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Single-Molecule Investigation of the Influence Played by Lipid Rafts on Ion Transport and Dynamic Features of the Pore-Forming Alamethicin Oligomer

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Abstract In this experimental work we employed single-molecule electrical recordings on alamethicin oligomers inserted in lipid bilayers made of brain sphingomyelin (bSM), palmitoyloleoylphosphatidylcholine (POPC) and cholesterol (chol) to unravel novel aspects regarding lipid raft interactions with pore-forming peptides. We probed the effect of lipid rafts on electrical properties of inserted alamethicin oligomers, and our data convincingly prove that the single-channel electrical conductance of various subconductance states of the alamethicin oligomer (1) increases in the presence of raft-containing ternary lipid mixtures (POPC–chol–bSM) compared to cases when bilayers were made of POPC–chol and POPC and (2) decreases in the presence of raft-containing ternary lipid mixtures compared to nonraft ternary mixtures which favor the fluid and liquid ordered phases alone. Our data demonstrate that the presence of lipid rafts leads to a slower association kinetics of alamethicin oligomers, seemingly reflecting a slower lateral diffusion process of such peptide aggregates compared to the case of nonraft, binary lipid mixtures. Furthermore, we show that the electrical capacitance of ternary lipid mixtures (POPC–chol–bSM) decreases in the presence of raft domains by comparison to nonraft binary phases (POPC–chol) or POPC alone, and this could constitute an additional mechanism via which macroscopic electrical manifestations of eukaryotic cells are modulated by the coexistence of gel and fluid domains of the plasma membrane.

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Introduction

As a result of the complex biochemical composition of eukaryotic plasma membranes, an interesting concept emerged even at the dawn of modern membrane biology which considered that most cell membranes are highly heterogeneous structures, whereby lipids and proteins are organized in functional domains (Singer and Nicolson 1972). During the past decade such domains, called "lipid rafts," have constituted a major focus of studies of eukaryotic membrane structure. Concisely speaking, the lipid raft paradigm is based on the fact that selected, naturally occurring lipids aggregate in the plane of the membrane due to intermolecular interactions, such as van der Waals interactions between the nearly fully saturated chains of sphingomyelin and glycosphingolipids as well as hydrogen bonds between adjacent glycosyl moieties of glycosphingolipids (Simons and Ikonen 1997); and this entails the existence of relatively highly organized "islands" of lipid patches that "float" in a liquid-crystalline two-dimensional lipid phase.

The very existence of such domains was originally promoted by the fact that assemblies of sphingolipids and cholesterol apparently survived Triton-X 100 extraction at 4°C (Brown and Rose 1992), whereas methyl-β-cyclodextrin-induced cholesterol depletion led to loss of detergent resistance (Ilangumaran and Hoessli 1998; Scheiffele et al. 1997).

Within a raft-containing membrane, cholesterol was shown to induce the existence of a so-called liquid ordered phase (lo) (Sankaram and Thompson 1990), in which lipid

acyl chains are mostly extended and rather tightly packed—as in the solid phase of a lipid membrane (so)—but the lateral diffusion is almost as high as in the fluid phase (ld) (de Almeida et al. 1992). Built of the fact that the essential lipid components in the outer leaflet of eukaryotic membranes are sphingomyelin (SM), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and cholesterol (chol), ternary mixtures of these lipids do mimic quite well the essential properties of the raft-containing membranes (Cullis and Hope 1985; Dietrich et al. 2001; de Almeida et al. 2003; Feigenson and Buboltz 2001). Furthermore, fluorescence and nuclear magnetic resonance measurements have demonstrated the existence of segregated domain sizes in the nanometer range (de Almeida et al. 2003, 2005; Bunge et al. 2008) in such ternary lipid platforms. Based on work undertaken with liposomes generated from lipid mixtures mimicking the composition of detergent-resistant membranes (Ahmed et al. 1997; Schroeder et al. 1994), rafts were proposed to be membrane regions rich in SM and chol in the lo phase, laterally diffusing in the ld phase of the surrounding plasma membrane. In addition, fluorescence microscopy visualization of domains and membrane morphology for a number of model membranes, as well as atomic force microscopy experiments, have demonstrated the presence of coexisting ld and lo or gel phases in both giant vesicles and supported membranes (Simons and Vaz 2004; Edidin 2003; Veatch and Keller 2005; Rinia et al. 2001). Notably, phospholipids with polyunsaturated acyl chains have been suggested to greatly support lateral segregation of membrane lipids in multicomponent mixtures as a consequence stemming from their low miscibility with saturated phospholipids as well as their low propensity to establishing close interactions with flat steroids such as chol (Litman et al. 1991; Shaikh et al. 2004).

From a dynamic standpoint and with relevance to this work, it has been established that the lateral diffusion coefficient of the lipid molecules in lo phase is only reduced by a factor of about 2–3 compared to that in ld phase (de Almeida et al. 1993), whereas the conformational order of the lipid hydrocarbon chains in lo phase assumes values closer to that of the gel phase (so) of a membrane (Gally et al. 1976). Due to their ability to interact in a specific manner with lipids and proteins, rafts are of great interest to cell biologists, immunologists and physical scientists since they are believed to be involved in a number of important cell functions, including signal transduction (Simons and Toomre 2000), lipid sorting (Simons and Ikonen 2000) and protein trafficking (Ikonen 2001). Also, the role of lipid rafts in the uptake of bacterial toxins represents an important factor in a number of bacterial infections including shigellosis and anthrax (Abrami et al. 2003; Lafont and van der Goot 2005; Zaas et al. 2005).

To date and in direct connection to conclusions derived from our study, there is a great deal of knowledge strongly suggesting that the physical state of lipid bilayers modulates the activity of ion channels and other membrane-lodged proteins (Keller et al. 1993; Chang et al. 1995b; Lundbaek et al. 1996; Somerharju et al. 1999; Chiriac and Luchian 2007). Other results have demonstrated the effect of lipid phase transition on the activity of the single cardiac calcium-release channel in vitro, providing evidence for regulation of calcium-channel activity by a composition-driven liquid-to-gel phase transition of surrounding lipid bilayer (Cannon et al. 2003). Importantly, the conductance of the sarcoplasmic reticulum channel from cardiac myocytes and alamethicin channels reconstituted in planar lipid membranes was shown to be affected by the lipid composition of the bilayer, despite earlier findings stating quite the contrary (Keller et al. 1993; Chang et al. 1995a; Cernescu and Luchian 2006). A nonspecific lipid bilayer regulation of ion channels function has been explained in studies performed on simple systems such as gramicidin and alamethicin channels, which established that channel function is modulated by the elastic properties of the bilayer (e.g., monolayer equilibrium curvature and compression and bending moduli) in which they reside (Keller et al. 1993; Lundbaek et al. 1996; Sawyer et al. 1989; Lundbæk and Andersen 1999; Bezrukov 2000).

One of the most important manifestations of the heterogeneous raft-containing lipid bilayer is the presence of phase boundaries, which appear due to the coexistence of gel and liquid-crystalline domains. The looser lipid packing in domain boundaries would assist conformational changes of an inserted ion channel compared to the situation of a more tightly packed gel or liquid-crystalline phase (Hamill and Martinac 2001), which would entail an increase in the frequency of channel openings. An additional factor that might contribute to changes in the electrical manifestations of a raft-containing lipid membrane is the bilayer thickness, which in the coexistence region should assume intermediate values between those encountered in the gel and liquid phases. As a direct result of the bilayer–protein hydrophobic mismatch that might occur, the bilayer deformation which takes place in the vicinity of the embedded ion channel (e.g., local compression or extension of the acyl chains, bending of the bilayer/solution interface and splaying of the lipid acyl chains) would entail a supplementary energetic cost in the bilayer deformation energy that contributes to the overall free-energy difference between different conformations of the ion channel. As a result, ion channels located in such domains would adjust their degree of occupancy of conformational states in favor of conformations with smaller hydrophobic mismatches (Andersen and Koeppe 2007; Huang 1986).

Yet another factor relevant in the sorting of transmembrane peptides in raft-containg bilayers with possible applicability in the present work is given by the difference in elastic deformabilities between raft and nonraft bilayers (McIntosh et al. 2003; Vidal and McIntosh 2005). Due to the high concentrations of chol and sphingolipids, raft–lipid bilayers are presumed to have a much higher compression modulus (K_a) compared to phosphatidylcholine–chol bilayers; previous data in this respect have shown that the compression modulus for SM–chol bilayers at room temperature is about 10 times larger than that of a phosphatidylcholine-based, nonraft bilayer (Allende et al. 2003).

Herein, we employed single-molecule electrical recordings on alamethicin oligomers inserted in planar lipid bilayers to unravel novel aspects regarding lipid raft interactions with ion-forming ion peptides. To ensure optimal conditions for the existence of raft domains, we used the ternary system brain SM (bSM)–POPC–chol and chose appropriate molar concentrations of the lipids to end up with a considerable fraction of both ld and lo phases at a room temperature of $\sim$23°C (de Almeida et al. 2003).

Although the binary combination POPC–chol forms the coexisting ld + lo phase with chol added at the molar fraction used in this work (de Almeida et al. 2003), the lo phase formed from such unsaturated lipids (i.e., POPC) may be different from that generated by saturated lipids (e.g., SM). Moreover, keeping in mind that ternary lipid mixtures like those emphasized above are more relevant, in compositional terms, to plasma membranes and better mimic biological lipid rafts, we deemed it appropriate to differentiate in this work between raft microdomains generated by ternary lipid compositions and the ld + lo phase generated by the POPC–chol binary combination.

The effect of lipid rafts on electrical properties of inserted alamethicin oligomers was probed by evaluating the transport properties of such model ion channels, and our data convincingly prove that the single-channel electrical conductance of various subconductance states of the alamethicin oligomer increases in the presence of raft domains (POPC–chol–bSM, lo + ld phase) compared to binary mixtures of POPC–chol or POPC alone and decreases in the presence of raft domains (POPC–chol–bSM, lo + ld phase) compared to nonraft ternary mixtures (i.e., POPC–chol–bSM, ld phase, and POPC–chol–bSM, lo phase). This we believe is a supplementary proof of principle in favor of functional modulations of ion channel permeation properties by longer-range effects caused by raft microdomain-forming lipids.

With respect to dynamic features of the alamethicin oligomer, our data demonstrate that the presence of lipid rafts in the support membrane leads to a slower association kinetics of alamethicin oligomers, seemingly reflecting a slower lateral diffusion process of such peptide aggregates compared to the case of nonraft, binary lipid mixtures. In addition, we found that the electrical capacitance of planar membranes containing lipid rafts decreases in the presence of raft domains (POPC–chol–bSM) by comparison to compared binary mixtures of POPC–chol or POPC alone, and this could constitute an additional mechanism via which macroscopic electrical manifestations of eukaryotic cells are modulated by the coexistence of gel and fluid domains of raft-containing plasma membrane.

Materials and Methods

Electrophysiological experiments were carried out in the folded bilayer membrane system obtained with the Montal-Mueller technique (Montal and Mueller 1972) as previously described (Chiriac and Luchian 2007), at a room temperature of $\sim$23°C. The lipids used were POPC (Avanti Polar Lipids, Birmingham, AL; code 850457P), chol (Avanti Polar Lipids, code 700000P) and bSM (Avanti Polar Lipids, code 860062P); solvents employed were *n*-pentane (Sigma-Aldrich, St. Louis, MO) for POPC, chloroform (Sigma-Aldrich) for chol and a 4:1 (v/v) chloroform/methanol mixture for bSM. The general view of the bilayer recording setup and molecular structures of the lipids used in this study are shown in Fig. 1.

The relative molar fraction used for binary mixtures of POPC–chol was (66.6/33.3) mol%, and for ternary mixtures, depending on the expected phase at a room temperature of $\sim$23°C (de Almeida et al. 2003), the following relative molar fractions were employed (mol%): POPC–chol–bSM (85/5/10), ld phase; POPC–chol–bSM (50/30/20) or POPC–chol–bSM (50/25/25), lo + ld phase; POPC–chol–bSM (15/55/30), lo phase.

The membrane-bathing solutions contained 1 M NaCl buffered at a value of pH $\sim$7 in 10 mM HEPES. When required by the experimental protocol, the capacitance of the bilayer was computed as the ratio of the charge stored across the membrane at an applied potential of -10 m*V*, by integrating the capacitive current flowing across the bilayer when the holding potential changes in a step-like manner from 0 to -10 m*V*. Alamethicin monomers (Sigma-Aldrich, code A4665, Rf30, $\geq$90% HPLC) were added from a stock solution made in ethanol (5 μm) to the cis chamber of the bilayer system, which was grounded; and mechanical stirring was initiated in this chamber for 20 s to ensure proper concentration homogenization.

Currents from the bilayer chamber were detected and amplified with an integrating headstage Axopatch 200 B amplifier (Molecular Devices, Palo Alto, CA) set to the voltage-clamp mode. Data acquisition of the amplified electrical signals was performed with a NI PCI 6014, 16-bit

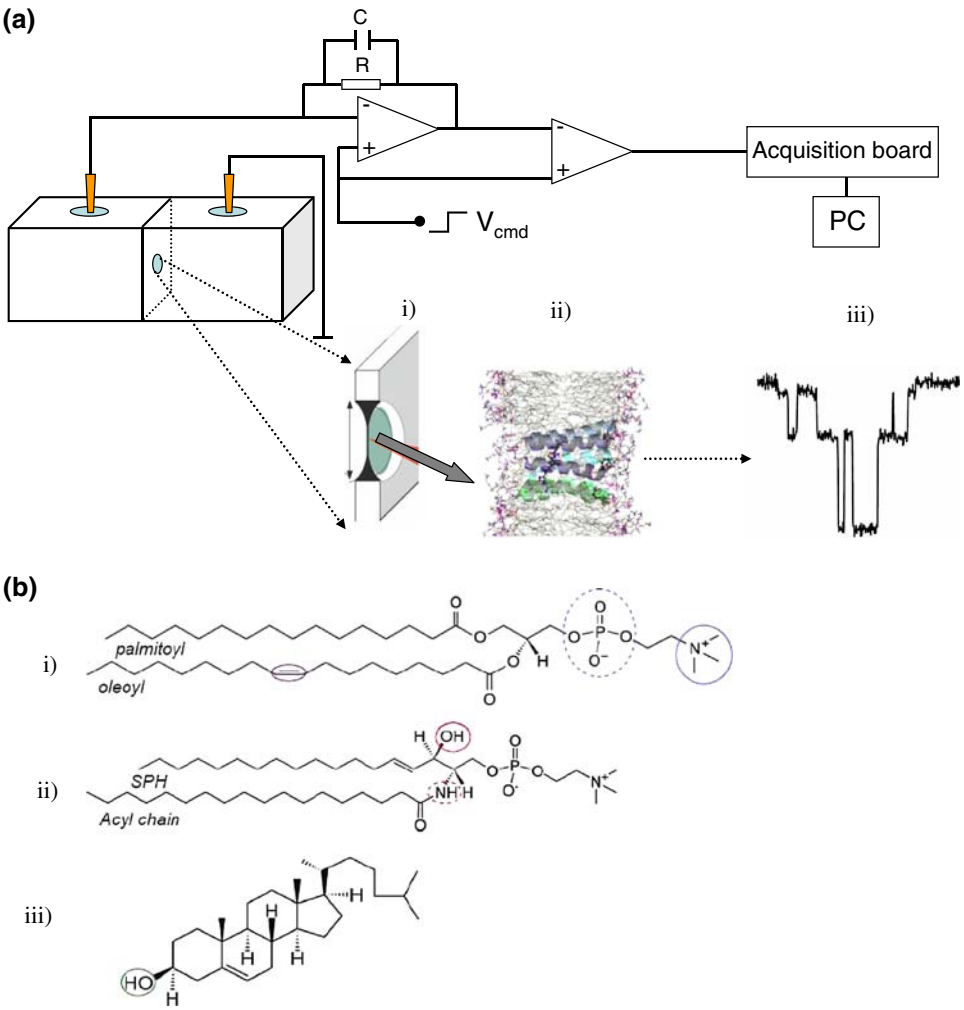

Fig. 1 **a** General layout of the experimental setup, which sketches the bilayer chamber and the general diagram of the amplifier and recording blocks. For clarity, in (*i*) we show a representative zoom-in of the Teflon partition where the bilayer forms (*ii*), serving as physical support for recording the electric activity (*iii*) of alamethicin pores. **b** Molecular structure of the three lipids used throughout this study: (*i*) POPC, (*ii*) bSM and (*iii*) chol. The important functional groups with major roles in lateral lipid segregation are indicated: the phosphate moiety (*dashed line*) and choline group (*continuous line*) of the PC headgroup, together with the cis-double bond of the monounsaturated chain. The two hydrogen donor groups of bSM are the amide group (*dashed line*) and the hydroxyl group (*continuous line*). For chol, the hydroxyl group is indicated

acquisition board (National Instruments, Austin, TX) at a sampling frequency of 10 kHz, within the LabVIEW 8.20 environment. The electrical conductance of the second conductive substate of the alamethicin oligomer inserted in planar bilayers consisting of various thermodynamic phases was computed using a nonlinear fitting algorithm of *I–V* diagrams recorded at applied potential differences ranging from −10 to −120 m*V*. All experiments were performed at a room temperature of ∼23°C. Numerical analysis, including time domain low-pass filtering and nonlinear fitting based on the Levenberg-Marquardt method for χ^2 reduction, was done with the help of Origin 6 software (OriginLab, Northampton, MA), and the numerical values represent averages ± S.E.M calculated from at least three separate experiments.

Results

On compelling observation derived from our study supports the idea of a strong influence exerted by the lipid phase upon transport features of membrane-embedded alamethicin channels. During one set of control experiments, we sought to determine the extent to which certain lipid mixtures, which are essential for cellular lipid raft formation, modulate electrical conductance of alamethicin channels. As seen in Fig. 2a, current levels for the most visible alamethicin substates increase in amplitude as the composition of the bilayer changes from POPC alone to binary mixtures of POPC–chol (lo + ld) (66.6/33.3) mol% and ternary mixtures of POPC–chol–bSM (50/25/25) mol%, which ensure the presence of lipid microdomains.

Interestingly, ternary mixtures of POPC–chol–bSM seem to distinctly regulate ion transport through alamethicin oligomers depending on the relative fraction of both ld and lo phases (Fig. 2b); i.e., in the presence of POPC–chol–bSM (50/30/20) mol%, which ensures the coexistence of lo and ld phases, the electrical current mediated by alamethicin is the smallest compared to other relative molar concentrations of POPC, chol and bSM which generate bilayers in either the ld (POPC–chol–bSM; 85/5/10 mol%) or lo (POPC–chol–bSM;15/55/30 mol%) phase.

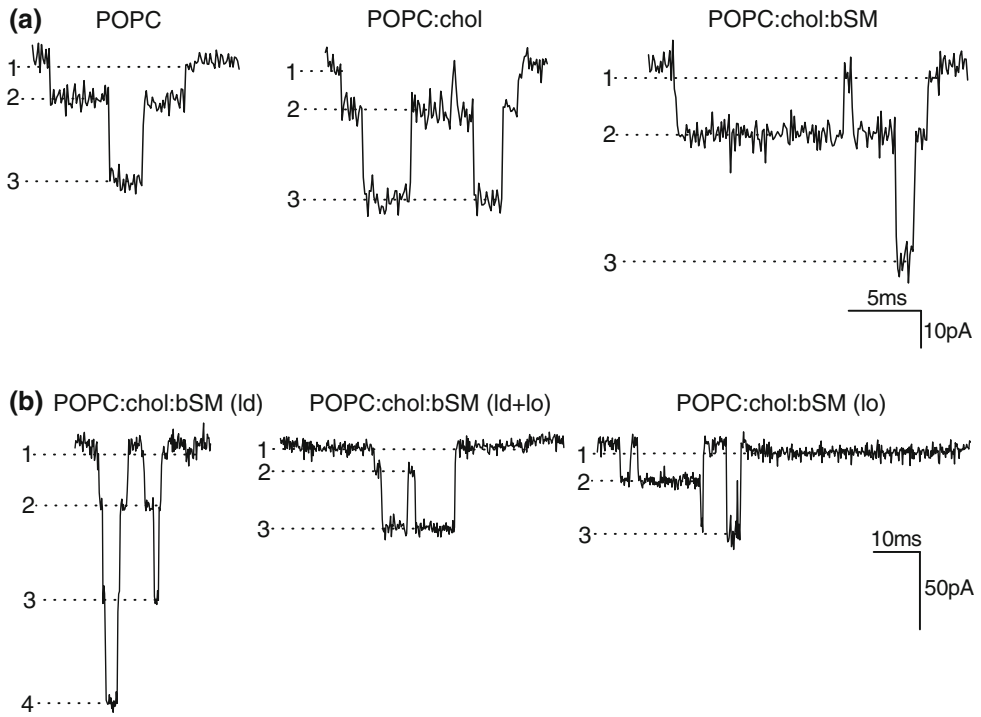

Fig. 2 **a** Original traces showing the activity of the alamethicin oligomer embedded on artificial membranes made of POPC, POPC–chol (66.6/33.3) mol% and raft-containing POPC–chol–bSM (50/25/25) mol%, measured at a holding potential of −30 m*V*. It is clearly seen that the presence of lipid microdomains ensured by the ternary lipid mixture further elevates ion transport through the alamethicin oligomer. **b** Typical current recordings of single ion channels formed by alamethicin measured at a holding potential of −50 m*V* in ternary lipid mixtures of POPC, chol and bSM with relative molar concentrations chosen to facilitate the presence of distinct phases (i.e., ld phase only, coexistence of raft-generating ld and lo phases and lo phase alone). To point out clearly the effect of various lipid combinations on the conductive features of the alamethicin oligomer, we marked distinctly the subconductive substate visible in this representation (denoted by *1–4*)

In quantitative terms, current–voltage diagrams drawn for the data shown before clearly suggest that membranes made of raft-containing ternary mixtures of POPC–chol–bSM (50/25/25) mol% lipids mediate the highest electrical conductivity for the second conductive states of the alamethicin oligomers compared to cases when the membrane was formed from either POPC alone or binary mixtures of POPC–chol (66.6/33.3) mol% (Fig. 3a). However, when working with ternary lipid mixtures under circumstances that allow ld/lo phase coexistence, ion flux through the alamethicin oligomers is reduced compared to cases when only ld or lo phase is present in the lipid system (Fig. 3b).

We next considered how the energy associated with the interface between various phases in a multiphase lipid bilayer alters the oligomerization of alamethicin monomers. In Fig. 4 we show, at an optimal time scale, selected original traces that illustrate a visible dampening effect of lipid microdomains generated in ternary lipid mixtures on alamethicin lateral dynamics. Namely, one can easily see that the time intervals needed for one alamethicin oligomer residing in the second conductive state to interact with another alamethicin monomer and assume a higher conductive state (i.e., the third conductive state as represented in our figure) increases in the presence of both chol (Fig. 4b) and lipid microdomains (Fig. 4c).

By quantifying the time intervals (τ_{2-3}) lapsing between states 2 and 3 of the alamethicin oligomers, we found that the presence of lipid rafts in planar membranes constructed from ternary lipid mixtures leads to a slower association kinetics of alamethicin oligomers, seemingly reflecting a slower lateral diffusion process of such peptide aggregates into such membranes (Fig. 5).

Since lipid bilayers in the solid phase are known to be thicker than those in the fluid phase, with an average height difference between solid domains and the fluid bilayer of $\sim$1 nm (Rinia et al. 2001), we asked whether the membrane capacitance of such planar lipid bilayers is modulated by ld/lo phase separation. Our data prove that the membrane capacitance decreases in ternary mixtures (POPC–chol–bSM) that allow for the segregation of lipid microdomains compared to either monophasic (POPC) or binary (POPC–chol) bilayers (Fig. 6a).

Interestingly, in POPC–chol–bSM ternary mixtures, our observations suggest that the coexistence of ld and lo phases, which ensure lipid raft macroscopic manifestation, lead to the smallest value of membrane capacitance (Fig. 6b).

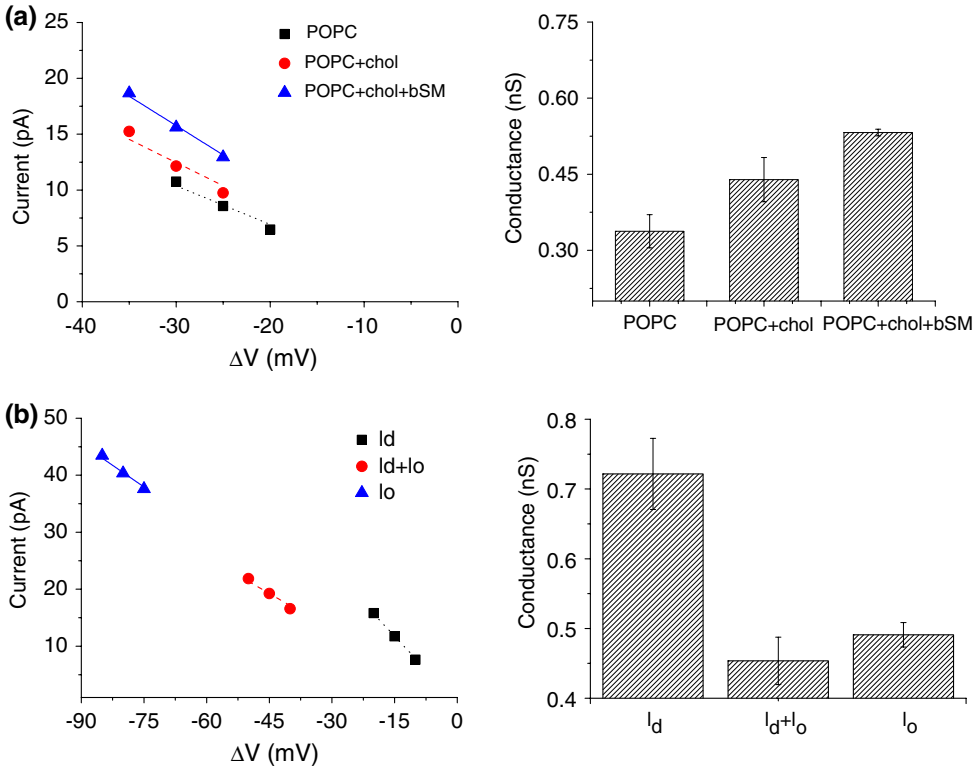

Fig. 3 *I–V* diagrams and computed conductance values of the second conductive state of the alamethicin oligomer inserted in various combinations of lipids: (**a**) POPC, POPC–chol and the raft-containing POPC–chol–bSM, (**b**) ternary lipid mixtures of POPC, chol and bSM with relative molar concentrations ensuring the presence of distinct lipid phases (i.e., ld phase only, coexistence of raft-generating ld and lo phases and lo phase alone). In order to facilitate the analysis of alamethicin subconductance states as they appear in various mixtures of lipids, we had to restrict our *I–V* analysis to different voltage ranges, as shown

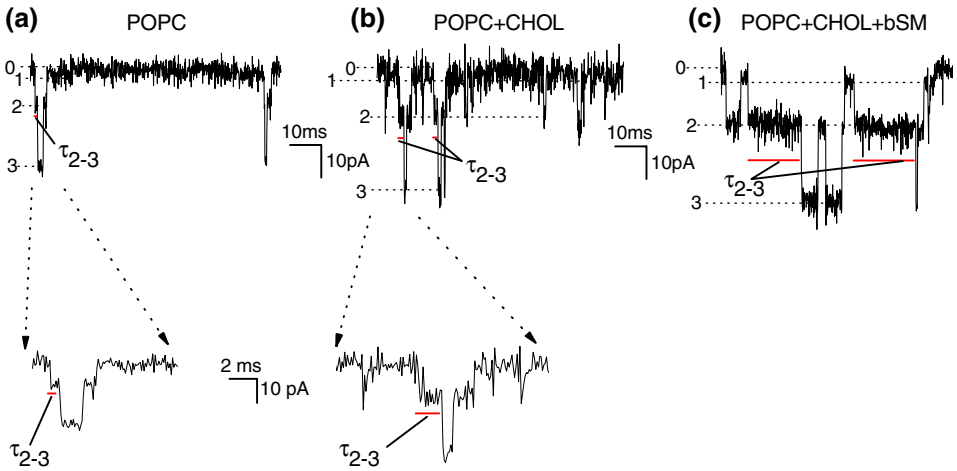

Fig. 4 Original traces recorded at −30 m*V* which emphasize the effect played by various lipid combinations on the kinetic activity associated with the reversible oligomerization of the alamethicin inserted in planar lipid membranes. Notably, the presence of lipid microdomains in the membrane consisting of POPC–chol–bSM (50/25/25) mol% (**c**) entails the lowest mobility of lateral displacement of alamethicin as reflected by the largest time needed for the alamethicin oligomer to gain one more monomer, via lateral diffusion in the membrane plane. For simplicity, we refer here specifically to the time needed for the alamethicin aggregate to oligomerize from its second conductive state to the third one (τ_{2-3}), following the lateral addition of one more alamethicin monomer

Discussion

Large bodies of evidence gathered so far suggest that the relative molar fraction of various lipids in ternary mixtures alter the intermolecular interactions among the bilayer lipids and consequently modulate membrane physical properties, such as the monolayer equilibrium curvature, compression and bending moduli. As a result, the energetic cost of a bilayer deformation which depends upon the distribution of lateral pressure across the width of the bilayer may vary considerably depending upon lipid composition, and certain membrane proteins whose function depends on conformational changes that involve the protein–bilayer boundary would be expected to be modulated by bilayer elasticity.

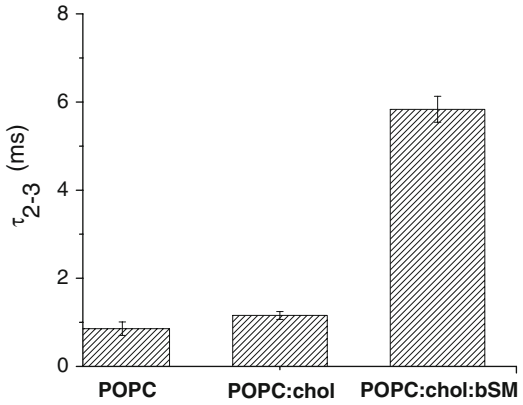

Fig. 5 Average time intervals needed for one alamethicin oligomer residing on its second conductive state to be taken up by another monomer and to move to its third conductive state (τ_{2-3}), computed as a function of various lipid mixtures: POPC, POPC–chol (66.6/33.3) mol% and raft-containing POPC–chol–bSM (50/25/25) mol%

Our data demonstrate that lipid microdomains modulate transport and kinetic activity of alamethicin embedded in lipid bilayer membranes. Specifically, in the presence of raft domains generated by ternary POPC–chol–bSM lipid mixtures, the values of the electrical conductance of the alamethicin oligomers increased compared to binary mixtures of POPC–chol or POPC alone and decreased in ternary, nonraft mixtures (POPC–chol–bSM, ld phase, and POPC–chol–bSM, lo phase).

At this point, we tend to dismiss as a possible explanation for this phenomenon changes in the electrical properties of the membrane brought about by various proportions of lipids used (e.g., the membrane dipole potential) since alterations in ion transport properties are also visible for the higher conductive states of the alamethicin oligomers. Due to their higher cross-sectional area, such states are also less ion-selective,and it would be expected that cations and anions are influenced to a rather similar extent by changes in the overall electrical profile across the lipid membrane.

However, our tentative conclusion derived from such experiments points to a possible involvement of a mechanical, lateral pressure effect within the lipid membrane, which in the presence of ternary lipid combinations leads to a lesser mechanical constriction of the alamethicin pore. As a result of changes in the pressure profile across the membrane entailed by various combinations of lipids whose relative concentrations dictate the specific phase for the lipid bilayer, we posit that the overall transmembrane conductive path created by the oligomerization of alamethicin monomers changes its cross-sectional size, which eventually leads to alterations of its electrical conductance. The mechanisms through which in the presence of lipid rafts the lateral pressure over the hydrophobic region of the membrane seemingly assumes higher values by comparison to conditions whereby ternary lipid combinations generate only ld and lo phases, as judged from the effects exerted on the alamethicin electrical conductance, remain to be fully determined.

As will be also emphasized below, it is clear from our data that the lo + ld phase coexistence in POPC–chol–bSM ternary lipid mixtures exerts distinct influences on the physical manifestations of the alamethicin oligomer from those described when a POPC–chol binary system

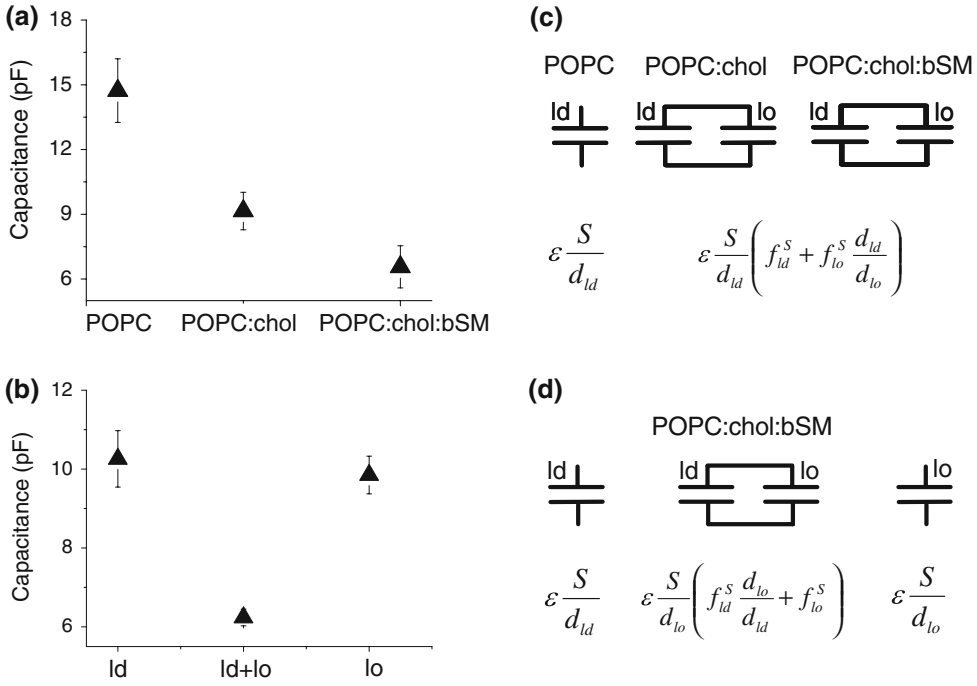

Fig. 6 Computed values of the electrical capacitance of membranes made of POPC, POPC–chol (66.6/33.3) mol% and raft-containing POPC–chol–bSM (50/25/25) mol% (**a**) and ternary lipid mixtures of POPC, chol and bSM with relative molar concentrations chosen to facilitate the presence of distinct phases (i.e., ld phase only, coexistence of rafts-generating ld and lo phases and lo phase alone) (**b**). In **c** and **d** we sketched the electrical equivalent representations of bilayers with various binary and ternary lipid mixtures, which facilitate distinct phases as presented in **a** and **b**, along with their theoretically estimated capacitance values (see "Discussion")

was used so as to favor lo + ld phase coexistence as well. Whether this is a reflection of how fatty acid composition and the unsaturation degree of raft-containing lipids affect raft physical properties, dynamics and chemical affinity toward transmembrane proteins is still an issue to be pursued in future work.

A lipid membrane containing microdomains is apparently less fluid with respect to lateral molecular diffusion, as indicated by an increase in the value of its average time intervals of one alamethicin monomer uptake by an oligomeric alamethicin protein residing in the second conductive state. The presence of lipid rafts in such lipid membranes leads to a slower association kinetics of alamethicin oligomers, thus reflecting a slower lateral diffusion process of such peptide aggregates in the membrane. It is likely that the lateral movement of individual alamethicin monomers and oligomers is hindered by the presence of lipid rafts since domain boundaries where lipid packing is perturbed compared to the either homogenous gel or fluid regions possess an attractive potential for various molecules (Cannon et al. 2003; Lipowsky and Sackmann 1995). Therefore, such "molecular traps" would perturb the otherwise Brownian motion of membrane-embedded alamethicin, thus determining a decrease in their lateral diffusion coefficient. This further supports the paradigm of activity regulation of pore-forming peptides by packing defects near the transmembrane regions of individual channels.

It is common knowledge that the presence of cholesterol condenses phospholipid bilayer membranes, which leads to an increase of their thickness. Cholesterol molecules are believed to induce such effects by intruding the hydrocarbon tails of phospholipids; as a result, an increase in the order of the acyl chains ensues, which eventually causes the membrane to thicken. Similarly, cholesterol may intercalate between the chains of SPM and thicken the rafts. Therefore, a height difference between the ordered lipid domains and the surrounding fluid bilayers exists, as was nicely unveiled by AFM measurements (Rinia et al. 2001; Johnston 2007). By evaluating the electrical capacitance of membranes containing binary (POPC–chol) and raft-generating ternary (POPC–chol–bSM) lipid mixtures, we noted that their values ranked in the order $C_{POPC/chol/bSM} < C_{POPC/chol} < C_{POPC}$. This tendency may be well accounted for if, for the sake of simplicity, one regards the equivalent capacitance of POPC–chol and POPC–chol–bSM bilayers as the sum of individual capacitance values attributed physically to the lo and ld phases, which are known to coexist in such membranes. Mainly due to the average height difference between the two phases (the hydrophobic thickness of the lo and ld phases is denoted by d_{lo} and d_{ld}, respectively) and the average amount of area occupied by these domains relative to the total area of the membrane (S) (the fractional area occupied by lo and ld phases is denoted by f_{lo}^S and f_{ld}^S, respectively), the lumped capacitance (C_{ld+lo}) equals

$$C_{ld+lo} = \varepsilon \frac{S}{d_{ld}} \left(f_{ld}^S + f_{lo}^S \frac{d_{ld}}{d_{lo}} \right) \quad (1)$$

In Eq. 1, ε stands for the absolute electric permittivity of the membrane hydrophobic core, assumed to be the same for both lo and ld phases. By a simple rationale, the average fractional areas occupied lo and ld phases are positive quantities less than 1, whose particular values ensure that

$$S\left(f_{ld}^S + f_{lo}^S\right) = S \quad (2)$$

Keeping in mind that lo phases are slightly thicker than lo phases (i.e., $d_{lo} > d_{ld}$), by inspecting Eq. 1 one may conclude that the lumped capacitance (C_{ld+lo}) of a bilayer containg both lo and ld phases should always be smaller than that of a bilayer composed only of POPC displaying the ld phase alone. Our experimental observation that supports the idea of a smaller lumped capacitance of bilayers made by POPC–chol–bSM compared to POPC–chol ones may point to fine structural differences between lo domains (e.g., their average occupied area and hydrophobic thickness) obtained in the presence or absence of bSM, which eventually translates into

$$\left(f_{ld}^S + f_{lo}^S \frac{d_{ld}}{d_{lo}} \right)_{POPC/chol/bSM} < \left(f_{ld}^S + f_{lo}^S \frac{d_{ld}}{d_{lo}} \right)_{POPC/chol} \quad (3)$$

Interestingly, in ternary lipid mixtures of POPC–chol–bSM, the lumped capacitance of bilayers containg rafts is smaller than that of those displaying the lo phase alone. By following a similar line of thought as described above and using similar notations, this would contradict the oversimplified theoretical estimations which indicate that, regardless of average fractional area occupied by lo and ld phases, the following applies since d_{lo} is always greater than d_{ld}:

$$C_{ld+lo} = \varepsilon \frac{S}{d_{lo}} \left(f_{lo}^S + f_{ld}^S \frac{d_{lo}}{d_{ld}} \right) > C_{lo} = \varepsilon \frac{S}{d_{lo}} \quad (4)$$

However, by taking into account that the voltage dependence of bilayer capacitance (C) may be approximated near its minimum (C_0) by

$$C = C_0\left(1 + \alpha U^2\right) \quad (5)$$

where α denotes the compliance of an elastic capacitor and U represents the potential difference at which the capacitance is being estimated, this may be an indication of the fact that in ternary mixtures raft-containg bilayers may be less compressible than those in the lo phase (Ermakov et al. 2001).

For future work and in conjunction with complementary techniques (e.g., fluorescence microscopy), this opens up

the possibility of time-monitoring the dynamics of raft domains as may arise upon changes in temperature, relative amounts of constituent lipids, lipid oxidation, equilibration of lipids between leaflets and domains merging.

Our data provide clear evidence for regulation of the alamethicin-channel activity by a composition-driven liquid-to-gel phase transition of the supporting lipid bilayer. The binary and ternary lipid systems studied in this work can provide a reliable framework that one may extrapolate toward understanding the complexity of cellular membranes and their interaction with pore-forming peptides and proteins.

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