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Jaburetox, a natural insecticide derived from Jack Bean Urease, activates voltage-gated sodium channels to modulate insect behavior

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ABSTRACT

Jaburetox (Jbtx) is an insecticidal peptide derived from *Canavalia ensiformis* urease, whose mechanism of action is not completely elucidated. We employed behavioral, electromyographical and electrophysiological protocols to identify the cellular and molecular targets involved in the Jbtx entomotoxicity in cockroaches and locusts. In *Nauphoeta cinerea*, Jbtx (32 μg/g) altered the locomotory behaviour inducing a significative decrease in the distance travelled followed by a significant increase in stopped time (52 ± 85 cm and 2573 ± 89 s, $p < .05$, $n = 40$). Jbtx (8 to 32 μg/g body weight, respectively) also increased the leg and antennae grooming activities ($p < .05$, n = 40, respectively). Jbtx (8 to 16 μg/g) induced a maximum neuromuscular blockade of 80.72% ($n = 6$, $p < .05$) and was cardiotoxic, decreasing the cockroach heart rate. The electrophysiological profiles of both muscle and nerve of *L. migratoria* showed that Jbtx (2.5×10^{-7} and 2.5×10^{-3} μg/ body weight) induced a significant increase in the amplitude of nerve action potentials ($n = 5$, $p < .05$). Voltage clamp analysis of Jbtx (200 nM) applied in *Xenopus laevis* oocytes heterologously expressed with Nav 1.1 channels showed a significant increase in the sodium currents. In conclusion, this work revealed that the entomotoxic activity of Jbtx involves complex behavioral alterations that begins with an initial activation of voltage-gated sodium channels.

1. Introduction

Chemical insecticides have long been used in a variety of situations, in our houses to kill domestic flies, cockroaches and other urban insects, in governmental programs to control insect-born diseases such as yellow fever, malaria and dengue or in agriculture to protect plants from herbivorous pests aiming to improve crop yield and food production (Zhu et al., 2016; Parween et al., 2016). In all these cases, there is an inherent concern related to the toxicity and bioaccumulation of these compounds, not only directly related to humans and domestic animals, but also in the environment (Damalas and Eleftherohorinos, 2011; Maurya and Malik, 2016). In 2007 about 3.5 million tons of chemical insecticides were used worldwide, mainly in developing countries, such as Brazil (EPA, 2017).These usages contribute to environmental threat affecting non-target beneficial species, therefore studies aiming to validate and/or elucidate the entomotoxic mechanisms of natural promising insecticide compounds are desirable.

Ureases (EC 3.5.1.5, urea amidohydrolase) are metalloenzymes that catalyze the hydrolysis of urea into ammonia and carbamate (Dixon et al., 1975). Ureases are natural insecticides widespread in plants and generally regarded as biosafe, since these proteins are present in large quantities in many edible species, particularly in legumes (Carlini &

Abbreviations: Jbtx, Jaburetox; JBU, Jack Bean Urease; CNTX, canatoxin; NCPS, *Nauphoeta cinerea* physiological saline; LMPS, *Locusta migratoria* physiological saline; Nav, voltage-gated sodium channel; BgNav1.1, *Blatella germanica* voltage-gated sodium channel; OA, octopamine; GABA, gamma-aminobutyric acid; SEG, subesophageal ganglion; GLU, glutamate; IGRs, insect growth regulator; mAPs, muscle action potentials; nAPs, nerve action potentials

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Ligabue-Braun, 2016). Jaburetox (Jbtx) is a recombinant peptide (91 aminoacids) representing an internal sequence of an isoform of jack bean (*Canavalia ensiformis*) urease (Mulinari et al., 2007; Postal et al., 2012). The entomotoxic effect of Jbtx was demonstrated using the cotton stainer bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae), the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae), the kissing bugs *Rhodnius prolixus* and *Triatoma infestans* (Hemiptera: Reduvidae). For a review on the entomotoxic effects of jaburetox, refer to (Stanisçuaski & Carlini, 2012; Carlini & Ligabue-Braun, 2016).

The mechanisms involved in the insecticidal activity of plant ureases or derived peptides have not been fully elucidated so far. *R. prolixus* and *T. infestans* fed on meals containing jaburetox exhibit impairment of diuresis and symptoms suggestive of neurotoxicities, such as uncoordinated movements of limbs and antenna and reversible paralysis in sub-lethal doses (Mulinari et al., 2007; Galvani et al., 2015). After injection into *T. infestans* hemocoell, the peptide was immunolocalized in the insect's central nervous system, where it caused pronounced inhibition of NO synthase and decreased NO levels (Galvani et al., 2015). In the *in vivo* cockroach (*Nauphoeta cinerea*) metathoracic coxal-adductor nerve–muscle preparation, a two-hour treatment with the peptide led to a complete neuromuscular blockade (Martinelli et al., 2014).

Studies on the behavioral effects of neurotoxicants are important not only to elucidate the modes of action of both novel or traditional insecticides, but also to understand how beneficial insects would be potentially affected by these compounds (Haynes, 1988). The evolution of behavioral resistance to insecticides is another important consideration, since selection may favor the target insects to avoid exposition to toxic compounds (Chareonviriyaphap et al., 2013; Dang et al., 2017). In this respect, cockroaches are primitive insects, easy to breed and maintain, and together locusts have been used in a wide range of studies of pharmacology and toxicology to understand the physiological aspects between insect and chemical compounds (Stanisçuaski & Carlini, 2012; Rodríguez et al., 2012; Stürmer et al., 2014; Carrazoni et al., 2018). In this work, we investigated, through a range of behavioral and electrophysiological protocols, the mechanism associated to the neurotoxic activity of the insecticide peptide Jbtx on the central and peripheral nervous system of two model insects, the cockroach *N. cinerea* (Blaberidae) and in the locust *Locusta migratoria* (Acrididae). We have identified possible functional cellular and molecular targets involved in the entomotoxic activity that could account for the lethal effect of this peptide.

2. Materials and methods

2.1. Experimental animals

All experiments were performed using both sexes of adult *Nauphoeta cinerea* cockroaches (3–4 month after adult molt) and *Locusta migratoria* (adult 7-weeks-old). The cockroaches were reared under laboratory conditions of controlled temperature (22–25 °C) on a 12 h: 12 h light: dark cycle, with water and dog chow *ad libitum*. *Locusta migratoria* were reared under crowded conditions at 30 °C and 50% humidity, with a12h:12 h light: dark regimen, and fed on a diet of fresh wheat seedlings supplemented with bran. Complete description of animal nutrition and care is found at the supplementary material file of this manuscript.

2.2. Expression of the recombinant peptide Jaburetox (Jbtx)

The recombinant peptide Jaburetox was expressed and purified as previously described (Broll et al., 2017; Fruttero et al., 2016), with modifications. Briefly, *Escherichia coli* BL21 (DE3) RIL harboring the plasmid pET-23a:Jaburetox was cultured in 20 mL LB (Luria Bertani) Broth, supplemented with 1% glucose, 100 μg/mL ampicillin and 40 μg/mL chloramphenicol. The culture was grown overnight at 37 °C and 200 rpm. The pre-inoculum was inoculated in 1 L of autoinduction medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l glycerol, 3.3 g/l $(NH_4)_2SO_4$, 6.8 g/l KH_2PO_4, 7.1 g/l Na_2HPO_4, 0.5 g/l glucose and 2 g/l lactose, with 100 μg/mL ampicillin and 40 μg/mL chloramphenicol). The inoculated medium was cultured at 37 °C, 200 rpm, until absorbance reached 0.7 at 600 nm, then the temperature was lowered to 20 °C, and maintained under stirring at 200 rpm overnight. The culture was centrifuged at 8000 ×*g* for 10 min. The cells were resuspended in 100 mL buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl and 20 mM imidazole), the cell suspension was sonicated (20 cycles of 1 min, 20 kHz frequency). The final cell lysate was centrifuged at 14,000 ×*g* for 40 min at 4 °C and the supernatant was submitted to affinity chromatography (Broll et al., 2017). As last step of purification, Jbtx was submitted to a size exclusion chromatography using Superdex 200 26/600 (GE Healthcare) in 50 mM sodium phosphate pH 7.5, 1 mM EDTA and 1 mM TCEP. The fractions were pooled and concentrated in 3 kDa Amicon Ultra Centrifugal Filter (Merk Milllipore) and stored at −20 °C. Before its use for incubations or injections, the peptide (Mr ~11 kDa) was dialyzed in 20 mM sodium phosphate buffer pH 7.4 using 3 kDa Amicon Ultra Centrifugal Filter.

2.3. Reagents and solutions

All chemicals and reagents used were of the highest purity and were obtained from Sigma-Aldrich, Merck, Roche, Life Technologies or BioRad. Test solutions were prepared daily by dilution in a physiological saline for *Nauphoeta cinerea* (NCPS) or *Locusta migratoria* (LMPS), immediately before use. The NCPS or LMPS were carbonate-buffered solutions prepared with the following composition in mM, respectively: NCPS - NaCl, 214; KCl, 3.1; $CaCl_2$, 9; sucrose, 50; HEPES buffer, 5 and pH 7.2 (Stürmer et al., 2014); LMPS -NaCl 150; $CaCl_2$ 10; KCl 4; $MgCl_2$ 2; $NaHCO_3$ 4; HEPES 5; Sucrose 90 and Trehalose 5 (Orchard & Lange, 1986).

2.4. Biological assays

2.4.1. Behavioral assays

2.4.1.1. Experimental design for behavioral assays. All animals used on behavioral assays were chosen randomly and had no previous exposition to the environment until tests. The standard protocol for animal manipulation was followed strictly. The animals were only handled by an experimenter wearing gloves. The insects were taken one by one, right before the begging of experiments to avoid stressful conditions to be far from the insectary. Before the beginning of assays there was a five-minute round for exploratory time for each individual. The experiments were performed by a double blind to avoid tendentious results. Treatments were codified by numbers and randomly arrayed during the exploratory time of the first animal of the repetition. The injections were applied after exploratory time. All experiments were performed under controlled conditions of temperature and luminosity, during light time from 9:00 a.m. to 6 p.m. Right after each experiment, the experimental chambers were cleaned with ethanol 70% and distilled water. In locomotory activity assays it was taken 20 min recordings divided in cleaning, exploratory and observation time, using four animals placed individually in each chamber. For the grooming behavior activity, one repetition was taken as the observation of one animal, during 40 min long (cleaning, exploratory and observation time).

2.4.1.2. Locomotory activity. The locomotory activity of *N. cinerea* was measured by the analysis of behavioral endpoints such as travelled distance, immobile episodes (number of times that the animal remained immobile during the experiment) and rate of stopped times (interval of time the animal interrupted its locomotion path during the experiment). The experiments were carried out essentially as describe in (Leal et al., 2018). The behavioral parameters from the control insects (treated with saline) and Jbtx-treated individuals were

measured during 10 min. All treatment groups were tested in a repetition. The recording groups for each repetition comprised four animals of the same treatment group. The complete experimental time was of 20 min (5 min of cleaning, 5 min of exploratory behavior and 10 min of locomotion recordings). Experiments were recorded by IDTracker (Software, Polavieja lab, Cajal Institute,M, Spain) and analyzed by Matlab (Natick, MA, USA).

2.4.2. Grooming behavior recordings

Grooming behavior was monitored in *N. cinerea* cockroaches using an opaque plastic box (29 cm × 18 cm × 13 cm), with a clear plastic cover and was recorded with a camera for posterior analysis essentially as described by (StuïRmer et al., 2014). The data were collected manually and analyzed by Graphpad Prism 7.0 (Software, San Diego, CA, USA).

2.5. Semi-isolated cockroach heart preparation

An *in vivo* semi-isolated cockroach heart bioassay was mounted essentially as described by (De Freitas et al., 2016). Heartbeat frequency was monitored for 30 min under a stereoscopic microscope. Nine cockroaches were used for each group. In the control group, the hearts were bathed in saline solution only.

2.6. Electromyographical recordings

2.6.1. In vivo cockroach metathoracic coxal-adductor nerve-muscle preparation

The *in vivo* cockroach metathoracic-coxal adductor muscle preparation was used to analyze the effect induced by Jbtx at insect neuromuscular junctions as previously described in (Martinelli et al., 2014) and by (De Freitas et al., 2016). A protocol using chloral hydrate as a pharmacological tool was also carried out. Chloral hydrate is a mild sedative and hypnotic drug which directly activates gama-aminobutyric acid (GABA) receptors (Garrett & Gan, 1998).

2.7. Electrophysiological recordings

2.7.1. Measurement of muscle and nerve potentials

To record and understand the process of neuromuscular paralysis induced by Jbtx, the conduction of muscle potentials was analyzed using *L. migratoria* leg preparation as described by (Carrazoni et al., 2018), with some modifications. The muscle potentials of the tarsal muscles of the leg were recorded from an isolated leg. One leg of an adult animal was removed and placed on a platform with attached electrodes. The stimulation electrodes were positioned in the femur and the recording electrodes were connected near the tarsus (foot) with a ground electrode inserted in between. The different treatments were administered through a small incision in the cuticle of the femur using a Hamilton syringe in a final volume of 2.5 µl. Five concentrations of Jbtx were assayed 2.5×10^{-7} to 2.5×10^{-3} µg/body weight and controls were injected with LMPS. All preparations were recorded for 15 min prior to injection of Jbtx, to verify the integrity of the signals. Each group consisted of 5 animals ($n = 5$). Muscle potentials were evoked by electrical stimulation of the nerve using a Grass SD stimulator at a voltage of 5 V, frequency of 0.2 PPS and a duration of 0.5 ms. Action potentials from the nerve in the leg preparation of the locust can also be evoked by placing the stimulating electrode in the tibia near the tarsus and the recording electrodes in the femur. In this way, the larger muscle potential does not interfere with the recording of the nerve action potentials. In this modification, the action potentials are evoked in the axon near the tarsus and travel anteriorly to the femur where they are recorded. The potentials were recorded using an AM Systems model 1700 differential amplifier to amplify the signal with a high-pass filter of 1000 Hz and a low pass filter of 100 Hz. The signal conversion was done using an 8-channel Powerlab IDE converter and the signals were recorded using Chart software version 4 and later analyzed in Chart version 8. The amplitude of the muscle and nerve potentials was measured by averaging 6 muscles and nerve potentials every 5 min during the 60 min of recording.

2.7.2. Sodium channel expression and two-microelectrode voltage-clamp

For the expression of the voltage-gated sodium (Nav) channel of the cockroach *Blattella germanica* ($BgNa_V$ 1.1), the cRNAs were synthesized from linearized plasmids using the large-scale T7 mMESSAGE mMACHINE transcription kit (Ambion, Carlsbad, CA, USA). The resected oocytes lobes from female *Xenopus laevis* frogs were used to obtain the stages V-VI oocytes. The frog surgery was performed in the Aquatic Facility at the KU Leuven University and the usage of the frogs was in accordance with the license number LA1210239. To induce anaesthesia the animals were immersed in Tricaine (Sigma Chemical Co, St. Louis, MO, USA) solution (1 g/L). The isolation procedure was described previously by (Koren et al., 1990; Liman et al., 1992). Oocytes (stages V-VI) were injected with 50 nL of the $BgNa_V$ 1.1cRNA using a micro-injector (Drummond Scientific, Broomall, PA, USA). ND-96 solution was used for the oocytes incubation (in mM): 96 NaCl, 2 KCl, 2 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES (pH 7.4), supplemented with 50 mg/L gentamicin sulfate (Sigma Chemical Co, St. Louis, MO, USA) and 180 mg/L theophylline (Sigma Chemical Co, St. Louis, MO, USA).

Sodium currents were recorded using the two-microelectrode voltage-clamp technique at room temperature (18–22 °C). The recordings were processed by a Gene Clamp 500 amplifier (Molecular Devices, Downingtown, PA, USA) and controlled by a pClamp data acquisition system (Axon Instruments, Union City, CA, USA). Whole-cell currents from oocytes were recorded 1–7 days after injection. Current and voltage electrodes had resistances from 0.7 to 1.5 MΩ and were filled with 3 M KCl. Currents were sampled at 20 kHz and filtered at 1 Hz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4 protocol.

For the assays, 200 nM of Jbtx was added directly to the recording chamber. For the activation protocols, 100 ms test depolarization, ranging from −90 mV to +70 mV, were applied from a holding potential of −90 mV, in 5 mV increments at 5 s intervals. For the inactivation protocols, double pulses were employed, with a conditioning pulse applied from a holding potential of −100 mV to a range of potentials from −90 mV to +60 mV, in 5 mV increments for 100 ms, immediately followed by a test pulse to 0 mV (or − 5 mV).

Each experiment was performed 5-times. Data were analyzed using Clampfit 10.4 (Molecular Devices, Sunnyvale, CA, USA), Excel 2010 (Microsoft Corp., Redmond, WA, USA, 2010) and OriginPro 9.0 (OriginLab Corp., Northampton, MA, USA, 2012).

2.8. Statistical analysis

Data were expressed as mean ± S.E.M. The experimental groups were analyzed by one-way/ two-way ANOVA and MANOVA, followed by Tukey's or Dunnet test as *post hoc*. Testing of variance-homogeneity and normal distribution were accomplished by using Shapiro-Wilk test. All the statistical analyses were performed by using GraphPad Prism 7.0. (Software, San Diego, CA, USA). The values were considered significant when $p \leq .05$.

3. Results

3.1. Locomotor behavior alteration induced by Jbtx in cockroaches

Overall the administration of Jbtx in different doses (8, 16 and 32 µg/g) caused an impairment of *Nauphoeta cinerea* natural exploratory behavior, during the 10 min trials (Fig. 1A–C). In this set of protocols, the values for the control saline group of total distance travelled, immobile episodes and stopped times were 267 ± 18 cm, 884 ± 84 and 78 ± 8 s, respectively ($n = 40$). When Jbtx 16 and

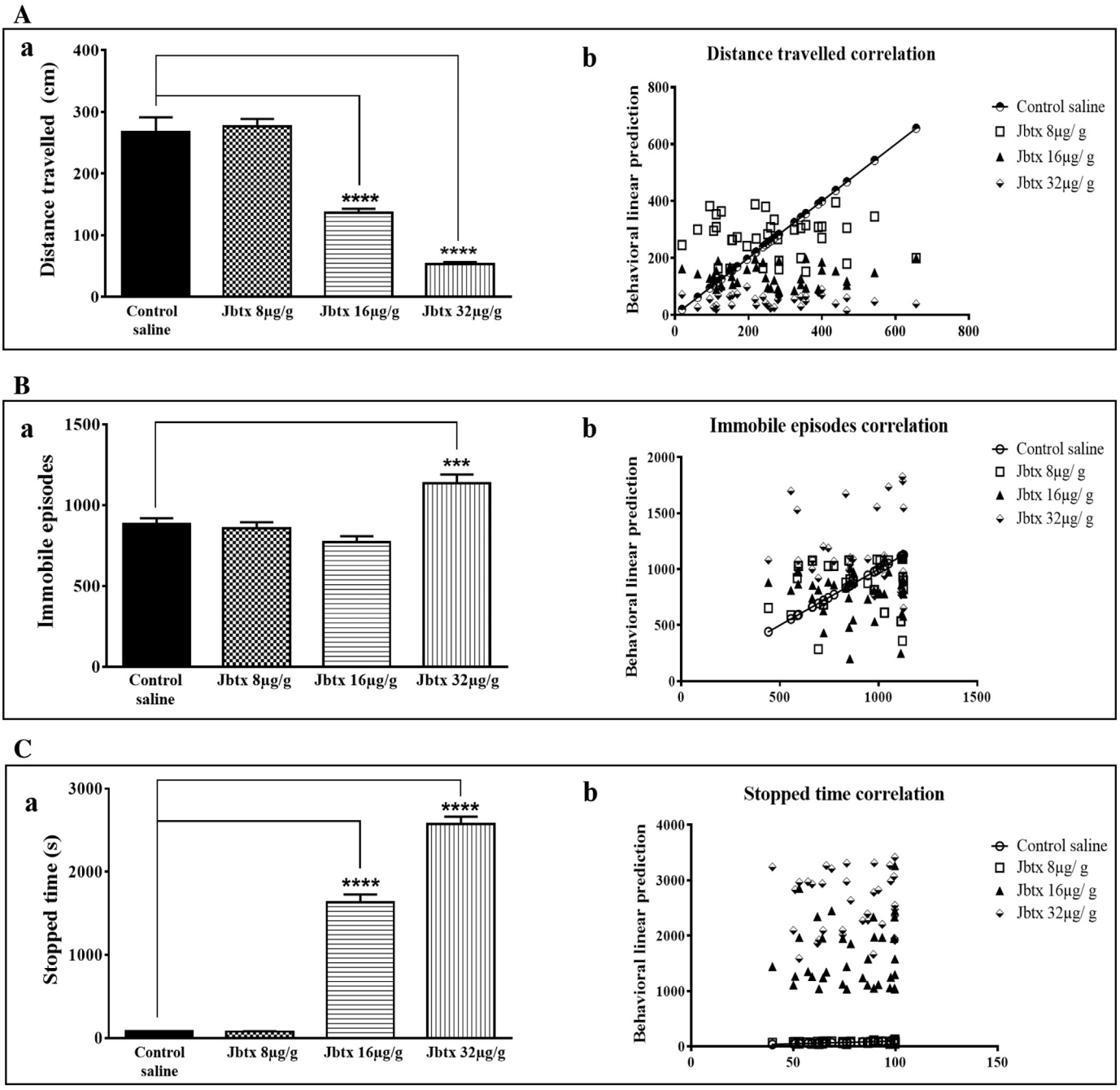


Fig. 1. Influence of Jbtx on the locomotory pattern of *Nauphoeta cinerea*. Figure shows the graphs related to the analysis of different parameters of the insect exploratory activity. (Aa) significant decrease in the distance travelled of cockroaches treated with Jbtx 16 µg/g and 32 µg/g body weight, respectively. (Ab) shows the MANOVA behavioral linear prediction for distance travelled interaction indicating a correlation between the highest doses of treatments staying below average when compared to the control. (Ba) shows a decrease in the parameters of immobile episodes of cockroaches treated with the highest dose. Bb: shows the MANOVA behavior linear prediction for immobile episodes indicating a significance only for the highest dose. (Ca) shows a decrease in the parameter of stopped time on cockroaches. Cb, MANOVA behavioral linear prediction for stopped time interactions indicating a significant effect for the highest dose of Jbtx. Data were expressed as mean ± S.E.M of recordings conducted with 40 animals per group. Statistical analysis was performed by One-Way ANOVA and MANOVA followed by the Tukey's test and a Shapiro-Wilk test, respectively. ***$p < .001$; ****$p < .0001$.

32 µg/g was administered, there was a maximum decrease in the animal distance travelled of 52 ± 85 cm and 136 ± 62 cm, respectively, in comparison with the control saline group (n = 40, $p < .05$) (Fig. 1Aa). The analysis of the number of immobile episodes and the stopped times also revealed a significant increase of 1135 ± 67and 2573 ± 89 s, with Jbtx 32 µg/g (Fig. 1Ba and Ca) (n = 40, $p < .05$, respectively). The figures 1Ab, 1Bb and 1Cb show the individual behavioral correlation events between treatments in all analyzed behavioral variables.

3.2. Effect of Jbtx on the cockroach heart rate

The addition of Jbtx (8, 16 and 32 µg/ 200 µl saline) to the cardiovascular preparations of *N. cinerea* induced cardiotoxicity, showing a dose-dependent negative chronotropic effect. In the control saline group the mean value for heart rate was 76.81 ± 5 beats/min, in 30 min recordings ($n = 9$) (Fig. 2). While no effect was seen at the lower dose, treatment with Jbtx 16 µg/ 200 µl induced a decrease of 30 ± 3% in chronotropic responses ($p < .05$ compared to the control

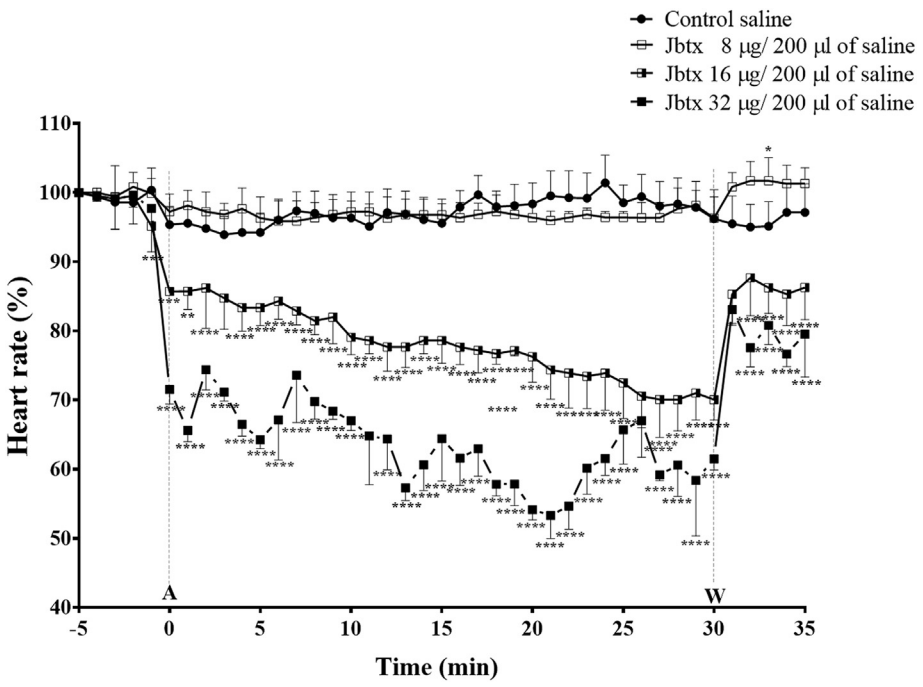


Fig. 2. Negative chronotropic effect induced by Jbtx on *Nauphoeta cinerea* hearts. In the graph, each point corresponds to the mean ± S.E.M. of the insect heart rate relative to the initial state (−5 min), measured during 30 min after exposition to Jbtx (8-32 µg/ 200 µl of control saline). Note that Jbtx (16 and 32 µg/ 200 µl) slows cardiac rhythm in 30 min of the experiment. The statistical analyses were performed by Two-way ANOVA followed by the Tukey's test. $^{*}p < .05$ ($n = 9$); $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$, (n = 9).

saline, n = 9), in 30 min recordings (Fig. 2A). The highest concentration of Jbtx (32 µg/200 µl) decreased even further the chronotropic response to reach 52.5 ± 2% at the end of the 30 min recordings compared to the saline control (n = 9, $p < .05$). For all treatments, the washout of the preparation with saline solution partially reversed the effects induced by the peptide.

3.3. Effect of Jbtx on grooming activity of N. cinerea

The effect of Jbtx on the insect grooming activity of antennae and legs were evaluated. In saline-injected cockroaches, the mean time of continuous leg grooming was 153.46 s/30 min and 70.63 s/30 min, for the antennae ($n = 32$, respectively) (Fig. 3). An increase of about 2.5 fold in the time spent on leg grooming activity was observed for all the tested Jbtx doses (8, 16 and 32 µg/g of body weight), but no difference among the tested doses was seen. The administration of Jbtx increased the time (~50%) spent on antennae grooming as well, seen at the doses of 8 µg and 32 µg peptide/g insect body weight, respectively. In insects, octopamine is a neurotransmitter responsible for modulating the leg grooming activity. As expected, treating the insects with octopamine increased the time spent on leg grooming to 198.4 s/30 min ($n = 32$; $p < .05$ compared to saline control) but did not alter the grooming activity of antennae (Fig. 3). These results have shown that Jbtx (8 µg/g) alters the leg grooming activity mimicking octopamine (15 µg/g), thereby suggesting that this peptide is interfering centrally on the octopaminergic signaling.

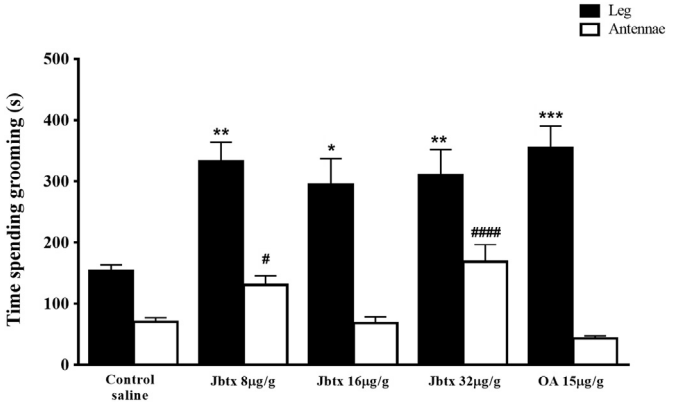


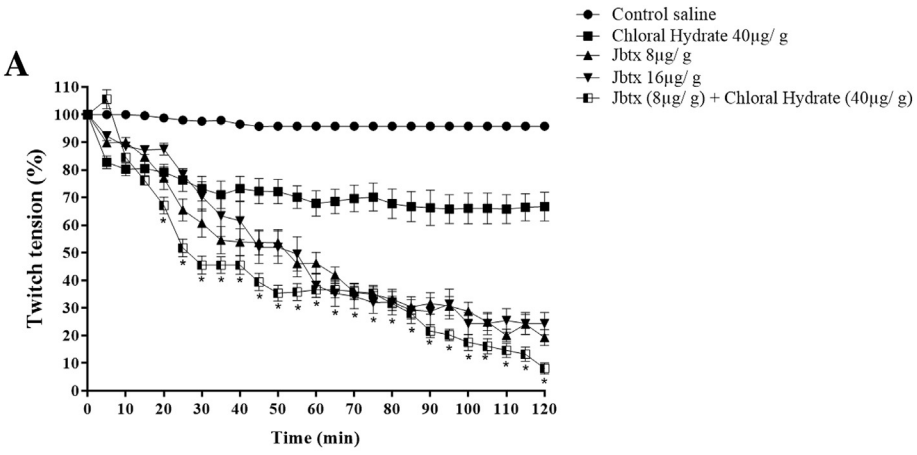
Fig. 3. Modulation of *Nauphoeta cinerea* grooming behavior by Jbtx. The cockroaches were injected with Jbtx (8, 16 and 32 µg/g body weight) and examined for grooming activity immediately after injection. To verify the octopaminergic interaction with grooming, octopamine (OA, 15 µg/g), was assayed. The grooming activity was recorded during 30 min and the results were expressed as mean ± S.E.M. of the total time of grooms (s) in 30 min. The data were analyzed by One-way ANOVA followed by the Dunnett's test. * comparison of leg grooming with the control; # compares antennae grooming with the control.$^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; $^{\#}p < .05$; $^{\#\#\#\#}p < .0001$, $n = 32$, respectively.

3.4. Neuromuscular blockade induced by Jbtx at in vivo cockroach nerve-muscle preparation

Jbtx was bioassayed on coxal-adductor nerve-muscle preparations. The administration of saline solution alone did not interfere with neuromuscular responses during 120 min recordings ($n = 6$) (Fig. 4A). The injection of Jbtx induced a dose-dependent inhibition of the twitch tension in 120 min of recording. At 8 and 16 µg/g insect body weight, there were 80.72% and 75.66% (n = 6 each dose, $p < .05$ compared to controls) blockade of the twitch tension, respectively. Co-treatment of the insects with Chloral hydrate (40 µg/g) and Jbtx (8 µg/g) induced 91.89% inhibition of the twitches ($p < .05$, n = 6) (Fig. 4B).

3.5. Jbtx effect on the muscle and nerve action potentials in tarsal muscle of Locusta migratoria

When Jbtx (2.5×10^{-7} µg/ body weight) was assayed, there was a gradual decrease in the muscle action potential amplitude reaching a significant decrease of approximately 20% at 15 min ($n = 5$, $p < .05$) (Fig. 5A–C). The muscle potential amplitude returned to pre-injection parameters after 20 min and then remained unchanged for the rest of the recording period. When the nerve action potentials were recorded, in the presence of Jbtx (2.5×10^{-3} µg/body weight) (Fig. 6A), there was a biphasic effect on the amplitude of the potentials, characterized by a significant decrease of about 20% and 30%, respectively (Fig. 6B). After 30 min the amplitude of the potentials returned to the resting parameters. The same biphasic effect was observed with Jbtx



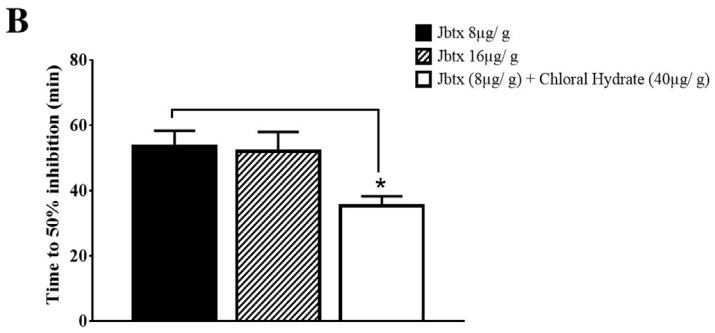
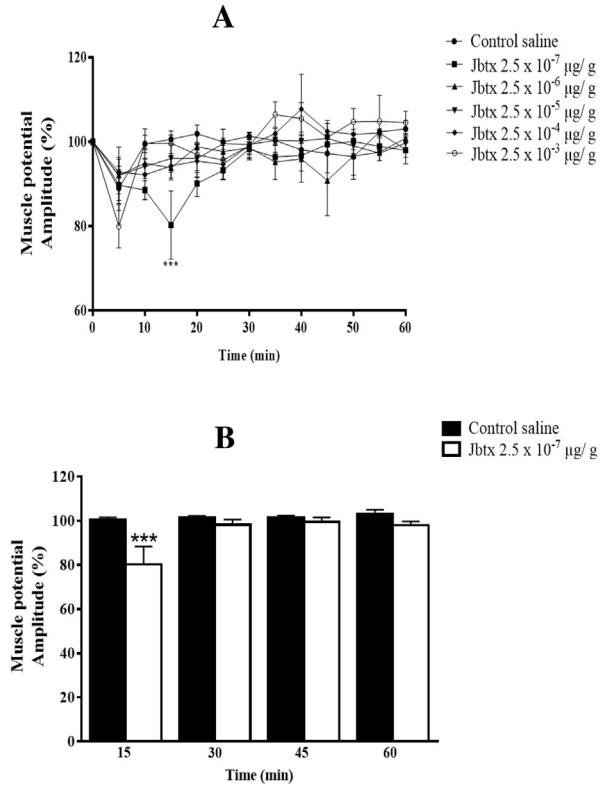


Fig. 4. *In vivo* neuromuscular blockade induced by Jbtx in *Nauphoeta cinerea* cockroaches. (A), shows the means ± S.E.M of the dose-response effect of Jbtx (8 and 16 µg/ g of body weight), and the treatment with Chloral Hydrate (40 µg/ g), in 120 min recordings. Statistics was performed by Student test. * $p < .05$ ($n = 5$). Panel (B) shows the time to 50% inhibition of the muscle twitches. Student test. * $p < .05$ ($n = 6$).



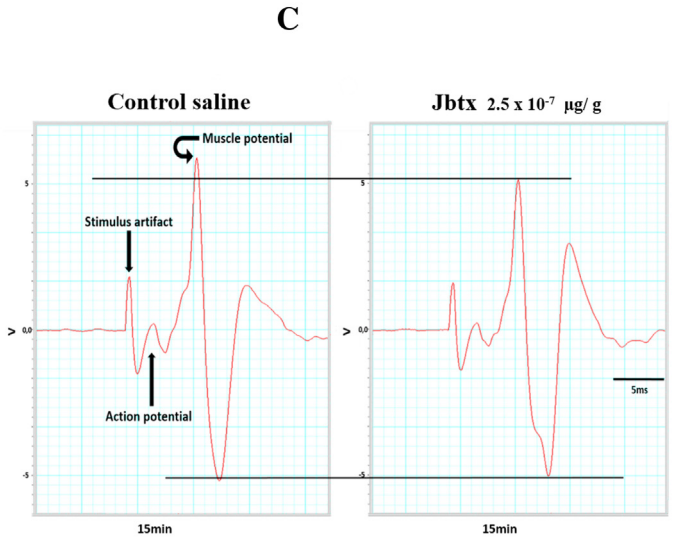


Fig. 5. Effect of Jbtx on the amplitude of the muscle action potentials (mAPs) in the leg of *Locusta migratoria*. Panel (A) shows the mean ± S.E.M of the percentage of the APs under Jbtx (2.5×10^{-7} to 2.5×10^{-3} µg/ body weight) treatment and control saline, during 60 min recordings. Statistics were performed by Two-way ANOVA, followed by the Tukey test as *post hoc*; *** $p < .01$ (n = 5). (B), 20% reduction on mAPs amplitude after 15 min of Jbtx (2.5×10^{-7} µg/ body weight) administration. Statistics were performed by One-way ANOVA, followed by the Tukey test. *** $p < .01$ (n = 5). Panel (C) shows representative recordings of mAPs from *L. migratoria* leg preparations in presence of Jbtx (2.5×10^{-7} µg / body weight) and saline, at 15 min.

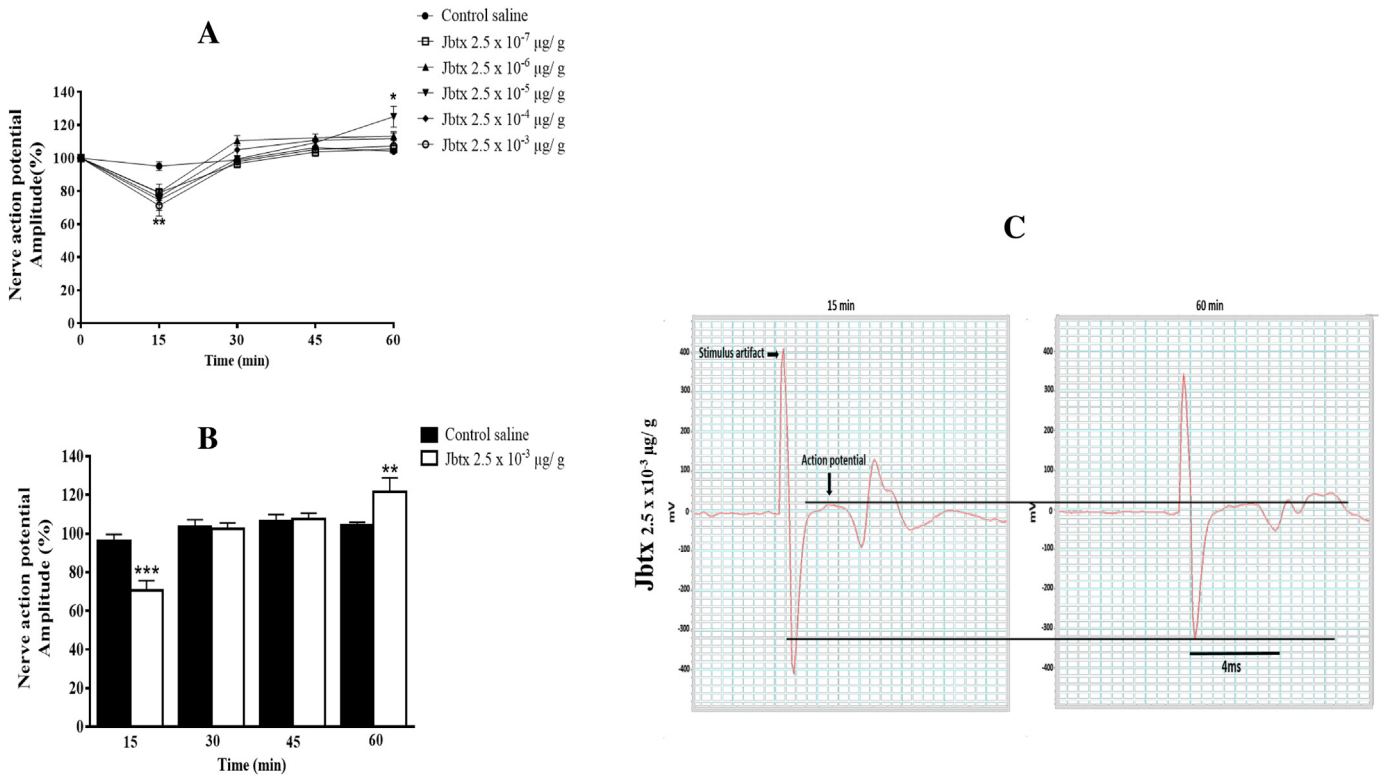


Fig. 6. Effect of Jbtx on the amplitude of the nerve action potentials (nAPs) from *Locusta migratoria* legs. On panel (A), each point represents the mean ± S.E.M of the percentage of the amplitude of the APs under the effects of Jbtx (2.5×10^{-7} to 2.5×10^{-3} µg/ body weight), in 60 min recordings. Statistics were done by Two-way ANOVA, followed by the Tukey test. *** $p < .01$ (n = 5). On (B), 30% reduction in the nAPs amplitude at the first 15 min after Jbtx (2.5×10^{-3} µg/ body weight), followed by a 20% increase in 60 min. Statistics with One-way ANOVA, followed by Tukey test. *** $p < .01$ (n = 5). Panel (C), shows representative traces illustrating the effects of Jbtx (2.5×10^{-3} µg/ body weight) on nerve action potentials recorded from *L. migratoria* leg preparations at 15 and 60 min.

(2.5×10^{-3} µg/body weight), in 60 min recordings (n = 5, $p < .05$). In these experiments it was worth to be noticed that the tarsus stopped twitching, even though the nerve and muscle action potentials were not greatly affected.

3.6. Jbtx effect on the Nav channel of the cockroach Blattella germanica ($BgNa_V$ 1.1)

In order to evaluate the interaction of Jbtx with insect voltage-gated sodium channels, electrophysiological recordings of voltage clamp, using *X. laevis* oocytes co-expressed with *Blattella germanica* $BgNa_V$ 1.1 were carried out (Fig. 7). The application of 200 nM of Jbtx resulted in a significant increase of about 50% in the peak current (Fig. 7A, B and C). The analysis of the I/V curves showed no alteration in the kinetic of activation, since no shift in the midpoint of the curves was observed in the presence of Jbtx (Fig. 7D).

4. Discussion

In this work, we have brought to light novel information about the mechanisms involved in the entomotoxic activity of Jbtx, a toxic peptide derived from *C. ensiformis* urease. The insect models *N. cinerea*, a cockroach, and the locust *L. migratoria* were chosen for this study. In sublethal doses, Jbtx caused profound alterations in the insect behavior and in the electrophysiological parameters that will be discussed in detail therein.

The importance of studying sublethal concentrations of an insecticide relies on the interference those compounds may cause on the chemical communication systems of an insect thereby promoting behavioral alterations that could disrupt reproduction, host-finding, feeding, locomotion and dispersal (Haynes, 1988). Although not lethal to *N. cinerea* (De Freitas et al., 2016) in our experimental approaches, treatment with Jbtx affected the insect's locomotory activity by decreasing the distance travelled and concomitantly altering immobile episodes and the stopped time parameters. We have previously shown that Jbtx and the jack bean urease (JBU), from which Jbtx is derived, interfere negatively on insect muscle twitch tensions (Martinelli et al., 2014; De Freitas et al., 2016). These effects suggested that Jbtx, besides acting directly on the insect nervous system, also targets insect neuromuscular junctions, thus confirming its neurotoxic nature.

Treatment with Jbtx increased the grooming behavior of *N. cinerea*, affecting more intensively grooming of the legs than of the antennae. Grooming in insects has the function of cleansing the external surface of the body as well as other functions which include cutting behavior, social signaling, displacement, and excitation activity (Spruijt et al., 1992; Zhukovskaya et al., 2013). The monoaminergic system is involved on insect's grooming behavior. This system controls locomotion, processing of sensory information in the periphery, central pattern generators, and processing of information within the brain (Zhukovskaya et al., 2013; Mustard et al., 2010). Although grooming behavior in insects is not fully elucidated, it has been shown that the neurotransmitter dopamine is more associated with antennae grooming activity, whereas octopamine is associated with prothoracic leg grooming (Fruttero et al., 2016; Orchard & Lange, 1986; Zhukovskaya et al., 2013; Mustard et al., 2010). In our experimental conditions, treatment of the animals with Jbtx mimicked the increase in leg grooming promoted by octopamine, suggesting an influence of Jbtx on octopaminergic signaling within the central nervous system. In this study the grooming of antennae was also significantly increased upon treatment with Jbtx, at certain doses. Recently, our research group has demonstrated that the antennae grooming was not altered by the treatment of *N. cinerea* with JBU, the urease from which Jbtx derives

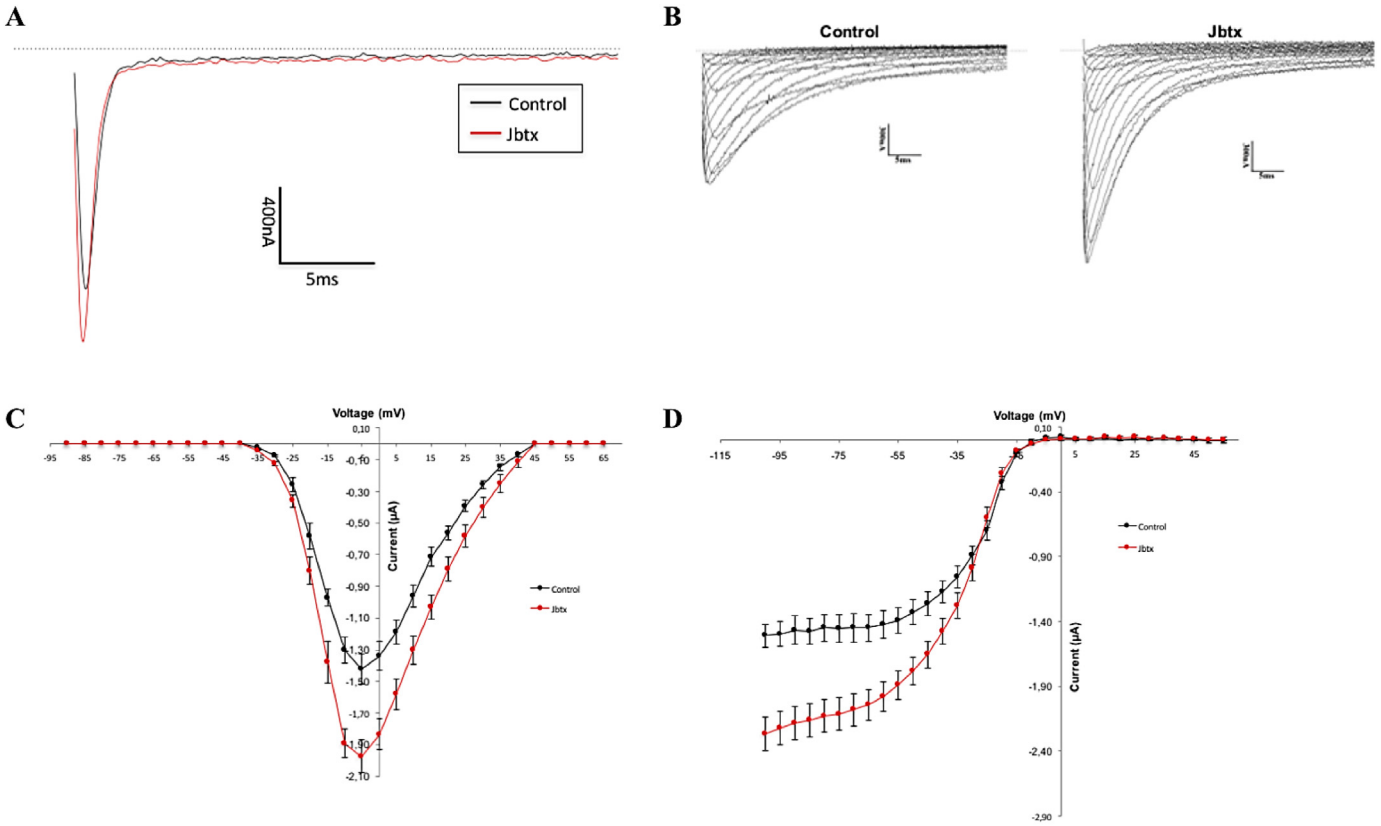


Fig. 7. Effect of Jbtx on sodium currents from *Xenopus oocytes* expressing BgNaV 1.1 channels using two electrode voltage clamp. (A), representative whole-cell current traces of BgNaV 1.1 in control (black trace) and in 200 nM Jbtx treatment. (B), current traces were evoked by 100 ms depolarizations stepping from a holding potential of −90 to +10 mV in 5 mV increments. Traces of a representative experiment are shown in control (left panel) and in 200 nM Jbtx-treated (right panel). (C), activation parameters; (D), inactivation parameters. The peak currents are plotted as a function of the test voltage in the absence (black trace) and the presence (red trace) of 200 nM Jbtx. Representative traces of five independent experiments (n = 5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(De Freitas et al., 2016). A possible explanation for this discrepant result is the much higher molecular mass of JBU (540 kDa) as compared to that of Jbtx (11.04 kDa), which could potentially prevent or reduce considerably the traversing of JBU into the insect brain to reach the sub-esophageal ganglion, which is likely to be involved with antennae grooming (Zhukovskaya et al., 2013; Gal & Libersat, 2010).

Jbtx is cardiotoxic to *N. cinerea* cockroaches, inducing a negative chronotropic effect. The heart of insects is essentially myogenic and its electrical activity is generated by striated muscle cells submitted to chronotropic and inotropic modulation by hormones, neurotransmitters and other cardioactive peptides (Feliciano et al., 2011). The cardiotoxic effect of Jbtx on *N. cinerea*'s heart is similar to those induced by the antidepressant mianserin and the alpha-adrenergic blocker phentolamine (Papaefthimiou & Theophilidis, 2011). Octopamine, a neuromodulator (Farooqui, 2012), is also involved in the regulation of heart beats in insects, since octopamine-containing axons have nerve endings in the pericardial organs (Enan, 2001; Evans, 1980). Besides the effect seen on grooming behavior, the alteration on the insect cardiac rhythm also suggests that Jbtx may specifically alter the octopaminergic neurotransmission (Papaefthimiou & Theophilidis, 2011). Our goal here did not include to explore in depth the pharmacology of Jbtx's effects on the heart of *N. cinerea.* However, at this point, it is possible to conclude that the insect heart (and possibly other pulsatile organs) is involved in the entomotoxic activity of Jbtx as a direct target of the peptide's effects.

Jbtx induced a progressive blockage of the muscle twitches in *N. cinerea*. This neuromuscular blocking effect of Jbtx was previously demonstrated in *Phoetalia pallida* cockroaches (Martinelli et al., 2014). Insect neuromuscular junctions work with two main neurotransmitters, glutamate (GLU), the main excitatory neurotransmitter and gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter, whose receptors appear to be spread throughout the muscle fiber (Enan, 2001; Evans, 1980). Here, treatment of cockroaches with chloral hydrate, reinforced the neuromuscular blocking effect of Jbtx. This result suggests a direct activity of Jbtx at the cockroach neuromuscular junctions, potentially by activation of GABA neurons (Garrett & Gan, 1998).

Our results also demonstrated that Jbtx caused a significant decrease in the amplitude of muscle and nerve action potentials in a *L. migratoria* leg preparation, to block muscle twitch. Alterations in the amplitude of a muscle or neuron action potential can be achieved not exclusively, but in part, by the interaction of neurotoxins with six different binding sites of sodium channels (Cestèle & Catterall, 2000; Dong, 2007; dos Santos et al., 2016; Wang & Wang, 2003). Blocking the sodium conductance, a negative shift in the voltage-dependence of activation, slowing down of inactivation or an alteration of ion selectivity are the most common results of the binding of neurotoxins to sodium channels (Stevens et al., 2011). Inhibition of activation is another mechanism involved in the effect of neurotoxins on sodium channels, as do brevetoxins on the binding site five of sodium channels (Stevens et al., 2011).

In this study, Jbtx induced a significant increase in the amplitude of sodium currents, evoked in *X. laevis* oocytes overexpressing BgNaV 1.1 channels from *Blattella germanica.* Therefore, the decrease in the amplitude of action potentials seen in the *L. migratoria* leg preparations can be a result of an increased sodium influx in excitable membranes (Stevens et al., 2011). These data offer a key for understanding the effects of Jbtx on the amplitude of action potentials and the peptide's

effects on the insect's central and peripheral nervous systems. However, since Jbtx did not induce any facilitatory effect on *Phoetalia pallida* and *N. cinerea* muscle twitch tensions nor an increase of the action potentials amplitude in *L. migratoria*, the effects of the peptide on voltage-gated sodium channels appear elusive. Because the electrophysiological techniques employed were not sophisticated, the increasing phase of the action potential in *in situ* preparations could be masked making only the secondary run down apparent.

Jbtx was previously shown to cause leakage of carboxy fluorescein entrapped inside liposomes but without lysing them (Barros et al., 2009). Later on we described that JBU and Jbtx form cation-selective ion channels in planar lipid bilayers with conductances in the range found for physiological potassium channels (Piovesan et al., 2014). Moreover, JBU and Jbtx are able to insert themselves into the lipid bilayers of multilamellar liposomes, thereby affecting physicochemical properties such as the number of lamellae and the membrane's fluidity (Micheletto et al., 2017). Our data showing the absence of a fast decrease in the resting membrane potential during the voltage clamp protocols suggest that, even if a pore forming activity could be involved in Jbtx's entomotoxicity, the main component to its neurotoxicity appears to involve alteration of the gating properties of sodium channels. Combining voltage clamp techniques and studies to explore pharmacologically sodium channels in the presence of Jbtx could pave the way to reveal the molecular site targeted by Jbtx's activity on insect sodium channels.

5. Conclusion

Our results indicate that the entomotoxic activity of Jaburetox involves significant changes in the behavioral patterns of insects and alterations in both central and peripheral nervous systems. The toxic effect begins with an initial depolarization of the metathoracic ganglion as a result of activation of voltage-gated sodium channels. Following depolarization of the ganglion there is a signal transmission to peripheral and central nerves that is orchestrated by the release of the association neurotransmitter octopamine. The alterations in behavior may be reinforced by a cardiotoxic activity. This initial electrophysiological alteration boosts the octopaminergic modulation resulting in a dopaminergic exacerbation, at the insect central nervous system and a concomitant GABAergic signaling at the insect neuromuscular junctions. Further biochemical, electrophysiological and molecular attempts are required to further elucidate the complexity of actions of Jbtx on the insect nervous system.

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Conflict of interests

The authors declare that there is no conflict of interests regarding this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2018.11.003.

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