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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).