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Membrane Phosphate Headgroups' Modulation of Permeation of Alkaline Cations in Gramicidin Channels

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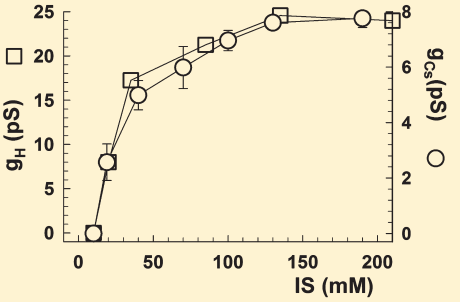
ABSTRACT: The function of membrane proteins is modulated by lipid bilayers. The permeation of ions in gramicidin A channels (gA) is markedly distinct in monoglyceride and phospholipid membranes. It was previously demonstrated that membrane phosphate headgroups accelerate the rate of proton transfer in gA. However, the permeation of alkalines in gA channels is considerably slower in phospholipid than in monoglyceride membranes. In this study, gA channels were reconstituted in various membranes of ceramides, monoglycerides, phospholipids, or sphingolipids. It is demonstrated that single channel conductances to alkalines are similar among bilayers consisting of phospholipids and sphingolipids, and ceramides and monoglycerides. The presence of phosphate headgroups in membranes (and not the double acyl chains in lipids) attenuates alkaline permeation and enhances the proton transfer permeation in gA channels. In ceramide membranes in low ionic strength (<250 mM) solutions, gA channels become dysfunctional. The experimental results are discussed in regard to membrane/solution and membrane/protein interfaces.



INTRODUCTION

Biological membranes are composed of distinct microdomains in which proteins are surrounded by specific lipids.[1] Even though not all membrane proteins have been studied in diverse lipid environments, it is reasonably assumed that the function of a protein is modulated by its surrounding lipids.[2−4] Some physical factors that underlie said modulation include the hydrophobic matching between protein and bilayer,[4,5] membrane surface tension and elasticity,[6,7] membrane surface charges,[8] and binding of specific lipids to a protein.[3,9,10] The physicochemical properties that account for the modulation of protein function in a native membrane are difficult to address. One method to address this issue consists in analyzing the function of a single protein in a well-defined lipid membrane. By modifying specific groups of atoms in lipids or protein (or both), insights are gained into protein−lipid interactions.[24,39−42]

Our experimental approach aims at reconstituting gramicidin A (gA) ion channels in well-defined lipid bilayers. gA is a highly hydrophobic pentadecapeptide (HCO−L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp−NH−$(CH_2)_2$−OH) secreted by *Bacillus brevis*. In lipid bilayers, this primary structure defines a right-handed $\beta^{6.3}$ helix in which the amino acid side chain residues are in contact with the hydrophobic core of the lipid membrane and the carbonyl and amide groups line the pore of the protein.[11,12] The association via six intermolecular H-bonds between the amino termini of the two native gA peptides, each located in a distinct monolayer of a lipid bilayer, forms a water-filled ion channel that is selective for monovalent cations.[13,14] Disruption of intermolecular H-bonds between the two gA monomers results in dimer dissociation and loss of channel function.

Although the structure of gA is markedly different from other ion channels, its "simplicity" offers an excellent opportunity to address at the molecular level the interaction between specific lipid components in the membrane and ion channel function. It has been known since earliest studies that the rate of ion permeation in gA channels depends on the nature of the lipid membrane.[15,16] In previous studies of H^+ transfer in gA channels and under nonsaturating H^+ concentrations in bulk solutions ($[H^+]$), the single channel conductance to H^+ ($g_H = i_H/V_m$, where i_H is the measured H^+ current through a single gA channel, and V_m is the applied transmembrane or trans-channel voltage) in distinct types of gA channels in phospholipids is ∼10-fold larger than in monoglyceride membranes.[17−21] Moreover, in phospholipid membranes, a simple adsorption isotherm accurately represents the $g_H-[H^+]$ relationship. By contrast, in monoglycerides, those relationships are complex and not well understood.[22−25]

Monoglycerides differ from phospholipids by the presence of a second acyl chain and a phosphate headgroup in the latter. Which of these molecular groups accounts for the modulation of gA channels? Adding a second fatty acid to a monoglyceride (diacylglycerols) or adding a phosphate group to monoglycerides could have provided an answer to this question. However, diacylglycerols and phosphorylated monoglycerides (detergents)

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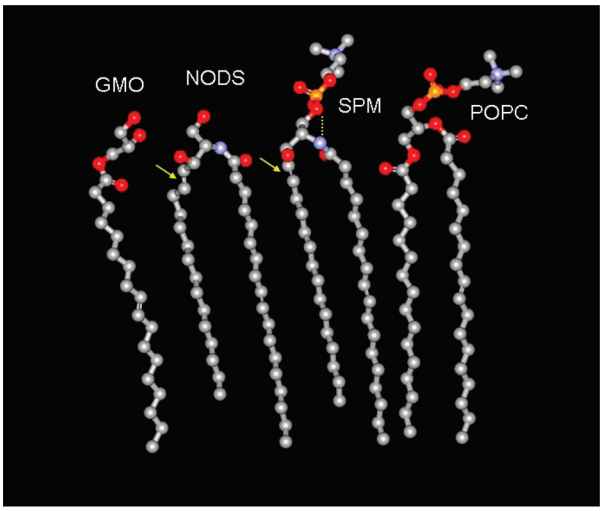

Figure 1. Structures of membrane constituents of bilayers used in this study. Arrows in NODS and SPM point out trans C_4-C_5 double bonds. Notice the H-bond in SPM between a phosphate oxygen and amide group. Hydrogens are not shown in the figure. C, O, N, and P are represented in gray, red, blue, and orange, respectively. See text for discussion.

do not form membranes. In fact, these lipids are membrane destabilizers. Consequently, this approach cannot address that significant question.

The effects of a second acyl chain and the presence of a phospholipid headgroup on the rate of H^+ transfer in gA channels were investigated using a new methodology with a distinct category of lipids. gA channels were incorporated into membranes formed by either ceramides or sphingolipids (see Figure 1).[24] The structural difference between a ceramide and sphingolipid is the presence of a phosphate headgroup in the latter. Those lipids formed stable membranes, and it was demonstrated that the rate of H^+ transfer in the water wire in gA channels and its dependence on $[H^+]$ were similar between ceramides and monoglyceride membranes and between phospholipid and sphingolipid membranes.[24] The inescapable conclusion is that phosphate headgroups (and not the second acyl chain in the lipid) enhance the rates of H^+ transfer in gA and are responsible for the major differences in $g_H-[H^+]$ relationships in distinct membranes.[24]

Interestingly, monoglyceride and phospholipid membranes have *opposite* effects on single-channel conductances to alkalines (g_{alk}). In contrast to g_H, g_{alk} values are significantly *larger* in monoglyceride than in phospholipid membranes. These observations prompted our measurements of g_{alk} in gA channels in ceramide and sphingolipid membranes. It is now demonstrated that g_{alk} values are similar between ceramide and monoglyceride membranes and between sphingolipid and phospholipid membranes. These new results are consistent with previous experiments on H^+ transfer[24] and may have significant implications for (a) the function of proteins that translocate protons in membranes (and in particular bioenergetic membranes) and (b) the modulation of membrane proteins surrounded by sphingolipids.

MATERIAL AND METHODS

Planar Lipid Membranes. Lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama). Lipid membranes were formed from decane solutions (~60 mM) of (a) *N*-oleoyl-D-erythrosphingosine or ceramide C18:1 (NODS) and (b) (2*S*,3*R*,4*E*)-2-acylaminooctadec-4-ene-3-hydroxy-1-phosphocholine (sphingomyelin, SPM). Bilayers made of NODS or SPM were similar in stability, thickness, and electrical characteristics to those formed with the more popular 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) and glycerylmonooleate (GMO).

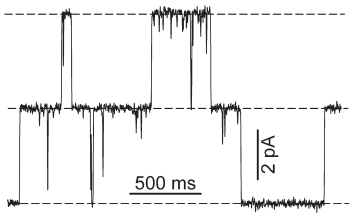

Figure 2. Single-channel gA recordings in 1 M Cs^+ in a GMO bilayer. Several openings of gA channels are shown. The bottom line indicates 0 pA (no channel openings), and the middle and upper dashed lines represent Cs^+ currents permeating through one and two open gA channels, respectively. Upper deflections of trace represent channel opening. These gA channels have a g_{Cs} of 77 pS. The transmembrane voltage was +50 mV.

The structural differences among GMO, NODS, SPM, and POPC are illustrated in Figure 1. NODS has a sphingosine backbone (notice the arrow pointing to the trans double bond between C_4 and C_5 in the sphingosine moiety). An oleate group is linked to the C_2 of the sphingosine molecule via an amino group, as in a peptide bond. This constrains the flexibility of the acyl groups and maximizes the intermolecular van der Waals interactions between adjacent NODS, contributing to the stability of the planar lipid bilayer.[26,44,45] As addressed in the Discussion, the trans $C_4{=}C_5$ bond is essential for assembling lipid membranes by (a) constraining the degrees of freedom in the alkyl chain and (b) allowing a network of intermolecular H-bonds between amides, carbonyls, and hydroxyl groups.[26,44,45] In fact, the saturation of $C_4{=}C_5$ (C18:1 dihydrosphingosine)[24] does not allow the assembly of a lipid membrane. Replacement of C_1-hydroxyl in NODS with a phosphocholine group results in SPM. In distinct MD simulations, several intra- and intermolecular H-bonds between SPM molecules in membranes were identified.[27−29] Those distinct networks of H-bonds in NODS and SPM could explain the significant differences in the function of gA channels.

Experimental Setup and Analysis. Experiments were performed in various concentrations of CsCl in both compartments across the membrane. Experimental results in NaCl and KCl solutions (not shown) were qualitatively identical to those in CsCl solutions. gA channels were added from a methanol stock solution to one bilayer compartment at a concentration of $\sim 10^{-9}$ M. Single channel conductances of hundreds of channels were plotted in histograms and fitted with simple Gaussian distributions. The experimental points in the graphs correspond to the median ± standard error of the mean of the distributions. Unpaired Student's *t* tests were applied for single channel conductances of gA channels in various lipid membranes. These results are shown in the figure legends.

RESULTS

A segment of a single gA channel recording is shown in Figure 2. This experiment was performed with a GMO bilayer in

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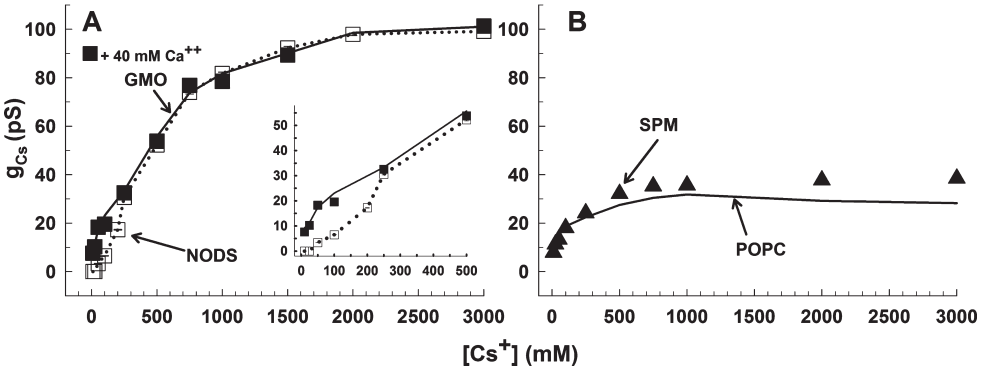

Figure 3. g_{Cs} versus $[Cs^+]$. Panel A: Solid line represents g_{Cs} values in GMO, and open squares, in NODS bilayers. Solid squares represent g_{Cs} values in NODS membranes with 40 mM Ca^{2+} added to CsCl solutions. The inset in panel A expands the plot at low $[Cs^+]$. Panel B shows g_{Cs} values in POPC (solid line) and SPM (triangles) membranes. g_{Cs} values are shown as mean ± sem. Error bars are usually smaller than the size of the symbols. Unpaired Student's *t* tests were used: (a) g_{Cs} in POPC (or SPM) are significantly larger ($p < 10^{-7}$) than in GMO (or NODS) membranes; (b) g_{Cs} in NODS (with and without Ca^{2+} at $[Cs^+] > 250$ mM) are not significantly different from those values in GMO bilayers; (c) g_{Cs} in NODS (with and without Ca^{2+} at $[Cs^+] < 250$ mM) are significantly different ($p < 10^{-5} - 10^{-4}$).

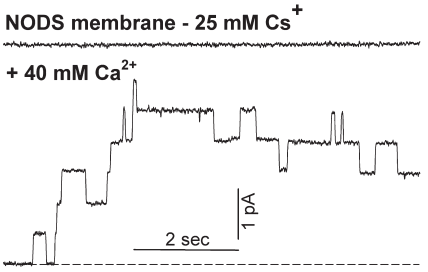

Figure 4. Single gA recordings in 25 mM Cs^+ in a NODS membrane. gA was added to the solution, and after 20 min, no signs of channel opening were identified (upper trace). After adding 40 mM Ca^{2+} to CsCl solutions, several channel openings were observed after a few seconds (bottom trace). Four levels of gA openings are shown in the bottom trace. The transmembrane voltage was +50 mV.

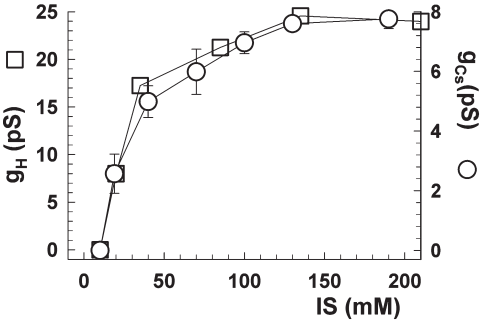

Figure 5. Single channel conductances of gA to H^+ (g_H, squares) and Cs^+ (g_{Cs}, circles) at various ionic strengths. In 10 mM HCl or CsCl (initial experimental conditions), no openings of gA channels were observed. The ionic strength of a 10 mM HCl or CsCl solution was increased by incrementing the concentrations of NaCl (10, 25, 75, 125, and 200 mM) or $CaCl_2$ (3, 10, 20, 30, 40, 60 mM).

1 M CsCl. The transmembrane voltage was 50 mV. Two gA channels are identified in this figure. The bottom dashed line is at the 0 pA level (closed channels), and the two other lines identify the open states of one (middle line) and two (top line) gA channels. Both gA channels have a g_{Cs} of 77 pS.

Panel A in Figure 3 shows g_{Cs} in GMO (solid line, no symbols), and in NODS membranes (open and filled squares). g_{Cs} values represented by solid squares were measured in solutions with various concentrations of Cs^+ and 40 mM Ca^{2+} (see below). Figure 3A shows that $g_{Cs}-[Cs^+]$ plots are almost identical between membranes formed by GMO (one acyl chain) or NODS (two acyl chains). The presence of a second acyl chain in NODS does not modify g_{Cs} values in GMO membranes. At $[Cs^+]$ below ~250 mM, g_{Cs} values (open squares) depart significantly from the GMO curve, and at concentrations smaller than ~50 mM, open gA channels cannot be observed. The addition of 40 mM $CaCl_2$ to solutions immediately caused gA channels to open. This is demonstrated in Figure 4. The top transmembrane current recording in Figure 4 was obtained in NODS membrane at 25 mM Cs^+. No open single gA channels were identified after addition of gA. After waiting for 20 min (enough time for incorporation of many gA channels in lipid membranes), addition of 40 mM Ca^{2+} opened gA channels immediately. This is shown in the bottom recording. It is likely that gA channels were already incorporated in NODS membranes but dysfunctional or inactivated at low concentrations of CsCl. Increasing the ionic strength of the solutions by adding $CaCl_2$ in this study or NaCl[24] immediately reactivated the gating (closed ↔ open) of gA channels (see Figure 5).

$g_{Cs}-[Cs^+]$ relationships in a POPC membrane (curve) and SPM (triangles) membranes are shown in Figure 3B. Compared with the graph in panel 3A, it is clear that the presence of phosphate headgroups in lipid membranes attenuates g_{Cs} values in gA channels. This effect is opposite to the effects of phosphate headgroups on g_H.[24] The major difference between single channel conductances in gA channels is caused by the presence of phosphate groups, *not* by the presence of a second acyl chain in the lipid membrane.

In Figure 5, g_H (squares) and g_{Cs} (circles) of gA channels in NODS membranes are plotted in solutions with various ionic strengths. In 10 mM HCl or CsCl (initial experimental conditions), no openings of gA channels were visible (see Figure 4). The ionic strength was increased by adding NaCl to 10 mM HCl or $CaCl_2$ to 10 mM CsCl (see legend). The addition of Na^+ to HCl solutions does not contribute to an increased g_H in gA channels. If anything, Na^+ should attenuate g_H by blocking proton transfer inside the channel.[38] Neither Ca^{2+} nor Cl^-

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permeates gA channels. As the ionic strength increases, the single channel conductances of gA channels become measurable, and at ionic strengths above ~250 mM, the values of g_H and g_{Cs} are very close to those measured in GMO membranes[24] (see Figure 3).

Despite the distinct experimental protocols in Figure 5, the recovery of g_H and g_{Cs} follow similar courses as ionic strength increases. This suggests that ionic strength is the factor that modulates the single channel conductance in NODS but *not* in other types of membranes. The enhancements of g_H or g_{Cs} from 0 pS to their full single channel conductances did not occur abruptly, as if the membrane had undergone a phase transition induced by ionic strength.[43] The overall single channel conductance is basically the *average* of the reciprocal of the residence times of cations in the channel. As such, the biophysical factors (see the Discussion section) that account for partially restoring single channel conductances in NODS membranes at various ionic strengths (between 10 and 250 mM) are a consequence of the equilibrium (channel inactivation) ↔ (channel activation). As the ionic strength increases, so does the probability of the channel's being activated, thus enhancing the single channel conductance. Above 250 mM ionic strength, the normal activation mode (closed ↔ open) of gA channels resumes.

DISCUSSION

Phosphate Headgroups, H^+ Transfer and Alkaline Permeation in gA Channels. The transfer of protons and the permeation of alkaline ions in gA channels are enhanced and attenuated, respectively, by phosphate headgroups in lipid membranes. Molecular dynamics simulations have shown that a free energy minimum for H^+ resides next to the surfaces of a phospholipid membrane.[30,31] This free energy minimum is a consequence of (a) the stabilization of hydroniums via H-bonds by solvated phosphate headgroups, and (b) the fact that the structure of bulk water is more likely to establish H-bonds with waters than with hydroniums. Further studies revealed that H^+ transfer in gA channels in GMO bilayers has a free energy barrier at the channel-membrane/solution interfaces significantly higher than in phospholipid membranes.[32]

One major difference between the GMO and phospholipid membrane interfaces is that waters are organized in the latter. In phospholipid membrane interfaces, the tendency for waters is to orient themselves with the oxygen pointing to the bilayer, which favors the solvation of hydroniums.[32] By contrast, waters at GMO interfaces are randomly and similarly distributed as in bulk solutions. The relatively small energy barrier for H^+ to enter and exit the mouths of the gA channel in phospholipid compared to GMO membranes could be a consequence of those structured waters at interfaces. The molecular dynamics studies are qualitatively consistent with experimental results.[18−20,32]

The transfer of H^+ (or OH^-) in solutions and across the membrane in gA channels is a consequence of the reorganization of H-bonds between hydroniums and water molecules in an electrochemical field.[19,22] The diffusion of other ions in solutions and in gA proceeds via hydrodynamic diffusion. The structured waters at phospholipid interfaces could stabilize the interaction with alkalines and Cl^-, their solvations in bulk water, or both. These would decrease alkaline mobility at the channel's mouths, attenuating single channel conductances in phospholipid compared with GMO interfaces.

The synthesis of ATP in multicellular organisms is an essential phenomenon in life. ATP synthesis is driven by the H^+ electrochemical gradient generated by membrane proton translocators in the mitochondria. The H^+ concentration in the cytoplasm or in the intermembrane space in mitochondria ($\sim 10^{-7}$ M) is 10^6-fold less than the concentration of the most abundant K^+. These distinct membrane electrochemical gradients would not favor the approach of protons to membrane interfaces. Following our results, the presence of membrane phosphate groups decreases (or increases) the free energy barriers[32] for H^+ (or alkaline) permeation. It is plausible that these phenomena and explanations could also extrapolate to H^+ transporters in bioenergetic and other membranes, thus incrementing ATP synthesis. In this context, it is interesting to note that cardiolipin (a diphosphatidylglycerol lipid) is abundantly present in virtually all membranes involved in ATP synthesis. A genetic mutation of tafazzin (an essential enzyme in cardiolipin synthesis) that causes Barth syndrome, leading to death, and in some types of heart failure and neurological diseases a significant decrease in cardiolipin concentration in the inner mitochondrial membrane has been linked to low ATP synthesis.[33,34] A cardiolipin-rich diet seems to improve some of these disease symptoms. Is it possible that cardiolipin interfaces favor the approach of protons and optimizes transfer across membranes because of its double phosphate headgroup?[31]

Dysfunction of gA Channels in Ceramide Membranes at Low Ionic Strengths. Because of the various effects of sphingolipids on distinct biological phenomena, these molecules have attracted an overwhelming amount of attention in the last 15 years.[35,36] One hypothesis[37] is that conversion of sphingolipids into ceramides by a phospholipase modulates the function of a membrane protein surrounded by these distinct lipids. However, direct experimental evidence has been lacking. The experimental results in this and in a previous study[24] clearly demonstrated that gA channels have distinct behavior in ceramide versus sphingolipid membranes and that the presence of phosphate groups in SPM is responsible for the distinct gA functions.

An unexpected finding that could be of significance for membrane protein function was that gA channels become dysfunctional in ceramide membranes at ionic strengths that are within the physiological range. This original observation was previously reported for the transfer of protons in gA channels[24] and has now been expanded to other monovalent cations. The cause of dysfunctional channels in ceramide membranes at low ionic strengths is not clear. It is essential to notice however, that (a) gA channels in low ionic strengths in various other membranes are functional; and (b) ceramide membranes are functional, behave similarly to other types of bilayers, and their electrical impedances, optical features, and thicknesses (in low or high ionic strengths) are similar to other membranes.[24] It is likely that the inactivation of gA channels is caused by molecular interactions between NODS and gA in low ionic strength solutions.

Significant intermolecular interactions account for the assembly of NODS into lipid bilayers. In addition to van der Waals interactions mentioned in the Methods section, there are also meaningful intermolecular H-bonds between NODS. The C_4═C_5 double bond in its sp^2 configurations is more electronegative than a saturated bond. The hydroxyl oxygen close to that double bond (an allyl alcohol) is very reactive, has a high negative polarity, and accepts H-bonds from OH groups in adjacent NODS.[26] In addition, intermolecular H-bonds also occur between amides and carbonyl groups in NODS. In view of these electrostatic interactions, it is not surprising that reduction of

C_4═C_5 in NODS does not allow the assembly of plain ceramide membranes.

Regarding the interaction between NODS and gA, it is reasoned that in high ionic strength solutions, the polar groups of NODS are screened,[8] and weaker interactions of H-bonds develop among OH, NH, and CO in NODS and the CO and NH in the outer loops of the gA channel. In low ionic strengths, however, the intermolecular H-bonds would be electrostatically strong enough to destabilize or unfold the outer loops of gA channels, leading to channel inactivation. Increasing the ionic strength of solutions would restore the secondary structure of gA channels, thus removing its inactivation. On the other hand, no inactivation of gA channels occurred in SPM membranes in low ionic strengths. Several intramolecular H-bonds were defined in distinct MD studies of SPM.[27−29] In particular, the OH and NH in the NODS portion of the SPM donate H-bonds with phosphate ester oxygens, associated waters, or both. It is possible that these H-bond interactions in SPM could prevent the gA inactivation.

■ CONCLUDING REMARKS

The main objective of this work was to analyze the effects of proton transfer and alkaline permeation in gA channels under distinct lipid membranes. Using classical (monoglycerides and phospholipids) and new (ceramides and sphingolipids) planar bilayer methodologies, the experimental results demonstrate that phosphate headgroups (and not double acyl chain in lipids) in bilayers account for the enhancement of proton transfer and attenuation of alkaline permeation in gA channels. These distinct effects relate to organized waters at phospholipid interfaces that decrease (or increase) the free energy for protons (or alkalines) to enter and exit gA channels. A novel experimental result that may be significant to understand the modulation of membrane proteins by ceramides and sphingolipids is that gA channels become dysfunctional in ceramide membranes at low ionic strengths. The modulation of the function of a membrane protein surrounded by sphingomyelin or ceramide membranes may be the consequence of the presence of phosphate groups in the former by (a) providing a preferential orientation for water molecules at the membrane/solution interfaces and (b) modifying the inter and intramolecular patterns of H-bonds at the membrane/protein interfaces.

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