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Probing membrane permeabilization by the antimicrobial peptide distinctin in mercury-supported biomimetic membranes

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ABSTRACT

The mechanism of membrane permeabilization by the antimicrobial peptide distinctin was investigated by using two different mercury-supported biomimetic membranes, namely a lipid self-assembled monolayer and a lipid bilayer tethered to the mercury surface through a hydrophilic spacer (tethered bilayer lipid membrane: tBLM). Incorporation of distinctin into a lipid monolayer from its aqueous solution yields rapidly ion channels selective toward inorganic cations, such as Tl^+ and Cd^{2+}. Conversely, its incorporation in a tBLM allows the formation of ion channels permeable to potassium ions only at non-physiological transmembrane potentials, more negative than −340 mV. These channels, once formed, are unstable at less negative transmembrane potentials. The kinetics of their formation is consistent with the disruption of distinctin clusters adsorbed on top of the lipid bilayer, incorporation of the resulting monomers and their aggregation into hydrophilic pores by a mechanism of nucleation and growth. Comparing the behavior of distinctin in tBLMs with that in conventional black lipid membranes strongly suggests that distinctin channel formation in lipid bilayer requires the partitioning of distinctin molecules between the two sides of the lipid bilayer. We can tentatively hypothesize that an ion channel is formed when one distinctin cluster on one side of the lipid bilayer matches another one on the opposite side.

1. Introduction

Antimicrobial cationic peptides secreted from amphibian dorsal granular glands are recognized as essential components of animal innate defense systems [1]. Most of these molecules assume a conformation in which clusters of hydrophobic and cationic amino acids are spatially organized into discrete sections, giving rise to the so-called amphipatic design. These peptides interact with negatively charged phospholipids, which are typical components of most target microbes.

Distinctin (D1), a bioactive peptide purified from *Phyllomedusa distincta*, a frog living in Brazil forests, shows an uncommon heterodimeric structure [2]. It consists of two linear peptide chains, one (chain A) of 22 amino acid residues and the other (chain B) of 25 amino acid residues, as shown in Fig. 1. The two chains are connected by a single intramolecular S–S bond. D1 has a net charge of +7, with 9 positively charged residues clustered in close proximity to the S–S bond. D1 exhibits a marked antimicrobial activity against both gram-positive and gram-negative bacteria, comparable with that of its individual chains [3,4]. In aqueous solution it forms a predominantly helical structure where its two chains are in close contact with each other [3]. To screen the hydrophobic surfaces formed by its two amphipatic helices, two D1 monomers associate in aqueous solution, forming a noncovalent compact 4-helix dimer $(A–B)_2$ that is more protected against proteolysis than the individual chains of the monomer [3,4]. The overall 3D structure of the D1 dimer in water is largely characterized by a bundle [3] of four left-handed helixes that have an almost parallel orientation and do not form a coiled coil. Oriented solid-state NMR and CD spectroscopies point to a quite different D1 conformation in lipid bilayers. In this case, chain B is adsorbed on the lipid bilayer surface, parallel to the bilayer plane, with the hydrophilic residues turned toward the aqueous phase and the hydrophobic ones turned toward the lipid. In contrast, chain A adopts a more tilted orientation that deviates from perfect in-plane alignment by about 20–25°, pointing toward the interior of the bilayer [4,5]. The flexible terminal of chain A inside the membrane is assumed to rearrange to optimize the interactions with the lipid bilayer [5]. The drastic conformational rearrangement induced by the membrane environment on the D1 dimer, with replacement of peptide–peptide interactions by peptide–membrane interactions, is quite probably preceded by dimer dissociation.

Interaction of D1 with a bilayer lipid membrane (BLM) interposed between two aqueous solutions gives rise to an ionic flux across the membrane, provided a high transmembrane potential ($\Delta\phi$) of about

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Fig. 1. Primary structure of distinctin.

250 mV is initially applied [3]. With increasing time, the transmembrane potential required to allow a detectable ionic flux decreases until ultimately, after about 45 min, the current vs. $\Delta\phi$ curve exhibits an ohmic behavior. Single channel currents recorded by the patch-clamp technique show different current levels that increase with an increase in $\Delta\phi$. This behavior led Scaloni and coworkers [3] to postulate D1 insertion into the BLM, with formation of ion channels consisting of monomer clusters of increasing size, according to the "barrel-stave" model [6]. After determining the D1 conformation in membrane environment, Scaloni and coworkers [4] then proposed ion channel formation by aggregation of four or five D1 monomers within the membrane, on the basis of molecular dynamics simulations; only a single D1 chain was considered to be inserted in the membrane, at the tilt angle estimated from solid-state NMR spectra, with the other chain lying flat on the lipid bilayer surface. In view of the small tilt angle with respect to the bilayer plane and the length of the A and B chains (about 1.7 and 2.7 nm, respectively), this model requires an appreciable local thinning of the lipid bilayer around the ion channel. Taking the latter features into account, Resende et al. [5] suggested that D1 intercalation into the membrane surface destabilizes the membrane packing locally. This may lead to local disintegration of the membrane by disrupting the bilayer curvature, according to the "carpet model" [7]. This model requires a high local surface concentration of the peptide monomers, so as to form a localized "carpet". A possible alternative model suggested by Velardi et al. [8] in a more recent paper does not require a high local D1 surface concentration. According to this model, an adsorbed peptide molecule perturbs the membrane surface by creating a mass imbalance across the bilayer, possibly because of an increase in local curvature strain. This perturbation enhances the probability of the peptide transiently inserting into the bilayer hydrocarbon tails and ultimately crossing the bilayer. The transient insertion state catalyzes ionic flux across the membrane. In the case of peptide molecules adsorbed on lipid vesicles, some vesicles may abruptly lose all of their contents due to peptide transient insertion, while other vesicles are not affected (all-or-none mechanism) [9]; at the other extreme, all vesicles lose their contents gradually (graded mechanism) [10].

The present work aims at clarifying the mechanism of membrane permeabilization by D1 by using two different mercury-supported biomimetic membranes, namely a lipid self-assembled monolayer (SAM) and a tethered bilayer lipid membrane (tBLM). The latter biomimetic membrane was obtained by tethering a thiolipid monolayer to the mercury surface. The thiolipid, called DPTL [11], consists of a tetraethyleneoxy hydrophilic chain, terminated at one end with a lipoic acid residue, for anchoring to the metal surface, and covalently linked at the other end to two phytanyl chains mimicking the hydrocarbon tails of a lipid. Self-assembling a phospholipid monolayer on top of the thiolipid monolayer gives rise to a lipid bilayer interposed between the aqueous solution and the hydrophilic chain, which acts as an ionic reservoir. This mercury-supported tBLM has been extensively employed for the investigation of ion channels [12–16].

2. Materials and methods

Water was obtained by an inverted osmosis unit; it was then distilled once and redistilled from alkaline permanganate. Merck (Darmstadt, Germany) suprapur® KCl was baked at 500 °C before use to remove any organic impurities. $TlNO_3$, $CdSO_4$, HCl and K_2HPO_4 from Merck and $NaIO_3$ from Sigma-Aldrich (Milano, Italy) were used without further purification. Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidic acid (DOPA) were purchased in chloroform solution from Avanti Polar Lipids (Birmingham, AL, U.S.A.). 2,3,di-O-phytanyl-sn-glycerol-1-tetraethylene-glycol-D,L-α lipoic acid ester lipid (DPTL) was provided by Prof. Adrian Schwan (Department of Chemistry, University of Guelph, Canada). Alamethicin was purchased from Sigma-Aldrich and was used without further purification. Solutions of 0.2 mg/mL DPTL in ethanol were prepared from a 2 mg/mL solution of DPTL in ethanol. Stock solutions of this thiolipid were stored at −18 °C. D1 peptide chains were independently synthesized by 9-flourenyl-methoxycarbonyl solid-phase (Fmoc) chemistry as previously described [3]. The two peptide chains were dissolved in basic aqueous solution and slowly mixed and dried under air flow to form the intramolecular disulfide bond [3]. The asymmetric dimer was purified by reversed phase HPLC (98.2% purity), with the molecular mass confirmed by MALDI-TOF mass spectrometry. D1 measured mass: 5479.6 Da (theoretical mass: 5478.6 Da). A detailed description of D1 synthesis and purification is provided in "Supporting Materials and Methods" of Ref. [3].

Small unilamellar vesicles (SUVs) were prepared by drying a single lipid or a lipid mixture in chloroform onto the walls of a test tube under flowing N_2 for 30 min, to ensure the removal of all the solvent. The dried lipid was resuspended in aqueous 0.1 M KCl by vortexing for about 5 min. Lipid vesicles were formed by sonicating the suspension to clarity, using the 3 mm diameter microtip of an Autotune Series High Intensity Ultrasonic Processor (Sonics, Danbury, CT). To prevent heating the sample, the sonication was carried out on ice in 5-s pulses separated by 5-s cooling periods. The total sonication time was typically 30 min. The SUVs were stored in small vials and were used over a period of one week. The hydrodynamic radius of the vesicles measured by dynamic light scattering was 50 nm, with a narrow distribution width.

All measurements were carried out with a home-made hanging mercury drop electrode (HMDE) described elsewhere [17]. A home-made glass capillary with a finely tapered tip, about 1 mm in outer diameter, was employed. Capillary and mercury reservoir were thermostated at 25 ± 0.1 °C in a water-jacketed box to avoid any changes in drop area due to a change in temperature. Mercury-supported lipid SAMs were obtained by spreading a lipid solution in pentane on the surface of a 0.1 M KCl aqueous solution, in an amount corresponding to five to six phospholipid monolayers. After allowing the pentane to evaporate, the HMDE was immersed into the aqueous solution across the lipid film. This procedure gives rise to a lipid monolayer with the hydrocarbon tails directed toward the mercury surface and the polar heads directed toward the aqueous solution, thanks to the hydrophobic nature of the mercury surface. Mercury-supported tBLMs were obtained by tethering a DPTL monolayer on the HMDE upon keeping the mercury drop immersed in a 0.2 mg/mL DPTL solution in ethanol for 20 min. A monolayer consisting of DOPC or a DOPC/DOPA mixture was then formed on top of the DPTL monolayer by two different procedures. One procedure was analogous to that employed for the preparation of mercury-supported lipid SAMs and consisted in spreading a lipid solution in pentane on the surface of a 0.1 M KCl aqueous solution. Immersing the DPTL-coated mercury into the aqueous solution across the lipid film causes a lipid monolayer to self-assemble on top of the DPTL monolayer, thanks to the hydrophobic interactions between the alkyl chains of the phospholipid and those of the thiolipid. The other procedure consisted in adding an aliquot of vesicle preparation, dosed to attain a vesicle concentration of 25 μg/mL, to a 0.1 M KCl aqueous solution containing a DPTL-coated mercury drop and in allowing the vesicles to fuse on the DPTL monolayer. With both the "spreading procedure" and the "vesicle fusion procedure", the tBLM was then subjected to repeated potential scans over a potential range from −0.20 to