Modulation of Proton Transfer in the Water Wire of Dioxolane-Linked Gramicidin Channels by Lipid Membranes

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ABSTRACT Proton conductance (g_H) in single SS stereoisomers of dioxolane-linked gramicidin A (gA) channels were measured in different phospholipid bilayers at different HCl concentrations. In particular, measurements were obtained in bilayers made of 1,2-diphytanoyl 3-phosphocholine (DiPhPC) or its ethylated derivative 1,2-diphytanoyl 3-ethyl-phosphocholine (et-DiPhPC,). The difference between these phospholipids is that in et-DiPhPC one of the phosphate oxygens is covalently linked to an ethyl group and cannot be protonated. In relatively dilute acid solutions, g_H in DiPhPC is significantly higher than in et-DiPhPC. At high acid concentrations, g_H is the same in both diphytanoyl bilayers. Such differences in g_H can be accounted for by surface charge effects at the membrane/solution interfaces. In the linear portion of the log g_H-log [H] relationship, g_H values in diphytanoyl bilayers were significantly larger (~10-fold) than in neutral glyceryl monooleate (GMO) membranes. The slopes of the linear log-log relationships between g_H and [H] in diphytanoyl and GMO bilayers are essentially the same (~0.76). This slope is significantly lower than the slope of the log-log plot of proton conductivity versus proton concentration in aqueous solutions (~1.00). Because the chemical composition of the membrane-channel/solution interface is strikingly different in GMO and diphytanoyl bilayers, the reduced slope in g_H-[HCl] relationships may be a characteristic of proton transfer in the water wire inside the SS channel. Values of g_H in diphytanoyl bilayers were also significantly larger than in membranes made of the more common biological phospholipids 1-palmitoyl 2-oleoyl phosphocholine (POPC) or 1-palmitoyl 2-oleoyl phosphoethanolamine (POPE). These differences, however, cannot be accounted for by different surface charge effects or by different internal dipole potentials. On the other hand, maximum g_H measured in the SS channel does not depend on the composition of the bilayer and is determined essentially by the reduced mobility of protons in concentrated acid solutions. Finally, no experimental evidence was found in support of a lateral proton movement at the phospholipid/solution interface contributing to g_H in single SS channels. Protein-lipid interactions are likely to modulate g_H in the SS channel.

INTRODUCTION

Gramicidin A (gA) is a highly hydrophobic pentadecapeptide secreted by *Bacillus brevis*. Its unusual primary structure consists mostly of an alternating sequence of D and L amino acids that determines a right-handed $\beta^{6.3}$ helix structure in different molecular environments (Andersen et al., 1999; Arseniev et al., 1985; Ketchem et al., 1993, 1997; Urry, 1971). In lipid bilayers, the head-to-head association of two gA molecules via H-bonds results in the formation of an ion channel that is selective for monovalent cations (Andersen, 1984; Busath, 1993; Koeppe and Andersen, 1996). The loss of intermolecular H-bonds between the gA monomers disrupts the channel.

Following the original work by Stankovic et al. (1989), two gA monomers were covalently linked with a dioxolane ring. The presence of two chiral carbons in the dioxolane linker offers the possibility to synthesize two different stereoisomers of dioxolane-linked gA dimers (the SS and RR dimers; see Quigley et al., 1999; Stankovic et al., 1989). In the SS stereoisomer, there is a constrained and continuous transition between the gA monomers without significant distortions in the β-helicity of the protein (Quigley et al., 1999; Stankovic et al., 1989). Ion channels formed by these dimers in lipid bilayers have an average lifetime significantly longer than native gA channels. Dioxolane-linked gA dimers provide an interesting model to explore structure-function relationships in ion channels. Of particular interest is the study of proton movement in proteins. Proton transfer in membrane proteins is an essential phenomenon in biology (Deamer and Nichols, 1989; DeCoursey and Cherny, 1999; DeCoursey et al., 2000). The synthesis of ATP is ultimately driven by proton transfer that occurs in bioenergetic proteins. Proton transfer between water molecules and/or amino acids in energy-transducing enzymes have been demonstrated (Baciou and Michel, 1995; Riistma et al., 1997). The tight coupling between proton transfer and redox potentials in these complex structures (Trumpower and Gennis, 1994) makes it difficult to perform a detailed analysis of proton transfer under different experimental conditions. In contrast, gA channels are water-filled proteins that have a very large single-channel conductance to protons (g_H) (Akeson and Deamer, 1991; Cukierman et al., 1997; Cukierman, 1999, 2000; Eisenman et al., 1980; Heinemann, 1990; Hladky and Haydon, 1972; Levitt and Decker, 1988; Myers and Haydon, 1972) and have proven to be quite useful as a model of proton transfer in proteins (Akeson and Deamer, 1991; Cukierman, 2000; Phillips et al., 1999; Pomès and Roux, 1996; Schumaker et al., 2000).

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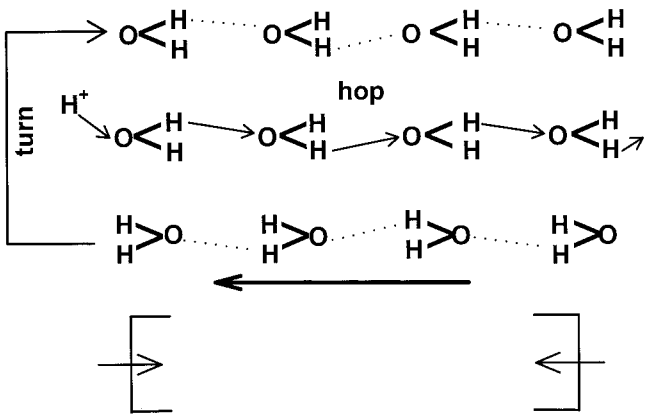

FIGURE 1 Schematic representation of four water molecules interconnected via H-bonds (water wire). The approach of a proton to the water molecule on the left triggers a reorganization of covalent and H-bonds inside the water wire with the result of transferring one proton to the right side (hop step). For another proton to be transferred in the same direction, the water molecules in the bottom row must rotate (turn step) to the original configuration in the upper row. These two different steps are the basis of a Grotthuss mechanism. Notice that the dipole moment of the water wire in the lower row is directed to the right (*bottom arrow*), whereas the upper row has an inverse dipole moment. Two monolayers of the same bilayer with their associated dipole moments are represented in the bottom of the figure. Notice the relative alignments between the dipole moments of the monolayers and the water wire inside the channel.

The mobility of protons in water (μ_H) is abnormally high in relation to other ions (cf. Bernal and Fowler, 1933). This suggests that μ_H in water is not determined by the hydrodynamic flow of a proton or by a molecular aggregate such as $(H_3O)^+$. An attractive hypothesis that became known as the Grotthuss mechanism could explain the transfer of protons along a chain of water molecules interconnected via H-bonds (water wire) (Nagle and Morowitz, 1978; Nagle and Tristam-Nagle, 1983). In Fig. 1, the basic steps of the Grotthuss mechanism are illustrated diagrammatically. In this figure the water wire is composed of four water molecules interconnected via H-bonds. The approach of a H^+ to the outermost water oxygen leads to the formation of a covalent bond between those two atoms. In consequence, one proton will now be shared between the two most external water molecules $(H_5O_2)^+$ in the wire. Proton hopping and reorganization of covalent bonds in waters will occur along the water wire with the final release of a proton from the water molecule on the other end of the wire. The hopping process leaves the water molecules in an orientation opposite to the initial one. For another proton to be transferred along the water wire in the same direction as before, each water molecule has to turn back to its initial configuration (turn step). The hopping process is not the rate-limiting step in the Grotthuss mechanism. Molecular dynamics simulations revealed that proton transfer between two adjacent water molecules inside the pore of the gA channel occurs in the sub-picosecond time scale (Pomès and Roux, 1996). In contrast, the reorientation step of the entire water wire has a higher energetic cost that is dependent on the number of water molecules (Pomès and Roux, 1998; Pomès, 1999).

The Grotthuss mechanism depicted in Fig. 1 can be applied to proton transfer only in relatively dilute solution of acids. As $[H]_{bulk}$ increases over 1 M, a significant reduction in μ_H occurs, and at $[H]_{bulk}$ of ~5 M, μ_H becomes close to the mobility of water (Cukierman, 2000; see Fig. 4), suggesting that the charged $(H_3O)^+$ is probably diffusing hydrodynamically.

It was demonstrated that proton transfer in gA channels occurs by a Grotthuss mechanism (Levitt et al., 1978). This explains the dramatically larger g_H in gA in relation to other monovalent cations. The log g_H-log [H] relationship of dioxolane-linked gA dimers in glyceryl monooleate (GMO) membranes was previously investigated (Cukierman, 2000). In the SS dimer, a linear relationship was found in those neutral lipid bilayers in the concentration range of 0.1–2000 mM $[H]_{bulk}$. Interestingly, the slope of that relationship (0.75) was significantly lower than that measured in water (1.00). At $[H]_{bulk} > 2000$ mM, g_H saturates and declines as the proton conductivity in bulk water, thus demonstrating that g_H has a significant component outside the channel itself (membrane/solution interface and bulk solution) (Cukierman, 2000; Decker and Levitt, 1988).

In this study we address several interrelated issues concerning proton transfer in the SS channel. We have focused our analysis on this gA dimer, because, in contrast to the RR stereoisomer of dioxolane-linked gA channels, it has a linear log g_H-$[H]_{bulk}$ relationship in GMO bilayers (Cukierman, 2000). This seems to simplify the interpretation of data.

Phillips et al. (1999) demonstrated that at a given $[H]_{bulk}$, the g_H of native gA channels was significantly larger in a diphytanoyl phosphatidylcholine (DiPhPC) than in a GMO bilayer. When a different phospholipid bilayer was used (a mixture of 1-palmitoyl 2-oleoyol phosphatidylethanolamine (POPE) and 1-palmitoyl 2-oleoyol phosphatidylcholine (POPC)), g_H in both native gA and SS channels was significantly smaller than in GMO bilayers (Cukierman et al., 1997; Quigley et al., 1999). It is of interest to investigate the effects of different phospholipid bilayers on g_H in the SS channel.

Both the SS and gA channels have high g_H. Under conditions in which the channel does not offer an appreciable resistance to current flow, the access resistance of the channel plays a crucial role in determining g_H (Quigley et al., 1998; Cukierman, 2000). An important component of the access resistance is determined by the membrane-channel/solution interface. A significant factor in that interface is the electrostatic potential (ψ_0) generated by fixed surface charges on the lipid bilayer (Rostovtseva et al., 1998). ψ_0 defines a chemical environment adjacent to the channel mouth that could modulate g_H. Because GMO and different

diphytanoyl bilayers have different surface potentials, they define different membrane-channel/solution interfaces. This effect was investigated on g_H-[H] relationships in the SS channel.

Does proton transfer in the plane of the membrane/solution interface contribute to g_H in the SS dimer? It has been proposed that a Grotthuss-like mechanism may occur at a phospholipid membrane/solution interface (Haines, 1983; Heberle et al., 1994; Leberle and Zundel, 1990; Morgan et al., 1989; Teissie et al., 1985). In such a model, protons would transfer between a protonated (HO-P—) and an adjacent unprotonated (-O-P—) phospholipid headgroup. This would occur at a rate significantly faster than proton transfer between water molecules in solution (Morgan et al., 1989; Teissie et al., 1985). This hypothesis was seriously questioned (Gutman et al., 1995; Menger et al., 1989) and has long been a matter of continuing debate in localized versus delocalized proton transport in bioenergetics (see Haines, 1983). Because g_H in the SS channel is very large, it is possible that proton depletion at the entrance of the channel pore occurs. This would have the effect of enhancing deprotonation of phospholipids adjacent to the channel entrance. Once these protons are released, they would be available to cross the channel. Protons could then be transferred between relatively distant protonated phospholipid headgroups and unprotonated phospholipids adjacent to the channel's mouth. If proton transfer at the membrane/solution interface is considerably faster than in solution (Teissie et al., 1985), then the phospholipid bilayer could be considered an additional pool of protons for the SS channel. In particular, such a mechanism could explain in part why g_H in the SS channel in phospholipid bilayers is considerably larger than in GMO (Cukierman et al., 1997; Phillips et al., 1999). In this regard, Antonenko and Pohl (1998) have recently provided experimental results consistent with this hypothesis.

In this study, we have measured g_H-[H] relationships in a wide range of $[HCl]_{bulk}$ in different phospholipid bilayers. One bilayer was made of diphytanoyl phosphatidylcholine (DiPhPC), and the other consisted of DiPhPC in which one of the phosphate oxygens was ethylated (et-DiPhPC). The properties of gA channels are dependent on the composition of the membrane. In using DiPhPC and et-DiPhPC, the differences between g_H in the SS channel in these bilayers must result from the marked differences between surface potentials in DiPhPC and et-DiPhPC. In addition, because DiPhPC (but not et-DiPhPC) is protonatable, it was of special interest to investigate whether the lateral proton movement at the bilayer/solution interface could contribute to g_H in single SS channels.

MATERIALS AND METHODS

Experimental

Proton conductances were measured in single SS dioxolane-linked gA channels reconstituted in different phospholipid bilayers. The synthesis, purification, and characterization of this channel have been previously described (Cukierman et al., 1997; Stankovic et al., 1989; Quigley et al., 1999). Voltage clamp ramps from 0 to ~200 mV were applied to single channels in bilayers. The resulting single channel *I-V* plots were subtracted from control *I-V* plots of bilayers that did not contain single channels. The g_H was measured from the initial linear portion of those final *I-V* plots. Each experimental point in this study is the average ± SEM of 4–10 different measurements of g_H (different single channels in different lipid bilayers). Experiments were performed at room temperature (22–24°C).

Lipid bilayers

The different planar bilayers used in this study were formed from the following phospholipids: 1) DiPhPC, 2) et-DiPhPC, 3) POPC, and 4) POPE. Bilayers were formed from a 60 mM solution of lipids in decane. Lipids were purchased from Avanti Lipids (Alabaster, AL). The decane was purchased from Sigma (St. Louis, MO) and was further purified with silica columns. In diphytanoyl bilayers, the single-channel proton conductances were measured over a wide range of acid concentrations. In POPE and POPC, proton conductances were measured in 1 M [HCl] only. Bilayers were formed on a polystyrene partition containing a hole (diameter ~0.1 mm) separating two small-volume (~3 ml) compartments.

Theory

Fig. 2 shows molecular models of DiPhPC (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; see also Hung et al., 2000) and et-DiPhPC (1,2-diphytanoyl-*sn*-glycero-3-ethyl-phosphocholine). The structural difference between these two phospholipids is the presence of an ethyl group covalently attached to one phosphate oxygen in et-DiPhPC. Et-DiPhPC has one net positive charge $(\text{-N-}(CH_3)_3)^+$. In dilute solutions of HCl, that positive charge gives rise to a very large positive surface potential (ψ_0) at the membrane/solution interface. As the concentration of HCl increases, ψ_0 decreases due to the screening effect of the Cl^- counterion. By contrast, DiPhPC is neutral and has no or a small ψ_0 in water or in very dilute acid solutions. As $[HCl]_{bulk}$ increases, so will the protonation of the phosphate moiety. Thus, the lipid bilayer acquires a positive potential. As $[HCl]_{bulk}$ increases, screening of the choline group by Cl^- and further protonation of the phosphate oxygen will occur. Evidently, the ψ_0 of those different lipid bilayers have different values as a function of $[HCl]_{bulk}$. The Gouy-Chapman model was used to quantify these interfacial phenomena.

A complete deduction of the following equations and assumptions of the model can be found in several publications (cf. Israelachvili, 1992; McLaughlin, 1989). Here we provide the basic equations used in this paper. Assume that the planar lipid bilayer contains fixed surface charges (σ) that are uniformly smeared over its entire surface and that H^+ and Cl^- ions can be treated as point charges. In equilibrium conditions, the application of the Poisson-Boltzmann equation with the appropriate boundary conditions leads to the simple expression (Grahame equation) for solutions containing H^+ and Cl^-:

$$\sigma = \{8[\text{HCl}]_{bulk}\varepsilon\varepsilon_o RT\}^{0.5} \sin h(F\psi_0/2RT), \qquad (1)$$

where ψ_0 is the potential at the membrane/solution interface ($x = 0$), σ is the charge density of the lipid bilayer assuming an area of 60 $Å^2$ for the phospholipid headgroup (Binder et al., 1998; Cseh and Benz, 1999), and ϵ and ϵ_o are the dielectric constant of solution (78) and the permittivity of free space (8.85×10^{-12} C^2 J^{-1} m^{-1}), respectively. *F*, *R*, and *T* have their usual meanings. Despite completely ignoring the microscopic structure of solutions and bilayer, the Grahame equation succeeds in correlating σ to ψ_0 in different systems (cf. Israelachvili, 1992; Rostovtseva et al., 1998). Fig. 3 *A* shows the calculated dependence of ψ_0 on $[HCl]_{bulk}$ for an et-DiPhPC

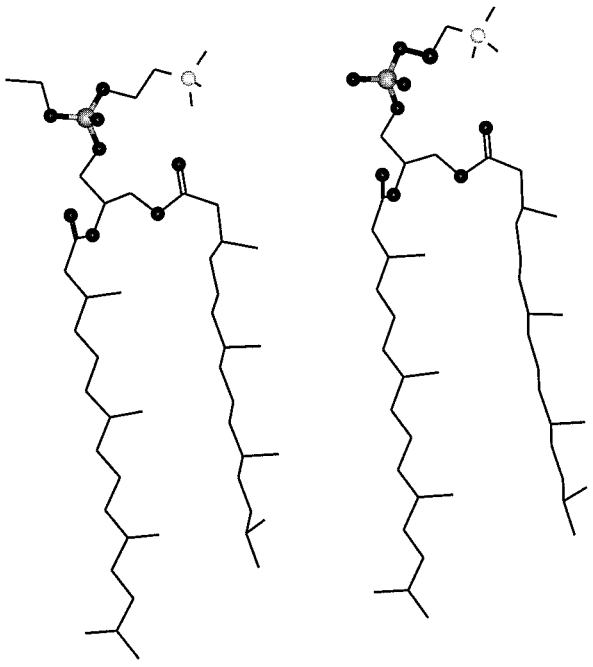

FIGURE 2 Molecular structures of et-DiPhPC (*left*) and DiPhPC (*right*). Hydrogens are not represented in this figure. O, P, and N are represented by black, gray, and light gray circles, respectively. Notice the ethyl group covalently linked to the headgroup in et-DiPhPC.

bilayer (solid line). ψ_0 is infinite in water and decreases monotonically with increasing $[HCl]_{bulk}$. Fig. 3 *B* is an expansion of Fig. 3 *A* for the concentration range of 0–0.5 M.

To calculate ψ_0 in DiPhPC bilayers, the protonation of the headgroup must be taken into consideration, and Eq. 1 has to be modified to account for the variation in protonation as a function of $[HCl]_{bulk}$. σ is then expressed as

$$\sigma = \frac{\sigma_{max} K_{assoc} [H]_{bulk} \exp(-F\psi_0/RT)}{1 + K_{assoc} [H]_{bulk} \exp(-F\psi_0/RT)}, \quad (2)$$

where K_{assoc} is the association constant of protons to DiPhPC:

$$K_{assoc} = [\text{DiPhPC} - \text{H}]/\{[\text{DiPhPC}][\text{H}]_{bulk}\}. \quad (3)$$

The relationship between ψ_0 and bulk [HCl] was calculated for DiPhPC bilayers assuming an association constant of 100 M^{-1} ($pK_a = 2$). This value is well within the range of pK_a values measured in different phospholipids (Marsh, 1990) and was found reasonably appropriate for fitting our experimental measurements of g_H-$[H]_{x=0}$ relationships (see Results). The relationships between ψ_0 and $[HCl]_{bulk}$ are shown as dotted curves in Fig. 3, *A* and *B*. Notice that differences between ψ_0 values in DiPhPC and et-DiPhPC are very large at low $[HCl]_{bulk}$ and become small or negligible at larger concentrations. Also in Fig. 3 *A* the relationship between σ/σ_{max} and $[HCl]_{bulk}$ is plotted as a dashed line with $pK_a = 2$.

Ionic concentrations at the mouths of the SS channel ($x = 0$) are determined by ψ_0 and were calculated by the Boltzmann equations (Fig. 4):

$$[H]_{x=0} = [H]_{bulk} \exp(-F\psi_0/RT)$$
$$[Cl]_{x=0} = [Cl]_{bulk} \exp(F\psi_0/RT) \quad (4)$$

There is an appreciable difference between $[H]_{x=0}$ for DiPhPC and et-DiPhPC at low $[HCl]_{bulk}$, and this difference decreases with increasing $[HCl]_{bulk}$. Except at very high concentrations (see Results), g_H is directly proportional to $[H]_{bulk}$. Thus, it is predicted that g_H is significantly larger in DiPhPC than in et-DiPhPC. Another point that will be evaluated in the Discussion is the high $[Cl]_{x=0}$ for both types of bilayers (Fig. 4, *C* and *D*). Notice in particular that for et-DiPhPC $[Cl]_{x=0}$ is larger than 20 M over the entire range of $[HCl]_{bulk}$.

RESULTS

Fig. 5 shows recordings of current-voltage relationships for the SS channel in two different $[HCl]_{bulk}$ concentrations (100 and 1000 mM). In each graph, plots with the larger and smaller proton currents were obtained in DiPhPC and et-DiPhPC bilayers, respectively. In 100 mM $[HCl]_{bulk}$, g_H in the SS channel is 198 pS in DiPhPC and 22 pS in et-DiPhPC. In 100 mM $[HCl]_{bulk}$, the ratio between $[H]_{x=0}$ in these different bilayers is ~10 (Fig. 4). By contrast, in 1000 mM $[HCl]_{bulk}$, that ratio is ~1.3

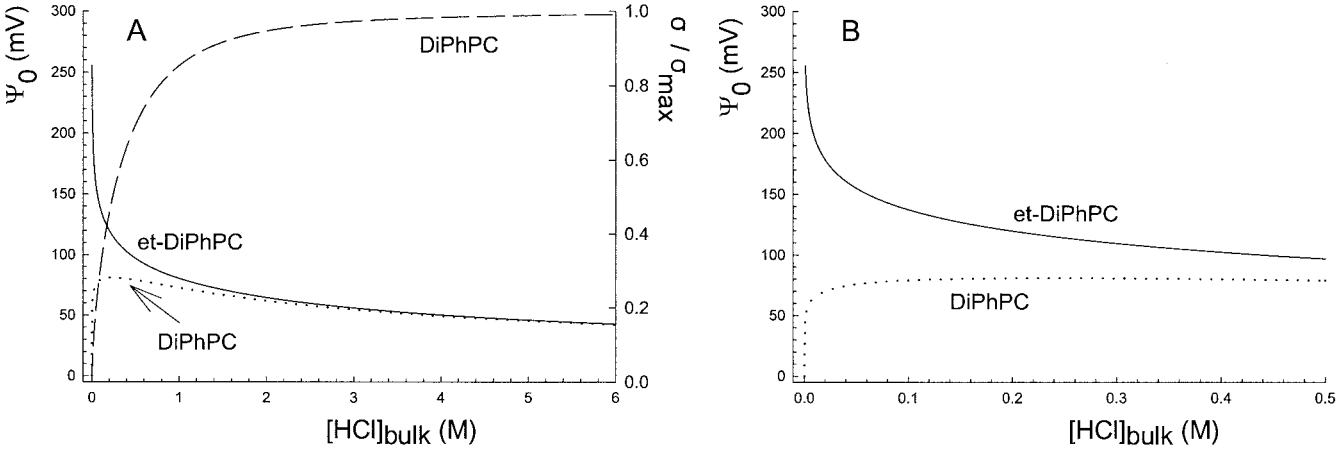

FIGURE 3 (*A*) Plots of ψ_0 versus $[HCl]_{bulk}$ in DiPhPC (· · ·) and et-DiPhPC (——). The dashed line applies to the *y* axis on the right and shows the protonation curve of DiPhPC (σ/σ_{max}). (*B*) Expansion of the curves in *A* to the low concentration range. See text for discussion.

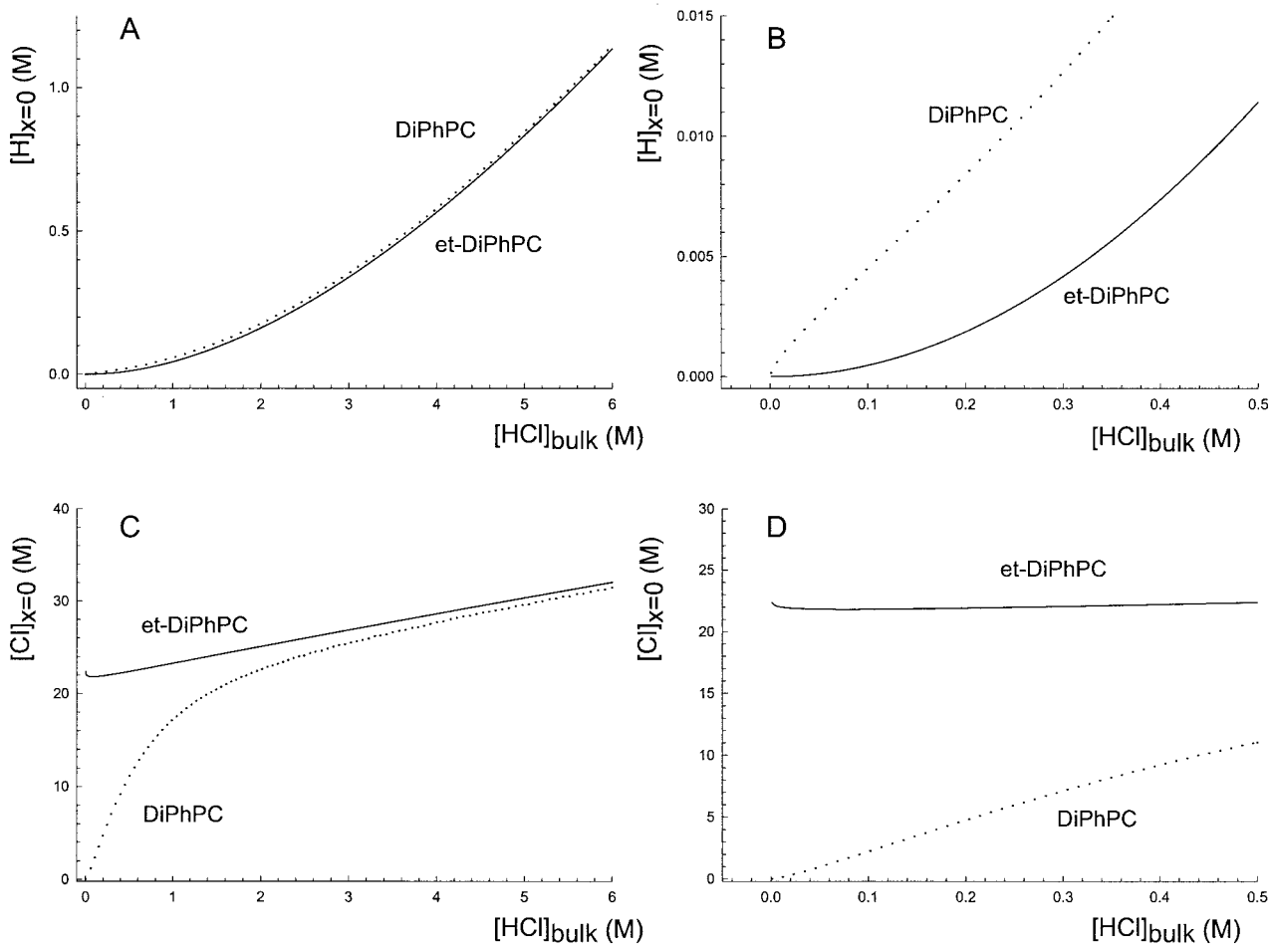

FIGURE 4 Calculated proton (*A* and *B*) and chloride (*C* and *D*) concentrations at the membrane/solution interface ($[H]_{x=0}$) were plotted against $[HCl]_{bulk}$ in different lipid bilayers. See text for details.

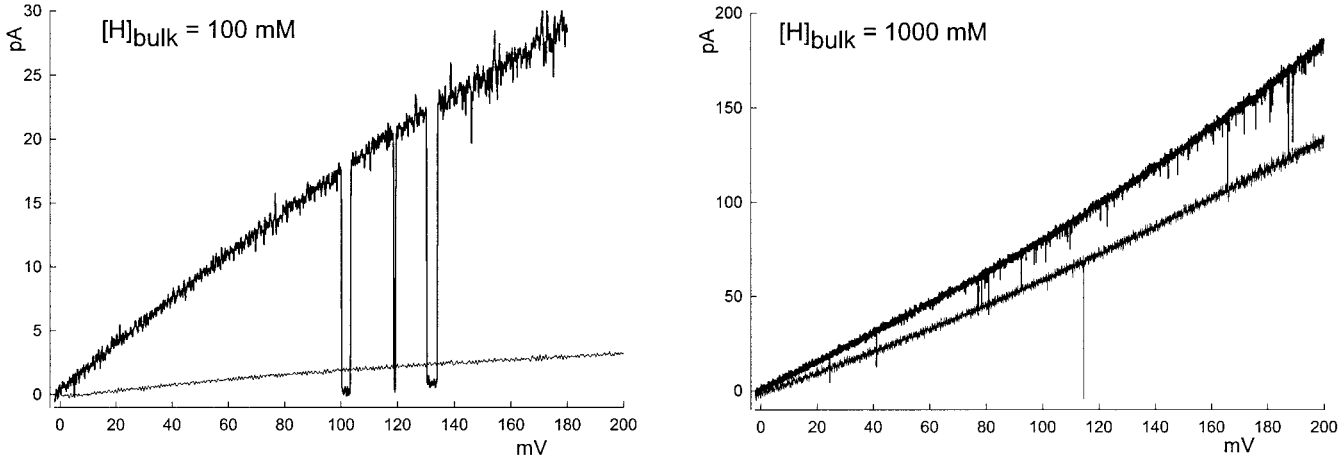

FIGURE 5 Single-channel current-voltage relationships at two different HCl concentrations. In each panel, the larger and smaller currents were obtained from SS channels in DiPhPC and et-DiPhPC bilayers, respectively.

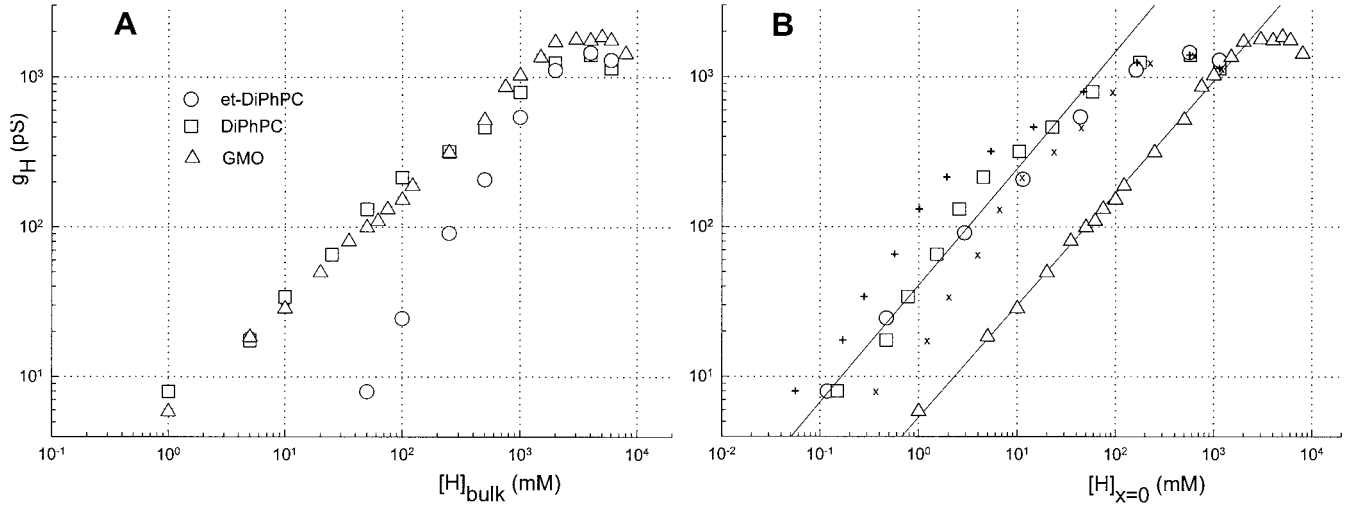

FIGURE 6 (*A*) g_H-$[H]_{bulk}$ relationships in DiPhPC (□), et-DiPhPC (○) and GMO (△); data from Cukierman (2000). (*B*) Data points in *A* were replotted against calculated $[H]_{x=0}$. See text for a complete description and discussion of this figure.

and the g_H values of the SS channel are closer (764 pS in DiPhPC and 573 pS in et-DiPhPC).

In Fig. 6 *A*, g_H measured in the SS channel in different bilayers was plotted against $[HCl]_{bulk}$. As suggested by the original recordings in Fig. 5, differences between g_H measured with the SS channel in DiPhPC or et-DiPhPC decrease continuously with increasing $[HCl]_{bulk}$. For $[HCl]_{bulk} > 1000$ mM, g_H of the SS channel was not significantly different in the different bilayers. Data points in Fig. 6 *A* were replotted in Fig. 6 *B* as a function of $[H]_{x=0}$. For et-DiPhPC bilayers (circles), $[H]_{x=0}$ was calculated using Eqs. 1 and 4. Between 0.1 and 100 mM, the log (g_H) − log $[H]_{x=0}$ is a straight line (Fig. 6 *B*). The experimental points obtained in DiPhPC bilayers (squares in Fig. 6 *A*) were corrected for $[H]_{x=0}$ using Eqs. 1, 2, and 4 with a K_{assoc} of 100 M^{-1} (squares in Fig. 6 *B*). In Fig. 6 *B*, the + and × symbols represent g_H in DiPhPC bilayers that had their corresponding $[H]_{x=0}$ calculated using a K_{assoc} of 500 and 20 M^{-1}, respectively. The upper straight line in Fig. 6 *B* (slope of 0.78) was obtained from a linear regression analysis of data points (circles and squares) between 0.1 and 100 mM $[H]_{x=0}$. The g_H measured in neutral ($[H]_{x=0} = [H]_{bulk}$) GMO bilayers are reproduced in Fig. 6 *B* (from Fig. 2 in Cukierman, 2000) as triangles. The straight line (slope of 0.75) connecting these triangles is the best fit for the points between $[H]_{x=0}$ of 1 and 2000 mM (Cukierman, 2000).

DISCUSSION

g_H-[H] relationships in the SS channel

It was previously shown that in the concentration range of 1–2000 mM, the log (g_H)-log $[H]_{bulk}$ relationship in the SS channel in GMO bilayers is linear (slope = 0.75; Cukierman, 2000). We have now extended this observation to the SS channel in diphytanoyl bilayers in the concentration range of 0.1–100 mM $[H]_{x=0}$.

In the concentration range where the log (g_H)-log $[H]_{x=0}$ is linear, g_H in diphytanoyl bilayers is considerably larger (~10-fold) than in GMO. This difference can be explained neither by surface charge effects (Fig. 6) nor by differences in internal dipole potentials of lipid monolayers (see the following section). We conclude that differences in g_H must be accounted for by different lipid-SS interactions. It may well be that lipid interactions with the SS channel inside the bilayer core modulates the dynamics of H-bonds between water molecules inside the channel and carbonyls at the channel wall. These interactions are thought to be essential for proton transfer between water molecules inside the channel (Pomès and Roux, 1996).

It was reassuring to confirm that the SS channel in diphytanoyl bilayers has practically the same slope in the log g_H-log $[H]_{x=0}$ relationship as in GMO bilayers. If this is not a mere coincidence, the application of the Gouy-Chapman model to estimate [H] at the membrane/solution interface (i.e., the mouths of the channel are effectively seeing $[H]_{x=0}$) seems to be validated.

In aqueous solutions of HCl, proton conductivity is directly proportional to $[H]_{bulk}$ with a slope of 1.00 (Cukierman, 2000). This suggests that the rate-limiting step of g_H in the SS channel is not in the bulk solution. We have previously suggested that the rate-limiting step for g_H may be at the membrane-channel/solution interfaces and not inside the SS channel that seems to be working in a single-occupancy mode by protons in a very wide range of bulk HCl concentrations. We now consider that the membrane-channel/solution interface is dramatically different in GMO and di-

phytanoyl bilayers. In particular, the $[Cl^-]_{x=0}$ in diphytanoyl bilayers is considerably larger than in GMO bilayers. In et-DiPhPC bilayers, for example, the ratio of Cl^- to H_2O at the plane of the membrane is 1:3 even at the low end of $[HCl]_{bulk}$. This ratio is not attained at the membrane solution/interface in neutral GMO bilayers. Proton mobility is strongly determined by the ionic composition of the solution, and thus the geometrical configuration of water-solute interactions (see Agmon, 1998). It is thus remarkable that despite dramatically different membrane/solution interfaces in GMO and diphytanoyl bilayers, the linear relationships in log g_H-log [HCl] and their slopes remained essentially the same in a wide range of concentrations. Despite our continuing ignorance on the basic mechanism that accounts for a slope of 0.75 in log (g_H)-[H] relationship, it is tempting to suggest that proton transfer along the water wire inside the channel may be responsible for it.

The proton mobility in concentrated (>2 M) HCl solutions decreases appreciably. In high acid concentrations, protons no longer move via a Grotthuss mechanism. Instead, the diffusion of protons must be determined predominantly by the hydrodynamic diffusion coefficient of $(H_3O)^+$ (Agmon, 1998; Cukierman, 2000; Lengyel et al., 1962; Lown and Thirsk, 1971; Owen and Sweeton, 1941). g_H attains the same maximum level in both GMO or diphytanoyl bilayers at different $[H]_{x=0}$ values. This indicates that at this high $[HCl]_{bulk}$, g_H is determined by proton transfer in bulk solution. If it were not for this limited proton mobility in bulk solution, a considerably larger g_H in different bilayers would be expected. The maximum g_H attained in the SS channel irrespective of bilayer composition is determined by the bulk conductivity of protons.

Differences between g_H values in phospholipid bilayers

In accordance with the Phillips et al. (1999) results with native gA channels, we have now determined for the SS channel that g_H in DiPhPC is significantly larger than in GMO bilayers, once surface charge effects are taken into account. Moreover, our experimental results compiled in Table 1 demonstrate that g_H in DiPhPC is significantly larger than in bilayers made of either POPC or POPE.

The structural difference between DiPhPC and POPC concerns the acyl chains: POPC has one palmitoyl and one oleoyl. The capacitances of bilayers made of DiPhPC or of dioleoyl (or dipalmitoyl) PE or PC are essentially the same (Janko and Benz, 1977). This suggests that bilayer thickness is not likely to account for differences in g_H in the SS channel. Despite the common headgroup in POPC and DiPhPC, there is a significantly larger internal (or interfacial or surface) dipole potential (IDP) in monolayers made of POPC. IDPs measured in monolayers of different phospholipids were compiled in Table 1 (see Smaby and Brockman, 1990). It is worth considering the relationship between IDP and g_H in view of an interesting hypothesis formulated by Phillips et al. (1999).

TABLE 1 Proton conductances in single SS channels and internal dipole potentials in different monolayers

	g_H in 58 mM $[H]_{x=0}$(pS)*	IDP† (mV)
DiPhPC	801 ± 12(7)	62
POPC	570 ± 12(7)	93
POPE-POPC	509 ± 8‡	
POPE	520 ± 17(6)	126
GMO	110 ± 5§	103

*This concentration corresponds to a $[H]_{bulk}$ of 1 M in a DiPhPC bilayer (Fig. 4). We assume that the same $[H]_{x=0}$ applies to the other phospholipid bilayers (POPC, POPE, and their mixture).

†Internal dipole potentials were measured in monolayers formed at a solution/air interface (Smaby and Brockman, 1990). There is considerable discrepancy between measurements of IDP by different investigators using different methodologies or experimental conditions. The IDP values reported in this table were obtained using the same methodology and experimental conditions. Such experimental conditions were, however, different from those used to study ion channel function such as composition of solutions and presence of decane in lipid bilayers.

‡Compiled from previous measurements in which the mole fraction for POPE was 0.8 (Cukierman et al., 1997; Quigley et al., 1998, 1999).

§Measured at 62 mM $[H]_{bulk}$ (Cukierman, 2000).

As discussed before, proton transfer in a Grotthuss mechanism seems to be limited by the reorientation of water molecules inside the water wire (see Fig. 1 and Introduction). After a proton had left the water wire inside the channel, the water column is aligned with its dipole moment in parallel (less stable conformation) with the IDP of the monolayer facing the side on which protons exit the channel. Evidently, on the side where proton enters the channel, the dipole moment of the water column is aligned in an anti-parallel (more stable) configuration with the IDP of the monolayer on that side (see Fig. 1). Based on their measurements of g_H in native gA and in gA that had their tryptophans fluorinated, Phillips et al. (1999) have proposed that the turn step in the Grotthuss mechanism inside gA channels starts with the reorientation of the water molecule nearest the channel exit. This turn step would then trigger the reorientation of the other water molecules (propagation of a Bjerrum D defect). It is likely that the interior of gA channels is appreciably shielded from the IDP (Jordan, 1983, 1984). It was reasoned that the water molecule nearest the channel exit should be more effectively influenced by the IDP (Phillips et al., 1999). Thus, an increased IDP of the monolayer facing the side on which proton exits the channel or a decreased peptide chain dipole should increase g_H. Although this hypothesis could explain differences in g_H measured in gA and fluorinated gA, data in Table 1 suggest that it cannot explain the effect of different phospholipid bilayers on g_H in the SS channel. In fact, there is an inverse relationship between g_H and IDP in different phospholipid bilayers. It could be argued (Phillips et al., 1999) that the experimental results in Table 1 could be explained if the

turn step of the water wire starts with the water nearest the channel entrance and not channel exit. This explanation would, however, conflict with the different g_H values measured in native and fluorinated gAs. Moreover, Table 1 also shows that the IDPs in GMO and POPC monolayers are comparable. Nevertheless, g_H in GMO is fivefold smaller. Our g_H measurements in different bilayers cannot support the conclusion that IDP by itself is a major modulator of the turn step in a water wire inside gA channels. In several experiments with different lipid bilayers (GMO, DiPhPC, PC, or PE), g_H was measured in 1 M HCl solutions containing 100 μM of phloretin, *p*-nitrophenol, or 6-ketocholestanol in both sides or in only one side of the bilayer. The first two substances are known to decrease IDP (see Andersen et al., 1976; Cseh and Benz, 1999) whereas the latter enhances IDP (Franklin and Cafiso, 1993; Gross et al., 1994). No significantly different g_H values were measured in the presence of those substances. Either these substances are not effective in modifying IDP in our experimental conditions and/or the IDP is not affecting g_H. It seems that other factors (such as the fluidity of the bilayers, for example) may have significant modulatory roles on g_H.

Another experimental observation concerns the smaller g_H measured in POPE in relation to POPC bilayers. The difference between these phospholipids is the presence of a choline group. It is commonly accepted that the PC headgroup is more hydrated and its waters more organized than in the headgroup of PE (Marrink and Berkowitz, 1995). Whether this may account for the differences between g_H values in those bilayers remains to be addressed.

In summary, there is a basic difference between g_H values measured in diphytanoyl, POPC, POPE, and GMO bilayers that cannot be explained by surface charge effects or IDP. Lipid-protein interactions are likely to affect proton transfer in the water wire inside the channel. Although these effects cannot be presently controlled or investigated at the experimental level, they will hopefully be addressed in the future using computational methods.

Does the lateral movement of protons at the membrane/solution interface contribute to g_H?

As mentioned in the Introduction, phospholipid bilayers have been considered as a potential source of protons for the SS channel. In high $[H]_{bulk}$ most phospholipids are protonated (Fig. 3) and g_H is quite high and is limited by diffusion of protons in bulk solution (see Discussion above). These conditions define an appropriate scenario for unraveling a possible contribution of lateral proton transfer at the bilayer interface to g_H. If lateral proton movement were an important source of protons for the SS channel, g_H at high $[H]_{bulk}$ would be larger in DiPhPC than in et-DiPhPC (which cannot participate in a Grotthuss mechanism) bilayers. The g_H in DiPhPC and et-DiPhPC are undistinguishable at $[H]_{bulk}$ larger than 1 M. At lower $[H]_{bulk}$, differences in g_H seen in those different membranes can reasonably be explained by surface charge effects. No clear experimental evidence was found in support of a lateral proton transfer in the membrane surface contributing protons to the SS channel.

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