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What planar lipid membranes tell us about the pore-forming activity of cholesterol-dependent cytolysins

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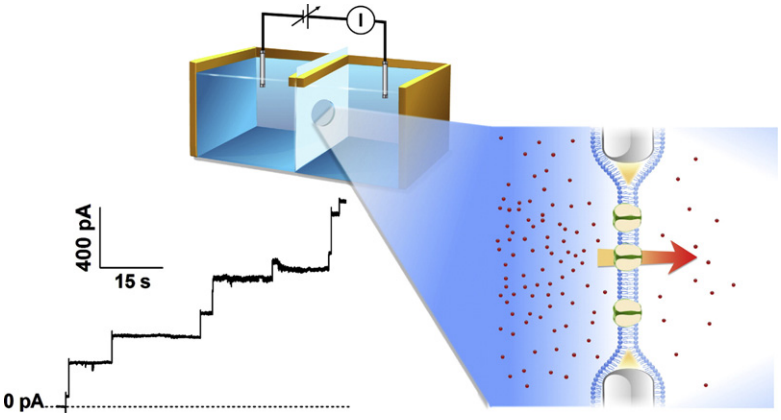
HIGHLIGHTS

- Pore-forming toxins (PFTs) are used for attack and defense by many organisms.
- Cholesterol-dependent cytolysins (CDCs) are an important toxin family from bacteria.
- The planar lipid membrane approach provides information on activity of PFTs.
- Published studies of CDC reflect different ways on how pores are formed.

GRAPHICAL ABSTRACT



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ABSTRACT

Pore-forming toxins are an important group of natural molecules that damage cellular membranes by forming transmembrane pores. They are used by many organisms for attack or defense and similar proteins are employed in the immune system of vertebrates. Various biophysical approaches have been used to understand how these proteins act at the molecular level. One of the most useful, in terms of monitoring pore formation in real time, is a method that employs planar lipid membranes and involves ionic current measurements. Here we highlight the advantages and possibilities that this approach offers and show how it can advance understanding of the pore-forming mechanism and pore properties for one of the most important families of natural toxins, the cholesterol-dependent cytolysins.

Abbreviations: AFM, atomic force microscopy; CDC, cholesterol-dependent cytolysins; Chol, cholesterol; D, domain; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPhPC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; LLO, listeriolysin O; PC, phosphatidylcholine; PFO, perfringolysin O; PFT, pore-forming toxin; PLM, planar lipid membrane; PLY, pneumolysin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TMH, transmembrane β-hairpin; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(N-morpholino)ethanesulfonic acid.

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1. Introduction

Pore-forming toxins (PFTs) are an important class of proteins produced mainly by bacteria, where they constitute the biochemical arsenal for attack or defense. PFTs are secreted as monomeric, water soluble proteins that oligomerize on the target cell membrane, thereby producing structured, nanometer-size pores (reviewed in [1–4]). Well-studied PFT families include aerolysin and related toxins, staphylococcal α-toxin and related toxins, cholesterol-dependent cytolysins (CDCs), colicins, actinoporins from sea anemones, and others [1,3]. PFTs induce cell death, either by directly impairing cell membrane integrity or by facilitating internalization of other toxic molecules. Much effort has been devoted to visualizing their structure directly and clarifying the mechanism of pore assembly on cell membranes. Pore-forming activity can easily be investigated by determining red blood cell hemolysis or the release from cells of lactate dehydrogenase, as well as by the more sophisticated electrophysiological technique of patch-clamp. The use of well characterized lipid membrane model systems has been employed successfully with PFTs, since the oligomerization of monomers and the coordinated conformational changes leading to assembly of pores are often triggered solely by the presence of a lipid bilayer. All environmental conditions can be finely controlled and changed with lipid model systems, which therefore become very useful and significant cell-membrane mimicking systems. Liposomes have been used in leakage assays to study membrane permeabilization, investigate special lipid requirements, estimate pore dimensions, etc. (reviewed in [5]). Electrophysiology on planar lipid membranes (PLM) has been extensively engaged for the functional characterization of many membrane interacting proteins and peptides, including PFTs as outlined below and in [6]. In this review we present (i) the PLM approach for studying the properties of pores and mechanism of pore formation, (ii) CDC protein family and (iii) how PLM helps to understand the pore-forming activity of CDCs.

2. Measuring pores directly

PLM is a highly sensitive method that enables single channel openings to be studied in real time (reviewed in [6,7]). The technique includes recording the ionic current passing through a stable lipid bilayer of controlled lipid composition. Typically, the bilayer is formed on a small aperture (100 μm diameter) made in a thin (25 μm) Teflon septum separating two chambers. The two chambers mimic the intra- and extra-cellular spaces. They are filled with an ionic solution and connected to an electronic system with two Ag–AgCl electrodes that permit the application of a stable electrical potential (in the range of tens of mV) and the recording of the ionic current passing through the membrane. The protein, added in one chamber only, can interact with the phospholipid bilayer, oligomerize and form pores (Fig. 1A). Typically, each single pore is detected in real time as an abrupt increase in the current. Successive pore insertions produce characteristic, step-like increments in current, as highlighted in Fig. 1B. PLM is a single molecule technique since each jump corresponds to the opening of a single active pore.

PLM allows several parameters that could influence PFT activity to be readily controlled and varied. Lytic ability of toxins can be observed under various environmental conditions, like salt concentration and composition, pH, temperature, transmembrane potential, as well as membrane lipid composition (either in a symmetric or asymmetric leaflet configuration). Any structural–functional correlation can be highlighted by including binding molecules and/or potential inhibitors of toxin activity.

PLM allows characteristic biophysical features of the pore to be determined, such as its size, ionic selectivity and voltage dependence. Pore size can be estimated from the single pore conductance (G), defined as the ratio of the amplitude of a single current jump (I) to the applied potential (V). Considering the pore as a cylindrical hole, its conductance is directly proportional to the pore area, as expressed in Eq. (1)

$$G = \frac{\sigma A}{L}, \tag{1}$$

where A is the area of the pore, L its length, and σ the conductivity of the buffer.

Pore selectivity can be analyzed by measuring the reversal potential, i.e. the electrical potential giving zero current, under asymmetric buffer conditions (e.g. different salt concentrations in the two chambers). Using the standard Goldman–Hodgkin–Katz equation [7], the permeability ratio between cations and anions (P_{K^+}/P_{Cl^-}) can be calculated as:

$$\frac{P_{K^+}}{P_{Cl^-}} = \frac{[a_{Cl^-}]_t - [a_{Cl^-}]_c\, e^{\frac{FV_{rev}}{RT}}}{[a_{K^+}]_t\, e^{\frac{FV_{rev}}{RT}} - [a_{K^+}]_c} \tag{2}$$

where RT/F is 25 mV at room temperature, V_{rev} is the reversal potential, a_i is the activity of ion i, and c and t correspond to the *cis* and *trans* chambers.

PLM can also be used to monitor the transport of macromolecules through the pores [8–10]. In this way, neutral polymers may be detected during their partition into the nanopore lumen. The decrease in the ionic current is related to polymer dimensions and provides information on the geometry of the pore [11]. The introduction of large molecules into the pore lumen may serve as convenient molecular adapters for increasing the sensitivity of detection of small specific analytes [12]. Interestingly, DNA can also be detected by nanopores reconstituted in PLM. The first and simplest approach for DNA sequencing is to transfer the charged molecule through the pore, driven by an applied electric potential. The passage of each nucleotide through the narrowest pore restriction partially occludes the pore, causing a characteristic reduction of the ionic current. The improved nanopore-based approaches exploit different chemical modifications or protein-derivatized nanopores in order to increase the yield of nucleotide transfer through the pore and to increase the residence time for permitting a more robust base recognition. In particular, an exonuclease has been linked to the top of the pore, allowing more specific interaction with DNA and the release of single nucleotides inside the pore [13]. Alternatively, a polymerase protein placed in the upper part of the pore structure has been used to synthesize a new RNA molecule from the template, thus releasing specific tags inside the pore [14]. More recently, nanopore technology has been exploited for protein detection [15] and drug screening. There have been some improvements on how this single molecule approach can be used in biomedical applications in high-throughput format, creating a nanopore 'micro-chip'. Another recent PLM application is the development of microfluidic devices that allow easier manipulation of environmental conditions (e.g. buffer exchange [16]) and also permit parallelization for drug screening at the single pore level [17]. The use of hydrogels is becoming popular for stabilizing the membrane bilayer in the devices. The hydrogel supported membrane itself becomes a micro-system for pore-forming studies at the molecular level, and even at the optical level [18], as well as for mimicking the electrical circuit [19]. The recently developed nanodroplet approach enables simultaneous optical and electrophysiological detection of single pores and ion movements (e.g. movements of Ca^{2+} ions through a single α-toxin pore [20]) and kinetics of multimeric pore assembly [21]. The nanodroplet system has been used by Fischer and coworkers to provide an elegant demonstration of transport of a macromolecular lethal factor through a single anthrax protective antigen pore [22].

A technique that allows simultaneous electrophysiological and structural analyses would be an effective method by which to study the mechanism of CDCs and PFT pore-formation. However, this remains a significant challenge. At present, the best option is to