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Voltage-dependent cationic channels formed by a cytolytic toxin produced by *Gardnerella vaginalis*

Oscar Moran[1], Olga Zegarra-Moran[1], Caterina Virginio[2] and Giandomenico Rottini[2]

[1] *Laboratorio di Neurofisiologia, SISSA, Via Beirut 2/4, I-34014 Trieste, Italy* and [2] *Istituto di Patologia Generale, Università di Trieste, Via A. Fleming 22, I-34127 Trieste, Italy*

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A cytolytic toxin produced by *G. vaginalis* was incorporated in artificial membranes and giant liposomes. The toxin formed ionic channels when incorporated in lipid bilayers. The electrical properties of such channels were studied. Current records revealed a unitary conductance of 126 pS (in symmetrical 150 mM KCl). The open state probability of the cytolysin formed channels was a function of the applied membrane potential. The permeability ratio of cations to anions was estimated to be 6.5.

Cytolytic toxin; Ionic channel; Patch clamp; *Gardnerella vaginalis*

1. INTRODUCTION

Gardnerella vaginalis is an opportunistic pathogen, which is closely associated with bacterial vaginitis [1,2]. A cytolytic toxin (CTOX) produced by *G. vaginalis* has been recently described [3]. This toxin is an amphiphilic protein with a molecular mass of 61–63 kDa [3]. The CTOX has a lytic activity towards human erythrocytes and nucleated cells, such as human endothelial cells and human neutrophils [3]. Almost no effect of CTOX towards other species erythrocytes has been demonstrated [3], indicating a target specificity of the toxin. The cytolytic mechanism of the *G. vaginalis* toxin is not linked to phospholipase activity [3].

We have studied a channel formed by incorporation of a purified fraction of CTOX in lipid membranes. We report here biophysical characterization of this channel by electrophysiological methods.

2. MATERIALS AND METHODS

2.1. *Bacterial culture and CTOX production*

The CTOX was obtained from a *G. vaginalis* stain, having biochemical markers identical to those of the reference stain ATCC 14018 [4,5]. The CTOX was isolated from the culture supernatant and purified in the presence of the non-ionic detergent Tween 80 (BDH) as described by Rottini et al. [3].

2.2. *Incorporation of CTOX in small liposomes*

Asolectin (Sigma) was suspended in 10 mM Tris and 100 mM NaCl solution, pH 7.2 (50 mg/ml). After sonication, the suspension was diluted 8 times in the same buffer solution, containing CHAPS, at a final concentration of 10%. The suspension was dialysed against the Tris/NaCl solution to remove the detergent. The CTOX was incorporated into the liposomes by sonicating a mixture of 50 μg protein with 50 μl of asolectin liposomes and 200 μl of Tris/NaCl solution. The mixture was dialysed against the Tris/NaCl solution.

Correspondence address: O. Moran, Laboratorio di Neurofisiologia, ISI/SISSA, Via Beirut 2/4, I-34014 Trieste, Italy. Fax (39) (40) 224600

2.3. *Incorporation of CTOX in artificial membranes*

Artificial lipid membranes were formed in the tip of a glass pipette (2–4 μm diameter), by the method of [6]. A solution of 10 mg/ml of asolectin in *n*-hexane was dispersed (8 μl) on the surface of a 3.5 ml Petri dish. Special care was taken to wait at least 4 min before formation of the membrane, to allow the solvent to evaporate. The lipid membranes had a typical resistance of about 40 GΩ. An aliquot of 12–20 μl of the small proteoliposomes was added to the bath solution. Incorporation of channels was continuously monitored by measuring the current under voltage-clamp conditions (see 2.5.).

2.4. *Incorporation of CTOX in giant liposomes*

Giant proteoliposomes were prepared by the dehydration-rehydration technique, as described in [7,8]. A mixture of 100 μl of asolectin liposomes and 50 μl of small proteoliposomes containing the cytolysin were centrifuged at 120000 × *g* in an Airfuge (Beckman). The pellet was resuspended in 20 μl 10 mM MOPS, pH 7.4, containing 5% ethylene glycol. The suspension was deposited in a glass slice and dehydrated in a desiccator containing $CaCl_2$ for 3–4 h at 4°C. The partially dehydrated film was covered with saline buffer and incubated in a wet chamber overnight at 4°C. Giant proteoliposomes (5–100 μm diameter) were usually formed after a few hours.

2.5. *Electrical recording*

Ionic currents of proteoliposome patches or from artificial membranes formed in the pipette tip were measured using a standard patch-clamp amplifier (EPC-7, List Medical Electronics). Single channel ionic currents were recorded from excised giant proteoliposome membrane patches [9]. Pipettes were pulled from borosilicate glass capillaries (Hilgemberg). Pipettes for forming artificial membranes had a resistance of 2–5 MΩ. Pipettes for patch clamping giant proteoliposomes were Sylgard (Corning) coated and fire-polished, and had a resistance of 10 to 15 mΩ. In most of the experiments a symmetrical solution, containing (in mM): 150 KCl, 0.1 mM $CaCl_2$, 20 HEPES-KOH, pH 7.3, was used. In some patch-clamp experiments, an asymmetrical solution, in which part of the KCl was isosmotically replaced by glucose to yield a final concentration of 30 mM KCl, was used in the pipette.

The stimulation and data acquisition were performed with a 12 bits AD/DA converter (M2-Lab, Instrutech), controlled by a microcomputer (Atari, 1040ST). Before digital acquisition the output of the patch-clamp amplifier was filtered by a low-pass 4-pole Bessel filter (Ithaco, 4302) set at a cut-off frequency of 1-5 kHz. The membrane current was sampled at 5-20 kHz.

3. RESULTS

3.1. *The CTOX produces a voltage dependent conductance in artificial membranes*

The artificial membrane formed in the tip of a pipette had a high resistance, typically in the range of 30-40 GΩ. This resistance was linear in the range of −100 mV to 100 mV. The addition of small proteoliposomes containing CTOX in the bath solution modifies the resistive properties of the artificial membrane. An increase in the membrane conductance was observed after 2 or 3 min of the proteoliposome addition. This indicates the incorporation of CTOX formed channels to the artificial membrane. This increase was stabilized after 5-6 min. The membrane current tended to increase when a positive pipette potential jump was applied, reflecting an increase of the membrane conductance. The conductance increase had a time constant in the order of 3 min, and reached its steady state value in about 6 min. On the other hand, when the potential jump was negative, the membrane current absolute value tended to decrease, with a time constant of about 0.5 min, indicating a decrease of the membrane conductance (Fig. 1A). Current fluctuations were observed in the current traces. These fluctuations corresponded to a conductance value of 120-150 pS, and were more frequent when the applied potential was positive (see Fig. 1A).

The current/potential relation was constructed by estimating the steady-state currents obtained at different applied pipette potentials, as shown in Fig. 1B. It shows a non-linear behavior of the current. The slope conductance of the membrane increased when the applied membrane potential was increased. It is consistent with an increase on the open channel probability at positive applied potentials. No steady-state conductance value was reached in a potential range of −80 to +80 mV. Measurements of the membrane current out of this range were precluded because of the instability of the CTOX containing bilayer when the applied membrane potential was outside this range.

3.2. *Patch-clamp of giant proteoliposomes containing CTOX*

In most of the experiments it was possible to record single channel currents (Fig. 2A). More than one active channel in a patch was frequently observed. The open state was more frequent at positive pipette potentials than at negative pipette potentials. Therefore, the presence of more than one channel in the patch was confirmed at positive pipette potentials. These single

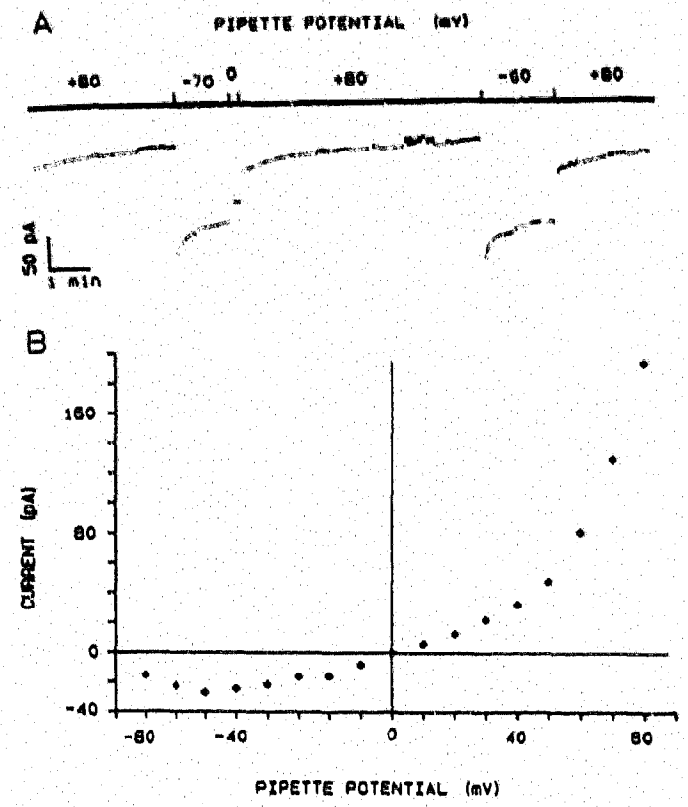

Fig. 1. (A) Macroscopic currents recorded from an artificial membrane formed in a pipette tip containing CTOX. The pipette applied potential V_m is indicated in the top of the figure. An increase of the absolute value of the membrane current is observed when V_m is positive. It reflects an increase in the open channel probability. Contrarily, a reduction of the membrane current absolute value is observed when V_m is negative, indicating a decrease of the open channel probability. (B) Current/voltage relationship of steady-state macroscopic membrane currents. An increase of the slope conductance is observed when V_m is increased. Symmetrical 150 mM KCl.

channel currents corresponded to a conductance of 128.6 ± 3.4 pS, as measured in 5 different experiments (Fig. 2B). This main conductance value was independent of the potential. Occasionally, a lower conductance value of 70-80 pS was also observed. The scarcity of the events of this lower conductance made further characterization difficult.

The duration of the open and shut states was significantly changed by the applied pipette potential. The open time was increased at positive applied pipette potential, while the shut time was increased at negative pipette potential. A mean open time of about 18 ms and a mean shut time of more than 400 ms were estimated at an applied pipette potential of −80 mV. These figures were roughly inverted when the pipette potential was inverted.

In order to investigate the ionic selectivity of the channels formed by CTOX, a set of experiments using asymmetrical solutions was performed. In these experiments, the total ionic concentration was isosmotically

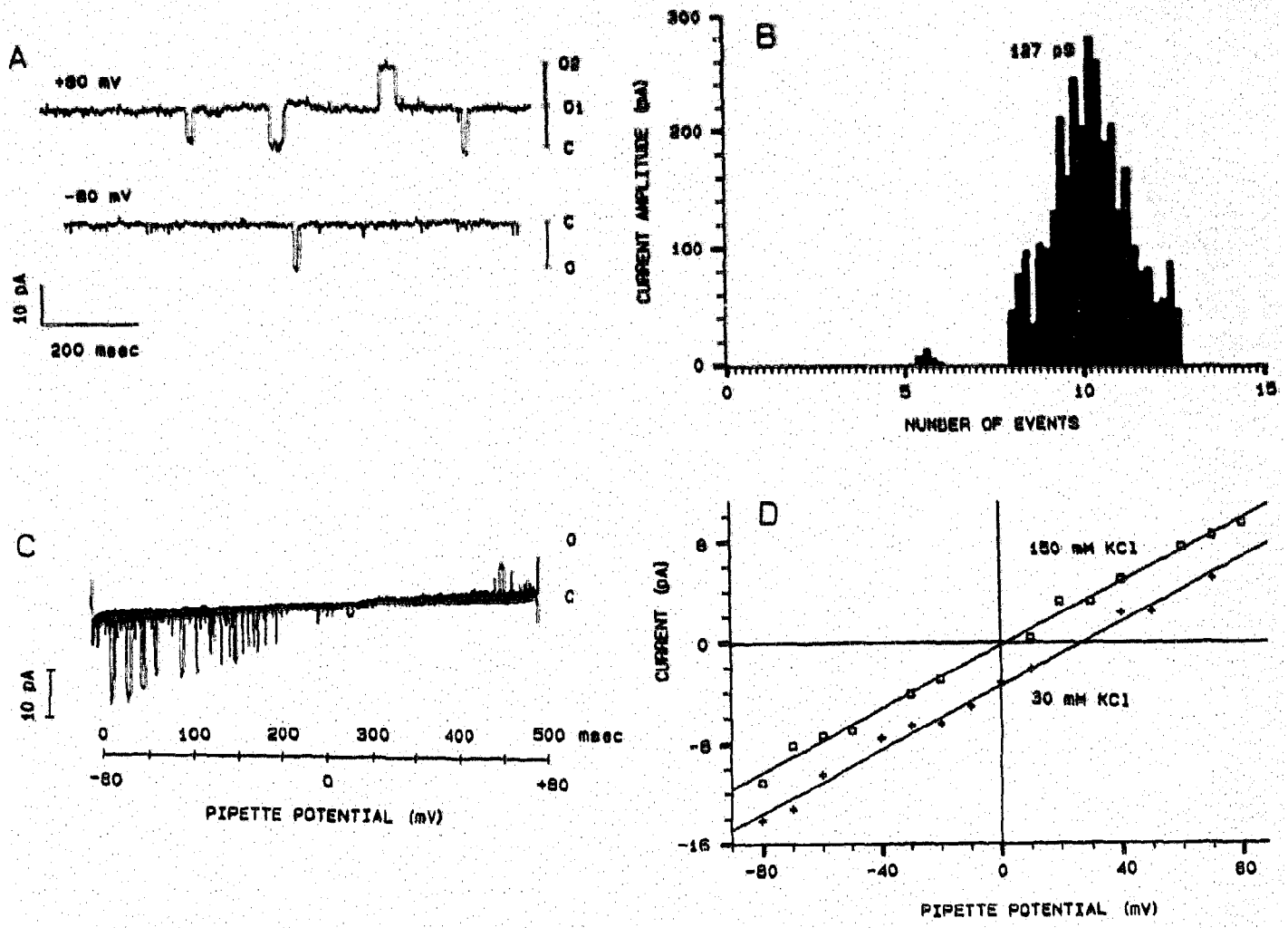

Fig. 2. (A) Single channel currents recorded from a giant proteoliposome membrane patch. The applied pipette potential is indicated in the figure. C and O correspond to the close and open states. (B) Current amplitude histogram of single channel currents recorded at an applied pipette membrane potential of +80 mV. (C) A superposition of single channel events evoked by a potential ramp from −80 mV to +80 mV (in 500 ms), in asymmetrical solutions. (D) Single channel current/pipette potential relations measured in 150 mM KCl symmetrical solution and with asymmetrical solution. The pipette solution is indicated in the figure.

reduced in the pipette, as described in Materials and Methods. In order to have a good estimate of the reversal potential, a ramp of potential was applied to the patch. The voltage dependence of the single channel events can be also easily visualized from this protocol (Fig. 2C). The reverse potential of 0 mV found in experiments using 150 mM KCl symmetrical solutions was shifted to +25 mV of pipette potential when the 30 mM KCl solution was in the pipette. This shift corresponds to a channel with cationic selectivity. The cation to anion permeability ratio was calculated, applying the Goldman-Hodgkin-Katz equation. It resulted in a cationic to anionic permeability ratio of 6.5.

4. DISCUSSION

We have described the channel-forming properties of the *G. vaginalis* cytolytic toxin. As the CTOX tends to aggregate in aqueous solution, a detergent solubilized fraction had to be used to incorporate it into small liposomes. The success of this incorporation demonstrates that, at least, the channel-forming conformation of the protein is not dependent on the possible target specificity mechanisms of the CTOX [3].

A voltage dependent cationic channel was formed when the CTOX was incorporated in artificial membranes or in giant proteoliposomes. When small proteoliposomes containing the CTOX were fused to artificial membranes, a massive incorporation of the protein, resulting in a voltage-dependent macroscopic current was observed. Under these conditions, it was also possible to record single channel events in some cases, with the same conductance values of those recorded by patch-clamping giant proteoliposomes.

Although the channel formation was obtained in ar-

tificial conditions, the possibility that a pore establishment would be the primary event of the lytic properties of the CTOX is quite attractive. This hypothesis can be put forward based on three arguments: (1) Other bacterial cytolysines are able to form pores in the plasma membrane in physiological conditions [10,11]. (2) The lysis mechanism of CTOX is not linked with phospholipase activity [3]. (3) The cationic selectivity of the channel formed by CTOX could imply a Ca^{2+} influx to the cell, with the consequent intracellular proteases activation [11]. In fact, a pore diameter estimation based on the single channel conductance [12] yields a value of 30 Å, which would fit well with a Ca^{2+} permeability.

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