.abb.2014.02.006.
[36] Y.M.S. Micheletto, C.F. Moro, F.C. Lopes, R. Ligabue-Braun, A.H.S. Martinelli, C.M. Marques, A.P. Schroder, C.R. Carlini, N.P. Silveira, Interaction of jack bean (*Canavalia ensiformis*) urease and a derived peptide with lipid vesicles, Colloids Surf. B Biointerfaces 145 (2016) 576–585, http://dx.doi.org/10.1016/j.colsurfb.2016.05.063.
[37] B.P. Fulton, P.N.R. Usherwood, Presynaptic acetylcholine action at the locust neuromuscular junction, Neuropharmacology 16 (1977) 877–880, http://dx.doi.org/10.1016/0028-3908(77)90152-6.
[38] T.N. Mellin, R.D. Busch, C.C. Wang, Postsynaptic inhibition of invertebrate neuromuscular transmission by Avermectin B1a, Neuropharmacology 22 (1983) 89–96, http://dx.doi.org/10.1016/0028-3908(83)90265-4.
[39] D.B. Elrick, M.P. Charlton, A-Latrocrustatoxin increases neurotransmitter release by activating a calcium influx pathway at crayfish neuromuscular junction, J. Neurophysiol. 82 (1999) 3550–3562.
[40] T.L. Schwarz, Transmitter release at the neuromuscular junction, Int. Rev. Neurobiol. 75 (2006) 105–144, http://dx.doi.org/10.1016/S0074-7742(06)75006-1.
[41] S.A. Desai, G.A. Lnenicka, Characterization of postsynaptic Ca2+ signals at the *Drosophila* larval NMJ, J. Neurophysiol. 106 (2011) 710–721, http://dx.doi.org/10.1152/jn.00045.2011.
[42] P.N.R. Usherwood, Transmitter release from insect excitatory motor nerve terminals, J. Physiol. 227 (1972) 527–551, http://dx.doi.org/10.1113/jphysiol.1972.sp010046.
[43] R.L. Cooper, C.C. Harrington, L. Marin, H.L. Atwood, Quantal release at visualized

terminals of a crayfish motor axon: Intraterminal and regional differences, J. Comp. Neurol. 375 (1996) 583–600, http://dx.doi.org/10.1002/(SICI)1096-9861(19961125)375:4<583::AID-CNE3>3.0.CO;2-Y.
[44] H.L. Atwood, S. Karunanithi, J. Georgiou, M.P. Charlton, Strength of synaptic transmission at neuromuscular junctions of crustaceans and insects in relation to calcium entry, Invertebr. Neurosci. 3 (1997) 81–87, http://dx.doi.org/10.1007/BF02480362.
[45] R.B. Clark, K.A.F. Gration, P.N.R. Usherwood, Desensitization of glutamate receptors on innervated and denervated locust muscle fibres, J. Physiol. 290 (1979) 551–568.
[46] C. Collet, L. Belzunces, Excitable properties of adult skeletal muscle fibres from the honeybee *Apis mellifera*, J. Exp. Biol. 210 (2007) 454–464, http://dx.doi.org/10.1242/jeb.02667.
[47] I. Orchard, A.B. Lange, The release of octopamine and proctolin from an insect visceral muscle: effects of high-potassium saline and neural stimulation, Brain Res. 413 (1987) 251–258, http://dx.doi.org/10.1016/0006-8993(87)91015-8.
[48] B.A. Stewart, Improved stability of Drosophila larval neuromuscular preparations in haemolymph-like physiological solutions, J. Comp. Physiol. 175 (1994) 179–191, http://dx.doi.org/10.1007/BF00215114.
[49] G.D. Sturmer, T. Carrazoni, M.A. Heberle, D.R. Assis, L. Vinadé, A.B. Pereira, J.L. Franco, C.A. Dal Belo, Modulation of dopaminergic neurotransmission induced by sublethal doses of the organophosphate trichlorfon in cockroaches, Ecotoxicol. Enviro. Saf. 109 (2014) 56–62, http://dx.doi.org/10.1016/j.ecoenv.2014.08.006.
[50] G. Hoyle, M. Burrows, Neural mechanisms underlying behavior in the locust *Schistocerca gregaria*. Physiology of identified motorneurons in the metathoracic ganglion, J. Neurobiol. 4 (1973) 3–41, http://dx.doi.org/10.1002/neu.480040104.
[51] C.T. Nguyen, B.A. Stewart, The influence of postsynaptic structure on missing quanta at the Drosophila neuromuscular junction, BMC Neurosci. 17 (2016) 1–11, http://dx.doi.org/10.1186/s12868-016-0290-7.
[52] R.F. Chapman, G. De Boer, Regulatory Mechanisms in Insect Feeding, Springer, 1995.
[53] L. Robertson, E.P. Rodriguez, A.B. Lange, The neural and peptidergic control of gut contraction in *Locusta migratoria*: the effect of an FGLa/AST, J. Exp. Biol. 215 (2012) 3394–3402, http://dx.doi.org/10.1242/jeb.073189.
[54] E. Sakal, S.W. Applebaum, Y. Birk, Purification and characterization of trypsins from the digestive tract of *Locusta migratoria*, Int. J. Pep. Protein Res. 34 (1989) 498–505, http://dx.doi.org/10.1111/j.1399-3011.1988.tb01391.x.
[55] W. Lam, G.M. Coast, R.C. Rayne, Characterisation of multiple trypsins from the midgut of *Locusta migratoria*, Insect Biochem. Mol. Biol. 30 (2000) 85–94, http://dx.doi.org/10.1016/S0965-1748(99)00103-4.
[56] K.E. Personius, R.F. Chapman, Control of muscle degeneration following autotomy of a hindleg in the grasshopper, *Barytettix humphreysii*, J. Insect Physiol. 48 (2002) 91–102, http://dx.doi.org/10.1016/s0022-1910(01)00149-4.
[57] R.K. Bharadwaj, S.K. Banerjee, The nervous system of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae) with a discussion on muscle innervation, J. Nat. Hist. 5 (2010) 37–41, http://dx.doi.org/10.1080/00222937100770111.
[58] C.A. Dal Belo, G.B. Leite, M.H. Toyama, S. Marangoni, A.L. Corrado, M.D. Fontana, A. Southan, E.G. Rowan, S. Hyslop, L. Rodrigues-Simioni, Pharmacological and structural characterization of a novel phospholipase A2 from *Micrurus dumerilii carinicauda* venom, Toxicon 46 (2005) 736–750, http://dx.doi.org/10.1016/j.toxicon.2005.07.016.
[59] V. Quintero-Hernandez, J.M. Jiménez-Vargas, G.B. Gurrola, H.H.F. Valdivia, L.D. Possani, Scorpion venom components that affect ion-channels function, Toxicon 144 (2008) 724–732, http://dx.doi.org/10.1016/j.toxicon.2013.07.012.
[60] M. Stevens, S. Peigneur, J. Tytgat, Neurotoxins and their binding areas on voltage-gated sodium channels, Front. Pharmacol. 2 (2011) 1–13, http://dx.doi.org/10.3389/fphar.2011.00071.
[61] I.E. Dulubova, V.G. Krasnoperov, M.V. Khvotchev, K.A. Pluzhnikov, T.M. Volkova, E.V. Grishin, H. Vais, D.R. Bell, P.N.R. Usherwood, Cloning and structure of d-Latroinsectotoxin, a novel insect-specific member of the Latrotoxin family, J. Biol. Chem. 271 (1996) 7535–7543, http://dx.doi.org/10.1074/jbc.271.13.7535.
[62] J.A. Umbach, A. Grasso, S.D. Zurcher, H.I. Kornblum, A. Mastrogiacomo, C.B. Gundersen, Electrical and optical monitoring of a-latrotoxin action at drosophila neuromuscular junctions, Neuroscience 87 (1998) 913–924, http://dx.doi.org/10.1016/S0306-4522(98)00664-2.
[63] G.G. Holz, J.F. Hebener, Black widow spider α-latrotoxin: a presynaptic neurotoxin that shares structural homology with the glucagon-like peptide-1 family of insulin secretagogic hormones, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 121 (1998) 177–184.
[64] S.G. Cull-Candy, Two types of extrajunctional ι-glutamate receptors in locust muscle fibres, J. Physiol. 255 (1976) 449–464.
[65] C. Barja-Fidalgo, J.A. Guimarães, C.R. Carlini, Lipoxygenase-mediated secretory effect of canatoxin, the toxic protein from *Canavalia ensiformis* seeds, Toxicon (Oxford) 29 (1991) 453–459, http://dx.doi.org/10.1016/0041-0101(91)90019-N.
[66] P. Renner, C.G. Caratsch, P.G. Waser, P. Lazarovici, N. Primor, Presynaptic effects of the pardaxins, polypeptides isolated from the gland secretion of the flatfish *Pardachirus marmoratus*, Neuroscience 23 (1987) 319–325, http://dx.doi.org/10.1016/0306-4522(87)90292-2.