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pH modulation of transport properties of alamethicin oligomers inserted in zwitterionic-based artificial lipid membranes

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Abstract

Electric features of biological membranes are major determinants of the function and physiological manifestation of membrane-penetrating peptides, and such features are prone to be modulated by the properties of the surrounding aqueous medium. In this work, we demonstrate that pH plays crucial roles in modulating electric characteristics of zwitterionic-based artificial lipid membranes. The effect of pH on electrical properties of such membranes was probed by evaluating the transport properties of embedded alamethicin oligomers over a wide range of pH values (i.e., 0.65, 2.08, 2.94, 7 and 10.1). Our data strongly support the paradigm of a pH-dependent variation of the surface and membrane dipole potential which, in conjunction with possible lateral pressure effects within the lipid membrane, lead to a non-monotonic modulation of the electrical conductance of alamethicin oligomers. As expected, pH modulation of transport properties through the alamethicin oligomer is more visible for narrower pores (that is, the 1st conductive state) with slightly better cation selectivity as compared to larger oligomers.

Keywords: Protein pores; Lipid membranes; Alamethicin; Electrophysiology; Membrane electrostatics

1. Introduction

The interaction of peripheral proteins with lipid membranes is known to be central to many cellular processes. In particular, studying the interaction between biological membranes and synthetic or natural peptides which possess the ability of forming transmembrane nanopores, is vital to understanding the functioning of ion channels and antimicrobial peptides. The Singer–Nicholson model-representation of a lipid membranes served as a conceptual pillar in cell biology and biophysics, and provided an extremely useful paradigm for the investigation of biomembranes properties, which began to be viewed as dynamic environments with the potential to affect protein structure and function [1,2]. Among others, electric features of biological membranes endow them with subtle and highly sophisticated modes of physiological behavior. The best known electrical potentials associated with lipid membranes are the transmembrane potential difference — associated with a gradient of electrical charge across the phospholipid bilayer — and the membrane surface potential, which is generated by the existence of net excess electric superficial charges at the membrane interface in contact with the aqueous medium [3,4]. Supplementary, a component of the electric membrane potential known as the dipole potential, was acknowledged to play important roles in protein–membrane interactions [5–8]. Physically speaking, the membrane dipole potential stems from the macroscopic manifestation of the polarized orientation of the electric dipoles in lipid head groups ($P^{\delta-}N^{\delta+}$), fatty acid carbonyl groups ($C^{\delta+}{=}O^{\delta-}$) and membrane-adsorbed water. A highly interesting paradigm which regards the zwitterionic, neutral lipids-based artificial membranes is related to the influence played by pH on its electrostatic manifestations, with particular emphasis in its ability to alter the membrane surface charge and the dipole potential value. It is well-known that pH affects a number of membrane-mediated biological processes, such as cholesterol domain formation, interactions manifested between various drugs and liposomes, and lipid membranes phase transitions (for a comprehensive reference, see Ref. [9]). Therefore, efforts aimed at characterization and understanding of interactions between protons, hydroxide ions and lipid membranes come to

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answer still open questions in membrane biophysics. On one hand, upon exposing a lipid membrane to a solution containing varying concentrations of counter-ions, including protons and hydroxide ions, phosphate and choline groups of lipid molecules may undergo changes with respect to their charge distribution at the membrane interface, which will reflect into alterations of their Debye length, membrane surface charge density and zeta potential [10,11]. In order to exemplify, is worth mentioning that liposomes made of neutral lipids do migrate along external electric fields lines, and this is a consequence of an accumulation of electric charge onto the external side of the membranes, stemming from the adsorption of aqueous ions on the zwitterionic liposomes [12]. Quantitatively, 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 points to a considerable association of protons at the membrane surface [9]. From the evaluation of the electrophoretic mobility of such vesicles, it has been inferred that the isoelectric point of phosphatidylcholine (PC) lipids — corresponding to a nearly zero zeta potential — is around pH 4, whereas at almost neutral pH values (6.5), the zeta potential becomes negative thus reflecting the existence of a negatively charged membrane surface. Consequently, a negative zeta potential at pH 6.5 suggests that under such circumstances hydroxide ions associate more consistently with the studied lipids than protons do [13]. The modulation of surface potential represents a highly relevant physiological task, since its value is critical in many macroscopic manifestation of a cell, such as: cell adhesion and spreading, chemotaxis, endo- and exocytosis processes, interaction with biological active cationic molecules (e.g., anesthetics, pore-forming peptides, various enzymes) [14,15]. Some of the most used methods to quantify changes in the membrane surface potential, include the electrophoresis method, the assessment of shifts in the I–V diagrams of embedded ion channels and voltage-sensitive styryl dyes in conjunction with the dual wavelength excitation ratiometric fluorescence measurements method [16–18]. Besides the potential of altering the membrane surface electrostatics, protons and hydroxide ions can also modulate the lipid membrane dipole potential. By knowing that in the case of PC lipids the pK_a for phosphate is less than 2, for choline approximately 11 and for the ester carbonyl groups is around-25 [9], pH changes that do not overlap these particular values leave the titration state of functional groups of such lipids largely un-modified, so that it can be stated that such pH induced changes on membrane electrostatics result from protons and hydroxide ions binding and partitioning into the membrane. Taking into account that the membrane dipole potential is positive towards the hydrophobic core of the membrane, the membrane partitioning of hydroxide ions is anticipated to lead to a decrease in the dipole potential; alternatively, at acidic pH values, the low concentration of hydroxide ions into the interfacial layer of the membrane lead to larger dipole potentials. Nonetheless, it should be kept in mind that anions and cations other than hydroxide and hydrogen ions were proven to have the potency of lowering the membrane dipole potential. Specifically, anions with the lowest free energy of hydration (i.e., the least hydrophilic ones) induce the greatest decrease in the dipole potential, whereas the most hydrophilic cations cause the greatest reduction in the membrane dipole potential [19]. As a possible model for the opposite behavior of cations in this respect, it was proposed that they either interact with specific polar sites found on the membrane surface, they may contribute to a partial dehydration of the membrane head group region, or both.

The specific purpose of this work was to demonstrate that pH plays crucial roles in modulating electric features of zwitterionic-based artificial lipid membranes. The effect of pH on transmembrane electrical properties of such membranes was probed by evaluating the transport properties of embedded alamethicin oligomers over a wide range of pH values (i.e., 0.65, 2.08, 2.94, 7 and 10.1). Such experiments involving single alamethicin oligomers, have demonstrated an unexpected, non-monotonic dependence of the single channel electrical conductance vs. pH within the 0.65–10.1 range, which reveals the involvement of various electric components of the electrified lipid membrane in setting ion transport through ion-selective pores. Specifically, at extreme acidic values, e.g. pH=0.65, the electrical conductance of the first and the second sub-conductive state of the alamethicin oligomer is reduced with about 20% and 11%, respectively, as compared to values seen at pH=2.08. We see this as a reflection of the fact that increasing pH values of the aqueous solution in contact with the zwitterionic lipid membranes lead to decrease of the membrane dipole potential, which combined with a monotonic decrease of the positive charge of the membrane surface, facilitates the transmembrane transfer of cations (at these acidic pH values, although alamethicin's glutamate-18 is mostly protonated, the channel is still cation selective — see Ref. [20]). At increasing pH values, from pH=2.94 to pH=10.1, the conductance of alamethicin increases, which is mostly visible for the first conductive state of the channel. This may be explained via a steric effect combined with an electrostatic one; namely, electrostatic repulsion among alamethicin monomers within a formed oligomer, facilitated at higher pH values due to the ionized state of glutamate-18, would cause a cross-sectional increase of the channel. In addition, increasing basic pH values promote a more negatively charged membrane interface, and this in turn lead to a higher local concentration of cations near the mouth of alamethicin, which will reflect in an elevated electrical conductance of it. Interestingly, across the studied acidic pH range (i.e., pH=0.65, 2.08, 2.94) and under conditions which would best favor cations transfer through the alamethicin channel, ensured by a lowest membrane dipole potential and associated with the smallest net positive charge onto the lipid membrane surface (e.g., pH=2.94), the electric conductance of the first and second conductive states of alamethicin are considerably 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 counterbalances the otherwise favorable electrostatic interactions between the membrane and incoming cations.

2. Materials and methods

Single-molecule electrophysiology experiments on alamethicin were carried out on the folded bilayer membranes system [8,21]. An artificial lipid membrane was formed by the apposition of lipid monolayers spread onto the water–air interface of the two chambers which made up the bilayer system, on a ~100 μm diameter aperture milled in a Teflon septum, that separated the two chambers and had been treated with 10% (v/v) hexadecane (Sigma–Aldrich) in highly purified *n*-pentane (Sigma–Aldrich). The membrane-bathing solutions contained NaCl 1 M buffered at different pH values (i.e., 0.65, 2.08, 2.94, 7 and 10.1) by using sodium phosphate (10 mM). The successful formation of a bilayer was assessed by monitoring the increase in the total capacitance of the system to a value of approximately 150 pF; it must be noted however, that only the presence of alamethicin activity was the ultimate proof a functional membrane. Alamethicin monomers (Sigma–Aldrich, code A4665, Rf30, ≥90% HPLC) were added from a stock solution made in ethanol (5 μM) to the cis chamber of the bilayer system, which was grounded. 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 an NI PCI 6014, 16-bit acquisition board (National Instruments) at a sampling frequency of 10 kHz, within the LabVIEW 8.20 environment. To monitor in real time changes induced by pH upon membrane dipole potential we resorted to the automated implementation of the inner field compensation (IFC) method. As described elsewhere [22], the core design of the IFC method lies in the use of an NI PCI 6014, 16 bit acquisition board (National Instruments, Inc., USA) operated via a graphical programming language, to monitor the time-evolution of the second harmonic component from the capacitive current generated through a lipid membrane measured with the integrating headstage amplifier. The simultaneous A/D and D/A operations of the PCI card used throughout, along with all-decision making steps, spectral analysis and data handling, have been implemented with the help of the LabView graphical programming language (National Instruments, Inc., USA) within the 'virtual-instrument' concept.

3. Results and discussion

Rather unexpectedly at the first glance, changes in the pH value of a buffer in contact with a lipid membrane made of zwitterionic lipids dramatically alter the single channel conductance of alamethicin in a non-monotonic manner. As it can be seen from the Fig. 1, current levels for the most ion selective alamethicin substates, denoted by '1' and '2', do vary in amplitude as the pH changes from a value of 0.65 up to 10.1.

It is apparent from these recordings that a pH value of about 2 ensures a highest flow of charge carriers through the alamethicin channel, and more acidic or less acidic aqueous solutions cause a visible drop in the single-channel current mediated by the oligomer. To better quantify the pH effect on ion transport properties of the alamethicin channel embedded in a zwitterionic lipid membrane, we next evaluated the conductance changes of the 1st and 2nd conductive states of the

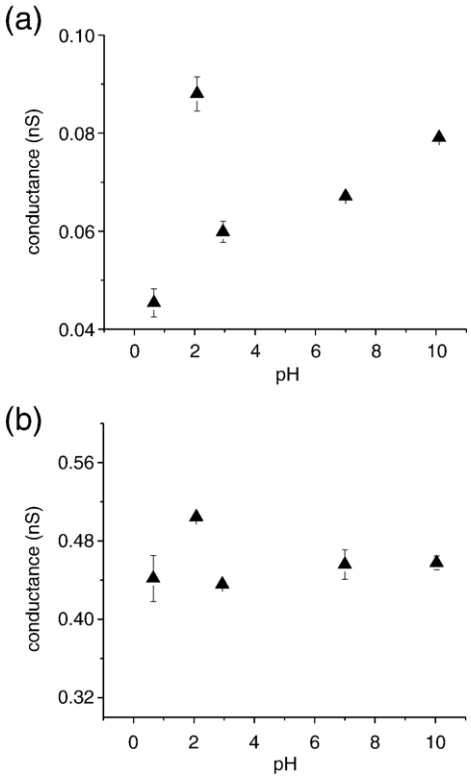

Fig. 2. Conductance values of the first (panel a) and second (panel b) conductive states of the alamethicin oligomer, estimated at various pH values (0.65, 2.08, 2.94, 7, 10.10), when buffer solutions contained NaCl 1 M. From such estimations it becomes clear that ion transport mediated by alamethicin depends non-monotonically vs. the bathing pH, and this phenomenon is apparently prevalent to the narrower sub-states of the oligomer (that is, sub-state '1'), which offers better ion selectivity.

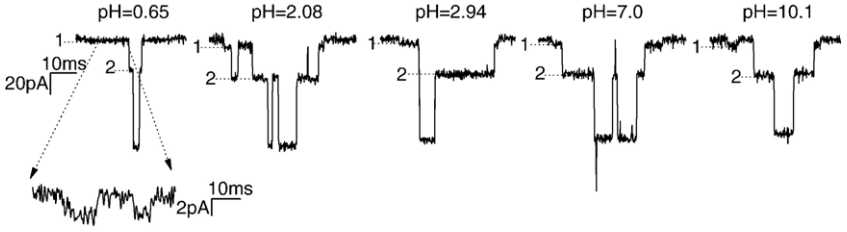

Fig. 1. Typical current recordings of single ion channels formed by alamethicin in PC bilayer membranes measured at a holding potential of −70 mV, when the membrane-bathing solutions were buffered to various pH values (i.e., 0.65, 2.08, 2.94, 7 and 10.1). It is clearly seen that the electrical current through various sub-conductive states of the alamethicin oligomer (we denoted by '1' and '2' the first two such states) vary non-monotonically with pH changes.

oligomers vs. pH, with the aid of current-voltage diagrams drawn for each specific case.

From the close inspection of data presented in Fig. 2, we conclude that there are two major regions within which pH plays an essential role in setting transport properties of alamethicin. That is, when pH varies from a value of 0.65 to 2.94, electrical conductance of both sub-states 1 and 2 go through a maximum located near the 2.08 pH value, and subsequently rise monotonically as the pH changes to less acidic values (i.e., pH=7 and 10.1). In our attempt to make sense of these data, we resorted to a close analysis of certain physical and chemical macroscopic manifestations of the lipid membrane within the studied pH domain, such as: head group charges of the lipid molecules, dipole potential of the membrane, surface charge density of the membrane and its transverse pressure profile.

In our attempt to grasp the physical reality, in Fig. 3 we represented the three main contributors to the overall 'electrostatic signature' of a zwitterionic lipid membrane: (a) the superficial surface charge (σ_s), which is known to give rise to the membrane surface potential (Φ_s) (b) the membrane dipole potential (Φ_d) and (c) the net charge of the lipid bilayer caused by the ionization state of the phosphate group linked to the choline group, symbolically denoted by P–N. In the current view and with direct relevance to the data presented above, pH changes do cause a shift in the macroscopic behavior of individual components listed above that lump together and set the electrostatic potential of the membrane, thus influencing the transport of ions through the alamethicin channel.

Specifically, zeta potential of a membrane composed by PC lipids is positive below pH 4 and this is due to the high concentration of protons in the solution which bind to PC lipids, thus generating a net positive charge on the membrane surface. As the pH changes to less acidic values, the concentration of the adsorbed protons on the membrane surface will decrease, entailing a consequent drop of zeta potential; around the isoelectric point of PC lipids (pH ~4), the superficial surface charge due to protons adsorption is expected to become zero. When changing the pH up to basic values, the concentration of hydroxide ions in solution will increase and therefore such ions will bind more effectively to PC head groups, generating a net negative charge on the membrane surface, which will be

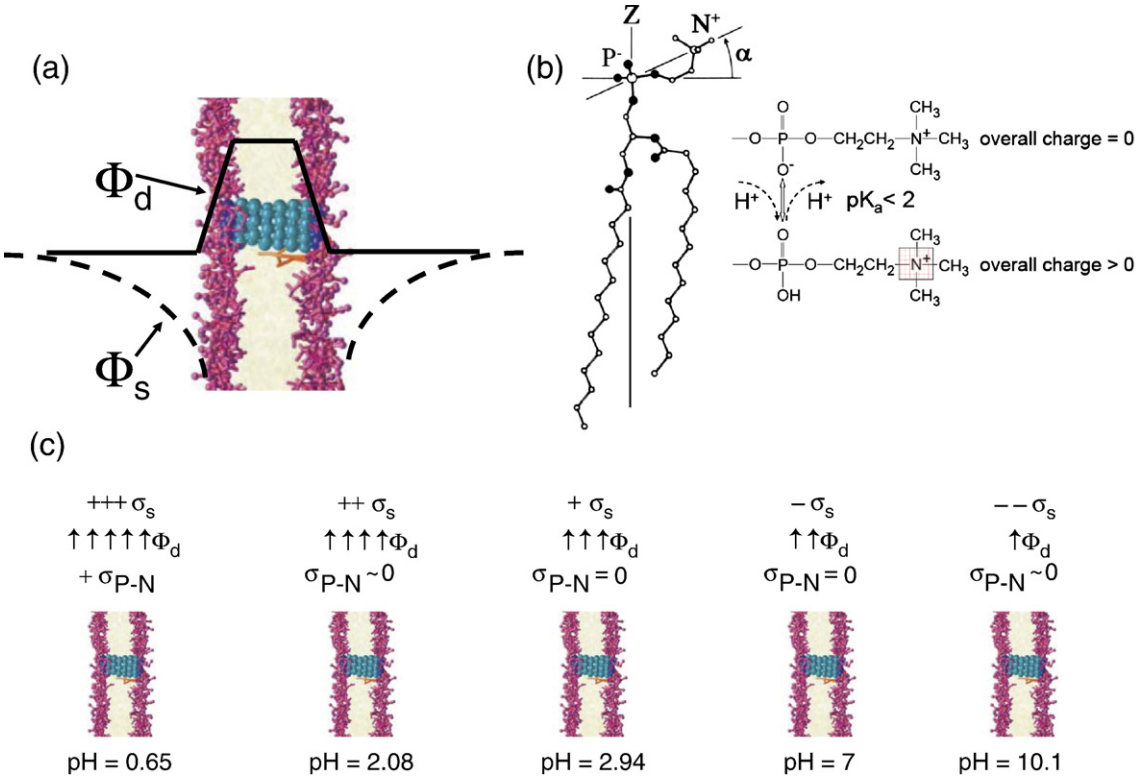

Fig. 3. Oversimplified representation of the main components which make up the lumped 'electrostatic signature' of a zwitterionic lipid membrane: (i) the superficial surface charge (σ_s), which is known to give rise to the membrane surface potential (Φ_s) (ii) the membrane dipole potential (Φ_d) and (iii) the net charge of the lipid bilayer caused by the ionization state of the phosphate group linked to the choline group, symbolically denoted by P–N. (a) The one-dimensional, spatial variation of the membrane surface potential and membrane dipole potential. The model membrane is being side-viewed, along with a very sketchy representation of an aqueous pore inserted into it. (b) The simplified view of a model PC lipid, where particular emphasis is being placed on showing the relative orientation-marked by the α angle — of the P–N group to the normal axis of the lipid (*Z*). On the inset there are shown the main protonation–deprotonation reactions underwent by lipid's head group — specifically, by the phosphate group — within the range of acidic pH's used in our experiments (c) Detailed description of how various electric components of the membrane electrostatics vary with the pH used in our experiments; the '+++' symbol denotes a higher positive superficial charge — at pH=0.65—than at pH=2.94 ('+'). Similarly, the '↑↑↑↑↑' symbol signifies a much elevated dipole potential present at pH=0.65, compared to the case at pH=10.1 ('↑'). With the clear exception encountered at the extreme 0.65 pH value, the overall charge of the P–N group is zero; by knowing that the pK_a of PC phosphate group is less than 2, a possibly small, non-zero value of the charge associated with the P–N group is present at pH=2.08 (see text).

reflected by a negative zeta potential of the membrane. In the case of our experiments, we can wrap up these arguments by concluding that below pH 2.94, the membrane surface is positively charged, with the highest superficial density at the extreme pH=0.65 (Fig. 3, panel c). As for the case of pH values of 7 and 10.1, respectively, the membrane surface becomes negatively charged, with a highest charge density at the more basic pH=10.1.

As we stated above, various ions in the aqueous solution can partition into lipid membranes and alter its electric internal properties, as well. With regard to the influence played by the pH value of the aqueous solution on the membrane dipole potential, previous data have undoubtedly proven that membrane partitioning of hydroxide ions leads to a decrease in the dipole potential, whereas at acidic pH values, the low concentration of hydroxide ions into the interfacial layer of the membrane lead to larger dipole potentials [9]. By employing the inner-field compensation method, we have been successful in monitoring the time-course of dipole potential changes entailed by a swift variation of the solution acidity, e.g. from pH=0.65 to pH=2 (data not shown). In our specific experimental conditions, it becomes safe to state that during the course of pH changes from the extreme acidic (pH=0.65) to more basic values (pH=10.1) the membrane dipole potential drops steadily, and this contribution would sum up with changes caused by the net membrane charge density when considering the so far lumped electric potential profile.

Lastly, another contribution to the electric field profile experienced by permeating anions and cations, stems from the net charge of the lipid bilayer caused by the ionization state of the phosphate group from the lipids structure; keeping in mind that the pK_a for PC phosphate group is less than 2 and for choline is approximately 11, one cannot overlook the fact that lipid head groups themselves will assume various charged states within the studied pH domain. Specifically, as a result of high likelihood of phosphate groups protonation at a pH=0.65, the lipid molecules will become mostly positively charged (see Fig. 3, panel b for a detailed reaction scheme underwent by the lipid head groups at acidic pH values); as for the other experimental circumstances when the pH of the aqueous solution was set to values above the pK_a of the phosphate group, the chemical scenario with respect to the protonation–deprotonation reactions underwent by such groups which are biased towards the deprotonation ones, supports the state of electric neutrality of the lipids head groups. With these in mind, one can provide a mechanistic interpretation of the data embodied by Figs. 1 and 2; the fact that at pH=2.08 the lipid membrane is less positively charged than at pH=0.65, and bearing in mind that such a pH value further entails a decrease in the superficial electric charge and a higher probability of PC head groups charge neutralization, would create premises for an increase in the local concentration of cations near the mouth of the alamethicin oligomer. The net concentration of ions close to the channel's mouth is sensitive to the membrane surface potential, whose value is intrinsically linked to the overall surface charge, nicely expressed by the Gouy–Chapman formalism [17]. Keeping in mind that at such acidic values alamethicin still retains its slightly elevated cationic selectivity, despite the fact that the only ionizable aminoacid residue (glutamate-18) is mostly protonated, it becomes reasonably well to conceive that alamethicin would exhibit a relatively higher conductance at pH=2.08. In addition to this, a lower value of the membrane dipole potential at pH=2.08 than at pH=0.65 would facilitate cations hopping across the lipid membrane.

As hypothesized above, under the acidic conditions used in our experiments the glutamate-18 residues are believed to be pronotated. Of course, at the first glance such an assertion should go without questioning since the pK_a of glutamate's carboxyl sidechain in solution is about 4.3. One should remember however that in a folded protein, the pK_a's can be shifted with respect to the solution values. Physically speaking, such shifts are caused by a number of factors, including the loss of interactions with water molecules, interactions with the protein's charged and polar groups, as well as possible structural reorganization of the protein in response to proton binding [23,24]. Consequently, development of theoretical frameworks to estimate protein pK_a's has been the focus of considerable undertakings in the past years [25–27]. More recently, in an experimental attempt towards quantifying the effect of the protein environment on the pK_a values of protein residues, protonatable side chains into the pore domain of the muscle nicotinic acetylcholine receptor were engineered, and large negative pK_a shifts for basic amino acid residues in non-aqueous environments were observed [28]. With regard to alamethicin, suspicions were raised as to whether ionization reactions of glutamate-18 residues from an oligomeric complex present in a lipid membrane would be affected as a result of electrostatic interactions manifested with its environment, both lipid and water. From molecular dynamics simulations of alamethicin complexes inserted in a model lipid membrane, it became clear that the glutamate-18 residue forms on average approximately five hydrogen bonds to water as well as fluctuating hydrogen bonds with lipids, thus suggesting that together with the glutamine-19 residue, glutamate-18 constitute the C-terminal 'anchor' of alamethicin to the lipid membrane [29]. Results of theoretical pK_a calculations for a hexameric alamethicin helix oligomer have hinted that at pH 7, either none or just one of the six glutamate side chains will be ionized [30]. In a more specific manner, from the pH dependence of the alamethicin conductance, the effective pK_a value of the glutamate-18 residue was estimated to be 4.5–5, therefore rather close to that of free glutamate, suggesting small electrostatic interactions between such residues in a functional channel [20]. It is thus safe to assume that under the acidic conditions used in our experiments the glutamate-18 residues are mostly pronotated.

By the virtue of the exact same physical considerations, it is perfectly feasible to explain the monotonic increase of alamethicin's conductance which we observed in the range of neutral and basic pH values, from 7 and up to 10.1. In addition, increasing basic pH values promote a more negatively charged membrane interface, and this in turn lead to a higher local concentration of cations near the mouth of alamethicin, which will reflect in an elevated electrical conductance of the

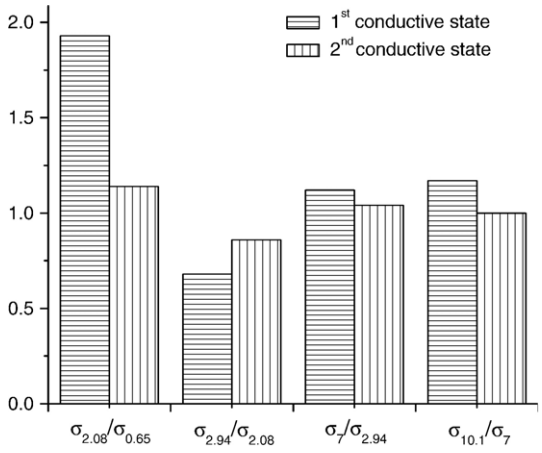

Fig. 4. Relative changes in the conductance of the alamethicin oligomer first and second conductive state, calculated when the pH of the buffer was changed from 0.65 to 2.08, 2.08 to 2.94, 2.94 to 7 and 7 to 10.1.

oligomer. As a result of the fact that at neutral and basic pH values glutamate-18 residues are ionized, electrostatic repulsions manifested among alamethicin monomers within a formed oligomer may cause an increase of the cross-sectional area of the channel, and this should also contribute in part to the higher alamethicin conductance seen under such experimental circumstances. As a matter of fact, based on our experience so far we can state quite safely that alamethicin oligomers are considerably less stable once the pH goes into the alkaline range, and this we believe may be another consequence of the electrostatic repulsions manifested among alamethicin monomers (unpublished observations). Based on sterical considerations and due to the fact that the second conductive state of the alamethicin oligomer is less cation-selective than the first substate, one would expect that the pH dependent behavior of alamethicin is less prevalent as the diameter of the alamethicin oligomer increases. Our quantitative estimations seem to be in good agreement with such an hypothesis; in Fig. 4 we represented the calculated values of the conductance ratios at any two consecutive pH values, for the first and second conductive states of the alamethicin oligomer.

When placed on a common reference scale, it becomes easy to notice that relative changes in the conductance of the alamethicin oligomer calculated when the pH of the buffer was changed from 0.65 to 2.08, 2.08 to 2.94, 2.94 to 7 and 7 to 10.1 are bigger for the most cation-selective sub-state of the channel (i.e., sub-state '1'). This result lends further support to the possibility that the pH modulation of transport features of the alamethicin channel takes place through electrostatic mechanisms described above, due to the larger impact seen on the most ion-selective state of the channel, which in turn is most sensitive to electrostatic changes of the bilayer membrane. To further test the prevalence of electrostatic interactions which we believe are the key factors to explaining the experimentally observed pH dependence of the alamethicin conductance, we reasoned that lower ionic strengths would provide improved conditions to observe it. As a result of a less effective screening of the surface potential by the surrounding electrolyte at 300 mM sodium salt than at 1 M, changes in the local concentration of cations and anions entailed by pH alterations of the overall membrane surface potential are expected to become more visible, thereby improving the chances of seeing them when alamethicin conductance variations are being estimated. In Fig. 5 we show original data that reflect conductance changes of alamethicin's substates manifested when the pH of the buffer solution containing 300 mM sodium salt was changed from an extreme acidic value of 0.62 to 3.05. We must stress here that we restricted our analysis only to the acidic regime, since this is the one within which most of the changes of the lumped surface potential occur, so a reasonable convincing point regarding the above-mentioned rationale could be safely made. By inspecting traces shown in Fig. 5, panel a, one can easily see that the general tendency of how alamethicin conductance varies vs. pH changes remains similar to that of the previous experimental circumstances, when the salt concentrations was 1 M. That is, by comparison to the pH's = 0.62 and 3.05, current flow through the alamethicin oligomers takes place optimally at the pH = 2.05. However, a straightforward conclusion regarding the salt screening influence of the surface potential upon the pH-dependent behavior of alamethicin is hardly visible only from such data, since a lower salt concentration will also diminish the electric conductivity of the buffer. Therefore, it is more interestingly to notice that relative modifications of alamethicin's first sub-conductance state when salt concentration is 300 mM vs. the pH changes are larger compared to the case of 1 M salt concentration. That is, when salt concentration is 300 mM, the calculated ratio of alamethicin's first conductive sub-state at

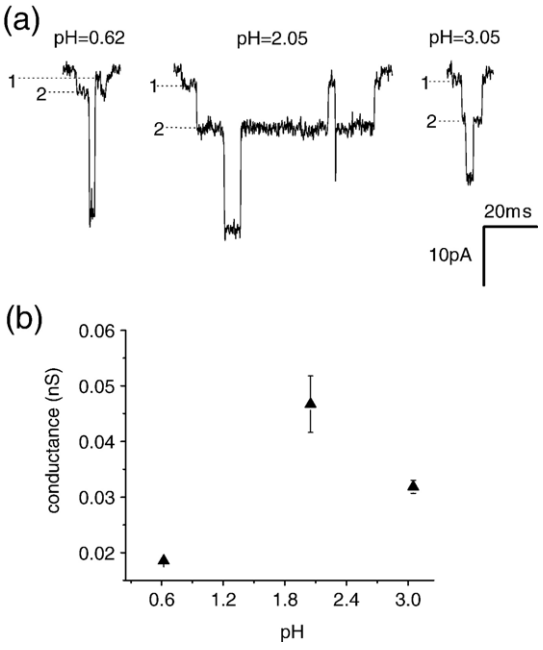

Fig.5. (a) Original recordings of single-ion currents through the alamethicin oligomer embedded in PC bilayer membranes when the membrane-bathing solutions contained salt at a 300 mM concentration. The pH of the buffer was set to the 0.62, 2.05 and 3.05 values by using sodium phosphate (10 mM). Similar to the previous case, it is obvious that the channel conductance of the 1st and 2nd conductive states (denoted by '1' and '2') vary non-monotonically with pH changes (b) Calculated conductance values of the first conductive state of the alamethicin oligomer at the various pH values (0.62, 2.05 and 3.05).

pH's=2.05 and 0.62 ($\frac{\sigma_{2.05}}{\sigma_{0.62(300mM)}}$) is 2.5, significantly larger than that when salt concentration is 1 M, at extremely close pH values ($\frac{\sigma_{2.08}}{\sigma_{0.65(1M)}}$=1.93). In keeping pace with this tendency, when moving up in the pH scale, $\frac{\sigma_{2.05}}{\sigma_{3.05(300mM)}}$ equals 1.7, significantly larger than $\frac{\sigma_{2.08}}{\sigma_{2.94(1M)}}$, which was estimated at 1.31. With such numerical estimations at hand and by corroboration with previously discussed data, we are in a strong position to assert that pH-modulation of alamethicin's conductance is, to an undeniable extent, caused by pH alterations of the overall membrane surface potential. We must also stress that in the estimations above we disregarded contributions stemming coming from the dipole potential, since electrolyte shielding leads to no quantitatively relevant changes in the dipole potential [31].

A rather unusual, yet interesting observation derived from our experiments is that within the studied acidic pH range (i.e., pH=0.65, 2.08, 2.94) and under conditions which would best favor cations transfer through the alamethicin channel-ensured by a lowest membrane dipole potential, associated with the smallest net positive charge onto the lipid membrane surface (e.g., pH=2.94) — the electric conductance of the first and second conductive states of alamethicin is actually considerably smaller than at pH=0.65 and 2.08 (see Fig. 2). Moreover, changes in the electric screening of the surface potential ensured by a smaller salt concentration, i.e. 300 mM NaCl, preserves this tendency (see Fig. 5). One plausible explanation for the observed phenomenon may rely upon alterations in membrane's curvature stress caused by pH-induced changes in the electrostatic energy of interactions among lipid head groups, manifested more visibly when the pH was changed from the 2.08 to 2.94. There is plenty of literature that support the paradigm according to which, in a lipid bilayer, the equilibrium curvature of the underlying lipid monolayers depends upon the net lumped result of the intermolecular forces among the lipid head groups and those among the hydrocarbon chains [32–34]. On the other hand, the equilibrium curvature of a lipid monolayer is intrinsically linked to intermolecular lateral interactions along the molecular axis, often giving rise to large lateral stresses that vary with depth within the membrane. Notably, there is good evidence which points to the fact that the function and conformation of many enzymes, ion pumps, and ion channels are quite sensitive to variation of lipid head groups and chain lengths, or to the concentrations of cholesterol, which are known to alter the equilibrium curvature of a lipid bilayer [35,36]. Changes of the molecular aspects of lipids themselves, including the length or degree of unsaturation of the hydrocarbon chains, lipid head group hydration or charge, the intensity of head group electrostatic interactions, incorporation of cholesterol, or even temperature changes do entail an alteration of the equilibrium curvature of the bilayer, and as a result cause a redistribution of the lateral stresses within the bilayer [37–39]. With direct relevance to our study, it has been proven recently that the bilayer electrostatic energy can alter membrane protein structure via a mechanism that takes into consideration electrostatic interactions among the phospholipid head groups in each monolayer, which are known to modify the bilayer curvature stress. That is, electrostatic repulsion among the negatively charged phosphatidylserine head groups in DOPS bilayers was decreased by increases in pH and ionic strength [40]; consequently and in accordance to previously known data [41,42] the thus alleviated electrostatic repulsion gave rise to a negative monolayer equilibrium curvature, which entailed a bilayer curvature stress which was shown to decrease the single-channel conductance of gramicidin A.

In this line of thoughts, we should stress that previous data have pointed to the involvement of either salt concentration or pH value on setting the elastic features of model lipid membranes. In one such representative study with mixed phosphatidylserine/phosphatidylcholine bilayers, addition of calcium ions was shown to induce lateral phase separations [43]. From the point of view of lipid structure, it is well known that changes in the pH or the ionic strength of the aqueous solution may cause phase transition of acidic phospholipids; moreover, cations such as magnesium and calcium are known to induce phase separation within mixtures of zwitterionic and acidic phospholipids (for a comprehensive work, please see Ref. [44]). Additionally, it has been shown that a change in pH from 7 to 9 increases the charge per polar group on the phosphatidic acid from one to two elementary charges, causing a lowering in the transition temperature by about 20 °C [45]; in the same study, it has been proven that divalent cations (magnesium and calcium) increase the transition temperature via charge neutralization and thus can be used to induce the phase transition from the fluid to ordered state at a constant temperature. In contrast, selected monovalent cations (lithium, sodium and potassium) were shown to lower the transition temperature and consequently make the bilayer structure more fluid at a given temperature. In a work more related to ours, elastic stress of lipid membranes containing alamethicin channels was varied by changing the pH of the bathing solution [41] and via X-ray diffraction it was shown that the decrease electrostatic energy of the polar surface of the bilayer — attainable at low pH values — shifts DOPS from a lamellar form seen at neutral pH to an inverted hexagonal H_{II} phase, characterized by a higher spontaneous curvature. As a direct consequence of changing membrane's surface charge and electrostatic interactions among lipid head groups via altering pH and salt concentration, dramatic changes in relative probabilities of channel conductance were seen. Nevertheless, there is still no full agreement regarding the mechanisms involved in the stress sensitivity of alamethicin conductance [32].

At this point, our tentative conclusion regarding the data presented herein points to a possible involvement of lateral pressure effects within the lipid membrane, which may increase as the pH changes from a value of 0.65 to ~3. Such an assertion would stand true, since by inspecting the sketch presented in Fig. 3 one can see that at pH 0.65, the electrostatic interactions manifested among lipid head groups are seemingly largest due to the net positive charge assumed by the choline moieties. In addition, at such a low pH value, the net accumulation of hydrogen ions onto the membrane surface is highest, which may contribute to even increasing electrostatic repulsions among lipid head groups. As the pH changes towards 2.94 and by taking into account the physical arguments presented above

regarding changes in the electrostatics of the membrane, such interactions will diminish in amplitude with a minimal contribution at pH 2.94. Seemingly, when the pH changes from a value of 2.08 to 2.94, lateral pressure modulations within the membrane caused by changes in its curvature, would lead to a prominent mechanical constriction of the alamethicin pore, which reflects in a decreased conductance of the channel. In terms of alamethicin ion conductive features, this mechanical effect thus counter-balances the electrostatic interactions between the membrane and incoming cations, which would favor cations diffusion through the pore at pH 2.94. As we specified above, by comparison to the cases when the buffer pH was set to either 0.65 or 2.08 values, such favorable electrostatic interactions are being ensured at pH=2.94 by a lowest membrane dipole potential, associated with the smallest net positive charge onto the lipid membrane surface. Although we regard this as a potentially nice mechanism to fully explain conductive features of alamethicin within the extreme acidic domain, more elaborate experiments are needed to place beyond any reasonable doubt our hypothesis.

4. Conclusions

In conclusion, our data demonstrate that: (i) when pH changes from extreme acidic (pH=0.65) to basic values (pH=10.1), visible changes occurs regarding the transport of ions through an alamethicin oligomer embedded on zwitterionic-based artificial lipid membranes (ii) membrane partitioning of hydrogen (favored at extreme acidic pH's) and hydroxide ions (favored at basic pH's) which lead to changes of the overall superficial charge onto the membrane, as well as of the membrane dipole potentials, are very good candidates to explaining the observed phenomena via the involvement of membrane electrostatics, that modulates the local concentration of cations near the mouth of the alamethicin oligomer, as well as cations hopping across the channel as well (iii) pH modulation of electrostatic interactions manifested among lipid head groups seemingly alter the curvature stress in the bilayer, and this would lead to a visible mechanical constriction of the alamethicin pore manifested by a drop in its conductance at pH=2.94. Such results strengthen the important paradigm of pH-induced modulations of ion channels transport properties through altering physical properties of zwitterionic-based lipid membranes. It is conceivable from our data that pH plays an important role in setting interfacial and internal electrical properties of such membranes, and this may prove useful in investigating functional properties of both lipid membranes and membrane proteins under aqueous pH stress.

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