

Effect of Gramicidin A on the Dipole Potential of Phospholipid Membranes

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ABSTRACT The effect of channel-forming peptide gramicidin A on the dipole potential of phospholipid monolayers and bilayers has been studied. Surface pressure and surface potential isotherms of monolayers have been measured with a Langmuir trough equipped with a Wilhelmy balance and a surface potential meter (Kelvin probe). Gramicidin has been shown to shift pressure-area isotherms of phospholipids and to reduce their monolayer surface potentials. Both effects increase with the increase in gramicidin concentration and depend on the kind of phosphatidylcholine used. Application of the dual-wavelength ratiometric fluorescence method using the potential-sensitive dye RH421 has revealed that the addition of gramicidin A to dipalmitoylphosphatidylcholine liposomes leads to a decrease in the fluorescence ratio of RH421. This is similar to the effect of phloretin, which is known to decrease the dipole potential. The comparison of the concentration dependences of the fluorescence ratio for gramicidin and phloretin shows that gramicidin is as potent as phloretin in modifying the membrane dipole potential.

INTRODUCTION

The pentadecapeptide gramicidin is one of the best studied substances that forms ion channels in artificial and natural membranes (Koepe and Andersen, 1996; Hladky and Haydon, 1984; Killian, 1992; Busath, 1993). Gramicidin channel is shown to be a transmembrane dimer formed by head-to-head association of monomers through hydrogen bonds that is stabilized by specific localization of tryptophan residues at the membrane-water interface (Arseniev et al., 1986; Ketchum et al., 1993). The position and orientation of the tryptophan residues are associated with the interaction of their indole dipoles with dipoles of polar groups and water molecules at the membrane-water interface (Becker et al., 1991; Providence et al., 1995; Busath, 1993; Seoh and Busath, 1995; Hu et al., 1993; Hu and Cross, 1995), which contribute to the origin of the membrane dipole potential (Brockman, 1994). It has been shown recently that the dipole potential profoundly affects the dissociation kinetics of gramicidin channels in bilayer lipid membranes (Rokitskaya et al., 1997). On the other hand, incorporation of gramicidin molecules carrying several dipoles into the membrane apparently may change the membrane dipole potential.

Gramicidin is a very potent channel former that induces a noticeable membrane conductance, even in the presence of a trace amount of permeable cations or anions (gramicidin is not perfectly selective for cations). Therefore, it is difficult to obtain unambiguous results on the dipole potential effect

through the influence on the conductivity of a bilayer lipid membrane for cations or anions (Liberman and Topaly, 1969; Andersen et al., 1976). However, the changes in the dipole potential can be monitored by the measurements of the monolayer surface potentials (see Brockman, 1994, and references therein). For example, the measurements performed with monolayers formed by gramicidin analogs themselves have shown that the monolayer surface potential is considerably lower in the case of gramicidin A, which contains four tryptophan residues, as compared to gramicidin M, which has phenylalanines instead of tryptophans (Van Mau et al., 1987, 1988). It still remains unclear whether incorporation of gramicidin affects the monolayer surface potential of phospholipids.

Recently the potential-sensitive fluorescent dyes RH421 and di-8-ANEPPS have successfully been applied to the measurement of changes in the membrane dipole potential (Gross et al., 1994; Clarke and Kane, 1997; Clarke, 1997) by the dual-wavelength ratiometric fluorescence method. In particular, this technique has been utilized to show that the mitochondrial amphipathic signal sequence peptide known as p25 is able to reduce the dipole potential of model membranes (Cladera and O'Shea, 1998). A similar effect was observed with a functionally active peptide, substance P, in the earlier work following the capacitance minimization technique (Sargent et al., 1989). These data support the idea of the involvement of proteins in the establishment of the dipole potential of natural membranes, which has been discussed in the literature (Franklin and Cafiso, 1993).

In the present work we have studied the ability of gramicidin A to change the membrane dipole potential by applying two complementary techniques: surface potential measurements of phospholipid monolayers and the dual-wavelength ratiometric fluorescence method using RH421. It has been shown that gramicidin A reduces the dipole

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potential of phospholipid membranes in a manner similar to that of the well-known dipole modifier phloretin.

MATERIALS AND METHODS

In this paper the term "monolayer surface potential" is used to designate the difference in the electrical potentials between the bulk subphase and the outer (top) surface of the monolayer. This term corresponds to "interfacial potential" (the difference between the inner part of the bilayer and the bulk water phase), which is commonly used in bilayer literature. The term "surface potential" should not be associated here with the diffuse double layer (or Gouy-Chapman) part of the interfacial potential.

Monolayers of phospholipids were formed by spreading their 0.5 mM solution in freshly distilled chloroform on the surface of aqueous solution. Surface pressure and surface potential isotherms were measured with a homemade Langmuir trough equipped with a Wilhelmy balance and a surface potential meter (Kelvin probe) described elsewhere (Shapovalov and Tronin, 1998). The monolayer surface potential was measured with respect to the potential of the pure subphase surface (the latter was taken as zero). The resolution and short-term stability of electronic balance were better than 10^{-7} N. For surface pressure measurements the resolution was mainly limited by occasional air flows and vibration of the water surface (~ 0.05 mN/m). Relative accuracy was limited by uncertainty in width of the Wilhelmy plate (filter paper) at the level of $\sim 2\%$. A three-electrode arrangement was used for the surface potential meter: a gold-coated polished vibrating electrode (1 cm in diameter) with a vibration frequency of 70 Hz, a Ag/AgCl reference electrode, and an auxiliary (grounding) electrode of stainless steel. This device has some advantages in accuracy and long-term stability (5 and 10 mV, respectively) compared to commercial surface potential meters for Langmuir monolayers. Subphase solutions were prepared by using double-distilled water that was further purified by passing through a column with activated carbon. The ionic strength in most experiments was fixed at 0.01 M by the addition of KCl (Fluka, Buchs, Switzerland).

N-(4-Sulfobutyl)-4-(4-(*p*-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt (RH421; for structure see Fig. 4) was obtained from Molecular Probes (Eugene, OR). For fluorescence measurements, 5 μ l of an ethanolic dye solution was added to 1 ml of aqueous buffer solution containing 0.2 mg/ml unilamellar lipid vesicles prepared by sonication and equilibrated for 30 min. The final solution contained 2.5 μ M RH421.

Dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC), and gramicidin were from Sigma (St. Louis, MO). Diphytanoylphosphatidylcholine (DPhPC) was from Avanti Polar Lipids (Alabaster, AL). Phloretin was from Fluka.

Measurements with the vesicles were performed in a buffer solution containing 30 mM Tris (Sigma) and 100 mM KCl (pH 7.5, adjusted by HCl).

Fluorescence excitation spectra were recorded with a Hitachi MPF-4 (Tokyo, Japan) fluorescence spectrophotometer (excitation bandwidth 1.5 nm, emission bandwidth 8 nm). Fluorescence was detected at 670 nm, i.e., at the red edge of the emission spectrum of RH421. According to Clarke and Kane (1997), this is one of the requirements for the correct measurement of the dipole potential changes by the dual-wavelength ratiometric fluorescence method.

All experiments were carried out at room temperature (21–22°C).

RESULTS

Dipole potential changes estimated from phospholipid monolayer surface potential measurements

According to Van Mau et al. (1987, 1988), spreading of the chloroform-methanol solution of gramicidin on the surface of aqueous solution leads to the formation of a stable

monolayer of the peptide. On the other hand, the addition of gramicidin to the subphase does not lead to the appearance of a noticeable amount of gramicidin at the interface after incubation for several minutes (Fig. 1, curve 3). However, gramicidin added to the subphase shifts considerably the π -*A* isotherm of DPPC monolayer, showing the insertion of gramicidin molecules into the monolayer (Fig. 1, curves 1 and 2). Subsequent cycles of compression-expansion do not result in an additional shift of the π -*A* isotherm under these conditions. The results of simultaneous measurements of the monolayer surface potential also favor the incorporation of gramicidin: the considerable reduction of the monolayer surface potential is seen at an area less than 60 \AA^2 per lipid molecule. It should be noted that exactly the same effect of gramicidin on the monolayer (the shift of the π -*A* isotherm and the reduction of the surface potential) is observed in the absence of KCl in the subphase.

Fig. 2 shows the dependences of the shift of the π -*A* isotherm (Fig. 2 *A*) and the decrease in the monolayer surface potential (Fig. 2 *B*) of DPPC on the concentration of

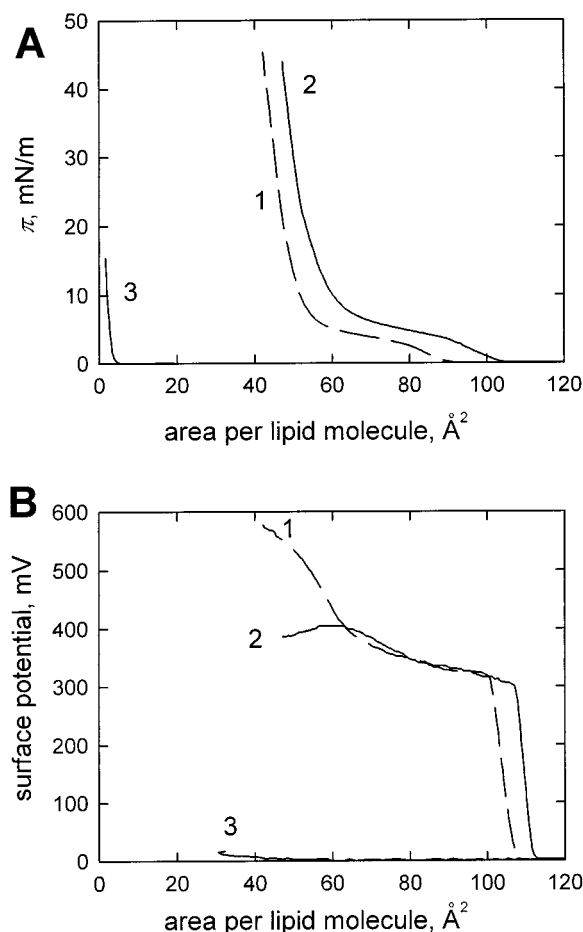


FIGURE 1 Surface pressure (*A*) and surface potential (*B*) versus area per lipid molecule for DPPC monolayer spread on the subphase without gramicidin (curve 1) and in the presence of 10^{-7} M gramicidin (curve 2). Curve 3 presents the control experiment: 10^{-7} M gramicidin in the subphase without spreading DPPC.

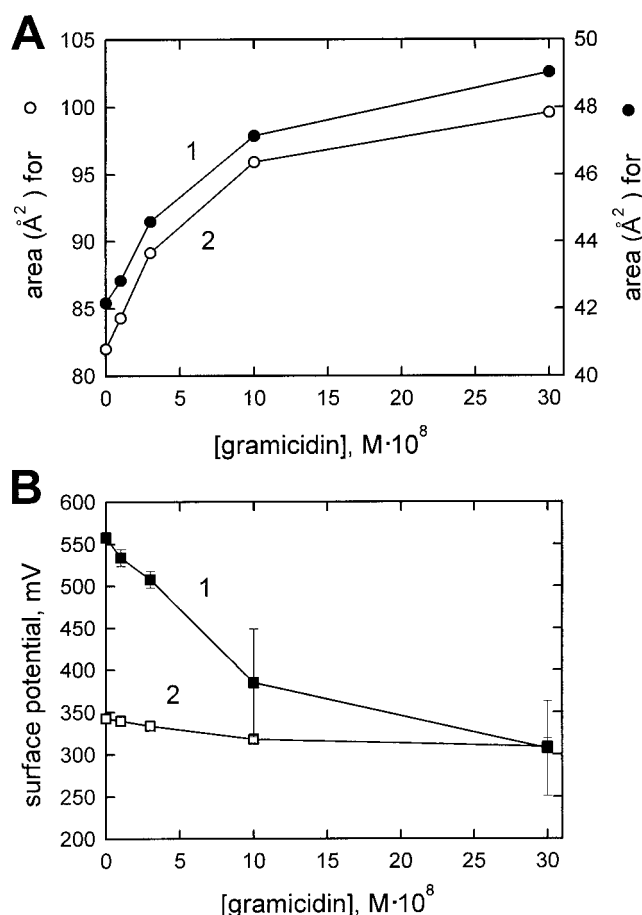


FIGURE 2 Area per DPPC molecule (*A*) and surface potential (*B*) versus gramicidin concentration in the subphase for $\pi = 45$ mN/m (curve 1) and $\pi = 2$ mN/m (curve 2).

gramicidin. The dependences reach saturation at a gramicidin concentration of $\sim 0.3 \mu\text{M}$.

The decrease in the monolayer surface potential induced by gramicidin is sensitive to the kind of lipid. As seen from Fig. 3, the effect of $1 \mu\text{M}$ gramicidin is most pronounced for DPPC, substantially less for DMPC, and even less for DSPC. The effect is hardly detectable in the case of DPhPC (data not shown).

It is worth noting that in contrast to π -*A* isotherms, the monolayer surface potential-area plots (Fig. 3, *A*-*D*) varied significantly from one compression-expansion cycle to another in the presence of gramicidin (the variation was less than 10 mV in the absence of gramicidin). This variation exceeds considerably the noise of our surface potential meter. Fig. 2 *B* presents the values of potentials averaged for five or six cycles of compression-expansion.

Dipole potential changes in unilamellar phospholipid vesicles as revealed by fluorescence of RH421

Fluorescent styrylpyridinium dyes, such as RH421 and di-8-ANEPPS, have been shown to respond to changes in the

membrane dipole potential by shifts in their fluorescence excitation spectra (Gross et al., 1994; Clarke and Kane, 1997; Clarke, 1997). Fig. 4 illustrates the normalized fluorescence excitation spectra of RH421 in DPPC liposomes in the presence of $5 \mu\text{M}$ gramicidin and $10 \mu\text{M}$ phloretin. The areas of the excitation spectra were normalized to the same integrated intensity. It is seen that the addition of gramicidin leads to a long-wavelength shift of the excitation spectrum, which is similar to the effect of the addition of phloretin.

To report the quantitative information of the changes in the dipole potential, it has been suggested by Gross et al. (1994), Clarke and Kane (1997), and Clarke (1997) the ratio of the fluorescence intensities detected at two excitation wavelengths on the blue and red edges of the excitation spectrum should be measured. Fig. 5 presents the dependence of *R*, the ratio of RH421 fluorescence excited at 440 nm to that excited at 540 nm, on the concentration of gramicidin and phloretin. It is seen that gramicidin causes a pronounced decrease in *R*. This effect also depends on the kind of lipid. For example, the decrease in *R* induced by gramicidin is more than four times less with DPhPC than with DPPC liposomes.

DISCUSSION

In our monolayer experiments gramicidin is initially added to the subphase, in contrast to the work of Van Mau et al. (1987, 1988), where it was added directly to a monolayer-forming solution. The process of gramicidin adsorption at the surface of the aqueous solution is rather slow in the absence of phospholipid. Additional experiments have shown that 2-h incubation of gramicidin-containing subphase in the absence of phospholipid makes it possible to measure the monolayer surface potential of gramicidin, which is ~ 250 mV at 15 mN/m. This value is in reasonable agreement with the value of 230 mV, which was obtained by Van Mau et al. (1987, 1988).

As is known from the literature, gramicidin can adopt different conformations depending on its environment, which leads to the "solvent history" effect in the case of channel incorporation (Killian, 1992; Bouchard and Auger, 1993; Abdul-Manan and Hinton, 1994). It can be proposed, therefore, that the differing ability of gramicidin to form a stable monolayer, depending on the manner of its addition to the system (spreading from chloroform solution or adsorption from the subphase), results from the existence of two (or more) stable conformations of gramicidin molecules, one of which is water-soluble and the other of which is hydrophobic and readily forms a monolayer from the organic solution.

The shift of π -*A* isotherms upon the addition of gramicidin to the subphase proves the incorporation of gramicidin into the phospholipid monolayer. Maximum area shifts reach 20% at a gramicidin concentration of $\sim 1 \mu\text{M}$. Because gramicidin and DPPC cross-sectional areas are equal to $\sim 250 \text{\AA}^2$ and $\sim 65 \text{\AA}^2$, respectively (Woelf and Roux,

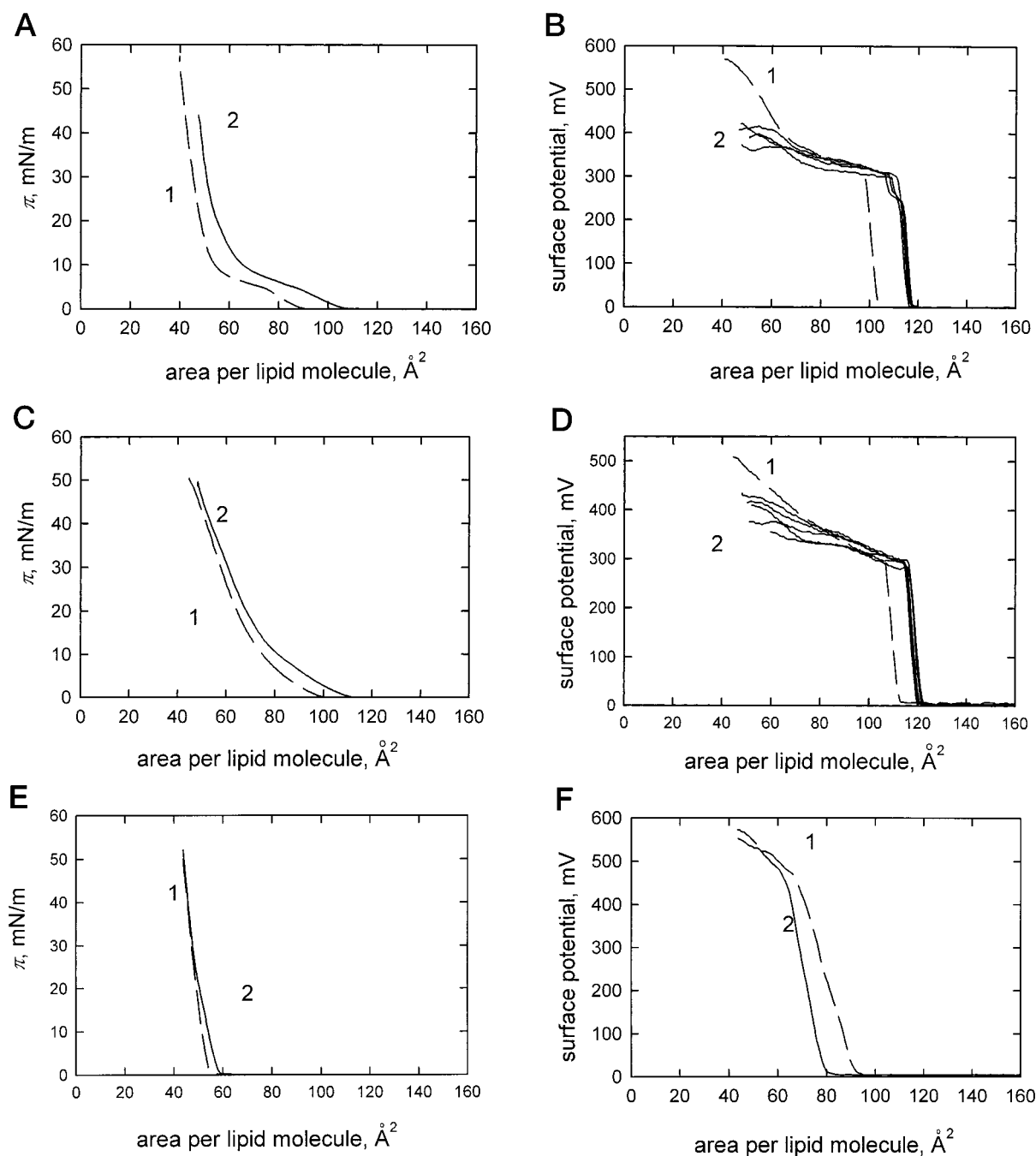


FIGURE 3 Surface pressure (A, C, E) and surface potential (B, D, F) versus area per lipid molecule for DPPC (A, B), DMPC (C, D), and DSPC (E, F) monolayer spread on the subphase without gramicidin (curve 1) and in the presence of 10^{-6} M gramicidin (curve 2). It is seen that in the case of DPPC and DMPC the surface potential-area plots varied significantly from one compression-expansion cycle to another in the presence of gramicidin.

1996, data for bilayers), a 20% increase in the monolayer area indicates that gramicidin molecules incorporated into the phospholipid monolayer comprise $\sim 5\%$ of the total molecules in it.

It is shown here that the incorporation of gramicidin into the phospholipid monolayer causes a decrease in the monolayer surface potential, which cannot be explained by a simple model of the coexistence of pure lipid and pure gramicidin domains. In fact, as seen from the shift of the π -A isotherm (Fig. 1), the area covered by gramicidin

cannot exceed 20% of the total area (10^{-7} M gramicidin is in the subphase). Assuming the value of the monolayer surface potential of pure DPPC to be 550 mV and that of pure gramicidin to be 250 mV, one can estimate the value of the area-weighted potential to be 490 mV. However, the value of the average monolayer surface potential observed experimentally was 380 mV (Fig. 2 B).

It should be noted that the analysis of surface potential-area plots is carried out here only for the range of areas corresponding to nonzero surface pressure. In the range of

In the recent years the dual-wavelength ratiometric fluorescence method, using potential-sensitive dyes such as RH421, has proved its validity for determining changes in the dipole potential produced by different agents (Gross et al., 1994; Clarke and Kane, 1997). Our experiments have shown that the addition of gramicidin A to DPPC liposomes leads to a decrease in the fluorescence ratio *R* of RH421 that is similar to the effect of phloretin, which is known to decrease the dipole potential (Andersen et al., 1976; Andersen, 1978; Melnik et al., 1977; Franklin and Cafiso, 1993; Pohl et al., 1997). The dependence of the fluorescence ratio on the concentration of phloretin obtained in our experiments (Fig. 5) is in agreement with the data of Clarke and Kane (1997). It follows from the comparison of the concentration dependences of the fluorescence ratio for gramicidin and phloretin that gramicidin is as potent as phloretin in modifying the membrane dipole potential, at least for DPPC bilayers. It is worth noting that the concentration dependence of the change in the dipole potential upon the addition of phloretin determined by the ratiometric fluorescence method is consistent with the results of the surface potential measurements on monolayers (Reyes et al., 1983; Cseh and Benz, 1998).

Concerning the mechanism of the gramicidin effect on the dipole potential, it can be supposed that gramicidin reduces the existing positive dipole potential of monolayers and bilayers by inducing reorientation of dipole-carrying groups, for example, phosphocholine headgroups of lipid molecules and/or water molecules forming the hydration shell of lipid headgroups.

It is known that the dipole potential of bilayer lipid membranes produces a pronounced effect on the processes of ion transport across the membranes (Brockman, 1994). It is reasonable to assume therefore that this parameter is also important for natural membranes (Zhang et al., 1996). Because natural membranes contain proteins (some of them contain more proteins than lipids by weight), it is essential to determine the contribution of the proteins to the dipole potential of the membranes. This work has shown that the presence of peptides in a bilayer lipid membrane can significantly affect the value of its dipole potential.

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