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Structure–function studies on jaburetox, a recombinant insecticidal peptide derived from jack bean (*Canavalia ensiformis*) urease

Anne H.S. Martinelli [a,b], Karine Kappaun [a], Rodrigo Ligabue-Braun [a], Marina S. Defferrari [a], Angela R. Piovesan [a], Fernanda Stanisçuaski [a,c], Diogo R. Demartini [a], Chariston A. Dal Belo [d], Carlos G.M. Almeida [d], Cristian Follmer [e], Hugo Verli [a,c], Celia R. Carlini [a,b,f,*,1], Giancarlo Pasquali [a,c,**,1]

[a] Graduate Program in Cellular and Molecular Biology, Center of Biotechnology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[b] Department of Biophysics, Institute of Biosciences, UFRGS, Brazil
[c] Department of Molecular Biology and Biotechnology, UFRGS, Porto Alegre, RS, Brazil
[d] Interdisciplinary Centre of Biotechnological Research, Universidade Federal do Pampa, UNIPAMPA, São Gabriel, RS, Brazil
[e] Department Physical Chemistry, Institute of Chemistry, Universidade Federal do Rio de Janeiro, RJ, Brazil
[f] Instituto do Cérebro (InsCer), Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

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ABSTRACT

Background: Ureases are metalloenzymes involved in defense mechanisms in plants. The insecticidal activity of *Canavalia ensiformis* (jack bean) ureases relies partially on an internal 10 kDa peptide generated by enzymatic hydrolysis of the protein within susceptible insects. A recombinant version of this peptide, jaburetox, exhibits insecticidal, antifungal and membrane-disruptive properties. Molecular modeling of jaburetox revealed a prominent β-hairpin motif consistent with either neurotoxicity or pore formation.
Methods: Aiming to identify structural motifs involved in its effects, mutated versions of jaburetox were built: 1) a peptide lacking the β-hairpin motif (residues 61–74), JbtxΔ-β; 2) a peptide corresponding the N-terminal half (residues 1–44), Jbtx N-ter, and 3) a peptide corresponding the C-terminal half (residues 45–93), Jbtx C-ter.
Results: 1) JbtxΔ-β disrupts liposomes, and exhibited entomotoxic effects similar to the whole peptide, suggesting that the β-hairpin motif is not a determinant of these biological activities; 2) both Jbtx C-ter and Jbtx N-ter disrupted liposomes, the C-terminal peptide being the most active; and 3) while Jbtx N-ter persisted to be biologically active, Jbtx C-ter was less active when tested on different insect preparations. Molecular modeling and dynamics were applied to the urease-derived peptides to complement the structure–function analysis.
Major conclusions: The N-terminal portion of the Jbtx carries the most important entomotoxic domain which is fully active in the absence of the β-hairpin motif. Although the β-hairpin contributes to some extent, probably by interaction with insect membranes, it is not essential for the entomotoxic properties of Jbtx.
General significance: Jbtx represents a new type of insecticidal and membrane-active peptide.

1. Introduction

Ureases (EC 3.5.1.5, urea amidohydrolase), are nickel dependent enzymes that catalyze urea hydrolysis into ammonia and carbon dioxide. Evolutionarily conserved [1], these proteins have been isolated from a wide variety of organisms including plants, fungi and bacteria. In plants, ureases contribute to the bioavailability of nitrogen and in defense mechanisms [2,3]. Ureases represent an unexplored group of plant proteins with potential use for insect control [3,4] and as antifungal agents [5]. Studies have shown that ureases from *Canavalia ensiformis* (jack bean) and *Glycine max* (soybean) display insecticidal activity (reviewed in [6]) and antifungal properties, inhibiting growth and affecting membrane integrity of filamentous fungi [7] as well as of yeasts [8] in the 10^{-7} M range. The urease from pigeon pea (*Cajanus cajan*) was recently described to exhibit insecticidal and antifungal properties at similar dose ranges [9].

The molecular basis of the insecticidal mechanism of action of plant ureases is not yet completely understood [6]. It has been demonstrated that the entomotoxic effect of canatoxin [10], an isoform of *C. ensiformis* (jack bean) urease [11], is partially due to an internal 10 kDa peptide

Abbreviations: Jbtx, jaburetox; JbtxΔ-β, β-hairpin deleted version of Jbtx; Jbtx N-ter, N-terminal domain of Jbtx; Jbtx C-ter, C-terminal domain of Jbtx; Jbtx-2Ec, a version of Jbtx containing a V5 epitope; LUV, large unilamellar vesicle; MD, molecular dynamics; RMSD, root mean square deviation; CD, circular dichroism

* Correspondence to: C. R. Carlini, Center of Biotechnology and Department of Biophysics, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Predio 43.431, Porto Alegre, RS, CEP 91501-970, Brazil. Tel.: +55 51 3308 7606.
** Correspondence to: G. Pasquali, Department of Molecular Biology and Biotechnology, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Predio 43.432, Porto Alegre, RS, CEP 91501-970, Brazil.
E-mail addresses: ccarlini@ufrgs.br, celia.carlini@pq.cnpq.br (C.R. Carlini), pasquali@cbiot.ufrgs.br (G. Pasquali).
[1] These authors share senior authorship.

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(pepcanatox), that is released from the protein upon hydrolysis by insect cathepsin-like digestive enzymes [12–16]. Jaburetox-2Ec (Jbtx-2Ec), a recombinant peptide analog to pepcanatox, exhibited a potent insecticidal effect on two economically important crop pests: *Spodoptera frugiperda* (fall armyworm) and *Dysdercus peruvianus* (cotton stainer bug) [17,18]. Jbtx-2Ec was also shown to both permeabilize large unilamellar liposomes (LUVs) [19] and to affect transmembrane potential of insect Malpighian tubules, causing inhibition of diuresis [20]. A β-hairpin motif in the modeled structure of Jbtx-2Ec has been proposed [17,19] and its presence has been confirmed in the crystallographic structures of jack bean [21] and pigeon pea [9] ureases. This motif is present also in one class of pore-forming peptides and neurotoxic peptides [22] such as charybdotoxin, which affect ion channels [23]. A variant form of Jbtx-2Ec lacking the fused V5-antigen, here called simply Jbtx, also exhibited antifungal activity [8].

Aiming to identify motifs possibly involved in the different biological activities of Jbtx, here we described the cloning and expression of mutated versions of the Jbtx-encoding cDNA. Truncated versions of the peptide, with deletions of the regions of the β-hairpin motif, the N-terminal or the C-terminal halves of the molecule, were tested on LUV permeabilization, for insecticidal and other entomotoxic effects. Structural analyses of the truncated peptides were also carried out.

2. Materials and methods

2.1. Jbtx cDNA constructs

Jaburetox-2Ec, the first version of the recombinant urease-derived peptide cloned in [17], harbored a V5-antigen with 18 amino acids derived from the pET101/D-TOPO plasmid. In order to eliminate this foreign sequence, the jack bean urease truncated cDNA encoding 93 amino acids, called simply jaburetox (Jbtx), was cloned and expressed in *Escherichia coli* via pET-23a vector (Novagen), as described in [8]. This sequence was used as template for site-directed mutagenesis and PCR amplifications of the mutant forms as described below.

2.2. Jbtx lacking the internal β-hairpin (JbtxΔ-β)

In order to delete the β-hairpin motif (residues 61–74) of the Jbtx peptide, site-directed mutagenesis was performed using the QuickChange Site-directed Mutagenesis Kit (Stratagene). As this method is often used to generate a few nucleotide deletions, some modifications in the primers' design were made, as described by [24]. Pairs of complementary primers were designed (Table 1), and site-directed mutagenesis was performed according to the kit manufacturer's instructions. The deleted gene version was confirmed by sequencing on an ABI Prism 3100 automated sequencer (Applied Biosystems) platform (ACTGene Ltd, Center of Biotechnology, UFRGS). Sequence comparisons were performed using the BLASTx software [25], available at (http://www.ncbi.nlm.nih.gov). The resulting peptide was called JbtxΔ-β.

Table 1
Primers used in this study.

Primer	Size	Sequence
5′ Del_β-hairpin	40-Mer	AGTATGGTCCGACTATTGGTGAAAAGGATTTTGCCCT TTA
3′ Del_ β-hairpin	40-Mer	TAAAGGGCAAAATCCTTTTCACCAATAGTCGGACCAT ACT
5′ Del_ α-helix	40-Mer	CTTTCACCAAAGCCATTCCTTATGGTCCGACTATTGGTGA
3′ Del_ α-helix	40-Mer	TCACCAATAGTCGGACCATAAGGAATGGCTTTGGTGA AAG
5′ N-terminal	25-Mer	CCAACATATGGGTCCAGTTAAATGA
3′ N-terminal	25-Mer	CCCCCTCGAGGGTGAAAGGACAATC
5′ C-terminal	25-Mer	CCAACATATGAAAGCCATTCCTCGT
3′ C-terminal	25-Mer	CCCCCTCGAGTATAACTTTTCCACC

2.3. Jbtx N-terminal (Jbtx N-ter) and C-terminal (Jbtx C-ter) domain versions

The Jbtx gene regions corresponding to the N-terminal (residues 1–44) and C-terminal (residues 45–93) halves of the peptide were amplified by PCR with specifically designed primers (Table 1) and products were cloned into pET23a (Novagen). PCRs were performed in a final volume of 50 μL containing 50 ng of the template plasmid DNA, 200 ng of each primer, 200 μM each dNTPs, 2.5 U *Pfu* taq DNA polymerase (Fermentas) and 1× *Pfu* reaction buffer. Amplification was carried out under the following conditions: denaturation at 95 °C for 3 min, annealing at 55 °C for 30 s and elongation at 72 °C for 2 min. After a total of 35 cycles, the final products were digested with *Nde*I and *Xho*I (Fermentas), dephosphorylated with thermosensitive alkaline phosphatase (Promega) and ligated into the expression vector pET23a (Novagen). The inserts of the recombinant plasmids were fully sequenced in order to confirm their sequences essentially as described above. The resulting peptides were called Jbtx N-terminal (Jbtx N-ter) and Jbtx C-terminal (Jbtx C-ter). A schematic representation of all Jbtx-related peptides is shown in Fig. 1.

2.4. Expression and purification of Jbtx recombinant peptides

Recombinant pET23a plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene) for Jbtx gene expressions following the provider's instructions. For the purification of the original Jbtx peptide and its mutated forms, 200 mL of Luria Bertani medium containing 100 μg/mL ampicillin and 40 μg/mL chloramphenicol were separately inoculated with 2 mL overnight cultures of each *E. coli* strain. Cells were grown for approximately 2 h at 37 °C under shaking until an optical density of 0.7 was reached. At this point, IPTG was added to cultures to a final concentration of 0.5 mM. After 3 h of additional culture, cells were harvested by centrifugation and suspended in 10 mL of lysis buffer (50 mM Tris buffer, pH 7.5, 500 mM NaCl, 5 mM imidazole), sonicated and centrifuged (14,000 *g*, 30 min). The supernatant was loaded onto a Ni^{+2} loaded Chelating Sepharose (GE Healthcare) column, previously equilibrated with the lysis buffer. After 30 min, the column was washed with 50 mL of the same buffer containing 50 mM imidazole. Bound protein was eluted with 200 mM imidazole in the lysis buffer. Samples were then dialyzed against buffer A (50 mM phosphate buffer, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol) in order to remove the imidazole. Protein concentration was measured by Bradford assay [26]. Predicted molecular mass of the peptides was obtained by submitting the deduced sequences to the ProtScale tool [27] available at the Expasy site (http://web.expasy.org/protscale).

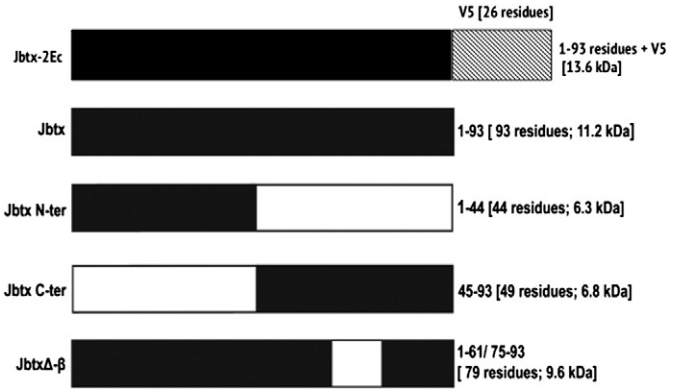

Fig. 1. Schematic representation of the sequences of jaburetox and mutants. The number of amino acid residues of each molecule (shown in black) is indicated on the right side.

Molar concentrations of peptides were calculated assuming a monomeric form in solution. Hydropathicity analysis of the peptides was carried out according to [28].

2.5. Tandem mass spectrometry (MS/MS)

In gel digestion of Jbtx peptides was performed for all samples analyzed, except for the positive control (C+), which was submitted to in solution digestion. For in gel digestion the protocol in [29] was followed, using 50 mM ammonium bicarbonate (AB) unless otherwise explained. Briefly, the bands on the gel were distained with 50 mM AB in 40% acetonitrile (ACN). Following, gel pieces were dehydrated with 100% ACN and lyophilized. Reduction was performed with 50 mM dithiothreitol (DTT) in 50 mM AB, for 30 min at 56 °C, in the dark, followed by the alkylation performed with 50 mM iodoacetamide (IAA) in 50 mM AB for 30 min, at room temperature, also in the dark. The gel pieces were washed with 50 mM AB + 40% ACN for 15 min, and a second dehydration step was done. Proteins in the gel were digested for 3 h (Jbtx N-ter and Jbtx C-ter) or 16 h (Jbtx and JbtxΔ-β) in 500 μL of 100 mM AB solution containing 100 ng of sequencing grade trypsin (Promega) at 37 °C. After digestion, supernatants were transferred to microcentrifuge tubes and gel pieces were washed with 1% formic acid in 60% ACN, and the supernatants were combined accordingly. Digested peptides were lyophilized and submitted to tandem mass spectrometry analyses. In the case of in solution digestion, sample of Jbtx peptide (30 μg in 20 mM sodium phosphate, 5 mM β-mercaptoethanol, 1 mM EDTA, pH 7.5) was reduced with 50 mM DTT and alkylated with 50 mM IAA (30 min each, at room temperature). Then, DTT to a final concentration of 10 mM was added for 15 min at room temperature. Digestion was performed in this case with 0.6 mg of trypsin (Promega) for 3 h at 37 °C. After the digestion process, the samples were desalted with ZipTip™ (Millipore®) according to the manufacturer's instructions. The eluted peptides were lyophilized and analyzed by tandem mass spectrometry.

The lyophilized digested peptides were suspended in 0.1% formic acid (10 μL) and 5 μL of each solution was subjected to reversed phase chromatography (NanoAcquity UltraPerformance LC-UPLC® chromatograph (Waters) using a Nanoease C18, 75 μm ID at 35 °C. The column was equilibrated with 0.1% trifluoroacetic acid (TFA) and the peptides were eluted in a 20 min gradient, ramping from 0 to 60% acetonitrile in 0.1% TFA at 0.6 nL/min constant flow. Eluted peptides were subjected to electrospray ionization and analyzed by mass spectrometry using a Q-TOF Micro™ spectrometer (Micromass). The voltage applied to the cone for the ionization step was 35 V. The three most intense ions in the range of m/z 200–2000 and +2 or +3 charges were selected for fragmentation. The acquired MS/MS spectra were processed using the Proteinlynx v.2.0 software (Waters) and the generated .mgf files were used to perform database searches using the MASCOT software version 2.4.00 (Matrix Science) against the NCBI database, and taxid was restricted to Viridiplantae (taxid:33090). Results were analyzed manually.

2.6. Electrophoresis

The peptide fractions of Jbtx and its mutant forms were visualized in SDS-Tricine gels [30]. The gels were stained with colloidal Coomassie G-250 (Sigma Chem. Co) according to [31].

2.7. Western blot

Western blots were performed according to [32]. Briefly, peptides were electrophoresed, transferred to PVDF membranes (Millipore) and immersed in a blocking buffer consisting of 5% nonfat dry milk in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl and 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.3). After washing, the membrane was incubated with rabbit anti-jaburetox-2Ec polyclonal antibodies (1:7500 dilution) for 2 h at room temperature, followed by a 2 h incubation with anti-rabbit IgG (1:20,000 dilution) alkaline phosphatase conjugate (Sigma Chem. Co.). Colorimetric detection was carried out using 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt and nitro-blue tetrazolium chloride.

2.8. Leakage experiment

Large unilamellar vesicles (LUVs) were produced and the leakage experiment was conducted as described previously [19]. LUVs were prepared using 10 mg of L-α-phosphatidic acid (egg chicken, Avant Polar Lipids), at a concentration of 20 mg/mL. The leakage promoted by Jbtx and its mutated forms at a final concentration of 5 μg/mL in 25 mM Tris, pH 7.0, was evaluated by the carboxyfluorescein release assay [19]. The concentration of LUVs in the experiment was estimated based on the absorbance of the fluorescent probe at 490 nm, and adjusted to a value of 0.1. In the leakage assays, fluorescence intensity of the reaction mixture (LUVs plus peptide or buffer) was recorded as a function of time. The samples were excited at 490 nm and the fluorescence was acquired at 518 nm. It was assumed that the absence of leakage (0%) corresponded to the fluorescence of the vesicles at time zero; 100% leakage was taken as the value of fluorescence intensity obtained after the addition of 1% (v/v) Triton X-100. All measurements were carried out in a Cary Eclipse fluorescence spectrophotometer (Varian).

2.9. Insecticidal activity

Fifth-instar *Rhodnius prolixus* were kindly provided by Dr. Hatisaburo Masuda and Dr. Pedro L. Oliveira (Institute of Medical Biochemistry, Universidade Federal do Rio de Janeiro, RJ, Brazil) and by Dr. Denise Feder (Universidade Federal Fluminense, RJ, Brazil). The phytophagous milkweed bugs (*Oncopeltus fasciatus*) were reared in our laboratory as previously described [15].

2.9.1. Injection assays

Fifth instars of *O. fasciatus* or *R. prolixus* were injected into the hemocoel using a Hamilton Microliter 900 series syringe (Hamilton). Group of 10 insects (*O. fasciatus*) or 5 insects (*R. prolixus*) were injected with 20 mM sodium phosphate buffer (pH 7.5) containing peptides at a final dose of 0.015 μg (*O. fasciatus*) or 0.05 μg (*R. prolixus*) per mg of insect body weight. Control insects received injections of buffer alone. Mortality rate within each group was recorded after 48 or 96 h. Two independent bioassays were carried out for each peptide on each insect model. Results shown are means ± standard errors.

2.9.2. Feeding assays

Fifth instars *R. prolixus* were fed on *R. prolixus* saline solution (150 mM NaCl, 8.6 mM KCl, 2.0 mM $CaCl_2$, 8.5 mM $MgCl_2$, 4.0 mM $NaHCO_3$, 34.0 mM glucose, 5.0 mM HEPES, pH 7.0) containing 1 mM ATP and enough peptide (tested individually) to give final doses of 0.1 μg per mg of body weight. Groups of 5 insects for each peptide were fed for approximately 30 min, at 37 °C, by placing their mouth apparatus inside glass capillaries containing the test solutions. Control insects fed solely on *R. prolixus* saline solution containing 1 mM ATP, under the same conditions. Mortality rate within each group was recorded after 24 h. One triplicated bioassay was carried out for each peptide. The results shown are means and standard errors.

2.10. Measurement of fluid secretion by Rhodnius prolixus Malpighian tubules

The assay was performed essentially as described in [20], using *R. prolixus* serotonin-stimulated Malpighian tubules. Secretion rate was expressed as the percentage of fluid secretion measured after the addition of Jbtx or mutated peptides as compared to serotonin (2.5×10^{-8} M) alone (control). For each peptide and dose, 5–6 replicates were done. The results shown are means ± standard error.

2.11. In vivo cockroach metathoracic coxal-adductor nerve–muscle preparation

The *in vivo* cockroach metathoracic coxal-adductor muscle preparation was used [33] to characterize further the entomotoxic activity of Jbtx and its mutated versions. Male adult *Phoetalia pallida* (3–4 months after molting) were reared in our laboratory at controlled temperature (22–25 °C) on a 12 h:12 h light:dark cycle. 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 coxae 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 micromanipulator to allow 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 covered with mineral oil to prevent dryness and stimulated at 0.5 Hz, 5 ms, with twice the threshold, during 120 min. Twitch tension was digitalized, recorded 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). Jbtx and peptides were dissolved in insect physiological solution (214 mM NaCl, 3.1 mM KCl, 9 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 5 mM HEPES, pH 7.2 [34]). The test solutions were prepared daily and 20 μL were injected into the insect's third abdominal segment using a Hamilton syringe.

2.12. Molecular modeling and simulation

The three-dimensional model for Jbtx was built by comparative modeling with MODELLER9v10 [35] employing the structure of the *C. ensiformis* major urease isoform (PDB ID: 3LA4), [21] as template. Ten models were built, stereochemically evaluated and theoretically validated for their three-dimensional profiles with PROCHECK [36] and Verify3D [37], respectively. The best scored model was then selected. The amino-terminal Met residue and the carboxy-terminal LEHHHHHH segment were added with SwissPDBviewer [38]. The Jbtx peptide was then subjected to molecular dynamics (MD) simulations with GROMACS 4.5 suite [39] using GROMOS96 53a6 force field [40] for 500 ns. The systems were solvated in triclinic boxes using periodic boundary conditions and SPC water models [41]. Counterions (Na^+) were added to neutralize the systems. The Lincs method [42] was applied to constrain covalent bond lengths, allowing an integration step of 2 fs after an initial energy minimization using Steepest Descents algorithm. Electrostatic interactions were calculated with Particle Mesh Ewald method [43]. Temperature and pressure were kept constant by coupling proteins, ions, and solvent to external temperature and pressure baths with coupling constants of $\tau = 0.1$ and 0.5 ps [44], respectively. The dielectric constant was treated as $\varepsilon = 1$, and the reference temperature was adjusted to 300 K. The system was slowly heated from 50 to 300 K, in steps of 5 ps, each step increasing the reference temperature by 50 K, allowing a progressive thermalization of the molecular system. The simulation was performed to 500 ns, with no restraint, considering a reference value of 3.5 Å between heavy atoms for a hydrogen-bond, and a cutoff angle of 30° between hydrogen-donor–acceptor [39].

2.13. Statistical analysis

Data were evaluated by ANOVA followed by the Bonferroni's or Student *t* test using GraphPad Prism software (Version 5.0 for Windows). See legends to figures for more details. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. MD simulation of Jbtx

It has been previously suggested that a prominent β-hairpin in the predicted model of the urease-derived peptide Jbtx could be responsible at least in part for its membrane-disturbing activity and some of its biological properties [17,19]. The presence of this β-hairpin was confirmed by x-ray crystallographic data of jack bean urease [21], and short simulations of the crystal-derived peptide were performed [45].

In order to establish if this β-hairpin would still be present in the peptide once it has been released from the urease molecule, a 3D-model of Jbtx was constructed using the crystal structure of jack bean urease as template and subjected to molecular dynamics for 500 ns (Fig. 2, panels A and B). The MD simulation indicated that Jbtx becomes more globular when in aqueous solution (Fig. 2, panel B), changing its conformation along the simulation with an increase of RMSD, as compared to the initial crystal-derived structure (Supplementary Fig. 1). The secondary structure of Jbtx changed in solution, with loss of many helix turns and formation of a minute beta sheet. The β-hairpin at Jbtx's C-terminal half was conserved despite the increase in coil content (Fig. 2, panels B and D).

3.2. Expression of recombinant jaburetox (Jbtx) and mutated forms

Aiming to identify motifs probably involved in the biological activities of Jbtx, mutated forms of the peptide lacking the internal β-hairpin (JbtxΔ-β), the N-terminal half (Jbtx C-ter) or the C-terminal half (Jbtx N-ter) domains were constructed. A schematic representation of these peptides is shown in Fig. 1.

All the His-tagged peptides were purified and analyzed by SDS-PAGE (Fig. 3A and B). The predicted molecular masses of the peptides based on their deduced amino acid sequences are 11,193 Da for Jbtx, 9625.6 Da for JbtxΔ-β, 6325.8 Da for Jbtx N-ter and 6772.5 Da for Jbtx C-ter. As it can be observed from the SDS-PAGE results, all the recombinant peptides showed the expected mass, except for the Jbtx N-ter peptide which behaved as a dimer with an estimated molecular mass of approximately 12 kDa (Fig. 3B).

Anti-Jbtx-2Ec polyclonal antibodies recognized equally Jbtx-2Ec, Jbtx and JbtxΔ-β (result not shown) and although with a weaker reactivity, also interacted with the two half-peptides (Fig. 3C).

All bands seen in the lanes corresponding to each peptide were excised from the SDS-PAGE gels, digested with trypsin and submitted to MS/MS analysis. The identities of the peptides Jbtx, JbtxΔ-β, and Jbtx C-ter (and aggregated forms of the peptides) were confirmed by MS/MS analysis as shown in Fig. 3D. On the other hand, the peptide Jbtx N-ter was not identified in the MS/MS assay. The band corresponding to the dimer of the peptide Jbtx N-ter in the SDS-PAGE (Fig. 3B) reacted positively with the anti-Jbtx antibodies (Fig. 3C), thus confirming its identity. The tendency to form aggregates previously described for jaburetox-2Ec [19,46] persisted in Jbtx, as well as in all the mutated forms of this peptide, as confirmed by the MS/MS analysis. After a few days in aqueous solution, all the peptides formed insoluble precipitates. These aggregates did not revert to the monomeric state under a number of tested conditions [19]. High ionic strength accelerates the aggregation of Jbtx (data not shown), suggesting hydrophobic interactions as a driving force for the oligomerization process. Because it was not possible to ascertain the oligomeric state of each peptide in solution, their monomeric states were considered when expressing molar concentrations in the subsequent assays.

Since all mutated peptides retained considerable antigenicity towards anti-Jbtx-2Ec polyclonal antibodies, they probably kept their tridimensional structures, resembling the corresponding portions in Jbtx. The CD spectrum of Jbtx (not shown) indicated the presence mainly of irregular structures, with a minor contribution of β-sheets and helices. This type of CD spectrum has been observed for Chab I,

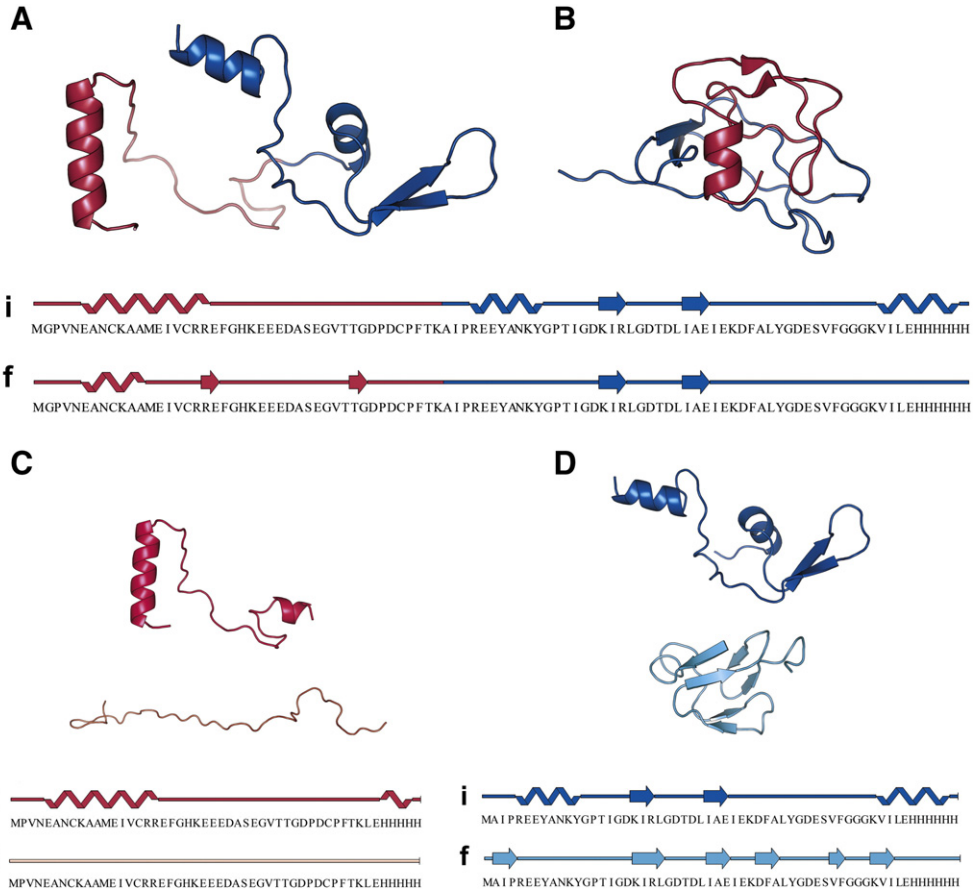

Fig. 2. Structural changes in jaburetox and its mutated versions after MD simulation of 500 ns. Three-dimensional representations of the full Jbtx peptide (A) initial and (B) final structures, with the N-terminal domain (residues 1–44) depicted in pink and the C-terminal domain in blue; (C) Jbtx N-ter (amino-terminal mutant): top, initial state; bottom, final state; (D) Jbtx C-ter (carboxy-terminal mutant): top, initial state; bottom, final state; Schematic representations of the secondary structure content of the (i) initial and (f) final structures are colored according to their three-dimensional counterparts. The corresponding amino acid sequences are also shown.

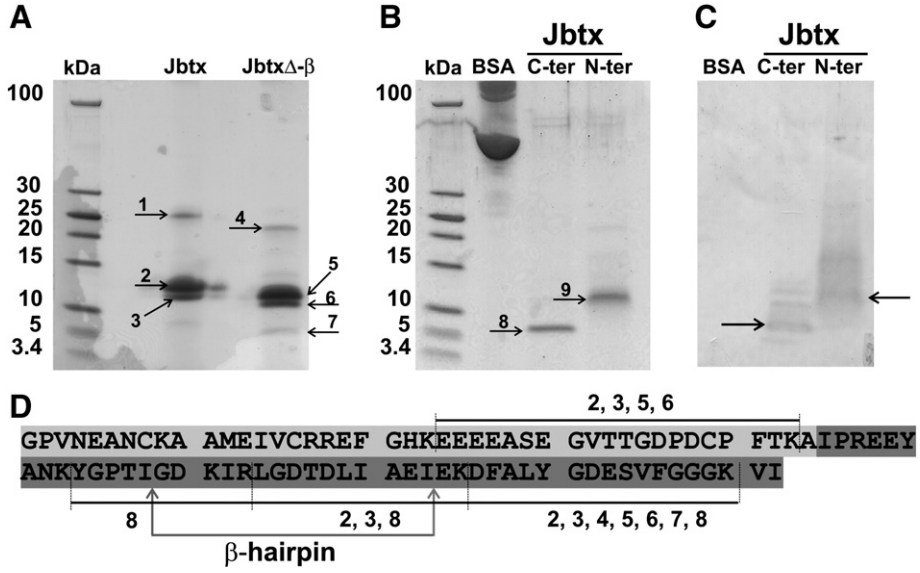

Fig. 3. (A and B) SDS-Tricine PAGE of jaburetox and their derived peptides. Numbered arrows indicate the bands that were excised and analyzed by mass spectrometry. *Lanes*: Jbtx, jaburetox; JbtxΔ-β, jaburetox with deleted β-hairpin motif; BSA, bovine serum albumin; C-ter, carboxy-terminal region of jaburetox; N-ter, amino-terminal region of jaburetox. (C) Western blot analysis with polyclonal anti-jaburetox antibodies. *Lanes*: BSA, bovine serum albumin as negative control; C-ter, carboxy-terminal region of jaburetox; N-ter, amino-terminal region of jaburetox; (D) amino acid sequence of jaburetox. The numbered lines above and below the sequence correspond to the arrows in panels A and B, showing parts of the jaburetox sequence identified by mass spectrometry. The sequence of Jbtx N-ter mutant is shown in light gray and that of the Jbtx C-ter in dark gray. The region corresponding to the β-hairpin is also indicated.

a charybdotoxin analog [47], and also for the acid unfolded state of equine β-lactoglobulin, which has residual helices and β-hairpins [48,49].

Simulations were carried out to establish the putative structures in solution of the mutated peptides representing the two half domains of Jbtx. The N-terminal mutant (residues 1 to 44) became completely unfolded after simulation (Fig. 2, panel C), while the C-terminal mutant (residues 45 to 93) showed propensity towards stabilization of a newly-formed β-sheet (Fig. 2, panel D).

3.3. Vesicle leakage promoted by Jbtx peptides

We employed LUVs composed by L-α-phosphatidic acid [19] as a membrane model to evaluate which part of the Jbtx molecule interacts with phospholipid membranes and induces vesicle leakage. Fig. 4 shows typical results. Vesicle leakage was more prominent when LUVs were treated with either Jbtx C-ter or Jbtx (5 μg/mL), although all peptides produced at least 80% of leakage at the end of the 10 min incubation period. Taken together, these findings showed that the β-hairpin is not essential for the membrane-disruptive activity of Jbtx. Moreover, the data indicated that all Jbtx-related peptides are able to induce LUV leakage, while the C-terminal region of the peptide seems to contribute the greatest effect. In fact, hydropathicity plots indicated the presence of prominent hydrophobic regions in both, the N-terminal and the C-terminal domains of Jbtx (Supplementary Fig. 2).

3.4. Insecticidal effect of Jbtx peptides

In order to compare the insecticidal activity of Jbtx to that previously described for Jbtx-2Ec [6,18], we tested the entomotoxic effect of Jbtx upon injection into *R. prolixus* nymphs. Employing a dose of 0.05 μg/mg of insect weight, 100% mortality was observed 48 h after injection (result not shown), indicating that the absence of the V5 epitope in Jbtx did not affect its insecticidal property. When the insecticidal activity of the β-hairpin deleted form (JbtxΔ-β) was assayed in *R. prolixus* nymphs, it produced an entomotoxic (mortality) effect equivalent to that of the original Jbtx, either by injection (Fig. 5A) or by feeding (Fig. 5B). Four days after injection into fifth instars *R. prolixus*, we have observed that the Jbtx N-term induced up to 60% mortality, while Jbtx C-ter caused less than 10% mortality

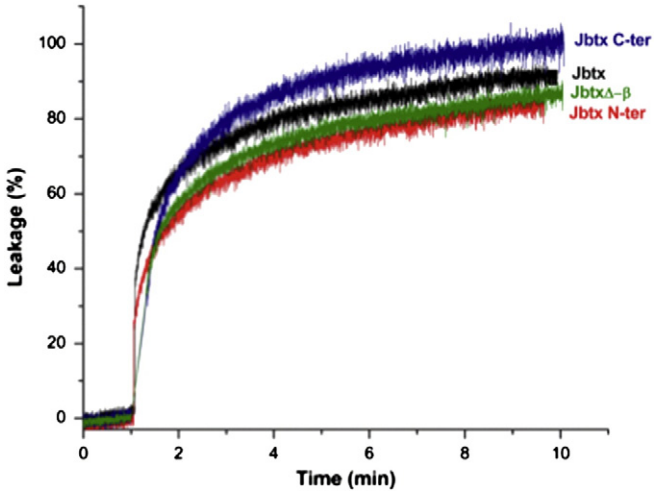

Fig. 4. Effect of jaburetox and derived mutants on LUVs composed by L-α-phospatidic acid. The carboxyfluorescein release assay was performed for each peptide at a final concentration of 5 μg/mL (Jbtx, 0.44 μM; JbtxΔ-β, 0.51 μM; Jbtx N-ter, 0.79 μM; and Jbtx C-ter, 0.73 μM) in 25 mM Tris, pH 7.0. The absence of leakage (0%) corresponds to the fluorescence of the vesicles at time zero; 100% leakage was taken as the value of fluorescence intensity obtained after addition of 1% (v/v) Triton X-100. The experiments were performed at 25 °C. The figure shows superimposed tracings of a typical result for each peptide to facilitate comparison.

(Fig. 5A). On the other hand, 24 h after feeding, both Jbtx N-ter and Jbtx C-ter had similar lethal effects on *R. prolixus* nymphs, ranging from 60 to 80% mortality (Fig. 5B).

Fifth instars *O. fasciatus* were also injected with Jbtx and its mutant variants. Similarly to what was observed for *R. prolixus* upon injections, Jbtx N-ter (Fig. 6A) and JbtxΔ-β (Fig. 6B) displayed lethal effects comparable to that of Jbtx, while Jbtx C-ter was near to inactive (Fig. 6A), suggesting that the N-terminal portion of the Jbtx carries its insecticidal domain.

3.5. Antidiuretic effect of Jbtx-related peptides on Malpighian tubules

We have previously described that, in the dose range of 10^{-16} to 10^{-15} M, Jbtx 2-Ec inhibited the serotonin-stimulated fluid secretion in *R. prolixus* Malpighian tubules [20]. Fig. 7 shows that Jbtx and all its variants, at a concentration of 1×10^{-15} M, were able to inhibit fluid secretion in the tubules producing similar antidiuretic effect.

3.6. In vivo neuromuscular blockade of cockroach nerve–muscle preparations induced by Jbtx-related peptides

The injection of Jbtx or its mutant versions (32 μg/g of animal weight) produced a time-dependent blockade of the cockroach nerve–muscle preparation (Fig. 8). Jbtx was the most effective and induced a complete neuromuscular paralysis at 35 ± 10 min followed by Jbtx N-ter at 80 ± 2 min (Fig. 8B). In contrast, the neuromuscular blockades induced by Jbtx Δ-β or Jbtx C-ter were only partial at the end of the 120 min recording time. The administration of insect saline alone did not interfere with normal neuromuscular responses during 120 min recordings (Fig. 8A). Thus, similar to what was observed in the case of the insecticidal activity (upon injection), these data suggest that the N-terminal half of Jbtx carries its entomotoxic domain. In this type of assay, however, there is a contribution of the β-hairpin to the effect.

4. Discussion

In this study we evaluated the jack bean urease-derived peptide Jbtx and three domain-deleted variants in order to identify the regions of the molecule that are critical for its entomotoxic activities. A previous version of Jbtx, harboring a large V5-antigen derived from the pET101/D-TOPO plasmid and called jaburetox-2Ec (Jbtx-2Ec), was shown to be lethal to *R. prolixus* by oral route and hemocoel injection [50,51] and to permeabilize vesicles composed of charged lipids [19]. Jbtx has the same 93 amino acid urease-derived sequence and the polyhistidine tail found in Jbtx-2Ec, but lacks the V5 epitope present in the later. Here we demonstrated that Jbtx displays insecticidal activity equivalent to that described for Jbtx-2Ec, evidencing that the epitope V5 is not implied in its entomotoxicity.

Comparing the structure obtained here for Jbtx with the model of jaburetox-2Ec generated by comparative modeling [19] (prior to the first description of the crystal structure of a plant urease [21]), important conformational similarities can be seen in the region correspondent to the short helix as well as in the large content of random coil conformation, even though jaburetox-2Ec exhibited a more well-defined β-hairpin than Jbtx. The differences in the models generated for these peptides might be attributed in part to the distinct initial structures used in the MD simulation, as *H. pylori* urease and jack bean urease served as template in the comparative modeling for jaburetox-2Ec [19] and Jbtx (this work), respectively.

Balasubramanian and Ponnuraj [21] were the first to report in 2010 the crystal structure of a plant (*C. ensiformis*, jack bean) urease at 2.05 Å of resolution. These authors confirmed the presence of an internal β-hairpin motif in the jack bean urease, previously suggested by our group to be present in the structure of jaburetox-2Ec [17,19], and proposed to be involved in the insecticidal activity of both urease and its derived entomotoxic peptide. The same group described insecticidal

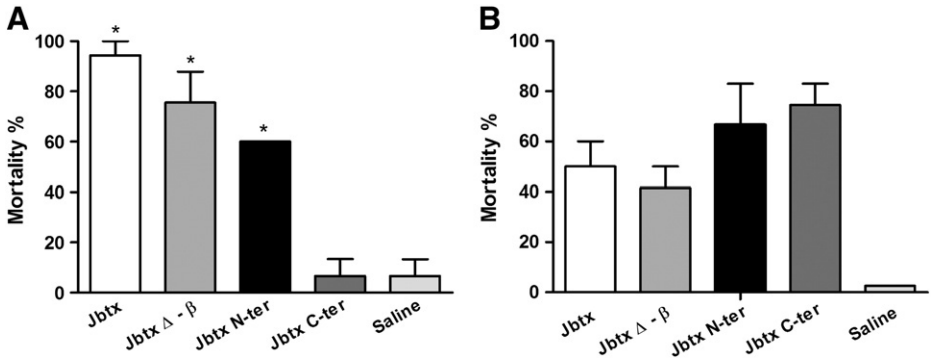

Fig. 5. Insecticidal effect of Jbtx and derived peptides on fifth instar *Rhodnius prolixus*. (A) Groups of 5 insects were injected with each peptide separately at final doses of 0.05 μg per mg of body weight. Control insects were injected with *Rhodnius* saline. The mortality was recorded after 96 h. Two independent bioassays were carried out for each peptide. Results shown are means and standard error. (B) Groups of 5 insects were fed on *R. prolixus* saline plus 1 mM ATP and the peptides separately at final doses of 0.1 μg per mg of body weight. Control insects were fed solely on *R. prolixus* saline plus 1 mM ATP. Mortality rate within each group was recorded after 24 h. Results shown are means and standard error.

and antifungal properties of the pigeon pea (*Cajanus cajan*) urease and reported the presence of a similar β-hairpin motif in the crystal structure of this urease [9]. Moreover, Balasubramanian and coworkers, using molecular modeling studies and short (5 ns) molecular dynamics simulations of Jbtx, suggested that its β-hairpin could self-associated into a β-barrel able to anchor into a membrane-like environment, and hypothesized an insecticidal mode of action of Jbtx based on pore formation [45].

Also present in bacterial ureases, the microbial β-hairpin motif is formed with contributions from the α- and the β urease chains while its counterpart in the single chain of plant ureases is formed exclusively by amino acids located in a region corresponding to the bacterial α-chain [21]. Our group reported that, contrasting to plant ureases, *Bacillus pasteurii* urease has no insecticidal activity against *Dysdercus peruvianus* [52]. Since the β-hairpin motif is present in the *B. pasteurii* urease as well [21], a plausible explanation for this could be the fact that part of the sequence corresponding to the N-terminal half of Jbtx is missing in bacterial ureases.

Here we demonstrated that Jbtx and JbtxΔ-β, its β-hairpin deleted version, behaved almost indistinguishably regarding LUV leakage, antidiuretic effect and insecticidal activity upon injection. These results strongly suggested that the β-hairpin motif is not involved in membrane-disturbing activity or in these biological properties of the peptide.

At that point we had no clues to any other possible motif in the Jbtx molecule that could be responsible for its biological properties, so we decided to produce two half-peptides, corresponding to the N-terminal and C-terminal half versions of Jbtx (Jbtx N-ter and Jbtx C-ter, respectively). The mutated peptides Jbtx N-ter and Jbtx C-ter were then tested for LUV leakage and for different types of entomotoxic activities. Two distinct groups of results were obtained depending on the assay: (i) Jbtx, JbtxΔ-β and Jbtx N-ter were equally active while and Jbtx C-ter was inactive or significantly less active; and (ii) all the peptides produced similar effects.

When tested for insecticidal activity upon injection into *R. prolixus* (Fig. 5A) or *O. fasciatus* (Fig. 6) nymphs, Jbtx, Jbtx Δ-β and Jbtx N-ter caused significant mortality after 96 h, while the survival rate of insects injected with Jbtx C-ter was equivalent to that of control group. It thus became clear from these experiments that the N-terminal half of Jbtx (Jbtx N-ter) has the insecticidal domain of Jbtx. This conclusion agrees with the fact that the deletion of the β-hairpin, which is present in the Jbtx C-ter, did not interfere on the entomotoxicity.

On the other hand, all the peptides were able to induce blockade of the cockroach neuromuscular junction *in vivo* (Fig. 8). The neuromuscular blockade induced by Jbtx resembles the effect of neurotoxins which act directly on receptor ion channels [53], among which are pore-forming neurotoxins [54]. In this work we did not attempt to elucidate the pharmacological interactions of Jbtx and related peptides at specific sites of insect neuromuscular junctions. The Jbtx N-ter peptide had an effect comparable to that of the intact peptide producing almost complete neuromuscular blockade after 40 min of recordings while JbtxΔ-β and Jbtx C-ter were clearly less active. The activity loss of JbtxΔ-β in this bioassay may reflect some critical alteration of the peptide 3D-structure affecting also its N-terminal domain, which alone is capable of producing full effect in the absence of the β-hairpin.

Upon feeding to *R. prolixus* (Fig. 5B) all the peptides were lethal, even Jbtx C-ter, contrasting with its lack of activity when injected into the hemolymph. This fact points to the presence of two active domains in the Jbtx molecule, with the amphipatic β-hairpin in the C-terminal domain probably interacting with insect's gut membranes as predicted,

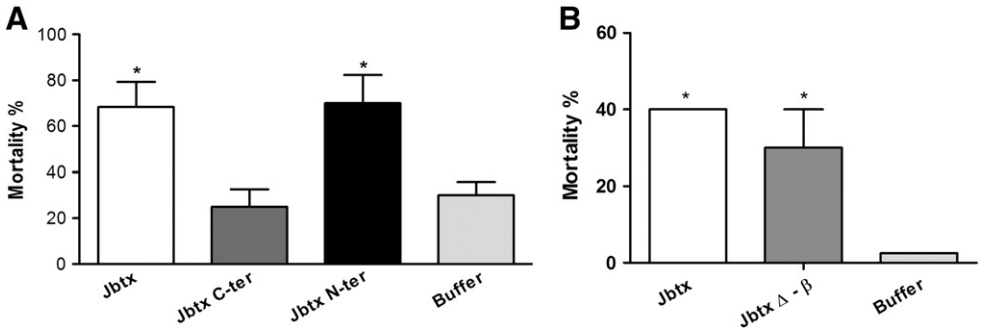

Fig. 6. Insecticidal effect of Jbtx and derived peptides on fifth instars *Oncopeltus fasciatus*. (A) Groups of 10 nymphs were injected with 1.5 μL of Jbtx, Jbtx N-ter or Jbtx C-ter into the hemocoel (dose of 0.015 μg/mg of insect body weight) or 20 mM phosphate buffer, pH 7.5 (control group). (B) Groups of 5 nymphs were injected with 1.5 μL of Jbtx or JbtxΔ-β peptides into the hemocoel (dose of 0.015 μg/mg of insect body weight) or 20 mM phosphate buffer, pH 7.5 (control group). The mortality rate was recorded after 96 h. Results are means ± standard error of triplicates of two independent experiments. (*) indicates statistically significant difference ($p \leq 0.05$) from the control group.

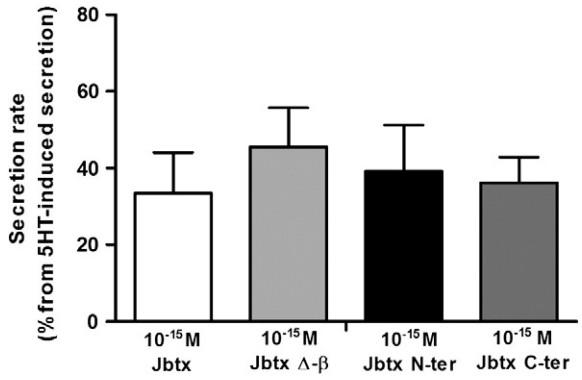

Fig. 7. Effect of jaburetox and mutants on secretion of *Rhodnius prolixus* Malpighian tubules. The assay was performed as described by Staniscuaski et al. [20]. Tubules were incubated with 2.5×10^{-8} M serotonin (5-hydroxy-tryptamine, 5-HT) for 20 min to record the maximal secretion. After washing, the tubules were incubated with the peptides (1×10^{-15} M) in the presence of serotonin for another 20 min. The secretion rate was expressed as a percentage from the control (serotonin without peptides). Results shown are means ± standard deviation of 5–6 replicates for each peptide.

when given by oral route. This conclusion could also be drawn from the facts that all the peptides had equivalent antidiuretic effects (Fig. 7) and that both terminal domains of Jbtx were able to induce leakage of vesicles (Fig. 4). The preliminary results using Planar Lipid Bilayers another artificial membrane composed only of lipids, also showed that all mutant versions of Jbtx form ion channels displaying membrane-disturbing properties (Piovesan A., unpublished data). On the other hand, Jbtx C-ter showed significantly lower activity than Jbtx or Jbtx N-ter, when its first contact within the insect was with the hemolymph probably due to a "saturating" effect of the lipid-rich medium on its membrane-disrupting ability.

All the peptides, including Jbtx C-ter, produced antidiuresis or were lethal given by oral route, circumstances where their first interaction happened with single cell layered tissues such as the Malpighian tubules [55] or the gut [56]. One hypothesis to explain the lack of specificity of these assays to discriminate the different Jbtx variants could be that biological multilayered tissue systems, such as the neuromuscular junction [57] and the whole insect (by injection, skipping the first contact with the gut), probably add additional levels of tissue- or cell specificity to the entomotoxic effects of Jbtx-related peptides. Altogether, our data indicate that the main entomotoxic domain of the urease-derived peptide Jbtx is located in its N-terminal half. However, depending on the bioassay, the C-terminal domain and/or its β-hairpin motif could also contribute part of the biological activity of Jbtx.

From the molecular dynamics simulation, it seems that the monomeric Jbtx peptide is mostly formed by coils (Fig. 2). Our simulation results confirm and expand previous theoretical observations [19], such as the compaction of the peptide in solution. Simulations of the half-peptides indicated that after 500 ns Jbtx N-ter adopts a random coil conformation while Jbtx C-ter acquires a newly-formed β-sheet (Fig. 2). These data may explain why Jbtx is highly prone to aggregation [19], and the instability of Jbtx N-ter in aqueous solution (unpublished results), possibly a consequence of the unfolding of the highly hydrophobic N-terminal of Jbtx that would require protein–protein (or protein–membrane) contact to stabilize.

Presently, to the best of our knowledge, it is not possible to compare the MD simulated structure of Jbtx to that of any other known insecticidal or membrane-disrupting peptide. The high level of coils, especially in the N-terminus, may be related to the peptide toxicity,

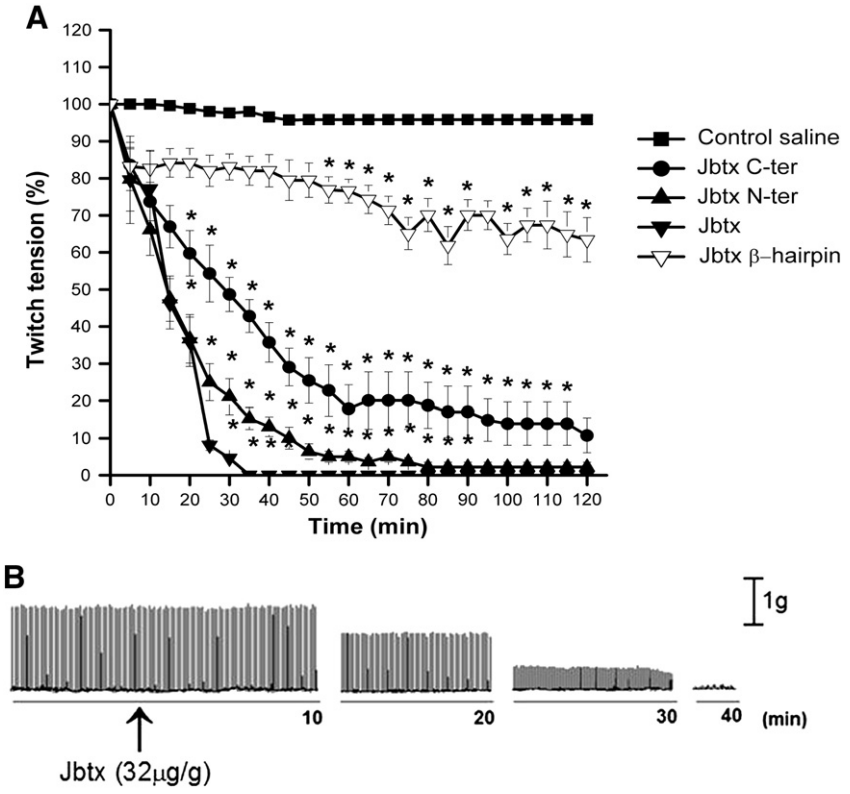

Fig. 8. Neuromuscular paralysis induced by Jbtx and peptides on *in vivo* cockroach coxal-adductor methatoracic nerve–muscle preparation. (A) Time course of the blockade of the neuromuscular activity in the presence of 32 μg/g of each version of Jbtx peptides against control insects treated only with saline (means ± standard error, n = 12). Note that Jbtx and Jbtx N-ter were able to induce complete paralysis. * indicates $p \leq 0.05$ in comparison to control saline, with ANOVA two way and Student *t* test. (B) Representative myographic 120 min recording of the coxal-adductor methatoracic nerve–muscle preparation of a Jbtx-treated cockroach.

since some toxins employ these unfolded states as recognition motifs. One example of such toxins is colicin, from *E. coli* [58]. These unfolded recognition domains may be advantageous for the toxins that carry them, since they allow these proteins to overcome steric restrictions while providing large average interaction surfaces per residue [58–60]. There are many reports in the literature of folding and oligomerization of proteins and peptides that acquire their biologically active state upon interaction with lipids or membranes. Examples are cecropin A, a 37-residue insect antimicrobial peptide [61,62], the Cyt1Aa toxin produced by *Bacillus thuringiensis* [63], anticancer β-hairpin peptides [64], antimicrobial, cell-penetrating peptides and fusion peptides such as the HIV fusion peptide FP23 [65], to cite a few.

The tendency to oligomerize and to interact with lipids exhibited by Jbtx brings the question whether the active form of the peptide (or its N-ter and C-ter versions) is an oligomer rather than a monomer. The oligomerization/aggregation phenomenon was also observed for Jbtx-2Ec, causing an enormous impact of the membrane-disruptive ability of the peptide [19].

Jbtx has promising biotechnological potential as a biopesticide. We are currently testing transgenic Jbtx expressing sugar cane (*Saccharum officinarum*) plants, and so far we have observed an increase of resistance to several species of lepidopterans in greenhouse conditions (Becker-Ritt et al., unpublished data). These data indicate the effectiveness of the peptide as an environment friendly insecticide with practical application, reducing crop losses while avoiding the use of chemical toxic agents.

We conclude that the urease-derived peptide Jbtx probably represents a new example of membrane-active peptide with insecticidal and fungitoxic activities. Its insecticidal activity was tracked down mostly to its N-terminal region and does not require the prominent β-hairpin present in the C-terminal region, although this part of the molecule probably contributes to its overall entomotoxic properties. Understanding the complex behavior of these peptides in solution as well as in the presence of lipids and biological membranes is a critical step towards unraveling their mechanisms of action and exploiting their potential as insecticidal agents.

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Authors' contributions

A.H.S.M. and K.K. constructed the mutated peptides, M.S.D. helped in the insect bioassays, A.R.P. and C.F. carried out LUVs leakage assay, F.S. run the Malpighian tubules assay, D.R.D. performed MS assays, R.L-B. and H.V. conducted molecular modeling and simulations, C.A.D.B. and C.G.M.A. tested the peptides on the cockroach neuromuscular junction, C.R.C. wrote the paper and together with G.P., conceived and supervised all the work.

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