Toxicology 368–369 (2016) 162–171

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Central and peripheral neurotoxicity induced by the Jack Bean Urease (JBU) in *Nauphoeta cinerea* cockroaches

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ARTICLE INFO

Article history:
Received 19 April 2016
Received in revised form 28 August 2016
Accepted 14 September 2016
Available online 15 September 2016

Keywords:
Plant ureases
Nauphoeta cinerea
Neurotoxicity
Acetylcholinesterase
Behavior modulation

ABSTRACT

Background: Ureases of *Canavalia ensiformis* are natural insecticides with a still elusive entomotoxic mode of action. We have investigated the mechanisms involved in the neurotoxicity induced by Jack Bean Urease (JBU) in *Nauphoeta cinerea* (Olivier).

Methods: To carry out this study we have employed biochemical and neurophysiological analysis of different cockroach organ systems.

Results and conclusions: The injection of the insects with JBU (0.75–6 μg/g animal), although not lethal within 24 h, caused significant inhibition of the brain acetylcholinesterase activity (60 ± 5%, $p < 0.05$, n = 6). JBU (1.5 μg/200 μL), acetylcholine (0.3 μg/200 μL) or neostigmine (0.22 μg/200 μL), induced a positive cardiac chronotropism (~25%) in the cockroaches ($p < 0.05$, n = 9). JBU (6 μg/g) increased the insects' grooming activity (137 ± 7%), similarly to octopamine (15 μg/g) ($p < 0.05$, n = 30, respectively). Pretreating the insects with phentolamine (0.1 μg/g) prevented the JBU- or octopamine-induced increase of grooming activity. JBU (6 μg/g) caused 65 ± 9% neuromuscular blockade in the cockroaches, an effect prevented by bicuculline (5 μg/g) ($p < 0.05$, n = 6). JBU (6 μg/g) decreased the frequency whilst increasing the amplitude of the spontaneous neural compound action potentials (1425 ± 52.60 min^{-1}, controls 1.102 ± 0.032 mV, $p < 0.05$, n = 6, respectively). Altogether the results indicate that JBU induces behavioral alterations in *Nauphoeta cinerea* cockroaches probably by interfering with the cholinergic neurotransmission. The neuromuscular blocking activity of JBU suggests an interplay between acetylcholine and GABA signaling.

General significance: The search for novel natural molecules with insecticide potential has become a necessity more than an alternative. Understanding the mode of action of candidate molecules is a crucial step towards the development of new bioinsecticides. The present study focused on the neurotoxicity of *Canavalia ensiformis* urease, a natural insecticide, in cockroaches and revealed interferences on the cholinergic, octopaminergic and GABA-ergic pathways as part of its entomotoxic mode of action.

Abbreviations: JBU, Jack Bean Urease; ACh, acetylcholine; CNTX, canatoxin; AChE, acetylcholinesterase; SNCAP, spontaneous neural compound action potentials; DTNB, (5,5′, dithiobis-(2-nitrobenzoic acid); TNB, thionitrobenzoic acid; GABA, gama-aminobutiric acid; GLU, glutamate; EPSPs, excitatory postsynaptic potentials.

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http://dx.doi.org/10.1016/j.tox.2016.09.007
0300-483X/

1. Introduction

Throughout the last century, the majority of natural insecticides were replaced by their synthetic counterparts. Amongst the main reasons for the popularization of synthetic insecticides were their low cost, broad spectrum of use covering different insect orders, and longevity in the field. However, although these characteristics appeared initially desirable, they revealed catastrophic consequences for the environment, since these chemical insecticides do not discriminate among insects species, affecting also beneficial ones. In addition, the simplicity of their chemical structures favors insect resistance (Hemingway et al., 2004; Li and Han, 2004; Suzuki and Hama, 1998), which to be overcomed normally requires increasing both dosage and frequency of application further endangering the environment. For these reasons, the search for novel natural molecules with insecticide potential has become a necessity more than an alternative.

Most insecticides exert their effects on insects and other arthropods inducing: (1) neuroexcitation that leads to hyperactivity; (2) spontaneous uncoordinated movements that lead to paralysis due to energy consumption; and (3) neuromuscular failure (Rattan, 2010). Acetylcholine (ACh) is an important neurotransmitter in the evolution of the nervous system (Wächtler, 1988), participating of cell-to-cell communication both in insects and mammals. Comparatively, there are more molecules of ACh in the CNS of insects than in that of a mouse (Sattelle and Breert, 1990). Acetylcholinesterase (AChE), the enzyme responsible for the breakdown of ACh and as such essential for CNS functioning, is one of the main targets of most of the modern insecticides (Leibson and Lifshitz, 2008).

The growing knowledge on the non-enzymatic properties of ureases, plant proteins crucial for the conversion of urea to ammonia, has unveiled their potential as biological insecticides (Carlini and Ligabue-Braun, 2016; Stanisçuaski and Carlini, 2012). Ureases (EC 3.5.1.5, urea amidohydrolase) are nickel-dependent metalloenzymes (Dixon et al., 1975) that catalyze the conversion of urea to two molecules of ammonia and one of carbon dioxide, enhancing the rate of the uncatalyzed hydrolysis by a factor of 8×10^{17} (Callahan et al., 2005). Ureases are widespread in plants, fungi, and bacteria but are not synthesized by animals (Mobley and Hausinger, 1989). *Canavalia ensiformis* displays several urease isoforms: the most abundant one Jack Bean Urease (JBU) (Sumner, 1926), canatoxin (CNTX) (Carlini and Guimarães, 1981; Follmer et al., 2001) and JBURE-II (Mulinari et al., 2011; Pires-Alves et al., 2003). The entomotoxic action of *C. ensiformis* ureases has been shown in different insect models (Stanisçuaski and Carlini, 2012). Afterwards, a recombinant peptide named Jaburetox, derived from the urease isoform JBURE-II of *C. ensiformis*, was produced in *Escherichia coli* and its direct paralyzing effect on the cockroach neuromuscular junction was reported (Martinelli et al., 2014). However, the specific mechanism(s) leading to this biocide activity are still not fully elucidated.

Cockroaches are quite diverse representing more than 4000 species comprising the suborder Dictyoptera. *Nauphoeta cinerea* is an ovoviviparous cockroach of the Blaberidae family. This species is frequently used in biomedical research not only because the easiness of breeding and maintenance but also due to their relative simplicity and suitability for certain experimental procedures (Huber et al., 1990). There are many similarities between the cockroach nervous system and that of other insect species (Huber et al., 1990). In addition their amenability to experimental manipulation and biophysical parallelism with vertebrates make their use very convenient for a number of neurophysiological approaches (Stankiewicz et al., 2012).

In this work we investigated the alterations induced by JBU on the *N. cinerea*'s central and peripheral nervous systems. To accomplish this aim we carried out electromyographic, electrophysiological and behavioral experiments using the cockroach as model.

2. Materials and methods

2.1. Experimental animals

All experiments were performed on adult male *Nauphoeta cinerea* (Olivier) cockroaches (3–4 months after adult molt). The animals were reared in laboratory conditions with controlled temperature (22–25 °C) on a 12 h:12 h L:D cycle. The cockroaches were provided with water and dog chow *ad libitum*.

2.2. Jack Bean Urease (JBU)

Highly purified crystalline urease of *Canavalia ensiformis* (type C3) was obtained from Sigma-Aldrich Brazil. The protein (hexameric molecular mass 545 kDa) crystals were dissolved in 10 mM sodium phosphate buffer and dialysed against deionized water to give 1 μM concentration. Protein solutions were kept at 4 °C and diluted in insect saline to appropriate concentrations before bioassays.

2.3. Reagents and solutions

All chemicals and reagents used 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 insect saline immediately before use. The insect saline is a carbonate-buffered solution prepared with the following composition in mM: NaCl, 214; KCl, 3.1; $CaCl_2$, 9; sucrose, 50; HEPES buffer, 5 and pH 7.2 (Stürmer et al., 2014). Except when stated otherwise, all drugs were injected into the abdominal hemocoel, in a 10 μL volume, by means of a Hamilton syringe.

2.4. Lethality assay

The insecticidal assay against adult *Nauphoeta cinerea* was carried out essentially as described by (Kagabu et al., 2007). Various concentrations of JBU dissolved in 10 μL insect saline were injected between the third and the fourth abdominal segments of *N. cinerea*. All the experiments were made in triplicate. Ten insects were used to test each dose. Survival rate was registered 24 h after injection.

2.5. Assay of acetylcholinesterase activity

Acetylcholinesterase activity was evaluated according to Ellman et al. (1961) as modified by Franco et al. (2009) and Stürmer et al. (2014). Briefly, six cockroaches were injected with JBU (1.5, 3 and 6 μg/g body weight), or with trichlorfon (0.03 μg/g), a well known inhibitor of acetylcholinesterase, six hours before the analysis. The animals were anesthetized by chilling for 5–7 min at −20 °C and after cuticle removal, their brains were collected. The material was mixed with 750 μL of potassium phosphate (KPi) buffer pH 7.0. After centrifugation (500 rpm/5 min/4 °C), 50 μL of the supernantant was mixed to 50 mM DTNB (5,5′-dithiobis-(2-nitrobenzoic acid), 500 mL Kpi (pH 8.0) and 2.5 mL acetylthiocholine. The rate of the hydrolysis of acetylthiocholine was measured through the release of thiolcholine, whose free sulphydryl group reacted with DTNB to produce the yellow compound thionitrobenzoic acid (TNB). The reaction was followed at room temperature during 60 s (s) at 412 nm using a UV–vis Spectrophotometer (model Evolution 60S, Thermoscientific, New Hampshire, USA) and analyzed by the software VISION lite (Thermoscientific). The

amount of protein in samples was measured according to Bradford, 1976. The results were expressed as miliunit of AChE per milligram protein (mU/mg protein). One milliunit of AChE was defined as the amount of enzyme able to produce 1 nmol of TNB per min under the specified conditions.

2.6. Semi-isolated cockroach heart preparation

A semi-isolated cockroach heart bioassay was mounted essentially as described by Rodríguez et al. (2012). Briefly adult male cockroaches were anesthetized by chilling (5–7 min) until immobile and placed ventral side up. The lateral margins of the abdomen were cut along each side, and the ventral abdominal body wall was pulled out to show the viscera. After moving the viscera carefully aside the heart was exposed, still contracting while attached to the dorsal body wall. The heart preparations were washed by bathing them in 200 μL of insect saline solution at room temperature (21–24 °C). After 5 min of heart beat stabilization, the treatments were delivered by exchanging the bathing solution. The mean beats.min^{-1} in the first 5 min was taken as a reference. Heart beat frequency was monitored for 30 min under a stereoscopic microscope. Nine cockroaches were used for each group. In the control group, only saline solution was used to bath the heart.

2.7. Behavioral assays

For behavioral studies, animals were placed in an open-field arena (300 × 300 mm, demarcated in 12 zones) with a video camera (Panasonic coupled to a 50 mm Karl-Zeiz lens) mounted overhead as previously described by Stürmer et al. (2014). The camera had a frame-by frame (60 frame/s) device and was connected to a PC (Infoway, ItauTec, Brazil). The insect's activities were recorded during 30 min and the video movies were later analyzed using a HD Writer AE 2.6T system (Panasonic) with variable speed control.

2.7.1. Grooming activity

Cockroaches were examined for grooming behavior (Stürmer et al., 2014) immediatly after injection with JBU (1.5, 3.0 and 6 μg/g). Pretreatment of the cockroaches with phentolamine (0.1 μg/g) or octopamine (15 μg/g) was done by injecting the drugs 10 min prior to JBU (6 μg/g) administration. Grooming behavior was recorded with a camera for later analysis. After treatments the time of continuous grooming in seconds was measured over a 30 min period. Animals had never been placed in the open-field previously, so it was a novel environment in all cases. Testing was performed 2–8 h after the beginning of the light cycle and the room was maintained at 22–25 °C. Control cockroaches were injected only with insect saline.

2.8. Electromyographic recordings

2.8.1. In vivo *cockroach metathoracic coxal-adductor nerve-muscle preparation*

To analyze the effect induced by JBU on insect neuromuscular junctions we used the *in vivo* cockroach metathoracic coxal-adductor muscle preparation essentially as described in Martinelli et al. (2014). Briefly, animals were immobilized by chilling and mounted, ventral side up, in a Lucite holder covered with 1 cm soft rubber that restrained the body and provided a platform to which the metathoracic thigh could be firmly attached using entomologic needles. The left leg was then tied at the medial joint with a dentistry suture line connected to a 1 g force transducer (AVS Instruments, São Carlos, SP, Brazil). The transducer was mounted in a manipulator which allowed adjustment of muscle length. The exoskeleton was removed from over the appropriated thoracic ganglion. Nerve 5, which includes the motor axon to the muscle, was exposed and a bipolar electrode was inserted to provide electrical stimulation. The nerve was stimulated at 0.5 Hz/5 ms, with twice the threshold, during 120 mim. After the insertion of the electrodes, the opening in the exoskeleton was covered with mineral oil to prevent dryness. Twitch tensions were recorded, digitalized and retrieved using a computer based software AQCAD (AVS Instruments, São Carlos, SP, Brazil). Data were further analyzed using the software ANCAD (AVS Instruments, São Carlos, SP, Brazil). The preparations were allowed to stabilize for at least 20 min before injection of drugs into the insect's abdominal hemocoel. Drugs (5 μg/g ACh, or 5 μg/g bicuculline, or 15 μg/g octopamine) were injected 15 min prior the application of JBU (1.5, 3 and 6 μg/g).

2.9. Electrophysiological recordings

2.9.1. In vitro extracellular recordings of spontaneous neural compound action potentials (SNCAP) of the cockroach leg

For the recordings of SNCAP male cockroaches were anesthetized by chilling during 5–7 min and the metathoracic leg was cut as close as possible to the body to ensure that the thigh, femur, tibia, and tarsus remained intact. The leg was then carefully fixed by means of three Ag/AgCl needle electrodes in a petri dish filled with a 10 mm Sylgard® layer. One of the electrodes was connected to the ground connector of the amplifier (Axoclamp 2B, Molecular Devices, USA) and the other to its indifferent (−) connector. The third electrode was placed into the femur as the active recording electrode (+). The signals were recorded at a sampling rate of 1 kHz and digitalized using a digitizer Digidata 1320A (Molecular Devices, USA). The action potentials were visualized, recorded and retrieved for later analysis in the computer-based software Clampex (Molecular Devices, USA), followed by the software WinWCP (John Dempster, University of Strathclyde). Using the described conditions, the preparation could be used for at least one hour without changes in the characteristics of the potentials. The treatments were injected in the leg by means of a Hamilton syringe and the doses were calculated in μg/g based on the weight of the isolated leg.

2.10. Statistical analysis

The results were expressed as mean ± SEM. Each experiment was repeated at least three times. For comparison between means of two different experimental groups the Student "*t*" test was employed. When data from more than two experimental groups were analyzed ANOVA was employed followed by Tukey (all groups were compared with each other) or Dunnet (the groups were compared with a positive control or saline) as *post hoc* tests. All statistical analyses were performed using the Graphpad Prism 6.0 (Software Inc., San Diego, CA). The values were considered significantly different when $p \leq 0.05$.

3. Results

3.1. Entomotoxic activity of Jack Bean Urease (JBU)

To determine the insecticidal activity of JBU, four doses were assayed (0.75, 1.5, 3 and 6 μg/g). After 24 h no lethality was observed. Although not lethal, the animals displayed a notorious grooming activity starting soon after the injection of urease. All insects were lethargic at the end of the 24 h observation period.

3.2. Acetylcholinesterase (AChE) activity in brain homogenates

Analysis of AChE activity of brain homogenates of cockroaches injected with JBU (1.5, 3 and 6 μg/g animal weight) or Trichlorfon (0.03 μg/g) revealed a dose-dependent enzyme inhibition, as compared to controls (insects injected with saline only) (Fig. 1). The control value of AChE activity was 186 ± 3 mU/mg protein/min. At 1.5 μg/g animal weight, JBU induced a significative decrease of the insect brain AChE activity (32.52 ± 3%, n = 6, $p < 0.0001$). At 3 μg JBU/g dose, the AChE activity decreased by 33 ± 3%, n = 6; $p < 0.0001$, and a greater inhibition was seen in brain homogenates of insects injected with JBU at a dose of 6 μg/g animal weight, which produced inhibition of 60 ± 5% (n = 6; $p < 0.0001$) of AChE activity. No difference in the AChE inhibition was seen between the doses of 1.5 and 3 μg JBU/g ($p > 0.05$). As expected Trichlorfon (0.03 μg/g) administration resulted in an AChE inhibition of 77 ± 6% (n = 6; $p < 0.0001$). These results are shown in Fig. 1. As it can be observed the effect of the largest dose of JBU was close to that seen in insects exposed to trichlorphon, a well known AChE inhibitor. Considering the difference in molecular mass of the two compounds, JBU (Mr 540.000) is about 10 times more active than trichlorfon (Mr 257.43) in inhibiting the activity of cockroach brain AChE.

3.3. Effects of JBU on the cockroach's heart rate

The *N. cinerea* semi-isolated heart preparation in the presence of saline had a control value of 76 ± 5 beats/min (n = 9) (Fig. 2A). The addition of JBU to the preparation produced a time-dependent and U-shaped dose-dependent effect. At the lowest dose of JBU (0.75 μg/200 μL) there was an increase of the chronotropic response of the heart, reaching a maximum (99 ± 4 beats/min; n = 9) after 20 min incubation ($p < 0.001$ compared to saline). Starting immediately after addition of 1.5 μg JBU/200 μL, the chronotropic response peaked at 5 min with 97 ± 5 beats/min ($p < 0.001$ compared to saline, n = 9) and lasted the whole 30 min of recording (Fig. 2A). In contrast, when the highest concentration of JBU (3 μg/200 μL) was assayed, no modulation of the chronotropic effect was seen (Fig. 2A). For comparison, ACh (0.3 μg/200 μL) or neostigmine (0.22 μg/200 μL) were assayed in the same preparation, producing a positive modulation of heart beats, with similar results of 92 ± 3 and 90 ± 4, respectively, in 30 min recordings (n = 6), (Fig. 2B).

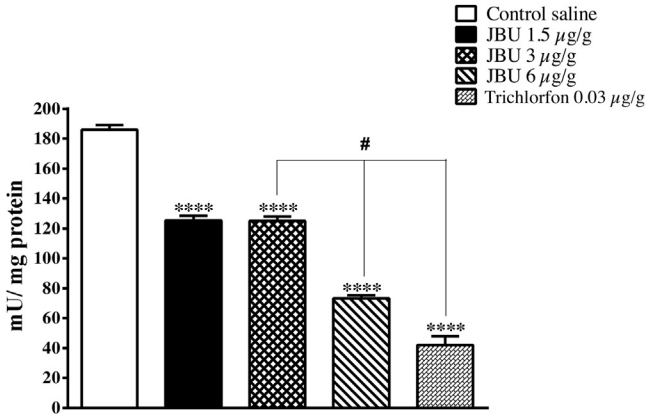

Fig. 1. Acetylcholinesterase inhibition in total cockroach brain homogenates by *in vivo* treatment with JBU.
Cockroaches were injected with JBU (1.5, 3.0 and 6 μg/g; n = 6 for each dose) or trichlorfon (0.03 μg/g) and total brain homogenates were prepared 6 h after the injections. Data were expressed as mU AChE/mg protein of brain homogenate. One mU of AChE is the amount of enzyme hydrolyzing 1 nmol of DTNB per min under the defined conditions (see Methods). The results are expressed as mean ± S.E.M. **** $p < 0.0001$ (n = 6) compared to the control saline by Anova followed by the Dunnett's test; #$p < 0.0001$ comparing each other with Anova followed by the Tukey's test.

3.4. Effects of JBU on the cockroach grooming activity

In saline-injected cockroaches the mean time of continuous grooming was 153 ± 8 s/30 min for the legs and 70 ± 6 s/30 min for the antennae (n = 32, respectively).

Injection of JBU (1.5, 3 and 6 μg/g of animal weight) produced a significant dose-dependent increase in the grooming activity of the leg but had no noticeable effect on the antennae. For 1.5 μg JBU/g animal weight only a tendency of increasing in leg grooming activity was seen (178 ± 24 s/30 min, n = 40; $p > 0.05$, compared to saline) (Fig. 3). At higher doses, JBU (3 and 6 μg/g) induced an evident increase in the leg grooming parameters to 253 ± 33 s/30 min (n = 29) and 363 ± 23 s/30 min (n = 30); $p < 0.002$ and 0.0001 respectively, compared to saline (Fig. 3). The values for antennae grooming were 48 ± 5 s/30 min, 57 ± 7 s/30 min and 81 ± 8 s/30 min, for insects injected with JBU at 1.5, 3 and 6 μg/g animal weight, respectively, (Fig. 3). Only seen at a dose of 1.5 μg/g animal weight, JBU induced a slight reduction of antennal grooming as compared to the control saline ($p < 0.001$), Fig. 3.

3.5. Effects of different pharmacological treatments on JBU-induced grooming activity

Leg grooming activity in insects is thought to be modulated mainly by the neurotransmitter octopamine (Weisel-Eichler et al., 1999). Here, we tested the effects of phentolamine, a selective octopamine receptor blocker, and of octopamine in modulating the behavioral changes induced by JBU. When octopamine (15 μg/g) was injected in the cockroaches, there was an increase in the leg grooming time (134 ± 10%; n = 40; $p < 0.05$ compared to saline; Fig. 4). The treatment of the animals with phentolamine (0.1 μg/g) alone decreased the normal grooming activity to 7.3 ± 2% and 20.3 ± 3.5, for leg and antenna, respectively ($p < 0.01$, n = 30). Administration of phentolamine (0.1 μg/g) 15 min before octopamine injection inhibited the octopamine-induced increase of leg grooming (52 ± 8%; n = 40; $p < 0.05$ compared to the control octopamine; Fig. 4). When phentolamine (0.1 μg/g) was administered 15 min before JBU (6 μg/g) injection, it reverted to control levels the time the insects spent in leg grooming (37 ± 5%; n = 40; $p < 0.05$; Fig. 4).

3.6. Neuromuscular blockade of a cockroach nerve-muscle preparation induced by JBU in vivo

To further analyze the effect of JBU on cockroach nervous system, we used the *in vivo* metathoracic coxal-adductor nerve-muscle preparation. The administration of insect saline alone did not interfere with neuromuscular responses during 120 min recordings (n = 6) (Fig. 5A). JBU (1.5, 3 and 6 μg/g of animal weight) induced a significative time-dependent neuromuscular blockade, which could be seen for the lower doses starting 1 h after injection. For the dose of 6 μg JBU/g body weight the inhibitory effect was noticeable after 30 min, and increased steadly to reach a maximal inhibition of 65 ± 9% (n = 6, $p < 0.05$) after 120 min (Fig. 5A). In the same set of experiments, the treatment of the animals with bicuculline (5 μg/g), a selective blocker of the gama-amino-butyric acid (GABA) receptor, did not affect the twitches' tension during the first 60 min recording, but produced a small (about 15%) although significative blockage after 120 min ($p < 0.0001$, n = 6) (Fig. 6A). When bicuculline (5 μg/g) was injected in the insect 15 min before JBU (6 μg/g), it reduced by ~40% the JBU-induced neuromuscular blockade ($p < 0.05$, compared to the

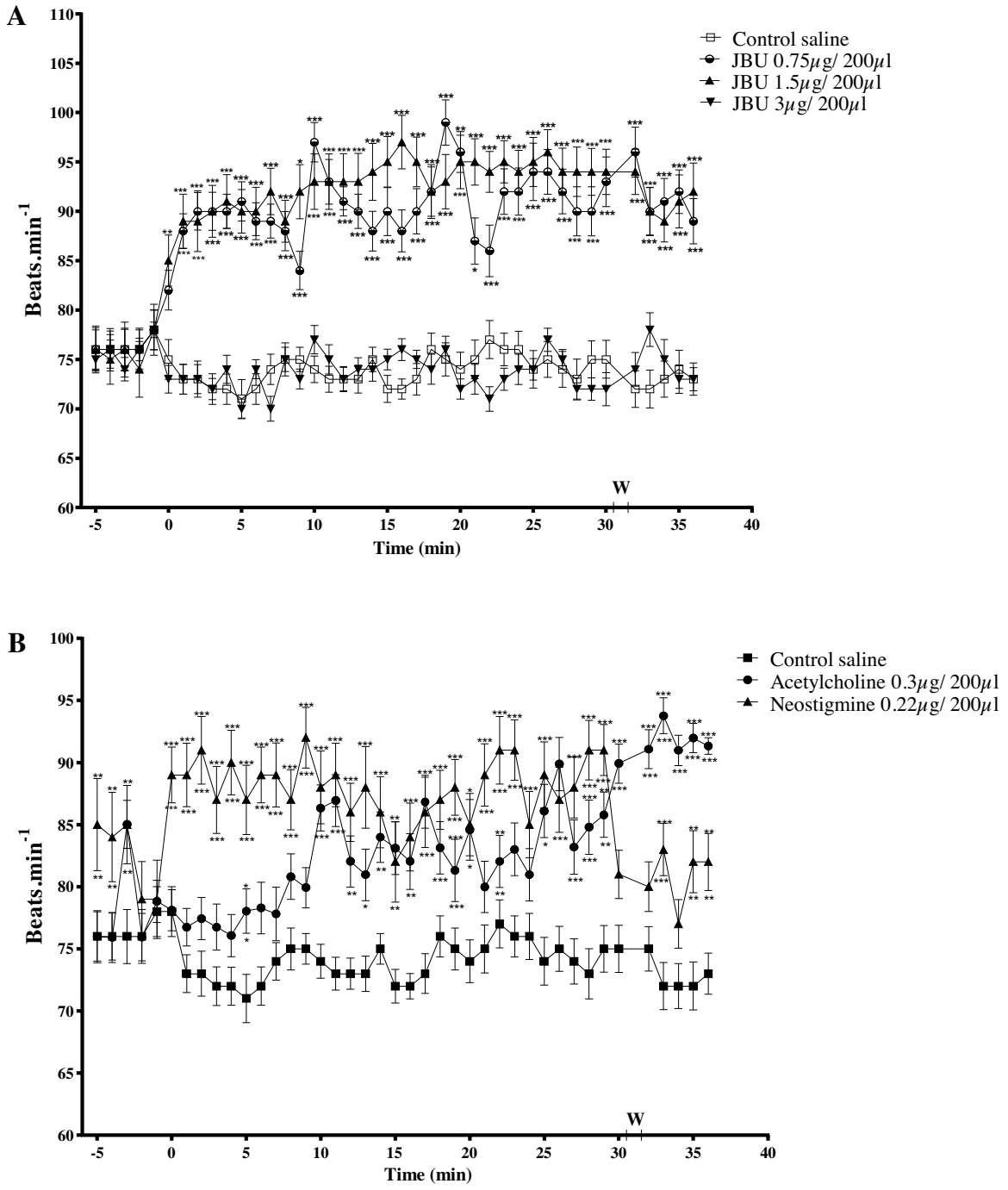

Fig. 2. Effect of different concentrations of Jack Bean Urease (JBU) on *Nauphoeta cinerea* heart rate.
In the graph each point corresponds to the mean ± S.E.M. of the insect heart beats/min relative to the initial state (−5 min), measured during 30 min after exposition to JBU (panel A) or drugs (panel B). Note that JBU (1.5 μg/200 μL) induced a cardio acceleratory activity similar to that induced by ACh (0.3 μg/200 μL) or neostigmine (0.22 μg/200 μL). For data in A and B, statistical analyses were performed by Two-way Anova followed by the Tukey's test. $^{*}p < 0.05$ (n = 9); ** $p < 0.01$; *** $p < 0.001$ (n = 6).

JBU alone) (n = 6, Fig. 6A). For comparison, ACh (5 μg/g) applied alone in the insects induced 52 ± 12% inhibition of the muscle twitches' tension in 120 min (n = 6, $p < 0.05$) (Fig. 6B). Previous application of bicuculline (5 μg/g) 15 min before ACh (5 μg/g) partially prevented (∼40%) the ACh-induced neuromuscular blockade ($p < 0.05$ compared to the ACh alone) (n = 6, Fig. 6B). The administration of octopamine (15 μg/g) alone led to 55 ± 9% neuromuscular blockade of the cockroach neuromuscular junction in 120 min (n = 6, $p < 0.05$) (Fig. 6C). When bicuculline (5 μg/g) was previously applied and followed by octopamine (15 μg/g) there was no prevention of the octopamine-induced neuromuscular blockade (n = 6, $p < 0.05$) (Fig. 6C).

3.7. Effect of JBU on the spontaneous activity of cockroach leg nerve-compound action potentials (SNCAP)

The cockroach leg nerve-compound action potential has a relative high rate of rise when compared to other neuronal models (Table 1). Injection of JBU (6 μg/g) into the cockroach leg caused a significant decrease in the frequency (1425 ± 52.6 min^{-1})

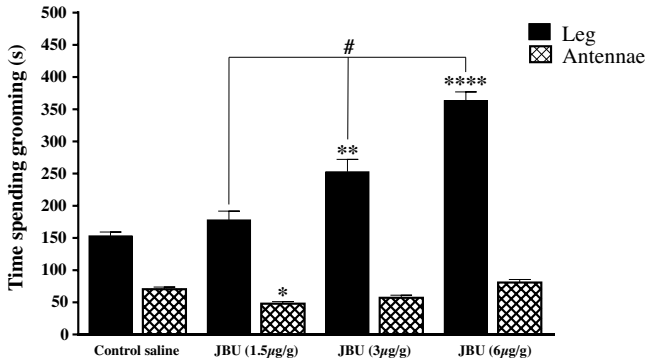

Fig. 3. Increase of grooming behavior induced by JBU in *Nauphoeta cinerea*.
The cockroaches were injected with JBU (1.5, 3.0 and 6 μg/g) and examined for grooming activity immediatly after injection. The grooming activity was recorded during 30 min and the results are expressed as mean ± S.E.M. of the total time of grooms (in s). 30 min^{-1}. The data were analysed by One-way Anova followed by the Dunnett's test.** $p < 0.01$, # $p < 0.001$ **** $p < 0.0001$, n = 28-32, respectively.

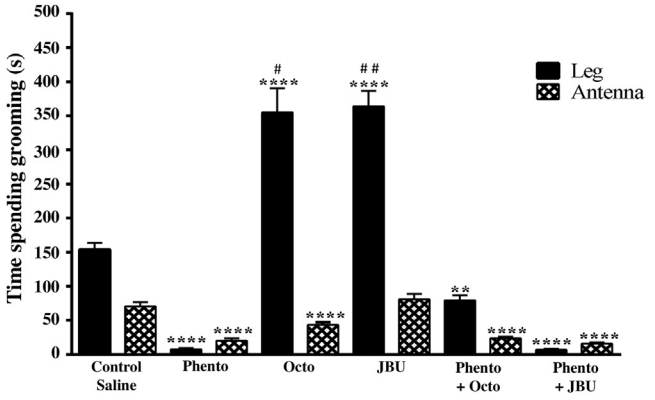

Fig. 4. Effect of different octopaminergic modulators on leg grooming induced by JBU in cockroaches.
The grooming activity was recorded during 30 min immediately after JBU (6 μg/g), octopamine or phentolamine injections. In the case of concomitant treatment with JBU or octopamine (15 μg/g), phentolamine (0.1 μg/g) was injected in the third abdominal segment 15 min before. In the graph, each bar represents the mean ± S.E.M. of the total time of grooms (in s) 30 min^{-1}. Statistical analyses were performed by One-way Anova followed by the Dunnett's test to compare the control saline group with the others. The Student "*t*" test was used to compare the positive control octopamine or JBU with the phentolamine-pretreated groups. **$p < 0.01$; **** $p < 0.0001$; #$p < 0.001$; ##$p < 0.0001$, n = 28-32, respectively.

accompanied by an increase in the mean amplitude (1.102 ± 0.032 mV) of SNCAP (n = 6, $p < 0.05$ respectively) in the 60 min period of observation (Fig. 7A, B). The rise time decreased (0.64 ± 0.044 ms) parallel to the decay time (3.72 ± 2.121 ms, $p < 0.05$ respectively). The treatment with JBU also induced a 9% decrease in the area of the action potentials (Table 1).

4. Discussion

In this work we have characterized the neurotoxic activity induced by the main urease isoform of the *Canavalia ensiformis* plant, the so-called Jack Bean Urease – JBU, in the cockroach *Nauphoeta cinerea*. Aspects related to the cellular and biochemical mechanisms involved in neuromodulation of the insect central and peripheral nervous system by JBU were elucidated in this study.

Although JBU has been proven to be insecticidal to different insects (Stanisçuaski and Carlini, 2012), it displayed no lethality in *Nauphoeta cinerea* cockroaches, at least not by intrabdominal administration in a 24 h observation time. In previous studies, we reported that the lethality of Jack Bean ureases given orally to insects depends on characteristics of their digestive system (Stanisçuaski and Carlini, 2012). At least part of the insecticidal effect is caused by toxic peptides derived from ureases upon cleavage by insect's digestive enzymes. Thus, only insects relying on cathepsin-like enzymes (cysteine and aspartic proteases) *e.g. Callosobruchus maculatus* and *Rhodnius prolixus*, died upon ingestion of ureases, while insects with digestion based on trypsin-like enzymes (serine proteases), such as *Manduca sexta*, *Schistocerca americana*, *Drosophila melanogaster* and *Aedes aegypti*, were not killed. Elpidina and cols (Elpidina et al., 2000) showed that the digestion in *N. cinerea* midgut is carried out with optimal activity at pH 11.5 by three types of serine proteinases and one cysteine proteinase, what could explain the absence of lethality of JBU in this insect model. On the other hand, it is already known that urease-derived peptides do not account for all the entomotoxic properties displayed by these proteins. The whole protein can be found circulating in the hemolymph of insects after feeding (Stanisçuaski et al., 2010). Urease itself was shown to produce entomotoxic effects in the hemipteran *Rhodnius prolixus* that were independent of enzymatic cleavage, such as impairment of diuresis in isolated Malpighiam tubules (Stanisçuaski et al., 2009) or increase in the frequency of crop contraction (Stanisçuaski et al., 2010). Here we have shown that, even in an insect model probably unable to cleave JBU to release its insecticidal peptides, thus not causing lethality, the whole protein induces profound alterations of the cockroach physiology, impacting both its central and peripheric nervous systems.

JBU-induced effects in *N. cinerea*, particularly the positive cardiac chronotropism and behavioral alterations, are consistent with inhibition of the cockroach's brain AChE activity (Stürmer et al., 2014). The precise molecular mechanism for this anti-AChE-like effect of JBU is presently unknown. A number of possibilities can be raised to explain this property of JBU, including but not limited to: (1) a direct interaction of JBU with the AChE molecule leading to its inhibition; (2) an "agonist"-type of interaction of JBU directly with ACh receptors; (3) an interaction of JBU with cell membranes in the vicinity of ACh receptors in a way that leads to their activation; (4) an interaction of JBU with sodium channels coupled to ACh receptors. It is already known that JBU is able to insert itself in lipid bilayers thereby alterating physicochemical parameters of lipid membranes (Piovesan et al., 2014) and that JBU activates sodium channels in a number of systems (unpublished data). Thus, one or more than one of the possibilities raised above could explain the anti-AChE of JBU in the cockroach brain homogenate as well as its other "cholinergic-like" effects.

The increase in leg grooming rather than that of antennae and its antagonism by phentolamine in JBU-treated cockroaches suggests the involvement of the neurotransmitter octopamine in the modulation of the cockroach's behavior (Weisel-Eichler et al., 1999) as triggered by the toxin. The observed inhibition of antennal grooming by phentolamine may be a result of its unspecific antagonistic action upon other monoaminergic receptors (Koons et al., 1983; Tayo, 1979). In insects, although the neural center involved in grooming behavior has not been identified so far, it is known that monoamines such as dopamine and octopamine modulate this behavioral activity (Libersat and Pflueger, 2004). Considering that cholinergic-octopaminergic signaling is a common physiological aspect of the insect CNS (Buhl et al., 2008), it is possible that the anti-AChE-like activity of JBU underlines the alterations in grooming behaviour evoked by the toxin in the cockroaches.

The anti-AChE-like activity of JBU could also account for the increase in the heart rate in our experimental model. In *Periplaneta americana*, the heart rate is determined by a neurogenic pacemaker with cholinergic properties (Wigglesworth, 1972), stimulated by ACh and by AChE inhibitors (Husmark and Ottoson, 1971a,

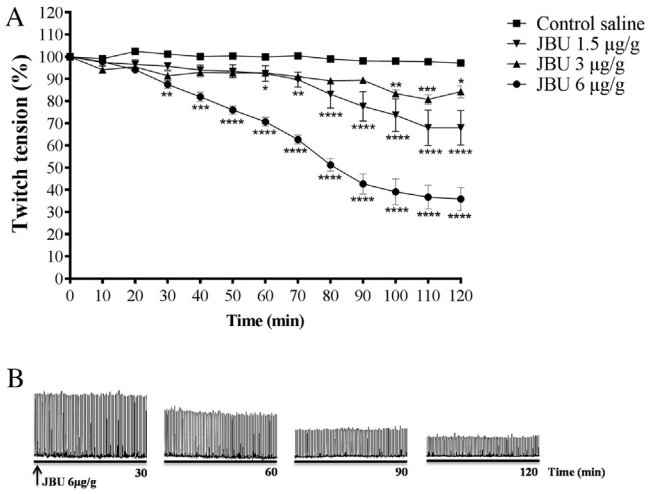

Fig. 5. Neuromuscular blockade induced by Jack Bean Urease (JBU) in *Nauphoeta cinerea* cockroaches.
In the graph (A) each point represents the mean ± S.E.M. of the twitch tension percentage relative to before the treatments. Panel B shows a representative recording of the JBU-induced neuromuscular blockage. Statistical analyses were performed by Two-way Anova followed by the Tukey's test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, n = 6, respectively.

bDahm, 1971). In a previous study, our group has demonstrated the increase of heart rate in *Leurolestes circunvagans* cockroaches by naturally occurring inhibitors of AChE (Rodríguez et al., 2012). The molecular mechanism(s) by which JBU leads to AChE inhibition was not investigated in the present work. Besides an interference of JBU on the cholinergic regulation of *N. cinerea*'s heart, there are other mechanisms that could account for the effects seen and that were not explored here. The cardioacceleratory peptide proctolin (Sliwowska et al., 2001), 5-hydroxytryptamine (5-HT) and, more relevant, octopamine (Tublitz and Truman, 1985) also function as cardioregulatory neurohormones (Miller, 1979). Since octopamine has a biphasic effect over the cardiac rhythm in other insects (Papaefthimiou and Theophilidis, 2011), it is also possible that the cholinergic overstimulation induced by JBU accounts for the modulation of the cockroach cardiac rhythm through an octopaminergic cotransmission.

The electromyographic recordings of cockroaches injected with JBU revealed that the whole protein has a neuromuscular blocking activity. This effect was previously reported for Jaburetox, a urease-derived recombinant peptide that corresponds to about one tenth the size of the whole protein (Martinelli et al., 2014). Several biological properties, but not all of them, are shared between the whole urease molecule and Jaburetox. Particularly relevant to the present data, it has been shown that in nanomolar concentrations both JBU and Jaburetox are able to insert themselves into artificial lipid bilayers creating cation-selective channels (Piovesan et al., 2014). The region of the JBU molecule comprising Jaburetox's sequence is well exposed at the protein's surface, hence it probably mediates most of the interactions of the urease molecule with its targets (Piovesan et al., 2014).

There is a considerable amount of work showing that insect neuromuscular junctions rely on glutamate (GLU) as the main

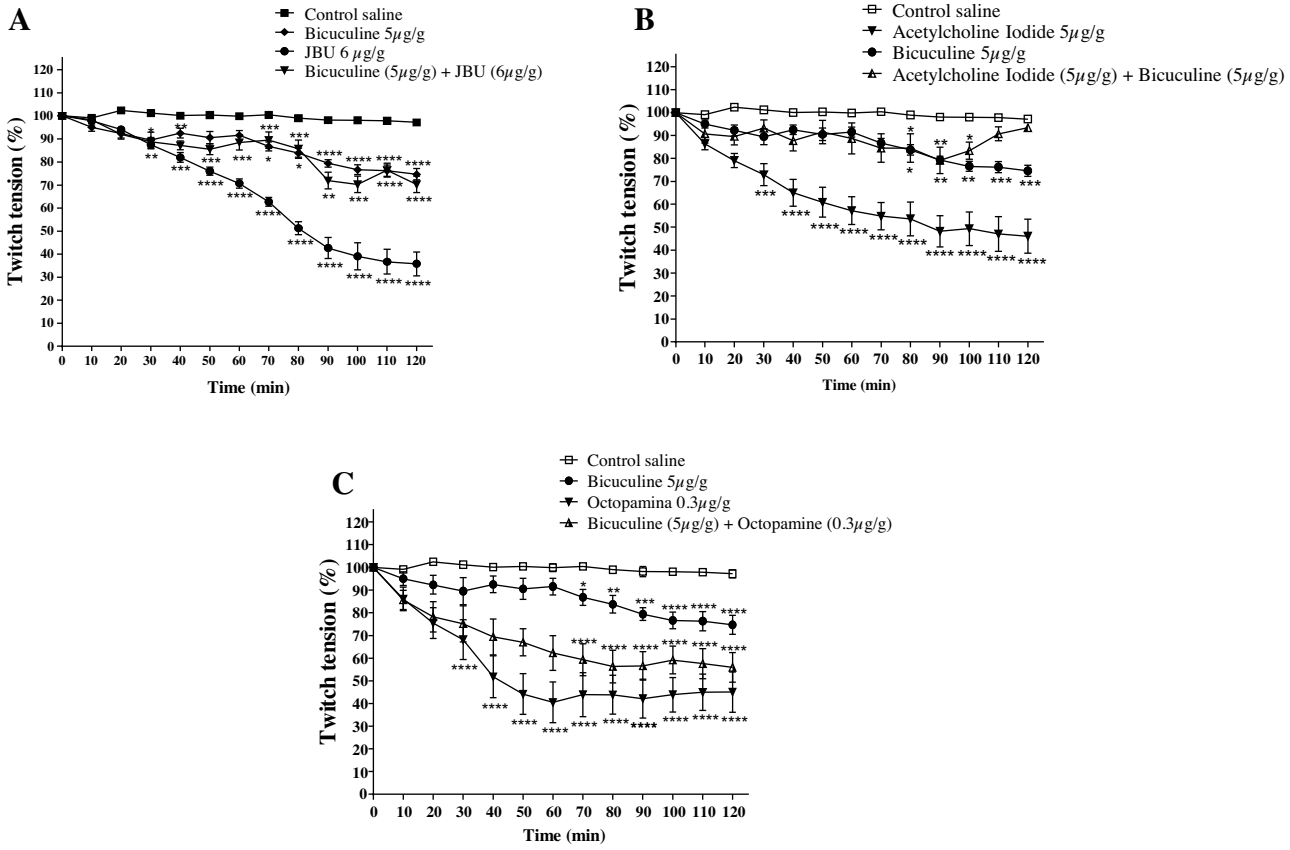

Fig. 6. Neuromuscular blocking activities induced by Jack Bean Urease, octopamine and acetylcholine and its prevention by bicuculline using *in vivo* essays with *Nauphoeta cinerea* cockroaches.
In A, B and C, note the similarity among the neuromuscular inhibitory activity of jack bean urease (6 µg/g), acetylcholine (5 µg/g) and octopamine (15 µg/g). In all cases, the administration of bicuculline (5 µg/g) 15 min before counteracted the neuromuscular blockade. The results are expressed as mean ± S.E.M. The statistical analyses were performed by Two-way Anova followed by the Tukey's test. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, n = 6, respectively.

Table 1
Effects of JBU on cockroach sensorial compound action potentials (SNCAP).

	Frequency (Events.60 min^{-1})	Amplitude Average (mV)	Rise time (ms)	Decay time (ms)	Area (mV.ms)
Control saline	3685 ± 273	0.0599 ± 0.0.19	3.72 ± 0.264	13.866 ± 8.03	0.939 ± 0.1339
JBU 6 μg/g	1425 ± 52.19*	1.102 ± 0.06*	0.64 ± 0.04*	3.72 ± 2.121*	0.724 ± 0.099

The SNCAP traces in Fig. 7 were analysed to extract their frequency (events. 60 min^{-1}), average amplitude (mV), rise and decay time (ms) and average area under the traces (mV.ms). Note that treatment with JBU induced a decrease in the frequency and an increase in the amplitude of the events in 60 min recordings. The rise and decay times and total area of the events were also reduced in JBU-treated preparation. Data are means ± S.E.M. of n = 9 replicates. The means of each parameter were compared using Student "*t*" test.
* $p < 0.05$.

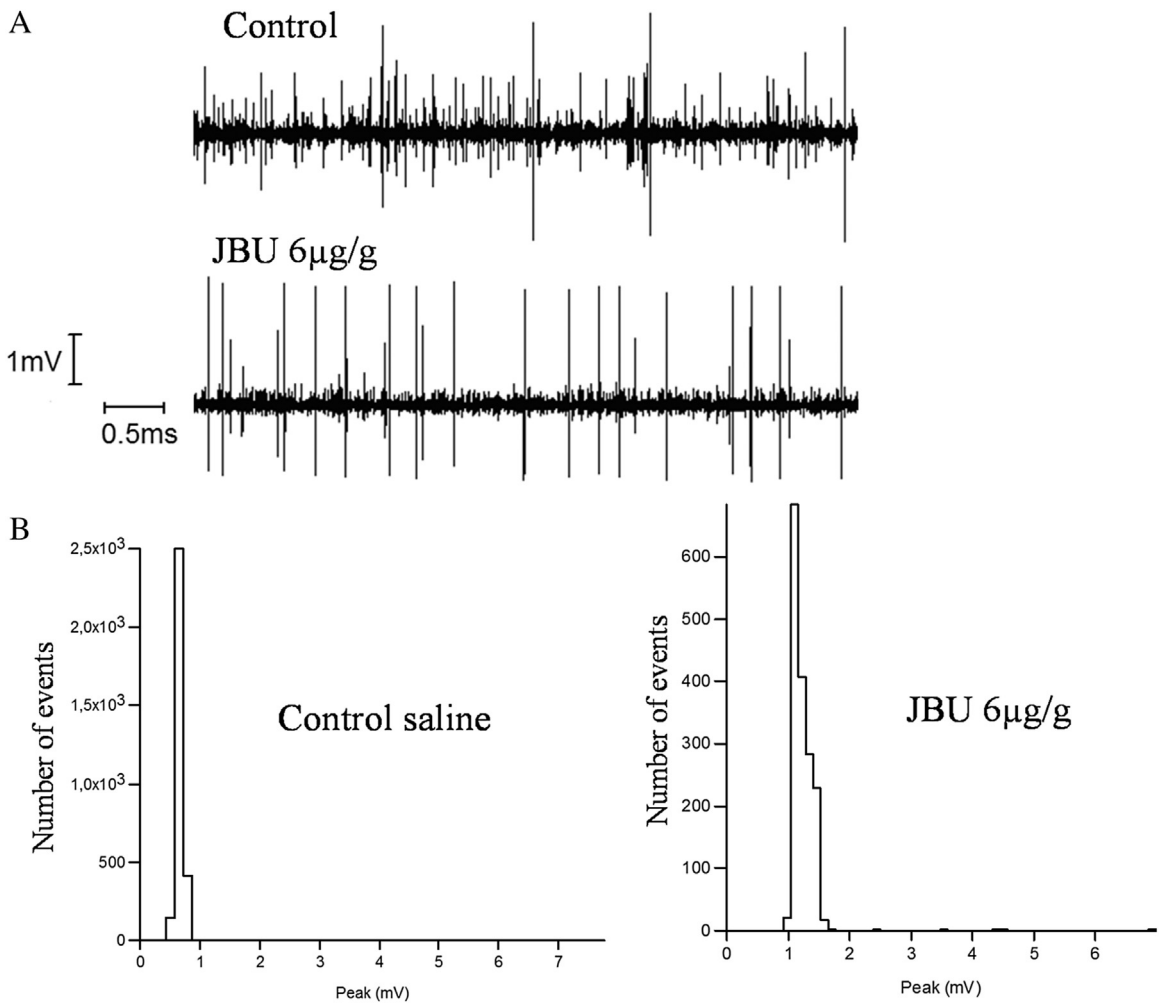

Fig. 7. Effect of Jack Bean Urease (JBU) on cockroach leg nerve-compound action potentials (SNCAP) kinetics.
Panel A shows representative traces of the SNCAP in control saline or JBU-treated preparations. Note the decrease in the frequency of the potentials during the 60 min recordings. In B, comparative histograms of control saline and JBU-treated preparations. Notice the increase in the frequency of higher amplitudes events upon exposure to JBU.

excitatory neurotransmitter and GABA as the main inhibitory one (Briley et al., 1982; Huber et al., 1990; Osborne, 1996). In insects the release of GLU or GABA in the synaptic cleft may induce either increase or decrease of muscle contraction strength, depending on the type of muscle and receptors present in the neuromuscular junctions (Chapman, 2013). Thus, the neuromuscular blockade produced by JBU in *N. cinerea* could result either from an increase of GABA-ergic neurotransmission or by inhibition of the glutamatergic counterparts. Considering that pretreatment of the insects with bicucculine decreased the level of neuromuscular blockade induced by JBU, it is suggested that GABA is involved in the inhibitory activity of the protein at the neuromuscular junction (Buckingham et al., 2005).

In our experimental model, the neuromuscular twitches were obtained by stimulating the nerve 5. In cockroaches, the axon of the slow depressor coxal motor neuron (Ds) leaves the ganglion via nerve 5 and innervates the coxal depressor muscle (muscle 177D) (Carr and Fourtner, 1980). Immunostaining studies with the locust *Schistocerca gregaria* revealed that at least two branches of inhibitory (GABA-ergic) neurons depart from nerve 5 at the metathoracic ganglion (Watson, 1986). Along nerve 5, the activity is conducted centrally (coming from afferent signals of sensila) or

peripherally, toward the methatoracic ganglion through monosynapticaly connected motorneurons via cholinergic synapses (Carr and Fourtner, 1980). Thus, we suggest that at least in part, the neuromuscular blockade induced by JBU in *N. cinerea* is consequent to an altered interplay between cholinergic motoneurons and GABA-ergic interneurons when in the presence of the protein.

JBU induced a decrease in the frequency of SNCAP concomitant with an increase in their amplitude. The hair plate neurons located on the animal's leg are connected to the ganglion via nerve 5, therefore the anti-AChE activity of JBU could also account for the increase in the amplitude of the spikes. Thus, it is possible that somehow JBU is inducing hyperpolarization of some branchs of nerve 5 either by increasing GABA-modulated activity or by direct interaction with neuronal membranes, resulting in an increased calcium-activated potassium conductance (French, 1986; Laurent and Hustert, 1988).

Finally, in some experiments we observed that higher doses of JBU produced less effect than lower doses. In fact, U-shape dose-response curves have been observed for JBU in other studies (Stanisçuaski et al., 2009). In Follmer and cols (Follmer et al., 2004), we reported that JBU undergoes a concentration-dependent oligomerization process which may lead to the formation of less active oligomeric states. Another possibility to explain the inverted dose-dependency of JBU effects could be that urease is acting as an inverted agonist. In such a condition, the effect attributed to the active form of a JBU's "receptor" with high affinity and efficacy would be counteracted by an increase of urease concentration and its consequent binding to an inactive form of the receptor displaying lower affinity and efficacy (Milligan, 2003).

5. Conclusions

Taken together our results indicate that Jack Bean Urease induces profound behavioral alterations in *Nauphoeta cinerea* cockroaches which may be related to the its anti-AChE-like activity and boosting of a secondary modulation of monoaminergic systems. The blockage of neuromuscular activity promoted by JBU is suggestive of an interplay between ACh and GABA signiling pathways in cockroaches. Further electrophysiological and biophysical studies on the direct interactions of Jack Bean Urease with the *Nauphoeta cinerea* nervous system are under way aiming to unveil more details of the molecular mechanisms involved in neurotoxic activity of this protein.

Conflict of interests

The authors declare no conflict of interests regarding this work.

Acknowledgments

The authors acknowledge the financial support by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES, Edital Toxinologia 063/2010. T. Carrazoni and M.A. Heberle were granted by CAPES fellowships. We also would like to thank Dr. Jeferson Luis Franco and Ana Paula Zemolin for technical assistance related to the acetylcholinesterase activity analysis. We acknowledge Dr. Angela Lange and Dr. Ian Orchard for all the inputs and assistance regarding the SNCAP essay.

References

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Briley, P.A., Filbin, M.T., Lunt, G.G., Donnellan, J.F., 1982. Binding and uptake of glutamate and -aminobutyric acid in membrane fractions from locust muscle. Ciba Foundation Symposium 88-Neuropharmacology of Insects. Wiley, 176-198 doi:http://dx.doi.org/10.1002/9780470720707 (ch13).
Buckingham, S.D., Biggin, P.C., Sattelle, B.M., Brown, L.A., Sattelle, D.B., 2005. Insect GABA receptors: splicing, editing, and targeting by antiparasitics and insecticides. Mol. Pharmacol. 68, 942–951. doi:http://dx.doi.org/10.1124/mol.105.015313.
Buhl, E., Schildberger, K., Stevenson, P.A., 2008. A muscarinic cholinergic mechanism underlies activation of the central pattern generator for locust flight. J. Exp. Biol. 211, 2346–2357. doi:http://dx.doi.org/10.1242/jeb.017384.
Callahan, B.P., Yuan, Y., Wolfenden, R., 2005. The burden borne by urease. J. Am. Chem. Soc. 127, 10828–10829. doi:http://dx.doi.org/10.1021/ja0525399.
Carlini, C.R., Guimarães, J.A., 1981. Isolation and characterization of a toxic protein from *Canavalia ensiformis* (jack bean) seeds, distinct from concanavalin A. Toxicon 19, 667–675. doi:http://dx.doi.org/10.1016/s0041-0101(81)90104-5.
Carlini, C.R., Ligabue-Braun, R., 2016. Ureases as multifunctional toxic proteins: a review. Toxicon 110, 90–109. doi:http://dx.doi.org/10.1016/j.toxicon.2015.11.020.
Carr, C.E., Fourtner, C., 1980. Pharmacological analysis of a monosynaptic reflex in the cockroach, *Periplaneta americana*. J. Exp. Biol. 86, 259–273.
Chapman, R.F., 2013. The Insects: Structure and Function, 5th ed. Cambridge University Press, New York.
Dahm, P.A., 1971. Toxic effects produced in insects by organophosphorus Compounds. World Heal. Organ. Bull. 44, 215–219.
Dixon, N.E., Gazzola, C., Watters, J.J., Blakeley, R.L., Zerner, B., 1975. Jack bean urease (EC 3.5.1.5), a metalloenzyme: a simple biological role for nickel? J. Am. Chem. Soc. 97, 4131–4133.
Ellman, G.L., Courney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetilcholinesterase activity. Biochem. Pharmacol. 7, 88–95.
Elpidina, E.N., Vinokuriva, K.S., Gromenko, V.A., Rudenskaya, Y.A., Dunaevskii, Y.E., Zhuzhikov, D.P., 2000. Midgut proteinases of the cockroach *Nauphoeta cinerea*. J. Evol. Biochem. Physiol. 36, 375–383.
Follmer, C., Barcellos, G.B., Zingali, R.B., Machado, O.L., Alves, E.W., Barja-Fidalgo, C., Guimarães, J., 2001. 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. 360, 217–224. doi:http://dx.doi.org/10.1042/0264-6021:3600217.
Follmer, C., Pereira, F.V., Da Silveira, N.P., Carlini, C.R., 2004. Jack bean urease (EC 3.5.1.5) aggregation monitored by dynamic and static light scattering. Biophys. Chem. 111, 79–87. doi:http://dx.doi.org/10.1016/j.bpc.2004.03.009.
Franco, J.L., Posser, T., Mattos, J.J., Trevisan, R., Brocardo, P.S., Rodrigues, A.L., Leal, R. B., Farina, M., Marques, M.R., Bainy, A.C., Dafre, A.L., 2009. Zinc reverses malathion-induced impairment in antioxidant defenses. Toxicol. Lett. 187, 137–143.
French, A.S., 1986. The role of calcium in the rapid adaptation of an insect mechanoreceptor. J. Neurosci. 6, 2322–2326.
Hemingway, J., Hawkes, N.J., McCarroll, L., Ranson, H., 2004. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem. Mol. Biol. 34, 653–665. doi:http://dx.doi.org/10.1016/j.ibmb.2004.03.018.
Huber, I., Masler, E.P., Rao, B.R., 1990. Cockroaches as Models for Neurobiology: Applications in Biomedical Research. .
Husmark, I., Ottoson, D., 1971a. Ionic effects on spindle adaptation. J. Physiol. 218, 257–269.
Husmark, I., Ottoson, D., 1971b. The contribution of mechanical factors to the early adaptation of the spindle response. J. Physiol. 212, 577–592.
Kagabu, S., Murase, Y., Imai, R., Ito, N., Nishimura, K., 2007. Effect of substituents at the 5-position of the pyridine ring imidacloprid on insecticidal activity against *Periplaneta americana*. Pest Manag. Sci. 63, 75–83. doi:http://dx.doi.org/10.1002/ps.
Koons, J.C., Flynn, J.R., Long, J.P., 1983. Antagonist properties of phentolamine at both presynaptic α2-adrenoceptors and presynaptic dopamine receptors using field stimulated right cat atria. Eur. J. Pharmacol. 88, 311–317.
Laurent, G., Hustert, R., 1988. Motor neuronal receptive fields delimit patterns of motor activity during locomotion of the locust. J. Neurosci. 8, 4349–4366.
Leibson, T., Lifshitz, M., 2008. Organophosphate and cCarbamate poisoning: review of the current literature and summary of clinical and laboratory experience in Southern Israel. Isr. Med. Assoc. J. 10, 767–770.
Li, F., Han, Z., 2004. Mutations in acetylcholinesterase associated with insecticide resistance in the cotton aphid, Aphis gossypii glover. Insect Biochem. Mol. Biol. 34, 397–405. doi:http://dx.doi.org/10.1016/j.ibmb.2004.02.001.
Libersat, F., Pflueger, H.-J., 2004. Monoamines and the orchestration of behavior. Bioscience 54, 17–25. doi:http://dx.doi.org/10.1641/0006-3568(2004)054[0017:MATOOB]2.0.CO;2.
Martinelli, A.H.S., Kappaun, K., Ligabue-Braun, R., Defferrari, M.S., Piovesan, A.R., Stanisçuaski, F., Demartini, D.R., Dal Belo, C.A., Almeida, C.G.M., Follmer, C., Verli, H., Carlini, C.R., Pasquali, G., 2014. Structure-function studies on jaburetox, a recombinant insecticidal peptide derived from jack bean (*Canavalia ensiformis*) urease. Biochim. Biophys. Acta 1840, 935–944. doi:http://dx.doi.org/10.1016/j.bbagen.2013.11.010.
Miller, T., 1979. Nervous versus neurohormonal control of insect heartbeat. Am. Zool. 19, 77–86.
Milligan, G., 2003. Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. Mol. Pharmacol. 64, 1271–1276. doi:http://dx.doi.org/10.1124/mol.64.6.1271.
Mobley, H.L.T., Hausinger, R.P., 1989. Microbial ureases: significance, regulation, and molecular characterization. Microbiol. Rev. 53, 85–108.

Mulinari, F., Becker-Ritt, A.B., Demartini, D.R., Ligabue-Braun, R., Stanisçuaski, F., Verli, H., Fragoso, R.R., Schroeder, E.K., Carlini, C.R., Grossi-De-Sá, M.F., 2011. Characterization of JBURE-IIb isoform of *Canavalia ensiformis* (L.) DC urease. Biochim. Biophys. Acta – Proteins Proteomics 1814, 1758–1768. doi:http://dx.doi.org/10.1016/j.bbapap.2011.07.022.

Osborne, R.H., 1996. Insect neurotransmission: neurotransmitters and their receptors. Pharmacol. Ther. 69, 117–142. doi:http://dx.doi.org/10.1016/0163-7258(95)02054-3.

Papaefthimiou, C., Theophilidis, G., 2011. Octopamine-A single modulator with double action on the heart of two insect species (Apis mellifera macedonica and Bactrocera oleae): acceleration vs. inhibition. J. Insect Physiol. 57, 316–325. doi:http://dx.doi.org/10.1016/j.jinsphys.2010.11.022.

Piovesan, A.R., Martinelli, A.H.S., Ligabue-Braun, R., Schwartz, J.L., Carlini, C.R., 2014. *Canavalia ensiformis* urease, Jaburetox and derived peptides form ion channels in planar lipid bilayers. Arch. Biochem. Biophys. 547, 6–17. doi:http://dx.doi.org/10.1016/j.abb.2014.02.006.

Pires-Alves, M., Grossi-De-Sá, M.F., Barcellos, G.B.S., Carlini, C.R., Moraes, M.G., 2003. Characterization and expression of a novel member (JBURE-II) of the urease gene family from jackbean [*Canavalia ensiformis* (L.) DC]. Plant Cell Physiol. 44, 139–145. doi:http://dx.doi.org/10.1093/pcp/pcg018.

Rattan, R.S., 2010. Mechanism of action of insecticidal secondary metabolites of plant origin. Crop Prot. 29, 913–920. doi:http://dx.doi.org/10.1016/j.cropro.2010.05.008.

Rodríguez, V., Mori, B., Dörr, F.A., Dal Belo, C.A., Colepicolo, P., Pinto, E., 2012. Effects of a cyanobacterial extract containing anatoxin-a(s) on the cardiac rhythm of *Leurolestes circunvagans*. Brazilian J. Pharmacogn. 22, 775–781. doi:http://dx.doi.org/10.1590/S0102-695x2012005000073.

Sattelle, D.B., Breert, H., 1990. Cholinergic nerve terminals in the central nervous system of insects. J. Neuroendocrinol. 2, 241–256. doi:http://dx.doi.org/10.1111/j.1365-2826.1990.tb00400.x.

Sliwowska, J., Rosinski, G., Nässel, D.R., 2001. Cardioacceleratory action of tachykinin-related neuropeptides and proctolin in two coleopteran insect species. Peptides 22, 209–217. doi:http://dx.doi.org/10.1016/S0196-9781(00)00384-3.

Stürmer, G.D., Freitas, T.C., Heberle, M.A., Assis de, D.R., Vinadé, L., Pereira, A.B., Franco, J.L., Dal Belo, C.A., 2014. Modulation of dopaminergic neurotransmission induced by sublethal doses of the organophosphate trichlorfon in cockroaches. Ecotoxicol. Environ. Saf. 109, 56–62. doi:http://dx.doi.org/10.1016/j.ecoenv.2014.08.006.

Stanisçuaski, F., Carlini, C.R., 2012. Plant ureases and related peptides: understanding their entomotoxic properties. Toxins (Basel) 4, 55–67. doi:http://dx.doi.org/10.3390/toxins4020055.

Stanisçuaski, F., Te Brugge, V., Carlini, C.R., Orchard, I., 2009. In vitro effect of *Canavalia ensiformis* urease and the derived peptide Jaburetox-2Ec on *Rhodnius prolixus* Malpighian tubules. J. Insect Physiol. 55, 255–263. doi:http://dx.doi.org/10.1016/j.jinsphys.2008.12.002.

Stanisçuaski, F., Te Brugge, V., Carlini, C.R., Orchard, I., 2010. Jack bean urease alters serotonin-induced effects on *Rhodnius prolixus* anterior midgut. J. Insect Physiol. 56, 1078–1086. doi:http://dx.doi.org/10.1016/j.jinsphys.2010.03.002.

Stankiewicz, M., Dbrowski, M., De Lima, M.E., 2012. Nervous system of *Periplaneta americana* cockroach as a model in toxinological studies: a short historical and actual view. J. Toxicol. 2012 doi:http://dx.doi.org/10.1155/2012/143740.

Sumner, J.B., 1926. The isolation and crystallization of the enzyme urease: preliminary paper. J. Biol. Chem. 69, 435–441.

Suzuki, K., Hama, H., 1998. Carboxylesterase of the cotton Aphid, *Aphis gossypii glover* (Homeptera: aphididae) isoeletric point variants in an organophosphorus insecticide resistant clone. Appl. Entomol. Zoo. 33, 11–20.

Tayo, F., 1979. Effects of dopaminoceptor agonists and antagonists on the rat vas deferens in vitro. West Afr. J. Phamarcol. Drug Res. 5, 1979.

Tublitz, B.Y.N.J., Truman, J.W., 1985. Insect Cardioactive Peptides I. distribution and molecular characteristics of two cardioacceleratory peptides in the tobacco hawkmoth, *Manduca sexta*. J. exp. Biol. 114, 365–379.

Wächtler, K., 1988. Phylogeny of the cholinergic synapse. In: Whittaker, V.P. (Ed.), The Cholinergic Synapse (Handbook of Experimental Pharmacology). Springer, pp. 762 (Softcover reprint of the original).

Watson, A.H.D., 1986. The distribution of GABA-like immunoreactivity in the thoracic nervous system of the locust *Schistocerca gregaria*. Cell Tissue Res. 246, 331–341. doi:http://dx.doi.org/10.1007/BF00215895.

Weisel-Eichler, A., Haspel, G., Libersat, F., 1999. Venom of a parasitoid wasp induces prolonged grooming in the cockroach. J. Exp. Biol. 202, 957–964.

Wigglesworth, V.B., 1972. The Principles of Insect Physiology, 7th ed. Springer, Verlag, New York.