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Interaction of Melittin with Negatively Charged Lipid Bilayers Supported on Gold Electrodes

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ABSTRACT

The interactions of melittin, a cationic antimicrobial peptide, with model lipid membranes consisting of negatively charged phospholipids: 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) or 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS) were investigated using electrochemical techniques and atomic force microscopy. Lipid bilayers were deposited on gold electrodes using a combination of Langmuir-Blodgett and Langmuir-Schaefer methods and the resulting membranes established a barrier for electron transfer between the electrode and the redox probe in the solution. After exposure to melittin, the blocking properties of the membranes were monitored using cyclic voltammetry and electrochemical impedance spectroscopy. It was observed that after treatment with peptide, the charge transfer through lipid bilayer is initially strongly inhibited. However, after longer exposure to melittin, the structure of the lipid film becomes less compact and the electrode reactions are facilitated due to the presence of numerous defect sites exposing bare substrate. We have assumed that such behavior reflects initial adsorption of melittin on top of the membrane and its further insertion which leads to formation of the pores or partial micellization of the lipid film. AFM imaging revealed that the exposure to 10 μM melittin solution induces significant structural changes in DMPG and DMPS membranes. However, melittin seems to affect their organization in a different manner. DMPG film appears to be more susceptible to peptide action compared with DMPS bilayer. In the latter case, long-time exposure to melittin does not result in the rupture of the membrane but rather leads to formation of pore-like defects. This observation is explained in terms of different nanomechanical properties of DMPG and DMPS films and different barrier for the reorientation and insertion of the peptide molecules into the membranes.

1. INTRODUCTION

Melittin is the principal active component of the venom of the European honey bee *Apis mellifera* [1]. It is a cationic, amphipathic peptide with the net charge of +6 at physiological pH [2]. Melittin displays high antimicrobial [3]. hemolytic [4] and anti-viral [5] activity and it is intensively studied as an anti-tumor agent [6]. Melittin can bind to neutral membranes as well as to membranes with negative surface charge and orient either parallel or perpendicular to the bilayer plane [7,8]. Numerous studies on the effect of melittin on lipid membranes of different composition showed that zwitterionic membranes are less resistant to melittin-induced lysis than membranes containing negatively charged lipids [9–13]. Although the affinity of melittin for negatively charged membranes was demonstrated to be significantly greater than for zwitterionic membranes, the latter ones were more susceptible to membrane leakage and micellization, despite the lower concentration of adsorbed melittin [14]. Higher susceptibility of zwitterionic membranes to melittin was observed in all ranges of peptide concentrations. The inhibition of melittin lytic activity in the presence of negatively charged lipids results from different orientation of melittin in zwitterionic and anionic membranes. Upon the adsorption on negatively charged membranes, melittin adopts parallel orientation since this orientation facilitates electrostatic interactions between cationic melittin and anionic headgroups of lipid. The parallel orientation was suggested to prevent the association of hydrophobic part of melittin with hydrophobic core of the membrane and consequently to inhibit the reorganization required to induce lysis. On the contrary, melittin adsorbed on zwitterionic membranes can easily reorient to perpendicular orientation and penetrate lipid membranes. These observations were confirmed by fluorescence studies, which showed that melittin penetrates deeper into the membrane

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containing zwitterionic lipids compared with the negatively charged membrane [15–17]. On the other hand, it was also demonstrated that the extent of quenching of intrinsic tryptophan residue fluorescence is similar when comparing lipid vesicles composed of phosphatidylcholine with analogous system enriched with phosphatidylserine [18]. This suggests, that the presence of negatively charged lipid does not affect insertion depth of the melittin into the bilayer. Interestingly, conductivity measurements performed by Feigin and coworkers showed that the active concentration of melittin for zwitterionic membrane was about 20 times higher comparing with negatively charged bilayers [19]. Thus the latter seem to be more sensitive to permeabilization by melittin. Similar conclusions can be drawn from the studies on bilayer membranes tethered to mercury electrode, where the highest melittin-induced conductance was observed for the systems containing phosphatidylserines [20]. It suggests that melittin is capable to insert into the lipid membrane even in the presence of negative charge in the polar headgroup region. Such assumption could be supported by the results of combined vibrational spectroscopic studies on negatively charged bilayers, which revealed that two orientations of melittin are observed at the same time with population of molecules oriented parallel three times higher than oriented perpendicularly [21].

It has been proposed that melittin forms toroidal pores in zwitterionic membranes and the size of the pore increases with the increasing peptide-to-lipid ratio [22–25]. We have demonstrated recently that melittin at 10 μM concentration causes rapid degradation of the supported membrane composed of zwitterionic lipid - 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) [26]. Under such conditions, we have observed micellization and subsequent removal of the bilayer from gold electrode. On the other hand, when the concentration of melittin was lowered down to 1 μM, the mode of the peptide action was substantially different. Initially, melittin was adsorbed on top of the membrane leading to fluidization of the lipid film. This was accompanied by reorientation and insertion of the peptide molecules into the bilayer. As a result, formation of numerous defect sites and pinholes was observed. These results demonstrate that melittin action cannot be described in terms of the single mode even if the same component of the bilayer is considered. The exact mechanism of melittin action on negatively charged membranes also remains elusive. Leakage experiments with different size markers demonstrated that in membranes composed of anionic lipids, the gradual efflux of markers did not occur and the correlation between melittin concentration and size of the pore was not observed [12]. It was proposed that melittin accumulates on the membrane and this asymmetric distribution leads to membrane fusion or aggregation accompanied by a nonspecific leakage. Ladokhin and White showed that melittin disrupts anionic membranes in a detergent-like manner, according to the carpet mechanism [27]. Interestingly, experiments on *Escherichia coli* cells revealed that none of these mechanisms is applicable to membranes of living bacteria [28].

In this paper, we present an integrated characterization of melittin action on negatively charged solid supported lipid bilayers using electrochemical techniques and atomic force microscopy. The model lipid membranes were composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) or 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS). We chose these lipids as a representative of anionic lipid components of bacterial and mammalian membranes, respectively. Phosphatidylglycerols are commonly found in membranes of both Gram-positive and Gram-negative bacteria [29,30]. Phosphatidylserines are the main anionic lipid in human red blood cells and they are localized predominantly in the inner leaflet of membranes. The externalization of phosphatidylserine into the outer leaflet of erythrocyte membrane was observed during normal aging of cells [31]. However, such externalization is also interpreted as an early marker of cell apoptosis as it was shown for cancer cells [32].

2. EXPERIMENTAL

1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS) were purchased from Avanti Polar Lipids Inc. Melittin**,** tris(hydroxylmethyl) aminomethane (TRIS), sodium chloride, ethylenediaminetetraacetic acid (EDTA), potassium hexacyanoferrate (III) and potassium hexacyanoferrate (II) were purchased from Sigma Aldrich. All reagents and compounds were used without further purification. The distilled water used in all experiments was passed through a Milli-Q water purification system and its final resistivity was 18.2 MΩ × cm.

Monolayers at the air-water interface were prepared using a KSV LB trough 5000 (KSV Ltd., Finland) and NIMA trough (NIMA Technology LTD., United Kingdom) equipped with two movable Teflon barriers and a paper plate was used as a surface pressure sensor. Before each experiment, troughs and barriers were washed using the mixture of chloroform and methanol and finally rinsed with Milli-Q water. The monolayers were formed on pure water. The compression of the monolayers was performed at the barriers speed of 10 mm/min at a constant temperature of 22 ± 1 °C. Lipid bilayers were transferred from the air-water interface on solid supports at a pressure of 35 mN/m. The first lipid layer was deposited using Langmuir-Blodgett technique, that is by vertical withdrawal of the substrate at the speed of 5 mm/min. The substrates were left to dry for approximately 1.5 hour and the second lipid layer was transferred at the surface pressure of 35 mN/m using the horizontal touch technique (Langmuir-Schaefer method). Then again the samples were left to dry for approximately 1.5 h. It should be noted that drying of the substrates coated with LB-LS bilayer does not affect the hydration of the final bilayer which is used in further experiments [33]. It is related with the fact that the bilayer deposited onto a gold electrode surface is fully rehydrated when brought into contact with an aqueous solution. As demonstrated by PM-IRRAS experiments the headgroups in a bilayer deposited at the Au(111) electrode surface using the same protocol are more hydrated than in the aqueous suspension of DMPC vesicles [34].

Lipid bilayers were transferred on two different substrates depending on the further experiments. For electrochemical studies, lipid membranes were transferred onto gold electrodes (11 × 11 mm slides, Arrandee), which were 200-300 nm thick gold films evaporated onto borosilicate glass precoated with a 4 nm thick adhesive layer of chromium. Prior to the deposition, gold substrates were flame annealed several times and cleaned in the mixture of $H_2O_2/NH_3/H_2O$ (1:1:5 v/v/v) at 70 °C, rinsed with water, dried and again flame annealed in order to improve the quality of the surface and obtain flat Au(111) terraces. The exemplary image of gold electrode surface subjected to above protocol is demonstrated in Supporting Information. For atomic force microscopy experiments, we used gold beads prepared according to Clavilier method [35]. The bead was attached to gold plate and atomically flat (111) terraces were used for image acquisition. This particular type of the substrate was proved to be well suited for lipid films deposition and high resolution imaging as it was demonstrated in several papers [36–38].

Electrochemical measurements were performed using CHI 750B bipotentiostat (CH Instruments Inc., Austin, TX) in a three-electrode cell with Ag/AgCl/sat.KCl as a reference electrode and platinum foil as a counter electrode. Potassium hexacyanoferrate (III) and potassium hexacyanoferrate (II) were used as electroactive probes. The electrochemical impedance spectra were

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