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DIPOLE POTENTIAL-INDUCED MODULATION OF THE INTERACTIONS BETWEEN RECONSTITUTED LIPID MEMBRANES AND CERTAIN PORE-FORMING PEPTIDES

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One biologically relevant parameter which modulates energetic couplings between a large variety of antimicrobial peptides and lipid membranes is known to arise from the dipole potential component present on both sides of a membrane. Due to the interfacial chemical and physical heterogeneity at the interface between the hydrophobic core of such membranes and the aqueous phase, membrane-penetrating peptides will sense a sizeable variation in environmental polarity. This in turn results in electrical interactions with the dipole potential of such membranes, prone to modulate the membrane insertion, reversible oligomeric interactions and folding of different peptides and proteins. In this work we review evidence stemming from electrophysiology experiments carried out at the single molecule level, demonstrating the modulatory role played be the membrane dipole potential upon transport features and membrane insertion of selected antimicrobial peptides (*e.g.,* alamethicin and HPA3). Such data further emphasize the interplay existing between membrane-based peptides and the membrane dipole potential, and bring new insights into the possibility that dipole potential could be employed to manipulate the in vivo susceptibility of various cells towards antimicrobial peptides and other membrane-penetrating proteins.

Tudor Luchian was born in Fălticeni, Roumania in February 26, 1968. In 1986 he graduated from the "Nicu Gane" High School, Fălticeni, Roumania and starting 1987 he attended the Faculty of Physics, "Alexandru I. Cuza" University Iaşi, Roumania, which he graduated in 1992 with the Bachelor of science degree in Physics/Biophysics. In 1994, following an international contest, he was offered a Ph. D. scholarship at the "Karl-Franzens" University of Graz (Austria) and in 1997 he completed his Ph. D. studies with the highest degree. While working abroad either as a research fellow or post-doctoral associate, Tudor Luchian was involved in several research programs carried out in prestigious laboratories, as follows: Biological Research Center, Institute of Biophysics (Szeged, Hungary), University of Queensland (Brisbane, Australia), Texas A&M University (College Station, Texas, USA) and University of Oxford (UK). He currently assumes the 'full professor' position in Physics/Biophysics at the Faculty of Physics, Department of Biophysics & Medical Physics, "Alexandru I. Cuza" University, Iaşi, Roumania and he is Ph. D. adviser in the field of "Physics". His scientific contributions are embodied in 29 ISI papers published in internationally renowned journals, some of which have resulted in a lumped number of 147 citations (self-citations excluded). He authored two books and co-authored two book-chapters. Three of his articles have been mentioned as exclusive 'Editorial Research Highlights' and one of his research figures has been chosen by the editor of ANGEWANDTE CHEMIE-INTERNATIONAL EDITION as 'cover page' for one of the journal's section. Among other synergistic activities, it should be mentioned that professor Luchian is the director and co-director of ten research projects (about 1.2 milion EURO in total), he is the vice-president of the "Roumanian Society of Pure and Applied Biophysics", member in the "Think-Tank Presidential Commission for the Analysis and Improvement of the Educational and Research Policies in Roumania" (Roumanian Presidency), and he is included in 'Marquis Who's Who in Science and Engineering' 10th Edition, USA. He currently advises seven Ph. D. students, two of which are expected to receive their degrees by the end of 2008.

INTRODUCTION

The interaction between biological membranes and macromolecules that exert their activity via the formation of membrane nanopores is at the core of the functioning of ion channels[1] and most antimicrobial peptides.[2,3] Despite the large plethora of studies undertaken, the insertion mechanism of amphiphilic molecules into and across biomembranes is still poorly understood.[4] It is currently known that most amphiphilic peptides bind to lipid membranes by a physical adsorption mechanism, while specific phospholipid–peptide interactions are very seldom. One particular example in this last regard is the interaction of nisin Z with lipid II.[5] From a computational point of view, the use of atomistic simulations has the potential to reveal insights into the interactions of membrane proteins with lipid membranes.[6] Unfortunately, despite many recent advances in computational power and algorithm deployment, the net result of such simulations is limited by the accessible system size and timescale.

With respect to the insertion mechanism, molecular dynamics simulations demonstrate that a model hydrophobic nanotube, possessing a hydrophilic functional group at its termini is capable of spontaneous insertion into a model lipid membrane, followed by its alignment across the membrane and thereupon facilitates molecules transport across the bilayer. In such examples, pore formation takes place in a two-stage process: (a) the nanotube gets first adsorbed into the membrane surface with its z axis aligned mostly parallel to the lipid–water interface and (b) the functionalized nanotube spontaneously reorients and turns into a transmembrane pore. From a physical standpoint, electrostatic forces (both coulombic and dipolar interactions), hydrogen bonds, and hydrophobic interactions play equally important roles into the membrane insertion of more complex, natural and artificial pore-forming peptides.[7]

It is currently established that the interaction of such peptides with the lipid membrane can be divided into at least three separate steps.[8,9] In a first step, membrane binding can be greatly promoted by the electrostatic attraction manifested between cationic peptides and negatively charged membranes. Obviously, depending on the peptide charge and the magnitude and sign of the membrane surface potential, electrostatic interactions have the potential to either increase or decrease the peptide concentration close to the membrane surface, as compared to the bulk concentration. Notably, electrostatic attraction is not a fundamental requirement for peptide adsorption to lipid membranes, which can also occur between a non-charged peptide and a neutral membrane. However, in such instances, the peptide concentration near the membrane surface equals that in bulk solution. The next step is the transition of the peptide into the plane of binding, whose geometry may be difficult to predict since it depends critically on the hydrophobic/hydrophilic balance of the molecular groups and forces involved. The third step in the peptide-membrane binding process is represented by a change of the conformation of the bound peptide. While in most cases peptides are in a random coil conformation in solution, they adopt a α-helical secondary structure following the interaction with lipid membranes. A classical example in this regard is represented by the bee venom melittin whose CD spectrum changes from almost completely random coil in buffer to approximately 80% α-helix upon addition of neutral phospholipids vesicles.[10] However, the α-helix formation is not the only membrane-induced secondary structure possible; in the case of Alzheimer peptides and at low lipid-to-protein ratios, binding to negatively charged lipid bilayers leads first to β-sheet structures, and at high lipid-to-protein ratios to α-helix formation.[11]

The relatively high cost of interfacial partitioning of a peptide bond (about 1.2 kcal per mol), may explain the origin of membrane partitioning-folding coupling events and thus answers to the question of why the membrane interface is a relevant catalyst for secondary structure formation. Previous data demonstrated that interfacial β-sheet formation greatly assisted by H-bond formation reduces the cost of peptide partitioning by about 0.5 kcal per mol, per peptide bond.[12] Similarly, the folding of melittin into an amphipathic α-helix on POPC membranes entails about 0.4 kcal per mol reduction, per residue, of the interfacial partitioning of a peptide bonds.[13]

Membrane insertion of peptides and proteins is poorly understood at the molecular level, but the membrane interfacial region is believed to play critical roles in the insertion and refolding of peptides, as well as in the macroscopic manifestations of the resulting transmembrane pores.[14] Consequently, the reversible and reproducible control of protein and peptide insertion is of considerable relevance for biological membranes, since it provides convenient ways for

altering membrane function. Structural information obtained through combined x-ray and neutron diffraction measurements on multilamellar bilayers dispersed in water or deposited on surfaces, have revealed that the combined thermal thicknesses of the interfacial layers of a lipid membrane is equal to that of the hydrocarbon core (30 Å). Therefore, a 15-Å-thick interfacial layer can easily accommodate a membrane-based protein helix lying parallel to the membrane plane. Equally important, such experiments have proven that the thermally disordered interfacial regions are highly heterogeneous both physically and chemically, and this is highly biologically relevant since these regions are of first contact in the folding and insertion processes of membrane proteins and peptides, such as toxins, antimicrobial peptides, pore-forming proteins and surface-binding enzymes. As it will be presented in what follows, because of interfacial chemical heterogeneity, a charged or dipolar macromolecule diffusing from the water phase to the bilayer hydrocarbon core will experience a very steep variation in environmental polarity over a short distance; consequently, peptide-bilayer interaction energies will greatly modulate amphipathic helices topology within membranes and alter their kinetic features.[15,16]

In a more general description, the lipids-associated parameters of a membrane modulate the functioning of membrane-residing proteins and peptides via bilayer surface hydration,[17] membrane curvature – induced lateral pressure along the normal to the membrane,[18,19] membrane phase state,[20,21] bilayer thickness,[22] and lateral organization of lipids into a membrane.[23,24]

As hinted above, one of the very interesting features that is associated with lipid membranes stems from their electric manifestation. Generally speaking, the overall electrical profile of a biomembrane consists of contributions from the transmembrane potential, dipole potential and the difference in the surface potentials on both sides of a membrane.[25,26]

While the transmembrane potential results from a charge gradient across the membrane and the surface potential from the net excess charge present at the membrane-water interface, the membrane dipole potential has its origin in the molecular dipoles located on the lipid molecules. Structural studies have revealed that two major factors underline the origin of the dipole potential: the orientation of dipolar groups located on the lipid molecule (i.e., the dipole of the carbonyl group of the ester bond and the P^- - N^+ dipole of the head group), and the dipoles of oriented water molecules at the membrane-water interface.[27,28] Its overall value (about 300 mV, positive toward the membrane interior) and the resulting extremely high electric field associated with it over the interfacial region ($10^8 - 10^9$ V m^{-1}), endow the dipole potential with major roles in the modulation of molecular processes which take place within a biomembrane. For instance, the dipole potential has been shown to play an important role for proteins insertion and functioning,[29] kinetics of the gramicidin channel,[30] modulation of the activity of phospholipase A2[31] and electrical conductance of certain aqueous protein pores.[32] Recently, dipole potential was shown to also affect the channel properties of the antifungal lipodepsipeptide syringomycin E (SRE), whereby a rise in the dipole potential triggers an increase in the minimum SRE concentration needed for the detection of single channels at a given voltage, as well as a decrease in the steady-state number of open SRE channels at a given SRE concentration and voltage.[33] Other data have demonstrated that the emission intensity of a widely used fluorescent moiety, 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) labeling covalently either the headgroup of DPPC lipids (DPPN) or the acyl chains of PC lipids (NBD-PC) aggregated as liposomes, is sensitive to the membrane dipole potential and that the rate of the reduction of NDB in these probes by dithionite can be controlled by the dipole potential.[34]

More interestingly, studies undertaken on peptides and proteins embedded on membranes with various compositions illustrated the interplay between the structure, dynamics, and function of such macromolecules and the properties of the lipid membrane. In the simplest terms and from an electrical perspective, a membrane-inserted peptide or protein may modulate the dipole potential of the membrane by affecting the hydration layer of the interface and/or by causing small variations in the orientation of the P^- - N^+ dipole. With respect to this last effect, it should be noted that even small alterations in the geometrical orientation of the P^- - N^+ dipole caused by the embedded protein or peptide, would lead to an increase in the probability of its orientations perpendicular to the interfacial plane, and induce an augmented dipole potential change in a direction normal to the membrane plane. In this line of argument and to name just one, there are a number of studies which specifically demonstrate that, for instance, melittin is able to modify the orientation of the P^- - N^+ dipole.[35]

In this short review we present some of our recent data gathered regarding the specific paradigm of the interactions manifested between the dipolar electric field of reconstituted phospholipid membranes and various antimicrobial peptides, from prospective changes imposed by the membrane upon kinetic and transport features of such model ion channels. Such data may prove useful as they shed more light into the mechanism of interaction between peptides and reconstituted lipid bilayers, resulting from electrophysiology experiments carried out at the single-molecule level, may help refine future approaches to better grasp the *in-vivo* putative mechanism of action of antimicrobial peptides and help understand biological membranes-drugs interactions.

RESULTS AND DISCUSSION

By using combined x-ray and neutron diffraction measurements, a relevant picture arises regarding the liquid crystallographic structure of a fluid lipid bilayer. As shown in Fig. 1, the time-averaged probability distribution functions of water and lipid groups – in fact, their projections of three-dimensional thermal motions onto the bilayer normal - reveal the great degree of thermal disorder of a fluid membrane. Most importantly, it is seen that the thermal thickness of a single interfacial layer of the model membrane represented in Fig. 1 assumes a value of ~ 15 Å, and therefore easily lodges a α-helix arranged parallel to the membrane plane. Of similar biophysical relevance is the fact that the thermally disordered interfacial layer are highly heterogeneous both physically and chemically; this reflects in the dramatic variation of charge density over the distance which encompasses such layers, thus putting in place conditions whereby peptides adsorbed to such layers will interact rather strongly via electrostatic forces with the membrane. It becomes therefore conceivable that the degree of insertion and interfacial mobility of peptides interacting with interfacial layers of a lipid membrane would dramatically be modulated by the very magnitude of electric field experienced over such regions (for a comprehensive review covering such aspect, the reader may consult the chapter of the biophysics books on line - Membrane structures, Membrane proteins - maintained by Dr. Stephen White (http://www.biophysics.org/education/topics.htm).

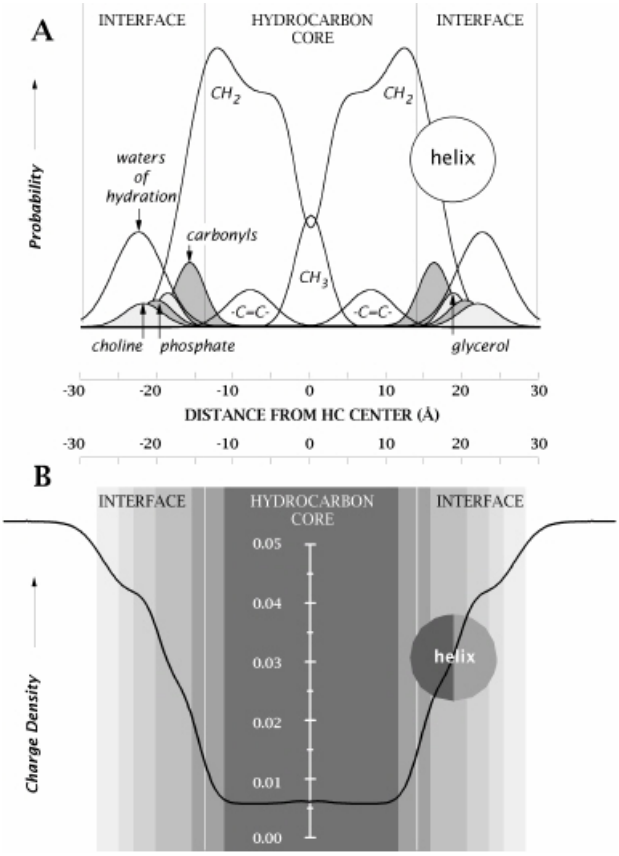

Fig. 1 – (A) General view of the liquid crystallographic structure of a DOPC fluid lipid bilayer, as inferred from X-ray and neutron diffraction data, along with its numerically estimated polarity profile. (B) The thickness of a single interfacial layer is about half of that of the hydrocarbon core, thus being able to comfortably lodge a monomeric α-helix arranged parallel to the membrane plane (adapted from http://blanco.biomol.uci.edu/Bilayer_Struc.html).

One particular interest in our lab was to shed more light into the less-explored paradigm of the energy coupling between membrane-based protein and peptides, and the membrane dipole potential. To do so, we sought to monitor quantitatively the extent to which alamethicin monomers alter the dipole potential of a model, reconstituted lipid membrane. Alamethicin is a 20 amino acid peptide isolated from the fungus *Trichoderma viride,* which has the propensity to form ion-channels in lipid membranes. Once added to the aqueous side of a lipid membrane, monomeric alamethicin strongly binds to lipid bilayers (partition coefficients of about 10^{-3} M)[36] exhibiting a cooperativity parameter of 5.5. It is now established that positive potentials on the addition side of alamethicin augment the probability of alamethicin oligomers formation within the membrane, due to a favorable interaction between the alamethicin monomers and the external electric field, which eventually results in a higher degree of membrane insertion of such monomers. Single-channel conductance measurements indicate that the alamethicin channels are characterized by multilevel bursts interrupted by prolonged periods of silence, and analysis of the alamethicin multiple channel kinetics indicates activation energies of 120 and 50 kJ/mole respectively.[37] By knowing that alamethicin monomers possess a rather large dipole moment of about 40 - 80 D[38], we believed that it may be possible for the dipole moment of the lipid monolayer where alamethicin monomers embed to undergo time-dependent changes, which would reflect alamethicin partitioning from the aqueous phase into that monolayer.

One of the powerful methods to time-resolve and characterize experimentally the effects induced by peptides and proteins insertion upon the dipole potential component of lipid membranes, is called the inner field compensation method (IFC), and it makes use of the well-known dependence of the membrane capacitance upon the effective potential difference applied.[39,40]

Our data presented in Fig. 2, panel a, show that upon alamethicin addition to the cis side of the lipid membrane, the amplitude of the dipole potential of the cis lipid monolayer undergoes a continuous changes, with a rather slow time constant. As emphasized before, the observed phenomenon reflects the partial partitioning of alamethicin monomers within the membrane.[41]

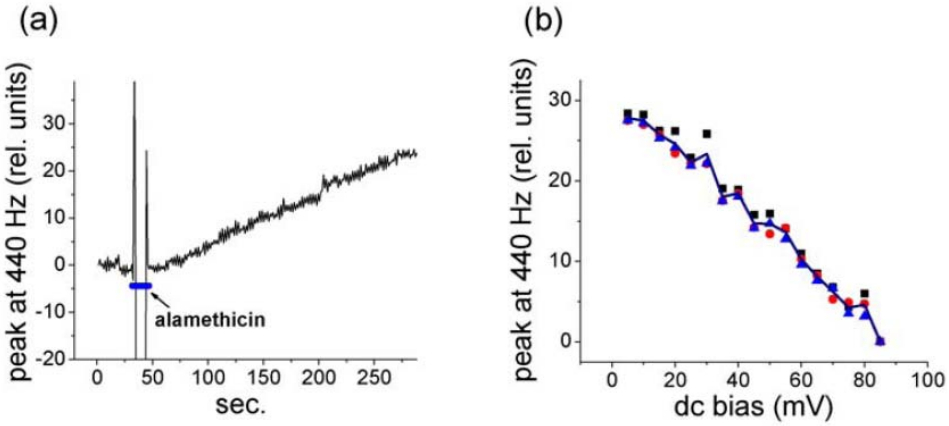

Fig. 2 – (a) Real-time monitoring of changes of the dipole potential of a lipid monolayer, brought about by alamethicin adsorption to a reconstituted lipid membrane. The time-unfolding of dipole potential changes is reflected by the continuous variation in the amplitude of the second harmonic component measured at 440 Hz, from the capacitive current through the artificial membrane subjected to a null dc bias of an applied sinusoidal time-varying potential difference with a main frequency of 220 Hz. (b) Small, continuous changes of the dc component from the applied potential difference which zero the amplitude of the second harmonic at ~ 85 mV, demonstrate the same magnitude net increase in the dipole moment of the lipid monolayer where alamethicin monomers get adsorbed.

We further quantified the change of the dipole moment of the cis monolayer as a result of alamethicin insertion into it, and concluded that ~ 85 mV positive on the trans side of the lipid membrane leads to an almost complete canceling of the amplitude of the second harmonic component from the capacitive current across the membrane. By simple analysis, this in turn points to the fact that the partial embedment of

alamethicin monomers into the cis lipid monolayer increases the dipole moment of it.[41]

Another important paradigm we considered in our investigations regarded the interactions manifested between the dipolar electric field of phospholipid membranes and alamethicin oligomers from prospective changes imposed by the membrane upon kinetic and transport features of such model ion channels. For this, we employed phlorizin, a chemical known for its ability to lower the magnitude of the dipole field only on the interface that is added to. As shown in Fig. 3, addition of 500 μM phlorizin to the cis side of a lipid membrane that contains alamethicin monomers, leads over time to a considerable enhancement in the activity of alamethicin oligomers. We proposed that such an increase in the alamethicin activity may be caused by a shift in the equilibrium of monomers that partition between the aqueous phase and the cis side lipid monolayer. That is, alamethicin monomers from the aqueous solution which partition into the lipid membrane would sense across the interfacial region of the cis monolayer a reduced value of the dipole potential. As a result, the reduced values of the dipole potential will result in a lower energy barrier for the initial adsorption of alamethicin monomers on the cis side of the membrane prior to their insertion into the membrane, which will eventually promote an elevated activity of alamethicin oligomers.[42]

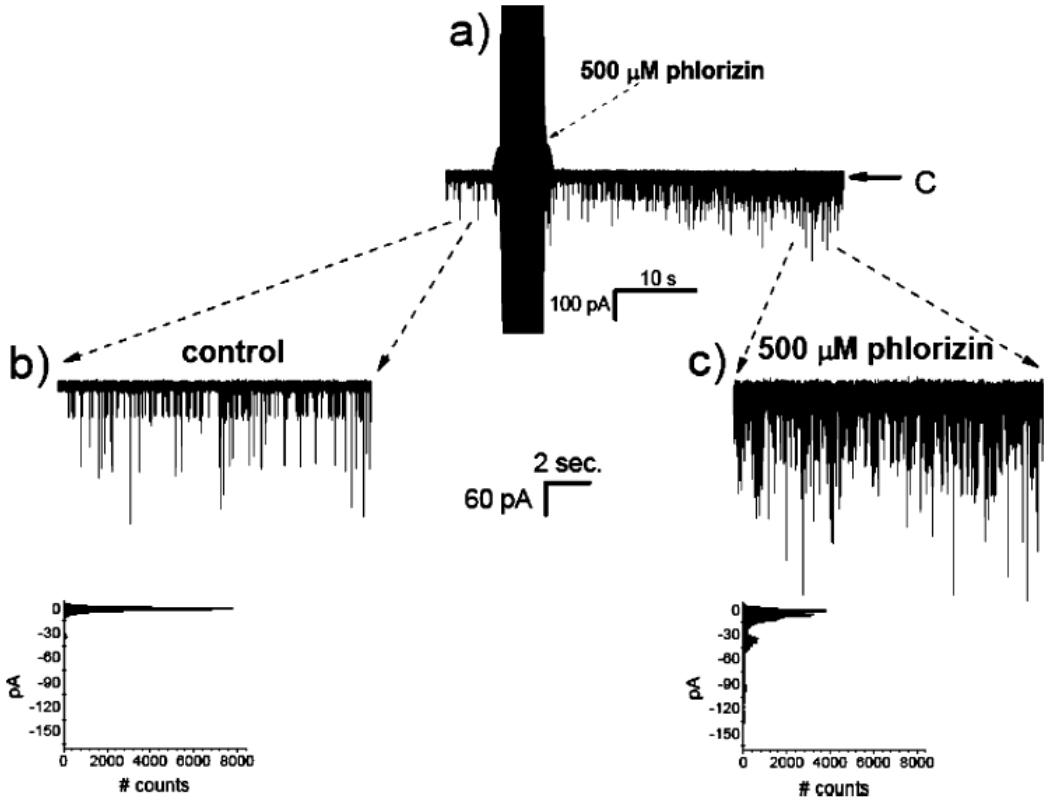

Fig. 3 – (a) Selected trace which shows the enhancement in the alamethicin oligomer activity recorded at -80 mV, brought about by the cis side addition of phlorizin (500 μM). The closed state of the channels is designated by C, and downward spikes reflect the electrical current mediated by various sizes alamethicin oligomers. (b and c) Magnified selections of alamethicin activity recorded at -80 mV, together with current amplitude histograms that correspond to channel's activity under control conditions and after the interaction of the membrane's cis side with 500 μM phlorizin.

Moreover and in relation to this, we proved that the actual energy barriers for alamethicin insertion become significantly smaller, leading to a 4-fold increase in the activity of ion-conducting oligomers across the membrane.[42] A similar effect has been observed by us before, using an alternative strategy based on the known potency of various inorganic and organic cations to decrease the dipole potential of lipid membranes.[43] Specifically, we have proven that at neutral pH and in the presence of calcium ions an increase in the number of alamethicin monomers that oligomerize within the membrane takes place.[44]

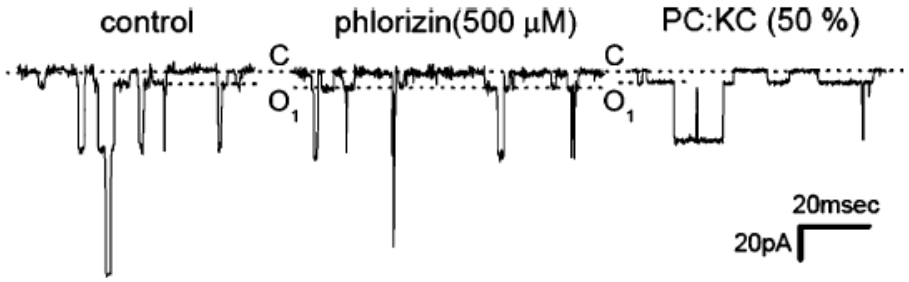

Fig. 4 – Original current recordings which reflect the single-channel activity of an alamethicin oligomer monitored at -110 mV, illustrating the increase in channel's conductance imposed by the decrease of the dipole potential of the cis membrane monolayer following its interaction with phlorizin. Complementarily, when added to the cis lipid monolayer, KC enhances the dipole potential of it and consequently leads to a decrease in the conductance of the alamethicin channel. In this figure C denotes the closed state of the channel, and O1 denotes its first conductive state.

As it would be expected for a channel with low selectivity for monovalent cations (i.e., alamethicin), the change in the electrical conductance of alamethicin channels caused by alterations of the dipole potential induced asymmetrically either by phlorizin or 6-ketocholestanol (KC- a chemical that enhances the dipole potential of the lipid monolayers where they reside) is expected to be low, yet still present. To evidence the antagonistic effects exerted by phlorizin and KC on the ion transport mediated by alamethicin oligomers, we displayed in Fig. 4 original traces of ion current fluctuations mediated by the alamethicin at an applied potential difference of -110 mV. It is thus seen that the single-channel current through the first conductive state of the alamethicin channel is slightly increased in the presence of 500 μM phlorizin, whereas on lipid membranes containing 50% (w/w) KC on the cis monolayer, the electrical current mediated by the same sub-state of alamethicin decreases[42].

In a recent attempt to unravel existing correlations between membrane binding and electric features of a model lipid membrane, we have undertaken a study involving HPA3, an analogue of the the HP(2–20) peptide (residues 2–20 parental HP derived from the N-terminus of *Helicobacter pylori* Ribosomal Protein L1), which showed more profound activity against yeast, Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria than did HP(2–20). Among others, HPA3 showed insignificant cytotoxic activity, suggesting that it would be a good candidate for the development of novel antibiotic agents.[45]

Data presented in Fig. 5 demonstrate that a lower electric dipole field of the interfacial region of a lipid membrane induced by phloretin, greatly assists the surface-bound HPA3 peptides to break free from one leaflet of the membrane, insert into the membrane and contribute to pore formation spanning the entire thickness of the membrane. We propose that phloretin leads to an enhanced peptide translocation across the membrane, contributing thus to accumulation of HPA3 peptides on the trans-side of the reconstituted membrane, upon disintegration of the conductive pores. This is substantiated by the fact that shortly after phloretin addition to the cis side of the membrane, the pore-forming ability of the HPA3 peptide becomes readily visible at positive-applied potentials, which can be explained by highly efficient peptide accumulation on the trans side of the membrane, which follows disintegration of phloretin-augmented density of HPA3 pores.[46]

Bearing in mind the relevance of the membrane dipole potential on cellular function, our data emphasizes that by manipulating its value, the influence of the dipole potential on protein-lipid membranes interactions may be explored and thereby may provide deeper insights into mechanisms of protein interactions with artificial and natural lipid membranes. We also extend the idea that the membrane dipole potential could be employed to manipulate the in-vivo susceptibility of cells towards various antimicrobial peptides. When working complementarily with chemical agents able to alter controllably the electric profile of the bacterial membrane interface, our data brings up the practical possibility of devising protocols aimed at destruction of bacteria by specific antimicrobial peptides, whose extracellular concentration could be kept at lower levels than their MIC's. Altogether, we believe that contributions stemming from the above experimental data pave the way toward a theoretical and practical understanding of how peptides interact with the heterogeneous membrane environment.

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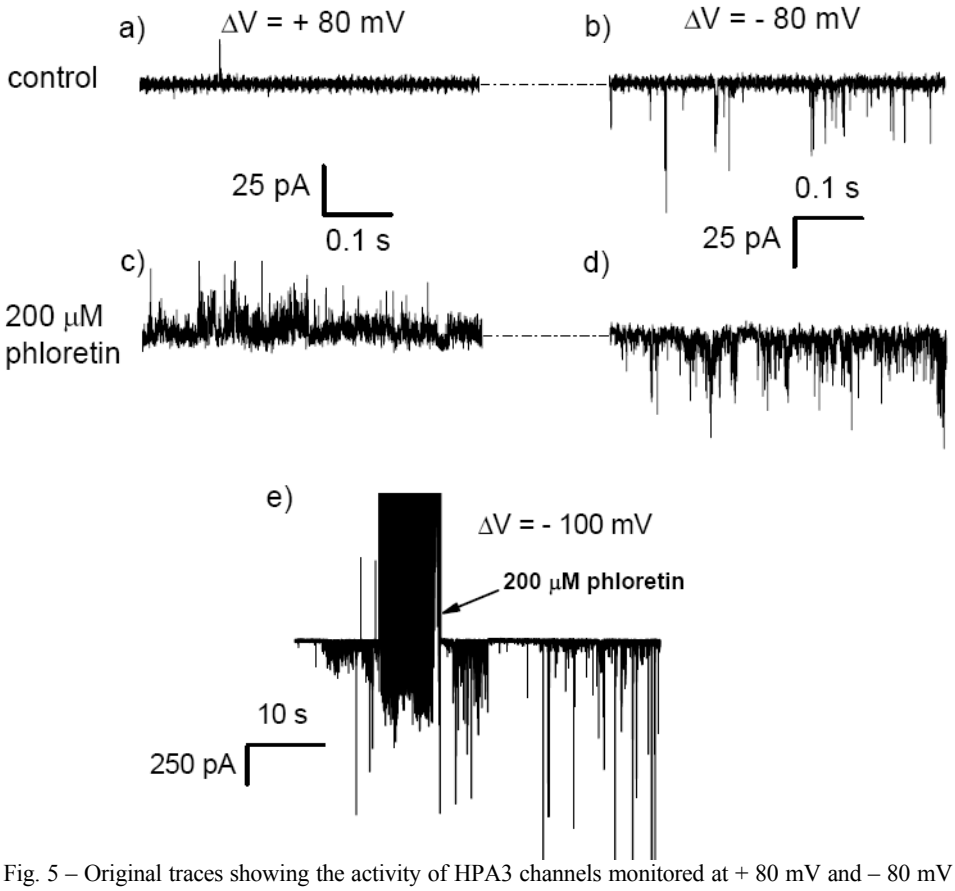

Fig. 5 – Original traces showing the activity of HPA3 channels monitored at + 80 mV and – 80 mV before (panels a and b, 'control'), and after the cis-side addition of 200 μM phloretin (panels c and d). The net increase in the number of active HPA3 channels induced by phloretin may be tackled within the paradigm of a decrease in the free energy penalty for partitioning of HPA3 peptides in the hydrophobic core of the membrane, caused by the phloretin-induced lowering the membrane dipole potential. e) Original trace which reveals, at a larger time scale, phloretin-mediated dipole potential changes in the activity of HPA3 monomers interacting with a reconstituted lipid membrane, measured at a potential difference of – 100 mV. Downward spikes designate electrical currents through the membrane mediated by transient pores formed by HPA3 monomers, whose number increase dramatically as a result of phloretin interaction with the lipid bilayer.

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