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Canavalia ensiformis urease, Jaburetox and derived peptides form ion channels in planar lipid bilayers

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ABSTRACT

Ureases catalyze the hydrolysis of urea into NH_3 and CO_2. They are synthesized by plants, fungi and bacteria but not by animals. Ureases display biological activities unrelated to their enzymatic activity, i.e., platelet and neutrophil activation, fungus inhibition and insecticidal effect. Urease from *Canavalia ensiformis* (jack bean) is toxic to several hemipteran and coleopteran insects. Jaburetox is an insecticidal fragment derived from jack bean urease. Among other effects, Jaburetox has been shown to interact with lipid vesicles. In this work, the ion channel activity of *C. ensiformis* urease, Jaburetox and three deletion mutants of Jaburetox (one lacking the N-terminal region, one lacking the C-terminal region and one missing the central β-hairpin) were tested on planar lipid bilayers. All proteins formed well resolved, highly cation-selective channels exhibiting two conducting states whose conductance ranges were 7–18 pS and 32–79 pS, respectively. Urease and the N-terminal mutant of Jaburetox were more active at negative potentials, while the channels of the other peptides did not display voltage-dependence. This is the first direct demonstration of the capacity of *C. ensiformis* urease and Jaburetox to permeabilize membranes through an ion channel-based mechanism, which may be a crucial step of their diverse biological activities, including host defense.

Introduction

Ureases are metalloenzymes (urea amidohydrolase; EC 3.5.1.5) that display many biological functions unrelated to their enzymatic activity. The primary function of these proteins is to hydrolyze urea into ammonium and carbon dioxide [1]. Plants, fungi and bacteria produce urease while animals do not [2]. The urease from *Canavalia ensiformis*, the so-called jack bean urease (JBU), was the first enzyme ever to be crystallized [3], thus confirming the protein nature of enzymes. JBU consists of a single polypeptide chain of 840 residues and forms either trimers or hexamers in solution [4–6]. Canatoxin[2] (CNTX), an isoform of JBU [7], was shown to display a number of toxic properties as reviewed in [8]. CNTX and JBU, as well as the bacterial enzymes from *Bacillus pasteurii* and *Helicobacter pylori*, disrupt Ca^{2+} transport across membranes [9–13], bind to sialylated glycoconjugates [7] and activate blood platelets [10,12–14] and pro-inflammatory cells [15,16]. These various effects point to interactions of ureases with cell membranes, either directly or via receptor modulation.

Besides contributing to the bioavailability of nitrogen, plant ureases might participate in host defense against insects and fungal pathogens [8,17,18]. CNTX and JBU display fungicidal activity [19,20] and insecticidal effects [21–24].

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[2] *Abbreviations used:* CNTX, canatoxin; JBU, jack bean urease; Jbtx, Jaburetox; Jbtx-2Ec, Jbtx containing a V5 epitope; Jbtx N-term, C-terminal deleted version of Jbtx; Jbtx C-term, N-terminal deleted version of Jbtx; Jbtx Δ–β, β-hairpin deleted version of Jbtx; LUV, large unilamellar vesicles; PLBs, planar lipid bilayers; PE, Phosphatidylethanolamine; PC, phosphatidylcholine; POPG, 1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); Ch, cholesterol.

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The latter is mostly due to the release of a 10 kDa internal peptide upon hydrolysis of the protein by cathepsin-like enzymes in the digestive tract of susceptible insects [23,25]. Two recombinant His-tagged versions of this peptide were developed, Jbtx-2Ec [26] and Jbtx [19], differing from each other only by the presence of a V5 viral epitope in the first one, both displaying potent insecticidal activity [27].

The insecticidal effect of JBU and its derived peptides has been extensively studied in the cotton stainer bug *Dysdercus peruvianus* (Hemiptera) and in the Chagas' disease vector *Rhodnius prolixus* (Hemiptera) [17]. At doses ranging from 10^{-15}–10^{-12} M, JBU and its derived peptides induce antidiuresis in isolated Malpighian tubules of *R. prolixus*, as part of the mechanisms which lead to the insect's death [28].

The structure of the Jbtx-2Ec peptide, predicted either *ab initio* [26] or by comparative molecular modeling [29], showed a prominent β-hairpin motif which is similar to the one found in a class of neurotoxic or pore-forming peptides, i.e., *charybdotoxin* (from the venom of the scorpion *Leiurus quinquestriatus*) or *protegrin* (from porcine leukocytes) [30,31]. The presence of this β-hairpin was later confirmed in the 2.05 Å resolved crystallographic structure of JBU [32]. Using computational simulation and data from experiments on liposomes, Barros and co-workers provided the first evidence of the interaction of Jbtx-2Ec with lipid membranes [29]. Their simulation study predicted that the peptide could anchor at a polar/non polar lipid interface. Furthermore, when carboxyfluorescein-loaded large unilamellar vesicles (LUV) were exposed to the peptide, leakage of the fluorescent probe was observed, suggesting that the peptide interacted with LUV's membranes either by disrupting the lipid bilayer or by pore formation, or both. Preliminary experiments conducted in planar lipid bilayers (PLBs) supported the channel mechanism of membrane permeabilization by Jbtx-2Ec [33]. Later, structure–function relationships studies were performed using mutated versions of Jbtx, either lacking the β-hairpin region (Jbtx -β), or corresponding to its N-terminal (Jbtx N-ter) or C-terminal (Jbtx C-ter) domains. These studies demonstrated that only part of the membrane-disturbing activity of Jbtx could be assigned to the amphiphylic β-hairpin and that the N-terminal domain alone is responsible for the insecticidal property of the peptide [27].

Based on the evidences of lipid membrane permeabilization by Jbtx and the several cellular effects involving membrane recognition displayed by CNTX and other ureases, it was hypothesized that these proteins interact with lipid membranes through a pore-forming mechanism. The present study was designed to investigate and characterize, using an electrophysiological approach and PLBs, the membrane permeabilization process induced by JBU and Jbtx. Furthermore, by using three Jbtx deletion mutants, we aimed to explore the structure–function relationships implicated in its ability to interact with membranes. The results of this work provide critical insights into the membrane bioactivity of these molecules, contributing to the overall understanding of the mechanism of action of JBU and related peptides.

Materials and methods

Chemicals

Phosphatidylethanolamine (PE), phosphatidylcholine (PC), 1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (POPG) and cholesterol (Ch) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Jack bean urease type C3 was purchased from Sigma Aldrich (St. Louis, MO, USA) and further purified as in [22]. It was dissolved in 20 mM sodium phosphate buffer, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 7.5, and quantified by absorbance spectroscopy at 280 nm (0.604 A_{280} was considered equivalent to a 1.0 mg/ml protein solution). Solutions of JBU (in its 540-kDa hexameric form) at 0.5–1 mg/ml were used as stock solutions for the assays. All other chemicals used were of analytical grade.

Expression and purification of Jbtx and derived mutants

Jbtx (wild-type, 92 amino acids, 11.2 kDa) and site-directed deleted mutants of Jbtx were produced according to [19] and [27], respectively. The mutated versions of Jbtx were: Jbtx N-ter, corresponding to the first 43 amino acids of the peptide, Jbtx C-ter, corresponding to the last 49 amino acids of Jbtx (AA 44–92), and Jbtx -β, a mutant without the β-hairpin central region and corresponding to the first 61 and the last 19 amino acids of the Jbtx sequence (AA 1–60–AA 73–92) [27]. All the mutants were ligated in Nde*I* and Xho*I* restriction site of pET-23a vector, resulting in peptides carrying two residual aminoacids (LE) of Xho*I* site and six histidine tag at the carboxi end. A schematic representation of these peptides (without the residues from restriction sites) is shown in Fig. 1. Solutions of the peptides (assumed to be in their monomeric form) at 1 mg/ml were used as stock solutions for the assays.

Planar lipid bilayers

The experimental technique used in this study was very similar to that described before [34,35]. Lipid mixtures of PE:PC:Ch (7:2:1, wt/wt) or PE:POPG (4:1, wt/wt) were dissolved in decane to a final concentration of 25 mg/ml. Lipid bilayers were painted on the 250 μm circular aperture of a Delrin cup constituting the *cis* chamber (800 μl) of the experimental apparatus. Bilayer painting was performed with tip-occluded, pre-pulled Pasteur pipettes dipped in the lipid mixture solution. This solution was also used to pretreat the aperture of the Delrin cup. In experiments with JBU, which proved to be extremely slow to insert into membranes using the regular procedure (it took one hour or more for channel activity to be observed), the pretreatment lipid mixture was enriched with the protein (4 μg JBU/mg lipids). Thinning of the membrane was observed with a low power binocular dissecting microscope. Bilayer formation was monitored by capacitance measurement. Typical membrane capacitance ranged between 150 and 200 pF. Once formed, the bilayers remained stable for several hours.

Electrophysiological assays

Electrical connections to the *trans* (1.6 ml) and *cis* (800 μl) compartments separating the membrane were made by Ag/AgCl electrodes and salt bridges made of glass pipettes filled with 1% agar and 3 M KCl to minimize liquid junction potentials. Voltage was applied to the *cis* compartment and the *trans* compartment was grounded. After bilayer formation, various levels of holding voltages were applied across the membrane for at least 30 min to ensure that there was no contaminant-induced channel activity. Incorporation of the test molecules into the lipid bilayer was performed by adding aliquots of the protein, peptide or mutants directly to the *cis* chamber. Channel activity was indicated by clearly resolved current jumps in response to voltage steps. Experiments were conducted either under symmetrical conditions in solutions containing 0.5 M KCl, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.5, or, for selectivity determination, under asymmetrical conditions in the above solutions in which the concentration of KCl was adjusted to 1.25 M (*cis*) and 0.5 M (*trans*). Experiments were performed at room temperature (22–25 °C).

Data recording and analysis

Single channel currents were recorded with an Axopatch-1D patch clamp amplifier (Molecular Devices, Sunnyvale, CA). Signals were digitalized with a Digidata 1200 analog-to-digital converter using Axoscope 10.2 software (both from Molecular Devices) at 50-kHz sampling frequency. They were low-pass filtered at 100 Hz and analyzed on a personal computer using Clampfit 10.2 software (Molecular Devices). Applied voltages are defined with respect to the *trans* chamber that was held at virtual ground. Positive currents, i.e., currents flowing from the *cis* to the *trans* compartment through the molecules inserted into the membrane, are shown as upward deflections in the figures.

For each applied voltage, an all-point amplitude histogram was generated and the amplitudes of the current jumps were determined by measuring the horizontal distance (in pA) between its successive peaks. The largest peak of the histogram was considered to represent the current in the main conducting state of the channels. Minor peaks were used to evaluate the amplitude of the current either in the subconducting states of the main channel or flowing through smaller channels. Current–voltage relation graphs (I/V curves) were then plotted and, when the relations were rectilinear, the channel conductances were estimated from the slopes of the linear regressions on the data points. For non-linear curves, the conductances at negative and positive voltages were obtained separately as the slopes of the linear regressions on the data points for voltages either inferior or equal to −40 mV, or superior or equal to +40 mV. The conductances obtained in each individual experiment were then averaged over the number of experiments performed under the same conditions. Ion selectivity was determined using the reversal potential values, V_R, obtained as the voltages for which the corresponding linear regressions intersected the horizontal axis of the I/V curves. The voltage dependence of the channels was evaluated by plotting the open probability of all channels (NP_o) vs the applied voltage. NP_o was obtained at each voltage by calculating the ratio of the area (A_{open}) under the Gaussian fit of the curve corresponding the open state of the channel over the total area under the all-point amplitude histogram, i.e., $NP_o = 100\text{-}A_{open}/(A_{open} + A_{closed})$.

Results are expressed as means ± SEM for the number of experiments given in the text and the figure captions.

Results

JBU, Jbtx and its three mutants formed ion channels in PLBs. Except for JBU, they partitioned easily into the membranes. In all cases, initial insertion was facilitated by applying a negative holding voltage to the bilayers. The biophysical properties of channels are listed in Table 1 and more details are provided in the following subsections.

Channel properties of JBU

Channel activity of JBU, either in PE:PC:Ch or in PE:POPG bilayers, was extremely difficult to observe without the addition of the protein to the lipid mixtures used to pretreat the aperture of the *cis* chamber. As the current records obtained in either type of membrane looked very similar, here only the JBU channel activity observed in PE:PC:Ch bilayers was analyzed. The protein formed well-resolved channels at concentrations of 10–20 μg/ml (18.4–36.8 nM). Representative traces of the current flowing through the channels under symmetrical conditions (0.5 M:0.5 M KCl, *cis*:*trans*) and the corresponding current–voltage relations are illustrated in Fig. 2, panels A and B, respectively. Three types of conductance were observed ($n = 4$), one main conductance displaying current rectification with conductance values of 79.15 ± 8.05 pS at positive voltages and 43.95 ± 3.10 pS at negative voltages, an intermediate non-rectifying conductance of 29.65 ± 5.34 pS and a smaller, non-rectifying conductance of 10.52 ± 0.22 pS. The open probability of the larger, rectifying channels is represented by the NP_o vs V plot (Fig. 2, panel C) which demonstrates that the main channels formed by JBU were voltage-dependent, being open quite more often at negative voltages. Ion selectivity was determined by conducting experiments under asymmetrical 1.25 M:0.5 M KCl (*cis*:*trans*) conditions. Under these conditions, the rectifying channels were rarely observed. The channels formed by JBU displayed a conductance of 49.95 ± 4.5 pS ($n = 4$) and their reversal potential, V_R, was −28.05 ± 7.66 mV ($n = 4$) (Fig. 2, panel D). Under the same ionic gradient

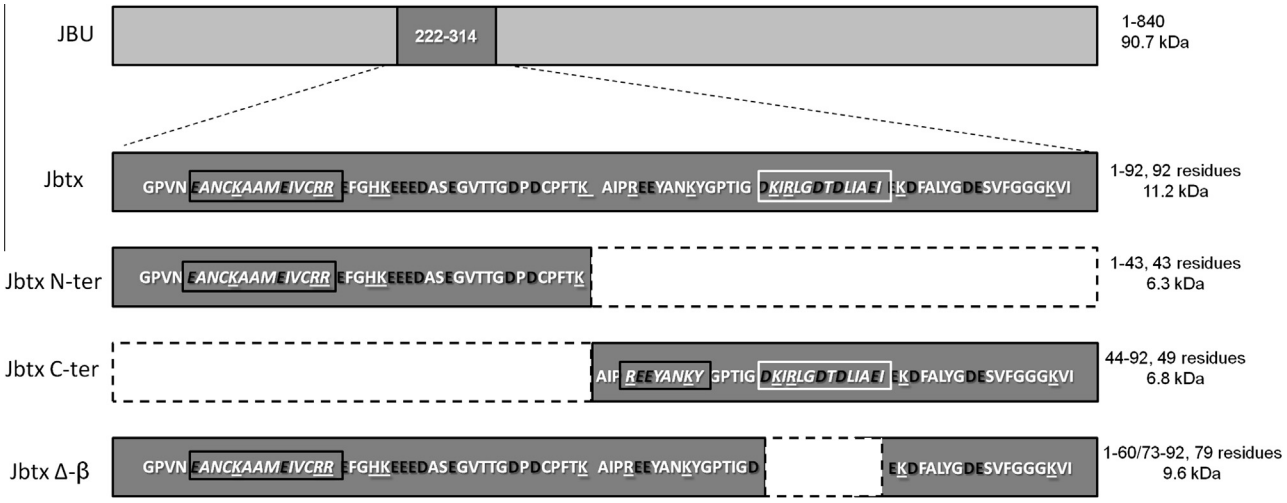

Fig. 1. Schematic representation of the primary sequences of JBU, Jbtx and the Jbtx N-ter, Jbtx C-ter and Jbtx Δ-β mutants. The sequence of Jbtx was derived from JBU isoform II, Genbank accession AF468788, as described in [26]. The location of Jbtx within JBU is shown as the darker grey area in the JBU box, starting at AA222 and ending at AA314 of the primary sequence of JBU. The AA sequences of Jbtx and its mutants are given in each of the corresponding boxes, with negatively charged residues shown in black and positively charged residues in white and underlined. The positions of the putative α-helices and β-hairpins, obtained by Swiss-Model molecular modeling [65] using JBU (3LA4) [32] as template, are indicated by black or white boxes, respectively. The number of residues, and the molecular mass of each molecule are indicated on the right side of the boxes (modified from [27]). The two residual aminoacids (LE) of restriction enzyme (Xho*I*) site and the six histidine tag at the end of all Jbtx peptides were removed for clarity.

Table 1
Biophysical properties of single ion channels formed by JBU, Jbtx, Jbtx N-ter, Jbtx C-ter and Jbtx Δ-β.

	JBU PE:PC:Ch	Jbtx PE:PC:Ch	Jbtx PE:POPG	Jbtx N-term PE:PC:Ch	Jbtx C-term PE:PC:Ch	Jbtx Δ-β PE:PC:Ch
Conductances[a] (pS ± SEM)						
Symmetrical condition	79.15 ± 8.05 (pos. voltages)	38.63 ± 4.48	45.52 ± 1.91	35.75 ± 1.15	32.10 ± 0.50	38.95 ± 3.97
*Main channel	43.95 ± 3.10 (neg. voltages)					
n[b]	4	8	4	4	5	4
*Intermediate channel	29.65 ± 5.34	–	–	–	–	–
n[b]	4					
*Smaller channel	10.52 ± 0.22	7.61 ± 1.15	18.20 ± 2.60	8.87 ± 1.27	9.30 ± 0.24	10.06 ± 0.88
n[b]	4	7	3	4	5	3
Asymmetrical condition	49.95 ± 4.50	52.62 ± 5.92	n.d.	39.10 ± 2.73	42.40 ± 5.06	n.d.
n[b]	4	4		3	3	
V_R, reversal potential (mV)[c]	−28.05 ± 7.66	−28.10 ± 5.03	n.d.	−21.80 ± 8.77	−23.15 ± 7.75	n.d.
Ease of partition[d]	+	++	+++	+++	+++	+++
P_o at −80 mV (%)[e]	83.56 ± 3.10	64.04 ± 8.87	68.80 ± 6.83	53.38 ± 4.87	66.72 ± 6.14	81.32 ± 3.31
P_o at +80 mV (%)[e]	52.67 ± 3.14	68.25 ± 9.61	73.75 ± 4.73	29.95[f]	45.21 ± 11.49	63.65 ± 13.22

[a] Determined either as conductance at full closure (when channel current returned to zero), or conductance to baseline (when at least one channel remained always open).
[b] Number of experiments used for analysis.
[c] Experiments were conducted under asymmetrical KCl conditions.
[d] Time to first detectable current step after protein or peptide addition to the PLB bath: +++ less than 15 min; ++ between 15 and 30 min; + more than 30 min.
[e] Open probability calculated following the equation $NP_o = 100A_{open}/(A_{open} + A_{closed})$. NP_o was obtained at each voltage by calculating the ratio of the area (A_{open}) under the Gaussian fit of the curve corresponding the open state of the channel over the total area under the all-point amplitude histogram.
[f] Data from one experiment only.

conditions, the Nernst potential for K^+, V_N, calculated at 25 °C, is −23.15 mV [36]. Therefore, the channels formed by JBU were strongly selective to cations.

Channel properties of Jbtx

To test the role of the membrane charge on Jbtx channel formation and properties, two different membrane compositions were used, PE:PC:Ch, as above, which has a neutral net charge, and PE:POPG, which is negatively charged. In both cases, Jbtx formed well-resolved channels within 30 min after addition of 5–15 μg/ml (0.45–1.36 μM) of the recombinant peptide to the *cis* chamber. Channel conductance was affected by the nature of the bilayer. Under symmetrical conditions (0.5 M:0.5 M KCl, *cis*:*trans*), in neutral membranes (Fig. 3, panels A and B), the main conductance of Jbtx channels was 38.63 ± 4.48 pS ($n = 8$) and the smaller one was 7.61 ± 1.15 pS ($n = 7$), while in negative membranes (Fig. 3, panel B), the main channel conductance was 45.52 ± 1.91 pS ($n = 4$) and that of the smaller one was equal to 18.20 ± 2.60 pS ($n = 3$). Neither current rectification nor voltage-dependence were observed in either type of membrane (Fig. 3, panels B, C and D). Ion selectivity of Jbtx channels was tested in PE:PC:Ch bilayers and under asymmetrical 1.25 M:0.5 M KCl (*cis*:*trans*) conditions (Fig. 3, panel E). The conductance of the main channel was equal to 52.62 ± 5.92 ($n = 4$) and V_R was shifted to −28.10 ± 5.03, which demonstrated the strong cationic nature of Jbtx channels, V_N being equal to −23.15 mV under these conditions.

Channel properties of Jbtx N-ter

Jbtx N-ter, the peptide mapping approximately the N-terminus half of Jbtx, promoted well-resolved current jumps within 30 min after addition of 5–15 μg/ml (0.85–2.55 μM) of the peptide to the *cis* chamber (Fig. 4, panel A). This mutant, contrary to Jbtx, was generally poorly active at positive voltages, which made current measurements difficult at these voltages. Under symmetrical conditions (0.5 M:0.5 M KCl, *cis*:*trans*), the channel conductances of Jbtx N-ter were equal to 35.75 ± 1.15 pS ($n = 4$) for the main channel, and 8.87 ± 1.27 pS ($n = 4$) for the smaller channel (Fig. 4, panel B). As illustrated by the NP_o plot (Fig. 4, panel C), Jbtx N-ter was highly voltage-dependent, with very little activity at positive voltages, as mentioned before. Under 1.25 M:0.5 M KCl (*cis*:*trans*) asymmetrical conditions, the channel conductance was 39.1 ± 2.73 pS ($n = 3$).and V_R was shifted to −21.8 ± 8.77 mV ($n = 3$), demonstrating the strong cationic selectivity of the N-terminal peptide (Fig. 4, panel D).

Channel properties of Jbtx C-ter

Like Jbtx and Jbtx N-ter, Jbtx C-ter, the peptide mapping the 49 C-terminus residues of Jbtx, partitioned easily in the bilayers at 5–15 μg/ml concentrations (0.76–2.29 μM) and formed well-resolved channels (Fig. 5, panel A). Under symmetrical conditions (0.5 M:0.5 M KCl, *cis*:*trans*), the main channel conductance was 32.1 ± 0.5 pS ($n = 5$) while the smaller channel conductance was equal to 9.3 ± 0.24 pS ($n = 5$). Channel activity was higher at negative voltage, but as shown on Fig. 5, panel C, this voltage-dependence was not as large as it was for Jbtx N-ter. Under asymmetrical 1.25 M:0.5 M KCl (*cis*:*trans*) KCl conditions, the conductance of the main channel was 42.4 ± 5.06 pS ($n = 5$) and V_R was shifted to −23.15 ± 7.75 mV (Fig. 5, panel D), not very different from V_N calculated under these ionic concentration conditions (Fig. 5, panel D). Thus, similar to JBU, Jbtx and Jbtx N-ter, the channels formed by Jbtx C-ter were highly cationic in nature.

Channel properties of Jbtx -β

Channels formed by Jbtx -β, the peptide derived after deletion of the β-hairpin region of Jbtx, inserted easily into the bilayers at concentrations comparable to those used with the three other peptides (Fig. 6, panel A). Similarly, two conducting states were observed under symmetrical conditions (0.5 M:0.5 M KCl, *cis*:*trans*), corresponding to one main channel conductance of 38.95 ± 3.97 pS ($n = 4$) and a smaller channel conductance of 10.06 ± 0.88 pS ($n = 3$) (Fig. 6, panel B). The channels were not voltage-dependent, with their opening probability remaining at about 60% at any voltage (Fig. 6, panel C).

Discussion

This study demonstrates, for the first time, that a plant urease and its insecticidal domain permeabilize phospholipid bilayers forming well resolved channels in planar lipid bilayers, and provides the initial characterization of the channels' signature (conductance, rectification, selectivity and voltage dependence). Our

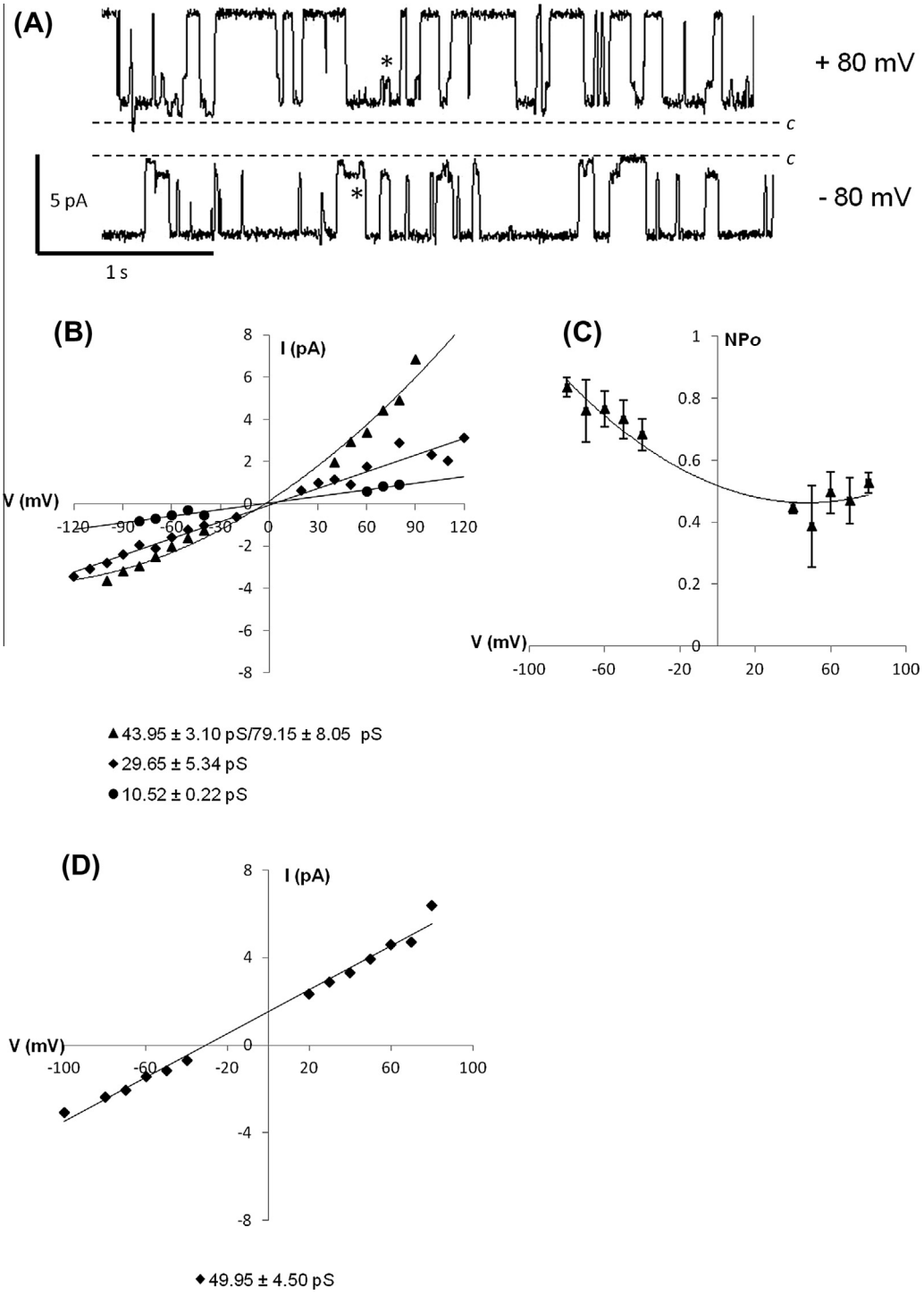

Fig. 2. Channels formed by JBU, the jack bean urease. (A) Single channel currents observed at +80 mV and −80 mV holding voltages in a typical experiment after JBU (9.2 nM) addition to the *cis* side of the bilayer under symmetrical (0.5 M:0.5 M KCl, *cis*:*trans*) conditions at pH 7.5. The letter *C* on the right side of the traces indicates the current level at which all channels are closed. Smaller channel current jumps are marked with asterisks. (B) Current–voltage relations of the channels formed by JBU in PLBs at pH 7.5 under symmetrical 0.5 mM KCl conditions. They show the presence of three conductances, two of them without rectification: 10.52 ± 0.22 pS (●, conductance corresponding to the current jumps indicated by asterisks in A) and 29.65 ± 5.34 pS (◆), and a third one (corresponding to the main channel) that rectifies the current, with conductances of 79.15 ± 8.05 pS at positive voltages and 43.95 ± 3.10 pS at negative voltages (▲), determined as described in section Materials and methods. The data obtained in 4 experiments was pooled together. (C) Open probability of the main channel formed by JBU. The data was derived from 4 experiments under symmetrical conditions as in A. (D) Under asymmetrical conditions (1.25 M:0.5 M KCl, *cis*:*trans*), at pH 7.5, the reversal potential was shifted to −28.05 ± 7.66 mV, which demonstrated the cationic selectivity of the main channel formed by JBU. The data was obtained from 4 experiments. All straight lines were fitted to the data points by linear regression (panels B and D), the rectifying line was fitted to the data points by a polynomial regression of order 3 (panel B) and the conductances (mean ± SEM) are given under the corresponding graphs. The NP_o data points were fitted by a polynomial regression line of order 2 (panel C).

group has shown that both the wild-type fragment of JBU and the corresponding recombinant peptide Jbtx are functionally similar in terms of insecticidal activity [26,27]. It is therefore clear that the LE residues and His tags of Jbtx and its mutated versions do not affect the peptide's function. But could they be responsible for channel formation of Jbtx and its mutants? This is unlikely, however,

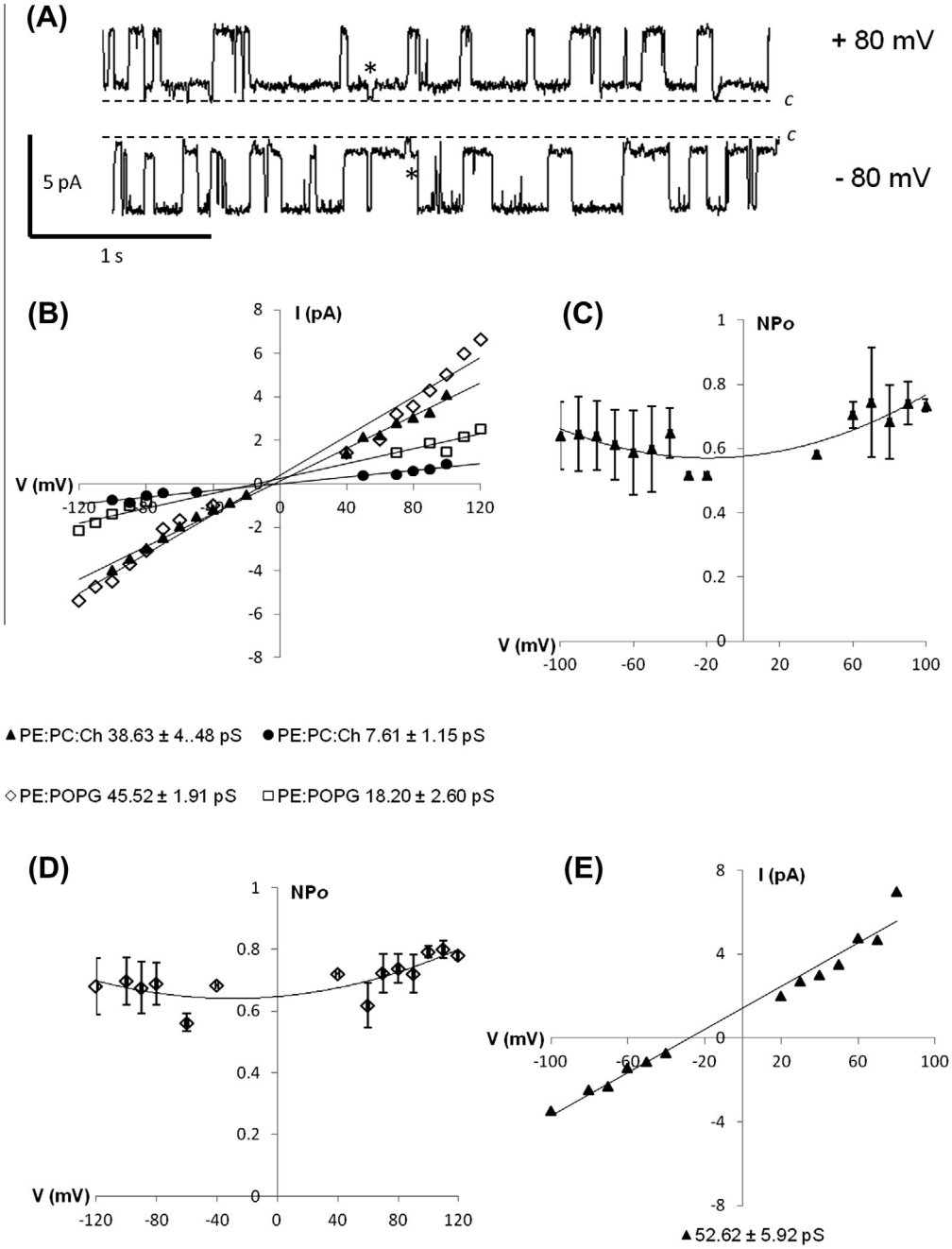

Fig. 3. Channels formed by Jbtx, the 93-residue recombinant peptide derived from JBU. (A) Single channel currents observed at +80 mV and −80 mV in a typical experiment after Jbtx (1.32 μM) addition to the *cis* side of a PE:PC:Ch bilayer under symmetrical (0.5 M:0.5 M KCl, *cis*:*trans*), pH 7.5. The letter *C* on the right side of the traces indicates the current level at which all channels are closed. Smaller channel current jumps are marked with asterisks. (B) Current–voltage relations of the channels formed by Jbtx in PE:POPG (◇ and □) or PE:PC:Ch (▲ and ●) PLBs, at pH 7.5 under symmetrical 0.5 M KCl conditions. They show the presence of four conductances: 45.52 ± 1.91 pS (◇), 18.20 ± 2.60 pS (□), 38.63 ± 4.48 pS (▲) and 7.61 ± 1.15 pS (●, conductance corresponding to the current jumps indicated by asterisks in A). The data was derived from 8 experiments in PE:PC:Ch bilayers and 4 experiments in PE:POPG bilayers under symmetrical 0.5 M KCl conditions in both cases, and was pooled together in each case. (C, D) Open probability of the main channel formed by Jbtx in PE:PC:Ch membrane (C) or PE:POPG membrane (D), respectively. The results were derived from 8 and 4 experiments under symmetrical conditions as in A, respectively. (E) In PE:PC:Ch membranes and under asymmetrical conditions (1.25 M:0.5 M KCl, *cis*:*trans*) at pH 7.5, the reversal potential was shifted to −28.10 ± 5.03 mV, demonstrating the cationic selectivity of the main channel of Jbtx. The data was obtained in 4 experiments. Straight lines were fitted to the data points by linear regression and the conductances are given under the corresponding graphs (panels B and E). The NP_o data points were fitted by polynomial regression lines of order 3 (panel C) and 2 (panel D).

because at the pH at which PLB experiments were conducted, the overall net charge of the peptides remained almost unchanged (theoretical isolectric point of 4.57 without His tag, and 5.14 with His tag) and therefore does not promote their interaction with membranes. This is also supported by studies showing that His tags do not affect membrane permeabilisation [37,38]. Finally, as demonstrated in our work, the full length urease JBU, which of course has no LE residues and no His tags, also forms channels in PLBs, that share several biophysical properties with those made by Jbtx.

This newly discovered activity of JBU reinforces its "moonlighting" nature [39], i.e., the fact that this protein has more than one function, adding ion channel activity to the other previously reported biological properties unrelated to its urea-hydrolyzing

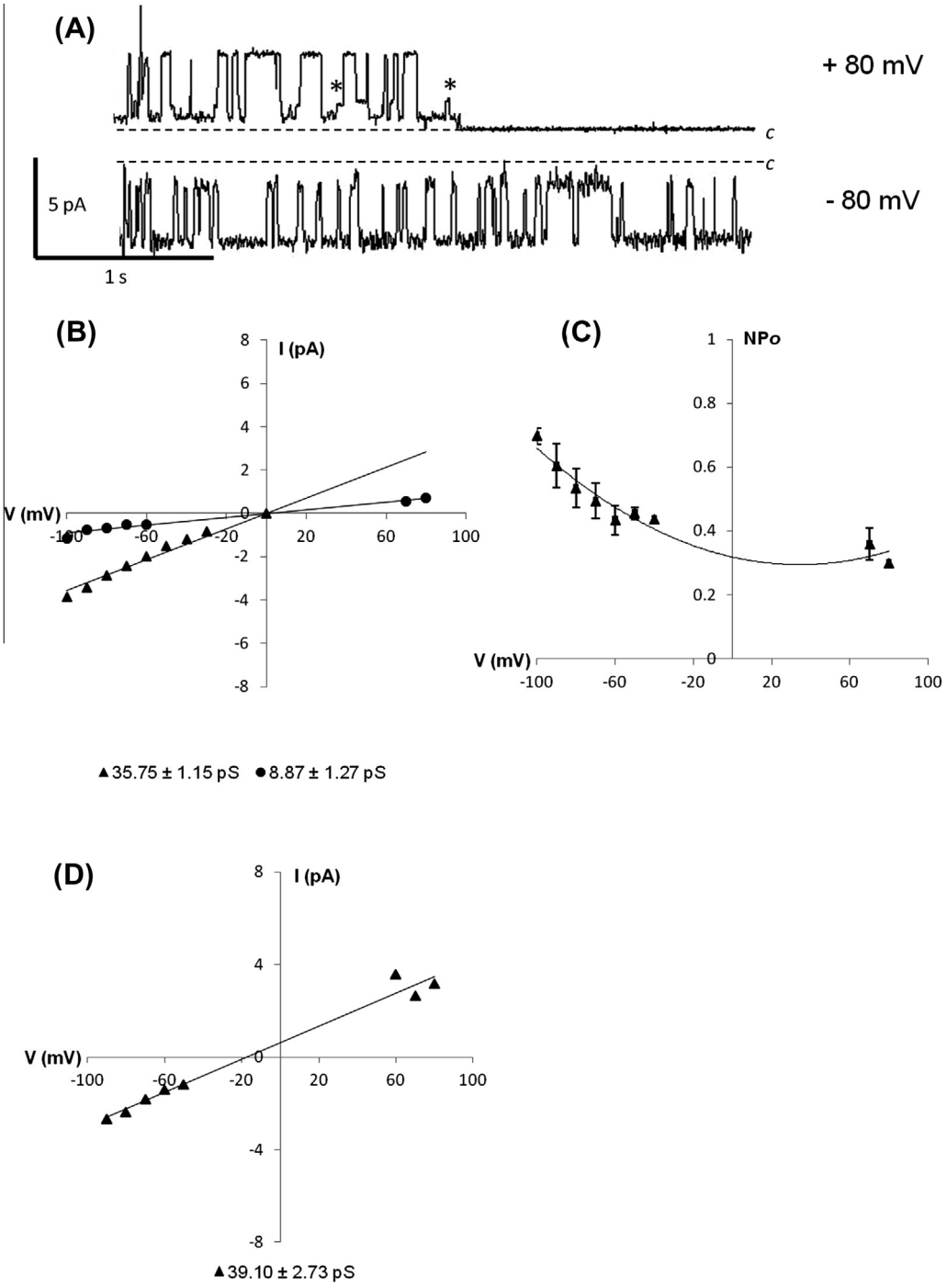

Fig. 4. Channels formed by Jbtx N-ter, the 44-residue N-terminal region of Jbtx. (A) Single channel currents observed at +80 mV and −80 mV in a typical experiment after Jbtx N-ter (2.36 μM) addition to the *cis* side of the bilayer under symmetrical (0.5 M:0.5 M KCl, *cis*:*trans*) conditions at pH 7.5. The letter *C* on the right side of the traces indicates the current level at which all channels are closed. Smaller channel current jumps are marked with asterisks. (B) Current–voltage relations of the channel activity formed by Jbtx N-ter in PLBs at pH 7.5 under symmetrical 0.5 M KCl conditions. They reveal the presence of two conductances of 35.75 ± 1.15 pS (⧫) and 8.87 ± 1.27 pS (●, conductance corresponding to the current jumps indicated by asterisks in A). The data obtained in 4 experiments was pooled together. (C) Open probability of the main channel formed by Jbtx N-ter. The data was derived from 4 experiments under symmetrical conditions as in A. (D) Under asymmetrical conditions (1.25 M:0.5 M KCl, *cis*:*trans*) at pH 7.5, the reversal potential was shifted to −21.80 ± 8.77 mV, which demonstrated the cationic selectivity of the main channel of Jbtx N-ter. The data was obtained in 3 experiments. Straight lines were fitted to the data points by linear regression and the conductances are given under the corresponding graphs (panels B and D). The NP_o data points were fitted by a polynomial regression line of order 2 (panel C).

function [8]. The channels formed by JBU, Jbtx and its derived peptides displayed similar biophysical properties. They had two conducting states ranging between around 7 pS and 18 pS (the "smaller channels") and 32 pS and 79 pS (the "main channels"), respectively, and were all highly selective to cations, represented here by potassium ions. The fact that JBU and Jbtx share similar channel properties strongly suggests that this peptide is within the pore-forming domain(s) of the urease. Indeed, structural analysis demonstrates that an extensive region of Jbtx is exposed at the surface of JBU (Fig. 7). *Bacillus thuringiensis* (*Bt*) toxin Cry3Aa is a

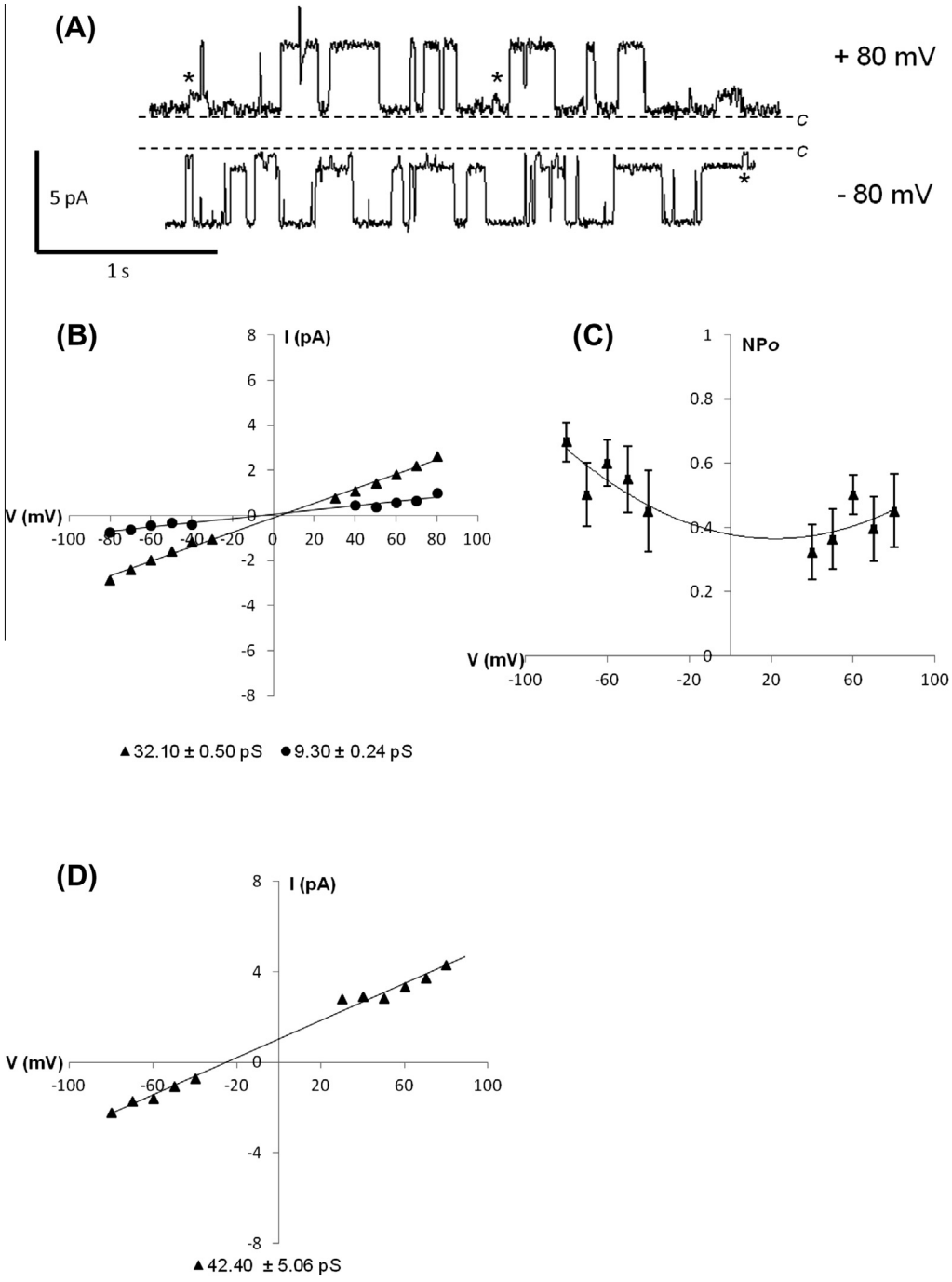

Fig. 5. Channels formed by Jbtx C-ter, the 49-residue C-terminal region of Jbtx. (A) Single channel currents observed at +80 mV and −80 mV in a typical experiment after Jbtx C-ter (2.19 μM) addition to the *cis* side of the bilayer under symmetrical (0.5 M:0.5 M KCl, *cis*:*trans*) conditions at pH 7.5. The letter *C* on the right side of the traces indicates the current level at which all channels are closed. Smaller channel current jumps are marked with asterisks. (B) Current–voltage relations of the channels formed by Jbtx C-ter in PLBs at pH 7.5 under symmetrical 0.5 M KCl conditions. They show the presence of two conductances of 32.10 ± 0.50 pS (♦) and 9.30 ± 0.24 pS (●, conductance corresponding to the current jumps indicated by asterisks in A). The data derived from 5 experiments was pooled together. (C) Open probability of the main channel formed by Jbtx C-ter. The data was derived from 5 experiments under symmetrical conditions as in A. (D) Under asymmetrical conditions (1.25 M:0.5 M KCl, *cis*:*trans*) at pH 7.5, the reversal potential was shifted to −23.15 ± 7.75 mV, which demonstrated the cationic selectivity of the main channel of Jbtx C-ter. The data was obtained in 3 experiments. Straight lines were fitted to the data points by linear regression and the conductances are given under the corresponding graphs (panels B and D). The NP_o data points were fitted by a polynomial regression line of order 2 (panel C).

good example of a protein in which the domain 1 region alone is responsible for pore formation in PLBs by the whole protein [40].

The range of conductance measured for JBU, Jbtx and its mutants is common for physiological potassium channels [36] and similar to that of channels formed by 100–500 nM concentrations of the *Bt* insecticidal protein Cry1Aa in receptor-free PLBs [41]. Small conductance channels were also described for other pore-forming proteins, such as colicin E1 from *Escherichia coli*, which forms small 30 pS channels, but also large, 480 pS channels [42]. Actually, conductances observed for many pore-forming toxin are usually larger, often in the 300 pS to 1–2 nS range. On the other hand, cation selectivity is shared by several pore-forming toxins,

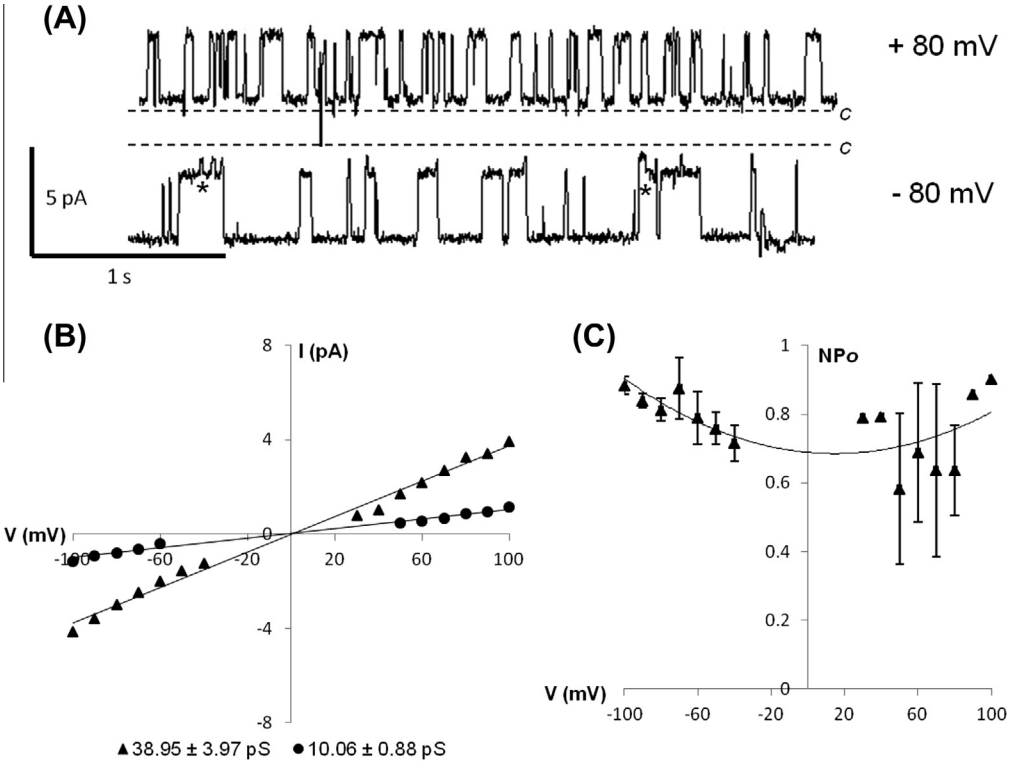

Fig. 6. Channels formed by Jbtx Δ-β, the 79-residue peptide derived from Jbtx in which the β-hairpin region was deleted. (A) Single channel currents observed at +80 mV and −80 mV in a typical experiment after Jbtx Δ-β (1.53 μM) addition to the *cis* side of the bilayer under symmetrical (0.5 M:0.5 M KCl, *cis*:*trans*) conditions at pH 7.5. The letter *C* on the right side of the traces indicates the current level at which all channels are closed. Smaller channel current jumps are marked with asterisks. (B) Current–voltage relations of the channels formed by Jbtx Δ-β in PLBs at pH 7.5 under symmetrical 0.5 M KCl conditions. They show the presence of two conductances of 38.95 ± 3.97 pS(⧫) and 10.06 ± 0.88 pS (●, conductance corresponding to the current jumps indicated by asterisks in A). The data from 4 experiments was pooled together. (C) Open probability of the main channel formed by Jbtx Δ-β. The data was derived from 4 experiments under symmetrical conditions as in A. The NP_o data points were fitted by a polynomial regression line of order 2 (panel C).

but the pores are usually not very selective [43], as exemplified by *Bt* toxins [34,44].

Jack bean urease is not the first insecticidal plant toxin described as capable to form pores. Thionins are small peptides (~5 kDa) toxic to fungi, bacteria and mammalian cells. They appear to act directly on membranes [45], either by disruption or by cation-selective ion channel formation [45–47]. Some plant defensins are also membrane-disturbing molecules [48,49], but despite its 3 cysteine residues, Jbtx shares no sequence identity to these polypeptides. The active doses reported in this work for JBU (18–30 nM) and for Jbtx and derived peptides (0.7–2.5 μM) are in the same range of those reported in the literature for plant pore forming proteins [50].

As indicated above, the conductances of JBU and the Jbtx peptides belonged to two classes: those of the main channels and those of the smaller channels. The latter may represent subconducting states of the main channels. The fact that more than one conducting state was observed is far from being unique in the field of pore-forming toxins (PFT) [43,51]. Whereas in the present study the analysis was restricted only to records corresponding to the activity of presumably one main channel, multiple levels of currents were observed at higher doses, which may have corresponded to the presence of several identical channels in the membrane, or, alternatively, to the simultaneous activity of oligomers of different sizes. Indeed, the tendency of JBU and Jbtx to oligomerize and how this affected some of their biological properties were previously reported [27,29,52]. Iacovache and coworkers [53] suggested that pre-oligomerization was an important step for some pore-forming proteins before inserting into membranes because of the resulting structural rearrangement that would provide the required hydrophobicity. On the other hand, oligomerization could also take place within the membrane after monomer insertion, as has been demonstrated for *Bt* insecticidal toxins [54,55]. Barros and co-workers [29] proposed that Jbtx-2Ec was able to interact in a parallel orientation at a polar/non-polar interface or to arrange itself in a transmembrane configuration. As noted in their work, Jbtx-2Ec (as well as Jbtx, [27]) is amphipathic, a feature considered essential for membrane activity of pore-forming peptides [56].

Jbtx formed ion channels in both zwitterionic (PE:PC:Ch) and negatively charged (PE:POPG) membranes. In the latter, insertion of the peptide was easier and the channel conductances were larger. The affinity of Jbtx for negatively charged membranes suggests that anionic lipids may constitute the actual receptors of the toxin in target cells. Previously, we reported the interaction of JBU with negatively charged polysialogangliosides (GD1b and GT1b) and with sialoproteins (mucin, tireoglobulin and fetuin) at the surface of erythrocytes and after absorption onto ELISA microplates [7,57]. For some pore forming toxins, lipids *per se* constitute the specific receptors responsible for binding of the toxins as an early step of intoxication. This is the case for cholera toxin, which binds very selectively to GM1gangliosides on the apical membrane of intestinal cells [58,59], and for the Cry5B *Bt* toxin, which docks on glycolipids of *Caenorhabditis elegans* nematodes [60].

Considering the data presented here and all previously available information, it is not yet possible to distinguish whether the protein or the peptide modulate pre-existing ion channels in biological membranes or interact directly with the cell membrane by forming ion channels *de novo*, or both. Further studies using lipids or gangliosides, or both, fused into phospholipid membranes, either PLBs

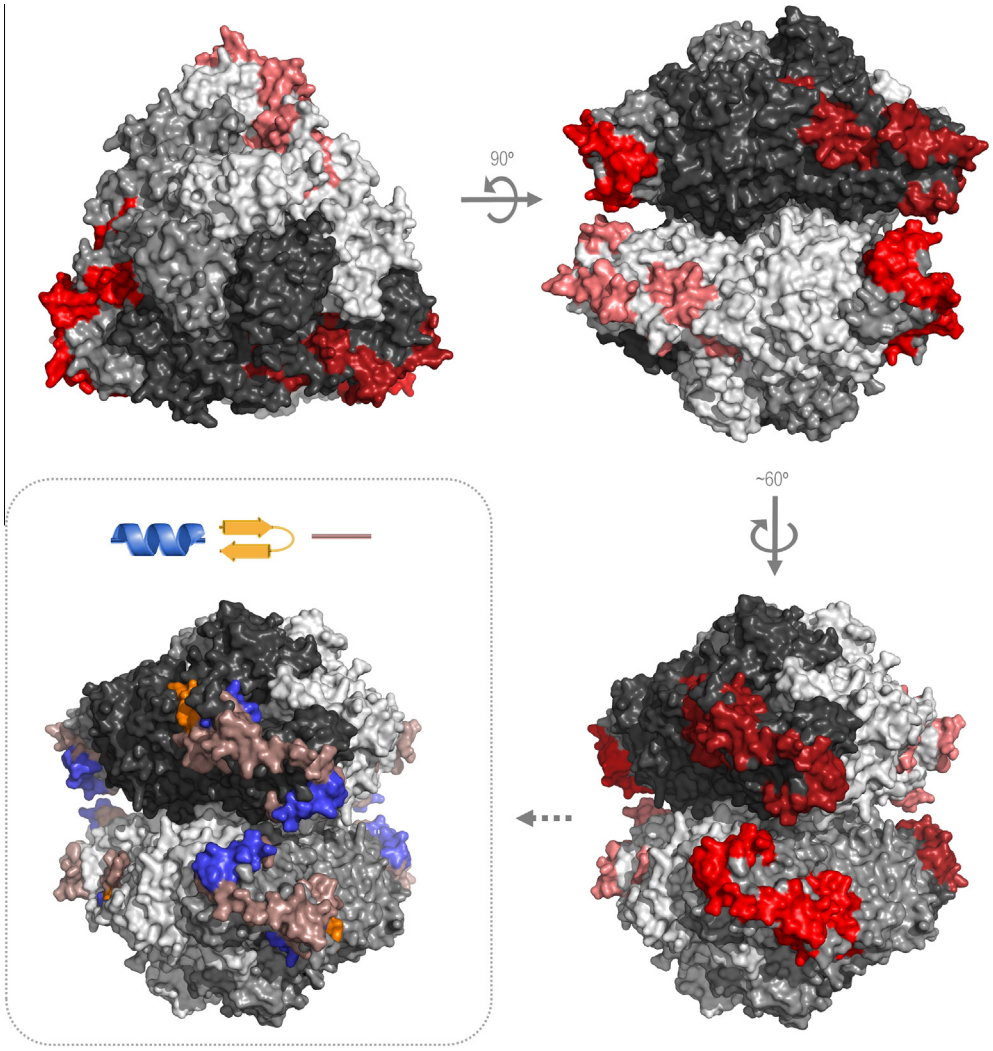

Fig. 7. Jbtx residues exposed at the surface of JBU. The region comprising the Jbtx peptide [26] was identified in the native 2.05 Å resolution atomic structure of JBU (PDB ID 3LA4, [32]) using PyMol (Schrödinger, LLC). The molecular surface of the JBU hexamer is shown in different orientations (two upper panels and right lower panel). Urease monomers are colored in different shades of gray, while the peptide regions are colored in reddish hues. The box (left lower panel showing the hexamer in the same orientation as in the right lower panel) highlights different parts of the secondary structure of the Jbtx moiety (colored according to icons above the structure). (For the colour version of this figure, the reader is referred to the web version of this article.)

or liposomes, will be required to better mimic the conditions found in actual biological membranes. Further work is also needed to investigate how voltage affects the insertion of the molecules into the bilayers and on the strong voltage-dependency of the channels formed by JBU and Jbtx N-ter.

Interestingly, Jbtx -β, the Jbtx mutant lacking the β-hairpin region, formed channels in PLBs like JBU, Jbtx and its two other mutants. This result was quite unexpected. Based on the 2.05 Å resolution atomic structure, molecular modeling and dynamics applied to Jbtx, Balasubramaniam and coworkers [32,61] proposed that a structural arrangement of the β-hairpin region (which maps similar regions in Jbtx and Jbtx C-ter, i.e., ^{281}DKIRLGDTDLIAEI^{294}, as shown in Fig. 1) would be responsible for channel formation through a β-barrel configuration, in which neighboring β-hairpins, each originating from a single monomer, would be associated. However, the present work demonstrating that the deletion from Jbtx of this β-hairpin did not affect its channel forming capability even though some properties of the channels formed were altered, provides a strong argument against the β-hairpin being crucial for membrane permeabilization. Actually, the 3-D structure of JBU [32] shows that the region that maps the Jbtx peptide contains also two short α-helices located on the N-terminal side of the β-hairpin, one of them present in Jbtx N-ter (^{226}EANCKAAMEIVCRR^{239}) and the other in Jbtx C-ter (^{268}REEYANKY^{275}) (Fig. 1). Interestingly, two identical α-helices corresponding to the region ^{226}EANCKAAMEIVCRR^{239} in each molecule are found in close proximity at the surface of the hexameric JBU (Fig. 7). This raises the possibility of a cooperative behavior of these short helices in the channel forming process and goes along with the fact that it is necessary for more than one of these short helices to cross the bilayer. It is therefore possible that an oligomeric rearrangement of such helical structures could do so, resulting in channel formation and membrane permeabilization by JBU, Jbtx or the N- and C-terminal halves, an unprecedented feature for a pore-forming protein. Whereas there are many examples of pore-forming toxins that cross biological membranes by means of β-barrel structures, α-helical transmembrane pores constitute an alternate strategy common to bacterial, fungal and plant toxins that permeabilize target cells [43,53,62].

Liposome permeabilization and biological activities of Jbtx peptides were also explored by Martinelli *et al.* [27]. In that study, the two terminal domains variants of Jbtx and the β-hairpin deleted

mutant were shown to behave very similarly in inducing leakage in lipid vesicles. Consistent with a neurotoxic effect, few micrograms of either JBU, Jbtx or its variants, particularly Jbtx N-ter, were able to block neuromuscular junctions when injected into cockroaches *in vivo*. This activity resembles the effects of neurotoxins which act directly on receptor ion channels [63], among which are pore-forming neurotoxins [64]. However, only Jbtx N-ter and Jbtx Δ-β displayed insecticidal activity while Jbtx C-ter (which contains the β-hairpin) was completely inactive [27]. A possible explanation for this contrasting effect of these peptides is that biological multilayered tissues systems, such as the neuromuscular junction and the whole insect, probably add additional levels of tissue- or cell specificity to the entomotoxic effects of Jbtx-related peptides, rendering Jbtx N-ter biologically active as opposed to Jbtx C-ter [27].

In summary, this study is the first direct demonstration of a cation-selective pore-formation mechanism employed by JBU, a *C. ensiformis* urease, to permeabilize phospholipid membranes. The region involved in this process is most likely the one that is mapped, at least partially, by the recombinant insecticidal peptide Jbtx. However the β-hairpin region of Jbtx does not appear to constitute a critical structure in the membrane permeabilizing function of the peptide, and therefore, of JBU itself. The diverse biological activities of urease and its recombinant peptide, including plant defense against insect pests, may well be related to their capacity to form pores in target cell membranes.

Authors contributions

A.R.P. and A.H.S.M. collected all experimental data. R.L.B. constructed JBU molecular models. Production of Jbtx and its mutated forms was performed in Dr. Carlini's lab, at the Center of Biotecnology, Universidade Federal do Rio Grande do Sul, and the electrophysiological work was conducted in Dr. Schwartz group, at Groupe d'Étude des Protéines Membranaires, Université de Montréal.

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