The Modulatory Effect of Calcium Ions Upon Alamethicin Monomers Uptake on Artificial Phospholipid Membranes

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Abstract. In this paper, we examined the influence exerted by calcium ions upon physical properties of lipids constituting an artificial membrane. Our strategy was to study changes on alamethicin oligomer kinetic features embedded into such an artificial membrane. At neutral pH and in the presence of calcium ions, we observed an increase in the number of alamethicin monomers that oligomerize within the membrane, forming a multi-substate nanopore. We make the argument that calcium ions binding within the interface between the hydrophobic and the hydrophilic regions of the biomembrane causes a sizeable alteration of the physical properties of neutral lipid membranes. This in turn is seen to influence the translocation rates of alamethicin monomers from the solution adjacent to the biomembrane and leads to an augmentation in the subunit composition of the alamethicin oligomers, leaving the electrical conductance of the substates and their kinetics mainly unchanged.

Key words: alamethicin, phospholipids, calcium, electrophysiology

1. Introduction

Biological membranes are mainly made of lipids and proteins, and their very existence translated early in evolution into cellular compartmentalization. Ions are known to have a relatively low permeability in biological membranes mainly due to two physical energy constraints [1–3]. Firstly, the Born energy or energy of the electric field around ions gets higher as an ion resides in a low relative electrical permittivity medium. Secondly, due to the difference in electrical polarizability of the two domains (i.e., water and biomembrane's core) and thin thickness of biomembranes (~ 40 Å), the Born energy barrier is further heightened by the interaction of ions with the induced polarization charges at the interface between the domains. A supplementary term needed to be taken into account regarding the nature of physical forces action on an ion when crossing the biomembrane core stem from the observation that anions permeate faster through biomembranes as compared to cations of comparable size. The interpretation given pointed to the presence of an internal dipole potential within the biomembrane, oriented such that the external part of the

biomembrane is negative. Structural studies on biomebranes revealed that carbonyl groups of the lipids and oriented, lipid headgroup bound-water molecules are the main contributors to the origin of dipole potentials of biomembranes [4, 5]. Its overall value (200–300 mV) and the ensuing extremely high electric field associated with it over the interface between the aqueous phase and hydrocarbon region of a biomembrane (10^8 – 10^9 V m⁻¹), endow the dipole potential with major roles in modulation of molecular processes which take place within a biomembrane. That is, dipole potential has long been suspected to play an important role for protein insertion and functioning [6, 7], for altering the kinetics of gramicidin channels [8], and modulating the activity of phospholipase A2 [9]. Moreover, dipolar potential was shown to affect the membrane insertion and folding of a model amphiphilic peptide [10], as well as the extent of the membrane fusion [11].

In order to overcome the energy barrier encountered by charged molecules and ions for the biomembrane translocation, nature helped evolve ‘proteins with holes down their middle that control access to biological cells and thus govern an enormous range of biological functions important in health and disease’ [12]. Electrical signals encoded by ion transport through such channels help cells communicate to one another via action potentials, coordinate muscle contraction, regulate salt composition in various organs. Such proteins are the best-known examples of natural nanotubes, which, properly engineered, became extremely powerful devices for many areas of nanotechnology, sensing, separation science [13]. The most common voltage-gated and ligand-gated ion channels with relevance for cell’s integrity belong to a particular class of helix bundle-forming channels [14–17]. There are, however, particular classes of nanochannels with a rather modest ion selectivity, which belong to the class of β -barrel-bundles forming channels. Of these, the most prominent natural channels that have been engineered as nanopores, have been porins, proteins that control the permeability of the bacterial outer membrane, and α -hemolysin (α HL), a pore-forming toxin secreted by the *Staphylococcus aureus* bacteria [13, 18, 19]. Another representative membrane-forming peptide, with a long and fruitful history as paradigm for membrane pores, is alamethicin.

Alamethicin is one of the best-studied models of membrane pore formed by amphiphatic α -helices. Studies regarding alamethicin and its biophysical properties date back to 1967 and, among others, represented one of the earliest examples of peptaibols, i.e., a peptide rich in α -aminoisobutyric acid (Aib) or α -methylalanine (α -MeA), capable of endowing an artificial membrane with properties encountered in living cells, that is a voltage-dependent conductance. Alamethicin is a 20 amino acid peptide, first discovered in cultures of the fungus *Trichoderma viride*, and it was in 1982 that [20] obtained high-resolution (1.5 Å) diffraction images of crystals of this peptide. The main structural piece of information inferred from the crystal structure, pointed to the existence of three independent monomeric structures which are mainly α -helices from the N-terminus down to the kink introduced by proline 14.

When added to one of the chambers of a planar lipid bilayer setup, alamethicin monomers become inserted into the membrane and generate highly

voltage-dependent pores. Firstly, alamethicin peptides generate pores with stark rectification features in the sense that the potential difference applied should be made positive in the chamber where peptide was added in order to get largest currents [21, 22]. This asymmetry was shown to vary with the lipid composition of the membrane and ceased to exist when alamethicin monomers are added simultaneously to both chambers of the bilayer system. Secondly, the relatively high conductance of pores induced by alamethicin was shown to be strongly voltage dependent, so that changes of only ~ 5 mV sufficed to modify the pore's conductance e -fold [23, 24]. Most probably, it is thought that it takes at least four helices to form the smallest conducting aggregate [25]. With respect to the voltage-driven formation of pores within a biomembrane, the current consensus favors the hypothesis of an initial, yet significant, partial embedment of the N-terminal part in the inner core of the biomembrane, up to the proline 14, making an angle of about 30° with the remaining part of the peptide which sticks almost parallel to the water-biomembrane interface [26]. In this configuration, the N terminal of the alamethicin peptide would sit around 10 Å down the membrane and thus able to experience part of the electric field existing across the biomembrane [26]. An optimal applied potential difference, that is positive at the peptide side, would tend to repel the N terminus even deeper into the hydrophobic core of the membrane until the peptide fully crosses it. This monomeric state of the 'to-be' conducting bundle is energetically rather unstable (due to hydrophilic residues forced into the bilayer), so that lateral diffusion of other monomers followed by collision among them lead eventually to relatively stable alamethicin oligomers [21]. It is well known that between the ionic solution, where the biomembrane is immersed and the lipid molecules that compose the biomembrane, electrostatic interactions do exist. These interactions were shown to be crucial for lipids phase transition, membrane fusion processes and ion transport across the biomembrane [27–29]. In a recent paper [27], authors have argued that monovalent ions change the membrane electrostatics and affect the lateral organization of artificial biological membranes. Aside from a large number of alterations in the physical and chemical properties of a biomembrane, it has been described that binding of sodium ions on the neutral lipid headgroups and subsequent complex formation leads to a drop in the dipole potential of lipid headgroup from ~ 4.5 to ~ 7 V [27]. The specific interaction between divalent cations, in particular calcium ions, and biological membranes received sustained attention in the past through the extensive use of various experimental methods. For instance, NMR experiments pointed to a possible conformational change in the polar region of dipalmitoylphosphatidylcholine (DPPC) bilayers [30]. Moreover, rearrangements or conformational changes in the carbonyl region of palmitoyloleoylphosphatidylcholine (POPC) lipids were disclosed through IR spectroscopy experiments [31]. Despite the lack of experimental hard evidence in its favor, the general consensus, as inferred from interpretation of data presented earlier, was that calcium ions get bound to the phosphate groups of two neighboring lipids from the membrane bilayer. Very recently, molecular dynamics simulations of solvated lipid bilayers

have brought attention to an interesting paradigm, namely that carbonyl oxygen of lipid headgroups bind in sequential manner calcium ions [32]. Very briefly, interaction with the first carbonyl oxygen requires the diffusion of calcium ions through the polar region of the lipid bilayer and this was shown to be a relatively fast process (30–40 ns). As the system evolves, after about 200 ns, nearly half of the ions get to the most energetically stable conformation, where an ion is coordinated by at least four phosphatidylcholines.

In this paper, we supplement the body of evidence gathered so far which favors the physiological relevance of aqueous ions, and in particular divalent calcium ions, for molecular processes mediated by and taking place at the biomembrane level. By measuring the activity of alamethicin oligomers within a biomembrane, we show that calcium ions seem to promote the transversal diffusion of alamethicin peptides across the membrane. Our data, gathered via single-channel recording, strengthen the fact that calcium ions binding within the interface between the hydrophobic and the hydrophilic region of the biomembrane cause a large number of phenomena, which may include: modification of the lipid density within the monolayer, alteration of the lipid dipole vector value and potential drop change across the monolayer they were added to. We show that in turn, this influences the translocation rates of alamethicin monomers from the solution adjacent to the biomembrane and lead to an augmentation in the subunit composition of the alamethicin oligomers.

2. Materials and Methods

Current recordings through alamethicin oligomers were carried out by using folded bilayer membranes obtained as previously described [13]. Briefly, a 25- μm thick Teflon septum was clamped between two Teflon chambers each of 1 ml volume. A bilayer was formed on an aperture of 100- μm diameter in the septum that had been pretreated with 10% (v/v) hexadecane (Sigma–Aldrich) in highly purified *n*-pentane (Sigma–Aldrich). Both chambers contained 1 M NaCl, pH 7.5. Initially, the level of electrolyte was set just below the aperture and 1% (w/v) L- α -phosphatidylcholine (Sigma–Aldrich) in pentane (6 μL) was spread on the surface of each chamber. After about 2 min, during which the solvent evaporated, the electrolyte level in the chambers was raised above the aperture. The formation of a bilayer was monitored by observing the increase in membrane capacitance to a value of approximately 150 pF. In order to suppress spurious electrical interference, the bilayer chamber was properly grounded and enclosed into a home-made Faraday cage.

Alamethicin monomers (Sigma–Aldrich) were added from a stock solution made in ethanol, in the *cis* chamber only, connected to the ground and mechanical stirring was initiated in this chamber for ~ 1 min to ensure proper concentration homogenization. All experiments were performed at a room temperature of ~ 25 °C. The electrical connection between the bilayer chamber and the rest of analogic devices was made via Ag/AgCl electrodes. Currents from the bilayer chamber were

detected and amplified with an integrating headstage Axopatch 200 B amplifier (Axon Instruments, Foster City, USA), set on the voltage-clamp mode. Data acquisition of the amplified electrical signals was performed with a 12-bit resolution, DAS1601 A/D card (Keithley Instruments Inc., USA) at a sampling frequency of 10 kHz. Data was then fed into a PC-compatible computer for further numerical analysis including time-domain low-pass filtering, spectral analysis and graphing, done mainly with the help of the Origin 6.0 (OriginLab Corporation, USA) and Matlab software (The Mathworks Inc., USA). The power spectra were computed from 2048 points and usually at least 15 data segments were employed to generate an averaged plot.

3. Results and Discussion

Following addition of alamethicin in the *cis* chamber of the bilayer setup and under conditions of negative potentials applied, monomers were seen to oligomerize within the bilayer and form conductive pores. As known, the very presence and dynamics of conductance substates in current traces of alamethicin-induced membrane pores most likely reflects the membrane-mediated docking and un-docking of individual alamethicin monomers to a minimum four-monomers oligomer, which gives the smallest conductance (Figure 1, panel a). The frequency of occurrences as well as the numbers of substates are dramatically enhanced by the increase in

Figure 1. Electrical recording of alamethicin activity inserted in a zwitterionic phospholipid biomembrane, studied in 1 M NaCl, pH 7.5 and -30 mV potential difference in the absence (panel a) and presence (panel b) of 1 mM calcium. Interaction between calcium ions and the polar region of the biomembrane is seen to lead to a significant increase in the probabilistic behavior of alamethicin oligomer.

the absolute value of potential difference across the biomembrane, and they almost cease to show up when the sign of the potential difference applied is reversed (data not shown). In the presence of 1 mM calcium added only on the *cis* chamber, we observed an augmentation in the stochastic behavior of the alamethicin oligomers, in the sense that the number of substates and implicitly the number of monomers present within the membrane were enhanced (Figure 1, panel b).

In order to further confirm the validity of this effect, we ran two different sets of control experiments. That is, when EDTA (3 mM) was added on the *cis* chamber, we observed a sizeable run-down in the channel's activity, as expected from a consequent decrease in the concentration of free calcium following its interaction with the chelator EDTA. Secondly, when calcium was added only on the *trans* side of the bilayer chamber, where no alamethicin monomers were present, no visible change in the activity of alamethicin oligomers was recorded (data not shown). These observations lead us to believe that calcium interactions with the biomembrane constitute the major cause for the alteration in the number of alamethicin monomers which diffuse transversally through the biomembrane and interact with an existing alamethicin oligomer. One should keep in mind that at the very start of their insertion process, alamethicin monomers sense an electric field caused mainly by the lumped value of the potential difference set across the membrane and dipolar field in the polar head region of lipids. Consequently, any change in the electrostatics of the biomembrane, large enough to shift the equilibrium constant of alamethicin monomers partitioning between the aqueous solution the membrane is bound to alter the stochastic behavior of the alamethicin oligomer.

Based on our data, we suggest that, among others, an alteration in the dipolar moment of lipids headgroups caused by ion calcium binding may one determinant factor for the observed run up in the alamethicin activity. However, changes in the biomembrane electrostatics ensued by calcium ion binding do not alter the intrinsic conductance of alamethicin substates (Figure 2), so that the increase in the standard deviation of alamethicin-mediated current fluctuations would mainly stem from a corresponding increase in the number of alamethicin monomers partitioning into the membrane (see also later). For five of the most visible conductance substates and at a 95% level of confidence, changes in the electrical current mediated by any intermediate oligomer are not statistically significant, as inferred from a Student's *t*-test analysis (see Table I).

It is however interesting that calcium-induced augmentation of the standard deviation of current fluctuations through the membrane, mediated by the dynamics of oligomeric composition of alamethicin inserted, seems to be dependent of the potential difference imposed. In Figure 3, we show representative power spectra of current traces calculated for the activity alamethicin in the absence and presence of calcium, recorded at -20 , -30 and -40 mV. In order to facilitate observation, we normalized spectra to their maximum value within the studied frequency range. For example, at -30 mV, the power spectrum of current fluctuations in the absence of calcium was normalized with respect to the one computed when 1 mM calcium

Table I. Absolute current values corresponding to five of the visible subconductance levels of the alamethicin oligomer in the absence and presence of 1 mM calcium

	Level 1 (pA)	Level 2 (pA)	Level 3 (pA)	Level 4 (pA)	Level 5 (pA)
Control (no calcium)	7.7 ± 0.4	19.2 ± 0.5	28.5 ± 1.1	38.9 ± 0.8	57.5 ± 1.1
Calcium (1 mM)	9.1 ± 0.5	16.7 ± 0.5	28.7 ± 0.5	39.7 ± 0.2	58.6 ± 1.6

Data are expressed as average values $\pm$ standard error and are based on at least five independent estimations.

Figure 2. Amplitude histograms of current fluctuation mediated by alamethicin in the absence and presence of 1 mM calcium. One should note that upon calcium addition an increase in the number of events corresponding to various amplitude levels is observed, which is an expected feature for the augmentation in the occurrence of intermediate conductive levels population. As described in the text, calcium does not seem to significantly change the values of any conductance intermediate level of the alamethicin oligomer.

was present on the *cis* side of the membrane. It becomes thus easier to compare the relative change in the standard deviation, given by the integral of power spectra over the chosen frequency domain, of current fluctuation upon calcium addition at a given potential difference, and to compare these changes for all cases (-20 , -30 and -40 mV, respectively). One of the main conclusions inferred from these data was that calcium-induced stimulatory effect of alamethicin activity is slightly, still statistically significant voltage-dependent, at all studied potential differences (-20 , -30 and -40 mV, respectively). Basically, at -40 mV, the relative increase of the current fluctuations through the membrane upon calcium addition is about nine-fold the one recorded at -20 mV.

Figure 3. Power spectra of current fluctuation mediated by the alamethicin oligomer at various potential difference values (-20 , -30 and -40 mV, respectively) in the control state (no calcium) and in the presence of 1 mM calcium. In order to better visualize the changes in standard deviation of current fluctuation, given by the area under any given spectrum, these traces were normalized to the maximum value, within the range, recorded in the presence of calcium.

This is a rather unexpected phenomenon, as from their simulations Bockmann and Grubmuller concluded that the back reaction for the molecular interaction between calcium ions and lipid carbonyl oxygen is virtually non-existing, rendering the entire reaction sequence uni-directional [32]. Under such circumstances, the magnitude of potential difference across the biomembrane would only affect the time until the uni-directional reaction between calcium ions and the polar heads of the biomembrane reaches equilibrium, leaving the value of the stationary probability of calcium ion-polar head complexes un-touched.

A tentative explanation for the observed voltage-dependent effect of calcium on the uptake of alamethicin monomers would be that higher electric fields across the biomembrane distort to a higher degree, via electrostriction forces, the physical state of the biomembrane and helps create local 'structural defects' which facilitate a higher, local concentration of calcium ions into the polar region of the biomembrane. Additionally, we show from our experiments that main effects entailed by calcium binding to the polar headgroups of lipids do not affect the kinetic features of alamethicin monomers binding and un-binding within a transmembrane oligomer. It is seen from Figure 3 that the slopes of the power spectra calculated for the kinetic activity of alamethicin oligomer in the absence and presence of calcium are rather coincidental. This, in turn, reflects the lack of influence exerted by tight ion-lipid headgroups complexes on the transversal diffusion of alamethicin monomers within the biomembrane plane.

Despite the fact that further experiments are needed to clarify the molecular mechanisms responsible for the calcium-induced modification of physical properties of neutral lipids, our work demonstrates the following interaction with zwitterionic lipids, calcium ions raise the uptake rate of alamethicin monomers from the solution and thus entail a larger kinetic activity of this voltage-dependent ion channel model. The two most obvious places where calcium ions may induce modification of physical properties of neutral lipids, would be the dipole and surface components of the boundary potential in the biomembrane. In order to properly distinguish between the two possibilities, our next plan is to conceive further experiments in which we will use phloretin, a sterol that reduces the magnitude of the dipole potential and 6-ketocholestanol, which increases the positive membrane dipole potential; if, as we presented it herein, calcium ions exert their influence on the biomembrane via alteration of the dipole potential mainly, we may be able to compensate this effect for or enhance it by having the biomembrane to interact with the aforementioned dipolar species. On the other hand, if the observed effect of calcium ions upon alamethicin monomers uptake would stem mainly from alteration of electrical properties of the biomembrane-aqueous solution interface, extreme values of the overall ionic strength of the buffer solutions should make a sizeable difference in the observed effect. Such data should be useful when considering the complex set of interactions taking place among intra- and extracellular ions, un-charged biomembranes and membrane peptide aggregates, which usually reflect in physiological alterations of cellular activity.

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