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Activity modulation of certain ion-pore forming proteins by electric properties of artificial lipid membranes

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Lipid-based artificial systems built to resemble closely biological membranes represent a hot-spot of today's biophysics research on lipid membranes-proteins interactions. Due to the interfacial chemical heterogeneity of the interface separating lipid membranes from aqueous media, membrane-penetrating peptides will sense a steep change in environmental polarity manifested via electrical interactions with the surface and dipole potential of membranes. We demonstrate that such interactions visibly modulate the membrane insertion of certain antimicrobial peptides. The effect of pH on artificial lipid membrane electrical properties was examined by studying the electrical conductance of alamethicin nanopores embedded in artificial lipid membranes. Our data strongly support the paradigm of a pH-dependent variation of the membrane dipole potential which, in conjunction with possible lateral pressure effects within the lipid membrane, lead to a non-monotonic modulation of ion transport mediated by alamethicin. By quantifying time-resolved discrete conductance fluctuations of the OmpF porin, our data point to a dipole potential-induced change of the protonation probability of acidic residues which define the constriction zone of the porin. Our interpretation points to a shift in the pKa values of such acidic residues caused most likely by alterations of the electric field profile through the OmpF pore, which in turn will lead to a change in the local concentration of hydrogen ions.

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1. Introduction

Biological membranes are polymers made mainly of lipids and proteins, and the most likely reason for their existence could be the evolution-driven quest for an efficient solution of mass and energy compartmentalization. Our understanding of the structure of biological membranes was tremendously aided by the now over-familiar, fluid–mosaic or Singer–Nicholson model. This model-representation of a biomembranes constituted a historic landmark in cell biology and biophysics and provided an extremely useful paradigm for the investigation of the properties of membranes, which started to be regarded as complex and dynamic environments with the potential to affect membrane protein structure and function [1]. Although the Singer–Nicholson model perceived the bulk of the bilayer as a homogeneous fluid, data gathered over the past decades strengthened the idea that plasma membranes contain phase separated domains of different lipid composition. Differential packing of lipids with various degrees of saturation leads to the formation of the so-called 'lipid rafts' which are found in a liquid-ordered (lo) phase characterized by tightly packed acyl chains, as compared to the liquid-disordered (ld) phase of the membrane [2]. The liquid-ordered (lo) phase has been shown to have a slightly increased translational order compared to the (ld) phase (e.g., the translational diffusion coefficient of lipids is about two times lower) and a configurational order that is comparable to that of an solid-ordered (so) phase [3, 4]. Notably, due to the increased trans/gauche ratio in a (lo) phase, this phase (and its domains) are thicker than an (ld) phase. It is worth mentioning that the raft hypothesis proposes that certain lipids aggregate in the plane of the membrane driven by intermolecular interactions, such as van der Waals interactions between the nearly fully saturated chains of sphingomyelin and glycosphingolipids as well as hydrogen bonding between adjacent glycosyl moieties of glycosphingolipids [5]. As hinted above, biological membranes possess additional properties caused mainly by their electric features, that further endow them with subtle and highly sophisticated modes of additional behavior. The most well-known electrical potentials associated with lipid membranes which were proven to play important roles on membrane behavior, are the transmembrane potential difference – associated with a gradient of electrical charge across the phospholipid bilayer - and the membrane surface potential, which is promoted by the existence of net excess electric superficial charges at the membrane interface in contact with the surrounding aqueous medium. One relatively recently acknowledged level of sophistication associated with 'electrified' biological membranes, is that a supplementary electric membrane potential, known as the dipole potential, appears to have important roles to play in protein-membrane interactions [6]. In a nutshell, the membrane dipole potential is the macroscopic manifestation of a nonrandom orientation of the electric dipoles in lipid headgroups ($P^{\square-}$ - $N^{\square+}$), fatty acid carbonyl groups ($C^{\square+}$ = $O^{\square-}$) and membrane-adsorbed

water. The first strong indication for the existance of the dipole potential came from experiments aimed at studying conductive properties of artificial lipid bilayers doped with hydrophobic ions. In order to explain the approximately 10^5 greater permeability of such membranes towards tetraphenylborate (TPB^-) than to tetraphenylphosphonium (TPP^+) ions, it has been proposed that the interior of the membrane must be positively charged [7]. Phospholipids, which are the most common class of membrane lipids, have two hydrophobic acyl chains and different hydrophilic headgroups, either charged or neutral. Even in phospholipid bilayers with neutral headgroups, the electrostatic interactions were shown to play vital roles in the structural properties of membranes. The phosphate group of such lipids, which is linked to the glycerol backbone, has a net negative charge, while the choline group, which constitutes the free end of the headgroup, bears a net positive charge; overall, these charges are spatially oriented such that there is a net headgroup dipole within the range 18.5–25 D [8]. However, the zwitterionic headgroups of most phospholipids, including phosphatidylcholine and phosphatidylethanolamine are thought not to be a major contributor to the magnitude of the dipole potential. On average, the headgroup dipoles lie approximately parallel, within 30°, to the membrane plane, and on average the $P^{\square-}$ atoms are actually located closer to the membrane interior than $N^{\square+}$ atoms, creating a negative potential in the membrane interior. Water molecules hydrating the sn-2 carbonyl and the phosphate group overwhelmingly cancel this effect and create a positive potential in the bilayer core. Along with the dipole moment of the carbonyl group of the sn-2 acyl chain which is directed towards the water phase with the positive charge inside the membrane, the oriented water molecules will lead to a considerable positive potential in the interior of the bilayer [9, 10]. Due to the extremely high electric field associated with it over the interface between the aqueous phase and the hydrocarbon region of a biomembrane (10^8 – 10^9 Vm^{-1}), the dipole potential has powerful influences on membrane-protein interactions [11, 12, 13]. A highly interesting concept which applies to zwitterionic lipids-based artificial membranes points to the influence played by pH on its electrostatic manifestations, with particular emphasis in modulating the dipole potential value. Broadly speaking, the pH affects a number of membrane-mediated biological processes, including here cholesterol domain formation, interactions manifested between various drugs and liposomes, and equal interesting membrane phase transitions (for a comprehensive reference, see 14). It comes therefore as no surprise that the work aimed at the characterization and understanding of the interactions between protons, hydroxide ions and lipid membranes comes to meet still virgin biophysical concepts. Upon exposing a lipid membrane to varying concentrations of counter-ions, including here protons and hydroxide ions, functional groups of lipid molecules (mostly phosphate and choline) may experience changes regarding their charge distribution at the membrane interface, which will reflect into alterations of their Debye length, membrane surface charge density and zeta potential [15, 16]. To substantiate this assertion, is worth mentioning that liposomes made of neutral lipids do electro-migrate when exposed to external electric fields, and this reflects an accumulation of electric charge onto the external side of the membranes stemming from the adsorption of aqueous ions on the zwitterionic liposomes [17]. In quantitative terms, it has been established that at pH 2 and 3, unilamellar vesicles made of 1-stearoyl-2-oleoyl-phosphatidylcholine (SOPC) possess a positive zeta potential, and this in turn points to a rather considerable association of protons at the membrane surface [14]. By measuring the electrophoretic mobility of such vesicles, it has been established that the isoelectric point of PC lipids is around pH 4 – corresponding to a nearly zero zeta potential, whereas close to an almost neutral pH (6.5), the zeta potential becomes negative pointing out the existence of negatively charged lipids. Provided that the acidity range within which one studies such pH-induced changes upon lipid membranes electrostatics does not overlap those values which may cause a change in the titration state of PC functional groups and induce the presence of net changes of the electric charge of lipid themselves- the pKa for phosphate is < 2, ~ 11 for choline and ~ -25 for the ester carbonyl groups- it can be stated safely that pH induced changes on membrane electrostatics result from protons and hydroxide ions binding and partitioning into the membrane. Due to the fact that, by definition, the zeta potential is physical reflection of the surface charge, a negative zeta potential at pH 6.5 very well makes the point that under such circumstances hydroxide ions associate more consistently with the studied lipids than protons do [14]. Besides altering the membrane surface electrostatics, aqueous ions including here protons and hydroxide ions can also modulate the membrane's dipole potential [14, 18, 19]. Due to the fact that the membrane dipole potential is positive towards the hydrophobic core of the membrane, the partitioning of hydroxide ions would lead to a decrease in the dipole potential. It should be kept in mind, however, that the potency of a given class of anions towards lowering the membrane dipole potential is controlled predominantly by the Gibbs free energy of hydration [19], which in turn rules the partitioning of ions between the inner region of interfacial part of the membrane and the aqueous phase. Alternatively, at acidic pH values, the low concentration of hydroxide ions into the interfacial layer of the membrane lead to larger dipole potentials.

In this work we review recent evidence from our lab which strengthens the possibility of the existence of strong interactions manifested between the dipolar electric field of phospholipid membranes and alamethicin oligomers, from prospective changes imposed by the membrane upon kinetic features of such model ion channels. Based on our data, we favor the paradigm that a lower electric dipole field of the interfacial region of the membrane provides a reduced repelling influence upon the positively charged N-terminus of the alamethicin peptides, as they move from the aqueous medium to lipid membranes. By employing phlorizin to selectively decrease the magnitude of the dipole potential on the membrane interface that is added

to, we show that the energy barrier for alamethicin insertion become significantly smaller, leading to a 4-fold increase in the activity of ion-conducting oligomers across the membrane. Supplementary experiments involving single alamethicin oligomers have revealed a non-monotonic dependence of the single channel electrical conductance versus pH changes within the 0.62 ÷ 2.94 range. Interestingly, under conditions which would better favor cations transfer through the alamethicin channel, ensured by a smaller net positive charge onto the lipid membrane surface (e.g., pH = 2.94), the electric conductance of the first and second conductive states of the slightly cation-selective alamethicin is actually smaller than at pH = 0.62 and 2.08. Our tentative conclusion derived from such experiments points to a possible involvement of lateral pressure effects within the lipid membrane, which may increase as the pH changes from a value of 0.62 to ~3 and therefore lead to a prominent mechanical constriction of the alamethicin pore, such that it counter-balances the favorable electrostatic interactions between the membrane and incoming cations. Supplementary, work performed on a structurally and functionally different protein nanopore inserted in artificial lipid membrane (i.e., the OmpF porin) pointed out to a dipole potential-induced modulation of the protonation probability of acidic residues which define the constriction zone of the porin. Our interpretation points to a shift in the pKa of values of such acidic residues, caused most likely by alterations of the electric field profile through the OmpF pore which result in a change in the local concentration of hydrogen ions and thus alter the protonation dynamics of Asp-113 and Glu-117 residues which constitute part of the constriction eyelet of the OmpF protein.

2. Materials and Methods

Electrophysiology experiments were carried out on the folded bilayer membranes system, obtained as previously described [13]. An artificial lipid membrane was formed on the ~ 100 μm diameter aperture milled in a teflon septum, that had been pretreated with 10% (v/v) hexadecane (Sigma-Aldrich) in highly purified n-pentane (Sigma-Aldrich). Both chambers of the bilayer cup contained 1 M NaCl and 10 mM sodium phosphate. The formation of a bilayer was monitored by observing the increase in capacitance to a value of approximately 90-130 pF. Alamethicin monomers (Sigma-Aldrich, code A4665, Rf30, ≥90% HPLC) were added from a stock solution made in ethanol (5 μM) to the cis chamber only, connected to the ground. When employed, phlorizin (Fluka) was added to the cis side of the membrane from an 80mM stock solution made in ethanol. Currents from the bilayer chamber were detected and amplified with an integrating headstage Axopatch 200 B amplifier (Molecular Devices, USA) set to the voltage-clamp mode. Data acquisition of the amplified electrical signals was performed with a NI PCI 6014, 16-bit acquisition board (National Instruments) at a sampling frequency of 5 kHz. When working with the OmpF protein, the buffer composition was as follows: NaCl 1 M, pH = 3.03 and 10 mM phosphate buffer. During experiments involving the OmpF porin, the sampling frequency was set to 50 kHz and data were low-pass filtered at 20 kHz with the help of an active low-pass filter (LPF-8, Warner Instrument Corp., USA). The purified OmpF protein was a precious gift from Prof. Mathias Winterhalter (Germany). Automatic data acquisition and analysis was done with the help of various virtual instruments created within the LabVIEW 8.20 environment.

3. Results and Discussion

As we show in figure 1, following the addition of a membrane dipole lowering agent to the side of a lipid membrane that contained alamethicin monomers (500 □M phlorizin), a vigorous increase in the activity of alamethicin oligomers across the lipid membrane was observed. As a possible explanation, we hypothesize that the elevation in the alamethicin activity may be caused by an alteration in the equilibrium of monomers that partition between the aqueous solution and the cis side lipid monolayer. That is, mostly due to their N-terminus vectorial insertion, incoming alamethicin monomers from the aqueous solution towards the lipid membrane are likely to experience over the interfacial region of the cis monolayer a reduced value of the dipole potential, which is being caused by the adsorbed phlorizin molecules. Consequently, it is very tempting to speculate that a reduced values of the dipole potential will result in a decrease in the energy barrier for the adsorption of alamethicin monomers on the cis side of the membrane before their insertion into the membrane. This in turn will lead to an elevated activity of alamethicin oligomers, since it is known that the overall conductance of the alamethicin-containing lipid membrane strongly depends on alamethicin concentration within the interfacial region of the cis monolayer [20].

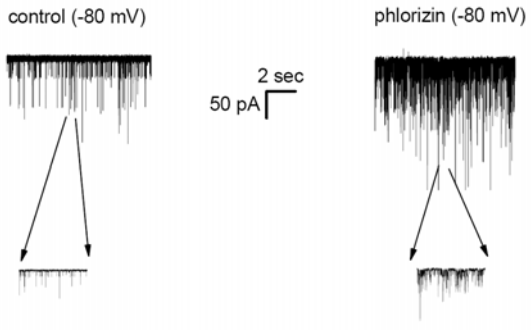

Fig. 1. Original traces of alamethicin activity in PC lipid membranes, in the absence (control) and presence of a dipole potential lowering agent (phlorizin). Downward electric current spikes reflect the reversible interactions between alamethicin molecules within the membrane plane, giving rise to oligomers of various size. Increased current 'noise' generated by membrane interaction with phlorizin were interpreted as an augmentation in the concentration of the interfacially adsobbed alamethicin monomers, which heighten the likelihood of ion-conducting alamethicin oligomers across the membrane

Another interesting piece of evidence which highlights the importance of membrane electrostatics, and of the dipole potential in particular, in setting ion transport properties of selective protein nanopores is being shown below.

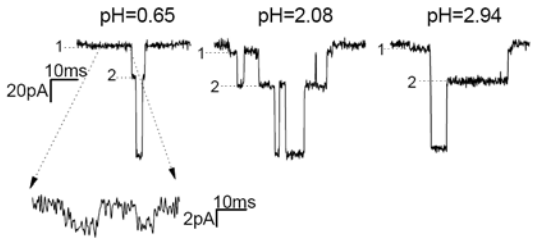

Fig. 2 pH modulation of the 1st and 2nd conductive state of a single alamethicin oligomer embedded on a artificial lipid membrane made of zwitterionic PC lipids, subjected to a -70 mV potential difference. Although the visible increase in the alamethicin's conductance when changing the pH from 0.65 to 2.08 would be easily explain by means of a decrease in the membrane dipole potential caused by hydroxide ions adsorption into the membrane hydrophilic region, the subsequent drop in the conductance at a even higher pH value (i.e., 2.94) still eludes a definitive explanation.

As it can be seen from figure 2, a monotonic change in the pH of the solution in contact with a artificial lipid membrane containing alamethicin oligomers causes a non-monotonic variation of alamethicin's sub-states conductance. At the studied pH values, Glu-18 which is the only ionizable aminoacid residue from the primary structure of the alamethicin oligomer, it is mostly protonated since its pKa hovers around 4.5 – 5. Apparently, even in this mostly-protonated state, the alamethicin oligomers still retains its weakly cation-selective property, as it can be inferred indirectly from studies involving the native Rf50 alamethicin isomer, in which the glutamate from the position 18 is replaced with a glutamine [21]. Therefore, electrostatic interactions manifested between the permeating anions, cations and the lipid membrane – alamethicin oligomer complex could in principle be used to explain the observed differences in alamethicin's conductance via a local increase in the cations concentration near the mouth of the channel and, alternatively, through a decrease in the energy barrier associated with cations translocation across the membrane. It is well-known that increasing pH values of the aqueous solution in contact with the zwitterionic lipid membranes lead to decrease of the membrane dipole potential, and this is being explained by a preferential adsorption of hydroxide anions close to the inner region of the hydrophilic domain of the membrane. Moreover, below pH 2, the phosphate functional group of PC lipids is mostly protonated (its pKa value is < 2) so that the net charged carried by lipids would be positive. In this line of arguments, it would be rather convenient to explain the mild, yet visible increase in the conductance of the first and second conductive states of the alamethicin oligomer when the pH changes from 0.65 to 2.08. However, this simple rationale seems to breakdown when trying to explain the decrease in alamethicin's conductance when the pH is further increased to a value of ~ 3; in considering a tentative explanation for this phenomenon, one may have to resort to a more in-depth analysis of membrane electrostatics and its modulation by the pH of the aqueous solution. Previous data from the literature has clearly pointed out that the zeta-potential of a zwitterionic lipid membrane is positive in the acidic range, and it decreases with increasing values of the pH up to the isoelectric point of PC lipids, which is ~ 4. With these in mind, the whole story becomes even tougher to tackle, since at a pH value of ~ 3 both the dipole potential and surface potential would facilitate cations transfer through the alamethicin pore, in stark contradiction with the experimental data. One possibility to reasonable explain our data which in turn certainly calls for more in-depth studies, resides in the yet not studied effects caused by the lateral pressure within the lipid membrane which may increase as the pH changes to ~3 and lead to a prominent mechanical constriction of the alamethicin pore in a way that counter-balance the favorable electrostatic interactions between the membrane and incoming cations.

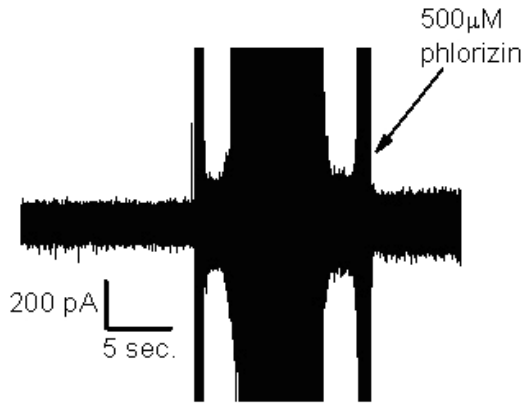

Fig. 3 Changes induced by phlorizin interaction with the lipid membrane upon current fluctuations measured through a single, fully open OmpF porin, at pH = 3 and 100 mV potential difference. Lower values of the membrane dipole potential lead to an increase in the 'on' reaction rate of acidic residues GLU 117 and ASP 113 which make up part of the constriction zone of the porin, and this will result in more pronounced electrostatic long-range effects upon ions permeation across the protein, and thereby more vivid current fluctuations.

One other interesting effect caused by dipole potential changes upon embedded protein nanopores is highlighted by data shown in figure 3. As it can be seen, a decrease in the dipole potential caused by 500 μM phlorizin interaction with a lipid membrane containing one single OmpF porin leads to a visible increase in the open-channel noise measured at acidic pH values (pH = 3) (the estimated standard deviation of current fluctuations was ~ 26 pA before, and ~ 37 after phlorizin addition). As documented by others [22], this electrical noise is Lorentzian in shape, pH-dependent and it reflects the fast protonation-deprotonation events of acidic aminoacid residues which make up part of the constriction eyelet of the OmpF protein.

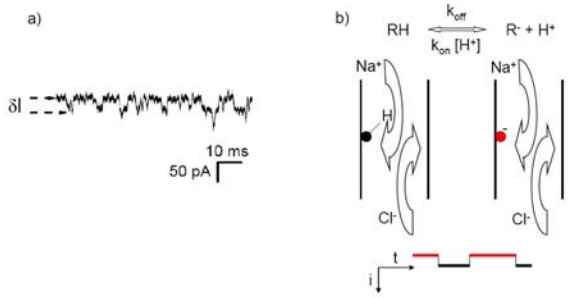

Fig. 4 (a) Transient sub-conductance states (I) resolved at pH = 3 in the current recorded through the OmpF protein at 100 mV, which stem from the reversible protonation of the Asp-113 and Glu-117 aminoacid residues; lower (downward pointing) electric currents can be associated with the temporary protonated state of acidic residues from the loop 3 (b) Schematic representation of protonation-deprotonation events taking place at one acidic residue (denoted by 'R', and identified as a black-filled circle on its protonated state) inside the OmpF pore, on the loop 3; lower pH values would promote higher protonation rates, and such events lead to more vivid downward stepwise current transients. The lower inset schematically shows the time dependent switch of the ion current mediated by the OmpF protein ('i') as the generic acidic residue ('R') flips between the protonated (RH) and un-protonated (R^-) states

That is, transient lower conductance substates of ~ < 10 ms duration (δI) can be resolved in the current recorded through the OmpF protein (Fig. 4), and would correspond to ionic flow through the mostly protonated OmpF protein. As it can be seen from Fig. 4 (a), on certain occasions double 'closing' evens could be seen, which may reflect the simultaneous protonation of both Asp-113 and Glu-117 residues. The power spectra of the stepwise flickering of the electrical current through the OmpF porin at such low pH values can be approximated by single Lorentzians which model rather reasonable the dynamics of the reversible protonation of the Asp-113 and Glu-117 residing on the loop 3 (Fig. 5).

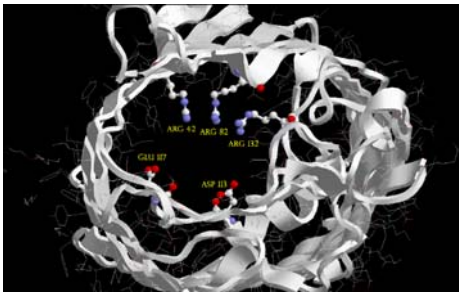

Fig. 5 Cross-sectional view from the cell-exterior side of one monomer from the OmpF trimer protein; the ionizable residues of the pore constrictions (glutamate-GLU 117, aspartate - ASP 113, arginine - ARG 42, arginine-ARG 82, arginine-ARG 132) are shown.

At this point, we posit that lower values of the membrane dipole potential lead to a local increase of the protons inside the OmpF pore close to the acidic residue GLU 117 and ASP 113 and subsequently increase the 'on' reaction rate of these aminoacids reversible protonation, fact which manifests itself through more pronounced electrostatic long-range effects upon ions permeation across the protein and thereby more vivid current fluctuations. Altogether, our experiments carried out with various classes of pore-forming protein s support the un-equivocal modulatory influence exerted by membrane electrostatics upon kinetic and transport properties of protein nanopores embedded in artificial lipid membranes, and possibly pave the way to a more comprehensive understanding of how membranes shape structural and functional properties of ion channels.

Acknowledgements

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