Membrane Dipole Potential Modulates Proton Conductance through Gramicidin Channel: Movement of Negative Ionic Defects inside the Channel

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ABSTRACT The effect of membrane dipole potential on gramicidin channel activity in bilayer lipid membranes (BLMs) was studied. Remarkably, it appeared that proton conductance of gramicidin A (gA) channels responded to modulation of the dipole potential oppositely as compared with gA alkali metal cation conductance. In particular, the addition of phloretin, known to reduce the membrane dipole potential, resulted in a decrease in gA proton conductance, on one hand, and an increase in gA alkali metal conductance, on the other hand, whereas 6-ketocholestanol, the agent raising the membrane dipole potential, provoked an increase in gA proton conductance as opposed to a decrease in the alkali metal cation conductance. The peculiarity of the 6-ketocholestanol effect consisted in its dependence on the H^+ concentration. The experiments with the impermeant dipolar compound, phloridzin, showed that the response of proton transport through gramicidin channels to varying the membrane dipole potential did not change qualitatively if the dipole potential of only one monolayer or both monolayers of the BLM was altered. In contrast to gA proton conductance, the single-channel lifetime changed similarly with varying the membrane dipole potential, regardless of the kind of permeant cations (protons or potassium ions). The results of this study could be tentatively accounted for by an assumption that one of the rate-limiting steps of proton conduction through gramicidin channels represents, in fact, movement of negatively charged species (negative ionic defects) across a membrane.

INTRODUCTION

Studying proton permeation through gramicidin channels in artificial bilayer membranes has attracted much attention during the recent years due to the key role of proton channels in performing a number of specific functions in different cells (DeCoursey and Cherny, 2000). A series of experimental results beginning from a remarkable fact that the H^+ conductance in gramicidin exceeds by more than an order of magnitude that of any other cation (Hladky and Haydon, 1972; Myers and Haydon, 1972; Neher et al., 1978; Eisenman et al., 1980; Busath and Szabo, 1988; Decker and Levitt, 1988; Heinemann and Sigworth, 1989; Woolley et al., 1997) have been explained in terms of the concept of proton conduction along a hydrogen-bonded chain (Nagle and Morowitz, 1978; Knapp et al., 1980; Nagle and Nagle, 1983), in other words, a refined Grotthuss mechanism (see Agmon, 1995; Zundel, 1997, and references therein). In the case of gramicidin, this chain is composed entirely of water molecules and called a water wire (Myers and Haydon, 1972; Levitt et al., 1978; Finkelstein and Andersen, 1981; Akeson and Deamer, 1991; Sagnella and Voth, 1996; Pomes and Roux, 1996, 1998; Schumaker et al., 2001). The idea of proton translocation via water wires was also implicated in theories of passive proton conductivity of lipid bilayers (Nagle, 1987; Deamer, 1987). To discriminate between different mechanisms of the passive H^+ transport, its sensitivity to the membrane dipole potential was considered to be critical (Perkins and Cafiso, 1986, 1987a; Gutknecht, 1987a,b; Fuks and Homble, 1996).

It is known that the alkali metal cation conductance of a gramicidin channel representing a transmembrane head-to-head dimer (Andersen et al., 1999) is strongly affected by the dipoles of four tryptophan residues that are located at both bilayer surfaces (Busath, 1993; Hu and Cross, 1995; Providence et al., 1995). The replacement of one or more tryptophans with nonpolar phenylalanines has been shown to decrease the alkali metal conductance (Bamberg et al., 1976; Heitz et al., 1982; Becker et al., 1991; Seoh and Busath, 1995), whereas increasing the dipole moment of tryptophan residues by their fluorination leads to an increase in the alkali metal conductance of gramicidin A (gA) in diphytanoylphosphatidylcholine bilayers (Andersen et al., 1998; Busath et al., 1998). Recent findings (Busath et al., 1998; Phillips et al., 1999) have revealed that the proton conductance of gramicidin channels is altered by changing tryptophan dipole moments inversely to alkali metal conductance: namely, it has appeared that 1) phenylalanine replacement analogs have an increased proton conductance compared with gramicidin A, and 2) analogs with fluorinated tryptophan side chains are characterized by a decreased proton conductance compared with gA.

A number of research works have shown that the cation permeation through gramicidin channels is sensitive to the membrane dipole potential (DP) (Bamberg et al., 1976; Andersen, 1978; Rokitskaya et al., 1997; Duffin et al., 2001). In particular, the potassium permeability of gramicidin B in glycerolmonooleate (GMO) is about twice that in

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dioleoylphosphatidylcholine membranes (Bamberg et al., 1976), the dipole potential of which is 120 mV higher than that of GMO membranes (Hladky and Haydon, 1973). According to Andersen (1978), Rokitskaya et al. (1997), and Duffin et al. (2001), the single-channel conductance of gramicidin A for potassium ions increases upon addition of phloretin, the well-known agent that lowers the membrane dipole potential (Andersen et al., 1976; Melnik et al., 1977; Reyes et al., 1983; Perkins and Cafiso, 1987b; Pohl et al., 1997; Cseh and Benz, 1999). On the contrary, the addition of RH421, which is known to increase the DP (Malkov and Sokolov, 1996) leads to the reduction of gramicidin single-channel conductance mediated by potassium ions (Rokitskaya et al., 1997; Antonenko et al., 1999; Duffin et al., 2001). Based on the above-mentioned properties of the gramicidin proton conductance, it was reasonable to suggest that the latter would also respond to variations of the membrane dipole potential, but the sign of the changes would be opposite to that observed with alkali metal conductance. This paper presents the results of studying the effects of agents modulating the membrane dipole potential (the dipolar compounds) on the proton conductance of gramicidin channels in comparison with the effects on the alkali metal conductance. The data obtained are discussed in relation to the mechanism of proton conduction through gramicidin channels.

MATERIALS AND METHODS

BLMs were formed from a 2% solution of diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, Alabaster, AL) or its 2:1 (w/w) mixture with 6-ketocholestanol (Sigma, St. Louis, MO) or diphytanoylphosphatidylglycerol (DPhPG, Avanti Polar Lipids, Alabaster, AL) in *n*-decane (Merck, Darmstadt, Germany) by the brush technique on a 0.55-mm-diameter hole in a Teflon partition separating two compartments of a cell containing aqueous solutions of KCl or HCl (see figure captions). Different solutions of HCl were prepared by diluting the stock (14 M) HCl solution with bi-distilled water. Gramicidin A (Fluka Chemie, Buchs, Germany) was added from stock solutions in ethanol to the bathing solutions at both sides of the BLM and routinely incubated for 15 min with constant stirring. All the experiments were carried out at room temperature (22–24°C). In photoinactivation experiments, aluminum trisulfophthalocyanine ($AlPcS_3$) from Porphyrin Products (Logan, UT) was added to the bathing solution at the *trans*-side (the *cis*-side is the front side with respect to the flash lamp).

The electric currents (I) were recorded under voltage-clamp conditions. Voltages were applied to BLMs with Ag-AgCl electrodes placed directly into the cell. The currents, measured by means of a patch-clamp amplifier (OES-2, OPUS, Moscow) in single-channel experiments and by a U5–11 amplifier (Moscow) in photoinactivation experiments, were digitized by using a LabPC 1200 (National Instruments, Austin, TX) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, Glasgow, UK). Single-channel currents were low-pass filtered with a cutoff frequency of 100 Hz, sampled at 1 kHz, and stored directly to the disk.

In photoinactivation experiments, BLMs were illuminated by single flashes produced by a xenon lamp with flash energy of about 400 mJ/cm^2 and flash duration < 2 ms.

RESULTS

It has been shown earlier that the addition of phloretin leads to an increase both in potassium permeability (g_K) and in the average lifetime of gramicidin A channels in the presence of 1 M KCl at neutral pH (Andersen, 1978; Rokitskaya et al., 1997). Here we performed a comparative study of the effects of phloretin and 6-ketocholestanol on proton (g_H) and potassium (g_K) conductance of gA channels as well as on the single-channel lifetime under the conditions when either proton or potassium cation dominated the conductance.

Fig. 1 presents gA single-channel recordings with BLMs formed of DPhPC when the bathing solution contained 150 mM HCl and no potassium ions. It is seen that the addition of 10 μM phloretin to the bathing solutions at both sides of the BLM decreased the proton single-channel conductance (g_H) and increased the channel lifetime. Including 6-ketocholestanol into the membrane-forming solution, which is known to increase the DP (Simon et al., 1992; Franklin and Cafiso, 1993), brought about an increase in g_H and a decrease in the lifetime. Fig. 1 *C* illustrates gA current-voltage dependences for 1) the BLM formed of pure DPhPC without additions (the control), 2) the same BLM after the addition of 10 μM phloretin to the bathing solution, and 3) the BLM formed of the mixture of DPhPC:6-ketocholestanol (2:1). The values of g_H were 154, 125, and 244 pS, and the channel lifetimes were 0.73, 3.8, and 0.07 s, respectively. The measurements performed in the presence of 1M KCl at pH 6 showed that the potassium single-channel conductance (g_K) amounted to 17.1 $\pm$ 0.9 pS in the control, 23.9 $\pm$ 2.0 pS in the presence of 10 μM phloretin in the bathing solution, and 11.2 $\pm$ 1.2 pS in the presence of 6-ketocholestanol in the membrane, respectively (the data not shown).

To probe further the effect of the dipolar compounds on the gramicidin channel lifetime under acidic conditions, we performed experiments on sensitized photoinactivation of gramicidin channels (Strassle and Stark, 1992; Rokitskaya et al., 1993; Kunz et al., 1995). We applied a previously developed method (Rokitskaya et al., 1996) that enabled us to calculate the rate constants of formation and dissociation of gramicidin channels from the time courses of the flash-induced decrease in the gramicidin-mediated current across BLMs in the presence of a photosensitizer. It has been shown in a series of works (Rokitskaya et al., 1996, 1997; Kotova et al., 2000) that the exponential factor of the current relaxation after a light flash, called below the characteristic time of gramicidin photoinactivation (τ), can be used to estimate the gramicidin channel lifetime. Fig. 2 displays the time courses of the flash-induced decrease in gramicidin-mediated current across BLMs sensitized by aluminum phthalocyanine in the presence of 80 mM HCl. The experimental kinetics were fitted well with monoexponential curves giving the following values of τ: 0.78 s in the

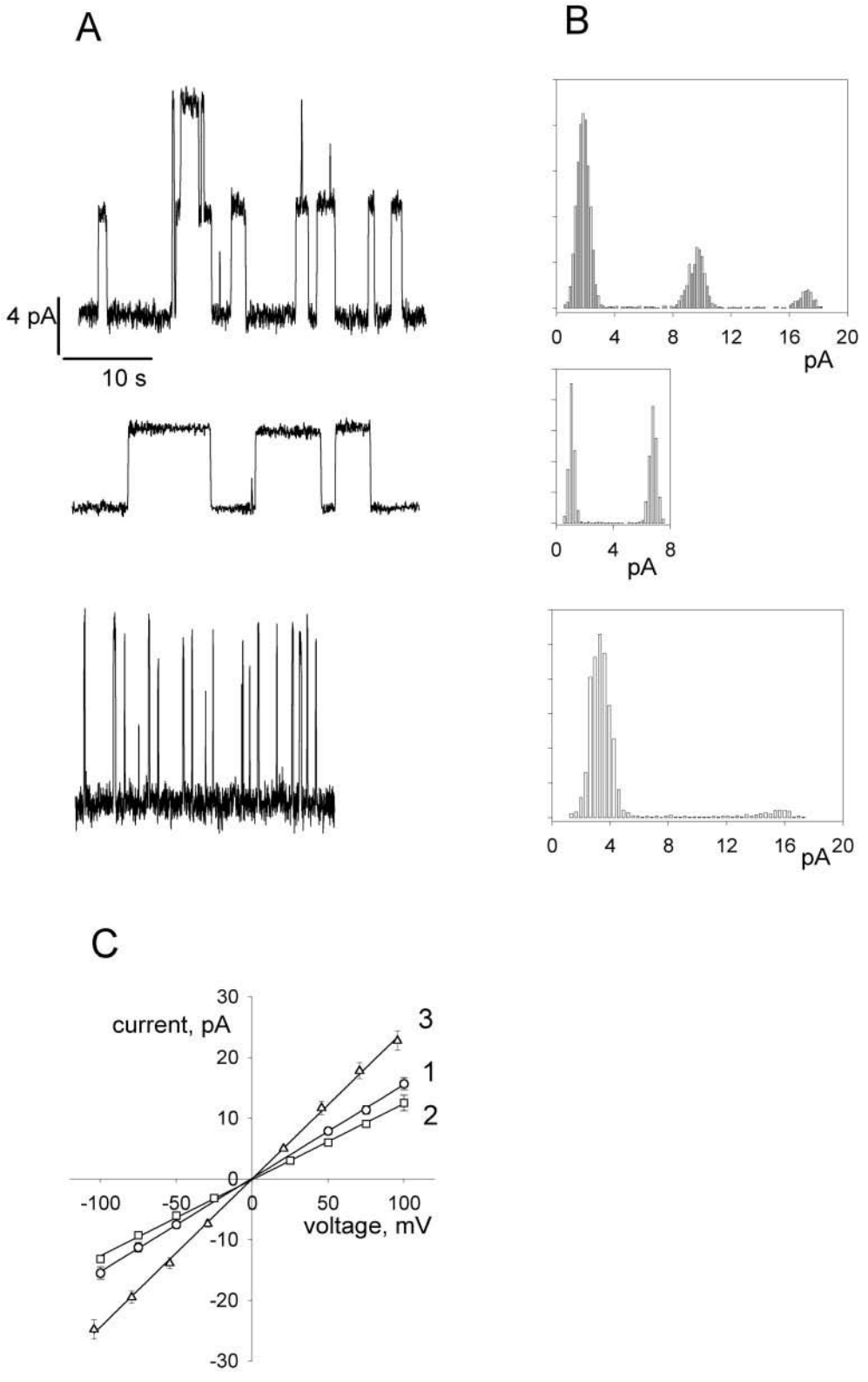

FIGURE 1 (*A* and *B*) Single-channel traces (*A*) and current histograms (*B*) of gramicidin A in DPhPC bilayers at 150 mM HCl, +50 mV in the absence of potassium ions in the bathing solutions. The upper trace is the control for the pure DPhPC membrane, the middle trace is recorded after the addition of 10 μM phloretin to the bathing solutions at both sides of the pure DPhPC membrane, and the lower trace is obtained with the membrane formed of the mixture of DPhPC:6-ketocholestanol (2:1). (*C*) Single-channel current versus applied voltage for the BLM formed of pure DPhPC without additions (the control) (*1*, ○), the same BLM after the addition of 10 μM phloretin to the bathing solutions (*2*, □), and the BLM formed of the mixture of DPhPC:6-ketocholestanol (2:1) (*3*, △).

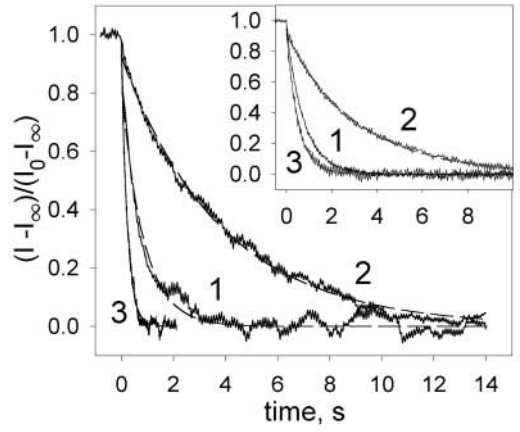

FIGURE 2 Time courses of the flash-induced decrease in gramicidin-mediated current (I) across DPhPC bilayers sensitized by aluminum phthalocyanine ($AlPcS_3$) at 80 mM HCl in the absence of potassium ions in the bathing solutions. Trace 1 is the current decrease for a pure DPhPC bilayer measured without additions (the control), trace 2 is after the addition of 10 μM phloretin to the bathing solutions at both sides of the bilayer formed of pure DPhPC, and trace 3 is the current decrease measured in the presence of 6-ketocholestanol in the bilayer (DPhPC:6-ketocholestanol, 2:1). Dashed curves represent monoexponential fits of the experimental time courses. The normalized values of the current decrease ($(I - I_\infty)/(I_0 - I_\infty)$) are plotted versus the time (t). The initial value of the current (I_0) was ~1 μA. The inset shows the corresponding kinetics of the current decrease measured at 1 M KCl and pH 6.0 (the initial value of the current was 0.8 μA in this experiment).

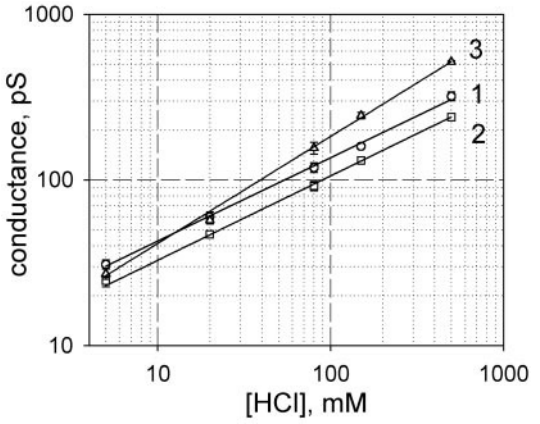

FIGURE 3 Dependences of the gramicidin single-channel conductance (g_H) on HCl concentration in the bathing solution for the BLM formed of pure DPhPC without additions (*1*, ○), in the presence of 10 μM phloretin at both sides of the BLM formed of pure DPhPC (*2*, □), and for the BLM formed of the mixture (2:1) of DPhPC and 6-ketocholestanol (*3*, △), respectively. Each point is the mean ± SD of four to five experiments on different membranes. Each value of g_H was calculated as a slope of the corresponding I-V curve measured at a low voltage (<50 mV).

control (curve 1), 3.52 s after the addition of 10 μM phloretin to the bathing solution (curve 2), and 0.23 s in the presence of 6-ketocholestanol in the membrane (curve 3). The inset to Fig. 2 shows the corresponding kinetics of photoinactivation measured at 1 M KCl and pH 6.0. In agreement with the data obtained previously (Rokitskaya et al., 1997; Antonenko et al., 1999), monoexponential approximation of the kinetics gave the following values of τ: 0.76 s in the control (curve 1), 3.11 s after the addition of 10 μM phloretin to the bathing solution (curve 2), and 0.52 s in the presence of 6-ketocholestanol in the membrane (curve 3). It should be mentioned that rather large fluctuations of the current observed in the experiments resulted from the discrete character of the channel operation, which manifested itself in the additional noise of the measured integral current having the Lorentzian spectrum (Zingsheim and Neher, 1974; Kolb and Bamberg, 1977; Bezrukov et al., 1984).

Fig. 3 demonstrates the results of measuring the effect of the dipolar compounds on the gramicidin single-channel conductance at different HCl concentrations. The control concentration dependence (*1*) of the gramicidin proton conductance plotted as $\log(g_H)$ versus $\log[H^+]$ is well approximated by a straight line with a slope of 0.5. The addition of 10 μM phloretin reduced the gramicidin conductance in the whole range of H^+ concentrations studied (*2*) retaining practically the same slope of the linear dependence, 0.509. However, the effect of 6-ketocholestanol appeared to depend substantially on the H^+ concentration; namely, there was no effect at 20 mM HCl, whereas at higher HCl concentrations the 6-ketocholestanol-induced increase in $\log(g_H)$ grew linearly with $\log[H^+]$. The values of g_H obtained in the presence of 6-ketocholestanol are well approximated by a straight line with a slope of 0.65 in the $\log(g_H) - \log[H^+]$ plot (*3*). At HCl concentrations lower than 20 mM, 6-ketocholestanol produced a slight decrease in the gA proton conductance; i.e., the sign of the effect changed.

It can be proposed that the variation of the 6-ketocholestanol effect on the gramicidin proton conductance with changing the pH is relevant to the fact that different steps may limit the proton current through gramicidin in different pH ranges. Actually, in agreement with the conclusions derived from the studies of gramicidin channel conductance for alkali metal cations (Andersen, 1983a,b), it has been shown that at low proton concentrations and high values of the applied voltage the gramicidin proton conductance is partially limited by the access from the bulk phase to the channel mouth, as judged from the sublinear current-voltage dependence (Eisenman et al., 1980; Akeson and Deamer, 1991; Cukierman et al., 1997; Phillips et al., 1999). With increasing the $[H^+]$, the rate-limiting step for the proton flux shifts from the access to a step within the gramicidin channel, which manifests itself in the superlinear current-voltage curve (Eisenman et al., 1980; Akeson and Deamer, 1991; Phillips et al., 1999). In line with this, the results shown in Fig. 4 demonstrate the transition from the sublinear behavior of the current-voltage dependence at 5 mM HCl to the superlinear behavior at 500 mM HCl. Thus, the

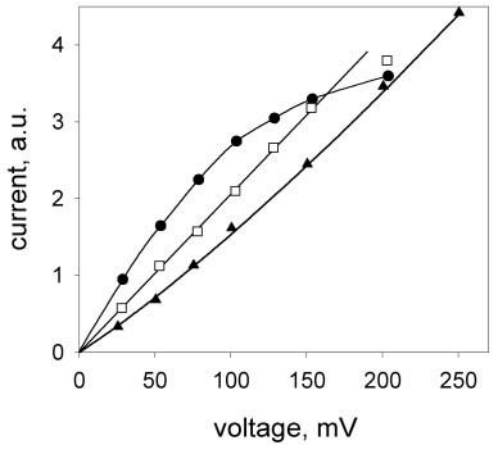

FIGURE 4 Single-channel current versus applied voltage for gramicidin A in pure DPhPC bilayers at 5 mM HCl (●), 80 mM HCl (□), and 500 mM HCl (▲). To show the data on the same plot, the values of the current in pA were scaled by a factor of 1, 6, and 26 for 5 mM, 80 mM, and 500 mM HCl, respectively.

shift of the rate-limiting step in the proton transfer mediated by gramicidin occurs in the pH range studied in Fig. 3.

To test possible differences in the effect of changing the dipole potential at only one side of the BLM compared with varying the dipole potential at both sides of the membrane, we performed experiments with phloridzin, a dipolar compound that cannot penetrate through the BLM. It is known that addition of phloridzin to the bathing solution at one side of the BLM results in the reduction of the dipole potential only in the monolayer facing this solution (Sokolov et al., 1984). As it is seen from the gramicidin current-voltage dependences obtained at 150 mM HCl (Fig. 5), the addition

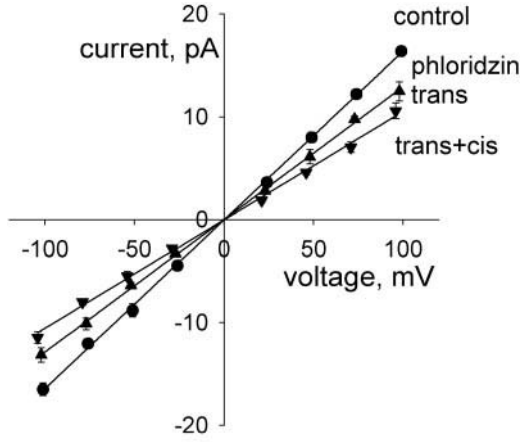

FIGURE 5 Single-channel current versus applied voltage for gramicidin A in pure DPhPC bilayers at 150 mM HCl in the absence of potassium ions in the bathing solutions: control, without additions; phloridzin *trans*, after the addition of 0.6 mM phloridzin at the *trans*-side of BLM (the side where the voltage was applied), and *trans*+*cis*, in the presence of 0.6 mM phloridzin at both sides of the BLM.

of 0.6 mM phloridzin at the *trans*-side of the BLM (the side where the voltage was applied) led to a decrease in g_H both at positive and negative values of the voltage. Subsequent addition of 0.6 mM phloridzin at the *cis*-side of the BLM brought about a further decrease in g_H. The calculated values of g_H were 164 pS in the control, 131 pS after the addition of 0.6 mM phloridzin at the *trans*-side (133 pS and 131 pS, if the values of g_H are calculated independently for the left (at negative voltages) and the right (at positive voltages) parts of the current-voltage dependence), and 113 pS in the presence of 0.6 mM phloridzin at both sides of the BLM (114 pS and 114 pS for the left and the right parts of the current-voltage dependence, respectively).

The effect of changing the dipole potential at one side of the BLM on the potassium conductance of gramicidin channels was also studied. The addition of 0.6 mM phloridzin at the *trans*-side of the BLM led to an increase in g_K both at negative and positive values of the applied voltage (the data not shown) with the slope of the current-voltage dependence being the same at voltages of different signs. The values of g_K were 17.1 $\pm$ 0.9 pS in the control, 18.7 $\pm$ 0.4 pS after the addition of 0.6 mM phloridzin at the *trans*-side of the BLM, and 22.5 $\pm$ 0.4 pS in the presence of 0.6 mM phloridzin at both sides of the BLM.

It is known that increasing the negative surface charge of BLMs leads to an increase in alkali metal conductance of gramicidin channels due to elevation of the cation concentration in the membrane vicinity (Apell et al., 1979; Alvarez et al., 1983; Rostovtseva et al., 1987, 1998). To compare the effects of varying the dipole and the surface potentials of BLMs on the gramicidin proton conductance, we examined sensitivity of the latter to the appearance of negative charges on the surface of BLMs. It was shown that admixing 30% DPhPG to the DPhPC membrane-forming solution caused an increase in g_H measured at 5 mM HCl from 29 pS to 43 pS (the data not shown). Besides, addition of 10 μM SDS also led to a 2.2-fold increase in g_H of the DPhPC membrane under similar conditions.

DISCUSSION

The results of our experiments showed that modulation of the membrane dipole potential produced effects of opposite signs on proton and potassium conductances of gramicidin channels (Fig. 1), whereas the channel lifetimes were affected similarly both under the conditions of proton and potassium predominant conductivity. Reducing the membrane dipole potential with phloretin led to the increase in g_K and a decrease in g_H. On the contrary, increasing the DP with 6-ketocholestanol caused the decrease in g_K and the increase in g_H. As to the channel lifetime, its value increased upon addition of phloretin and decreased in the presence of 6-ketocholestanol in both cases. It has been suggested previously (Rokitskaya et al., 1997; Antonenko et al., 1999) that the effect of the membrane dipole potential

on the gramicidin channel lifetime originates from the interaction with tryptophan dipoles moving near the water-membrane interface in the course of formation and dissociation of gramicidin channels. The results presented in Fig. 2 show that the influence of the dipolar compounds on the process of gramicidin dimer-monomer equilibration does not differ qualitatively for K^+ and H^+ being the permeant ions.

In view of the high 6-ketocholestanol concentration used, it should be noted that the effects, other than those related to the dipole potential, of these compounds on membranes have been reported to be quite small; namely, 6-ketocholestanol does not appreciably modify the bilayer thickness (Simon et al., 1992) and produces much smaller changes in acyl chain order than cholesterol (Franklin and Cafiso, 1993). According to the literature, this holds true also with phloretin; in particular, the NMR study (Bechinger and Seelig, 1991) has found no evidence that phloretin significantly alters the acyl chain order or lipid packing of the membrane. Taking into account the recent data of Alakoskela and Kinnunen (2001) and the earlier results of Andersen et al. (1976), the dipolar compounds might produce changes in membrane fluidity, but these changes apparently have the same direction for phloretin and 6-ketocholestanol (Alakoskela and Kinnunen, 2001), in contrast to the opposite signs of their effects on the dipole potential. Thus, possible fluidity changes seem unlikely to explain the effects of dipolar compounds on gramicidin proton conductance observed here.

The present data on the influence of dipolar compounds on the proton transport by gramicidin channels support the results of Busath et al. (1998) and Phillips et al. (1999) demonstrating the anomalous proton conductance effects in gramicidin. To explain these effects, Phillips et al. (1999) put forward a dipole/water-dipole interaction hypothesis assuming that 1) proton transport through the gA channel occurs by means of Grotthuss conductance, 2) water reorientation after proton translocation is the rate-limiting step of the process, and 3) reorientation of the water column is initiated at the channel exit. The exit-initiated water reorientation model qualitatively explains the dipole effects reported by the authors; namely, the increased membrane DP and decreased peptide side-chain dipoles facilitate proton transport because, in terms of this model, they destabilize the waters at the exit, increasing the rate of water reorientation and thus the H^+ conductance. Increasing tryptophan dipoles upon the side-chain fluorination reduces the proton conductance, in accord with this hypothesis.

Our experimental results shown in Fig. 1 also can be accounted for by the water-reorientation model of Phillips et al. (1999). On the other hand, it is not quite easy to explain the increase in proton flux with raising the bulk H^+ concentration in terms of this model. Besides, the exit-initiated water reorientation model implies that only for the exit-side lipid monolayer, a change in the dipole potential would result in the alteration of the channel conductance, whereas modulation of the dipole potential of the entry-side monolayer would not affect the water reorientation rate and thus the proton conductance. Therefore, this model predicts that a change in the slope of the gramicidin current-voltage dependence at zero voltage would occur if the dipole potential of only one of the monolayers in the BLM is altered. However, the data presented in Fig. 5 demonstrate that the slope of the gA current-voltage dependence is independent of the sign of the applied voltage under asymmetrical conditions, i.e., if the dipole potential of a single monolayer is modulated by the addition of the impermeant agent, phloridzin. Thus, it has appeared that the asymmetric change in the dipole potential does not produce a rectifying current-voltage curve, which is incompatible with the water-reorientation model of Phillips et al. (1999).

The remarkable fact that proton and potassium conductance of gramicidin channels exhibit changes in opposite directions in response to changes in the membrane dipole potential can be explained readily if negative charge movement is rate limiting for H^+ translocation, in contrast to the case of alkali metal cation conduction. This alternative model called dipole/negative-charge interaction hypothesis was discussed by Phillips et al. (1999). Based on certain observations, they considered it to be less plausible than the water reorientation hypothesis. For example, Phillips et al. (1999) reported that in asymmetrical 1 M guanidinium chloride/1 M KCl solutions, gA channels are completely impermeable when positive potential is applied to the guanidinium chloride bath. However, this observation does not rule out the involvement of negative charge movement in the mechanism of gramicidin proton conductivity. As seen from the scheme presented in Fig. 6, a substantial concentration of hydrogen ions in the membrane vicinity is required to maintain the proton current across the membrane, because only protons are capable of completing the water wire after the translocation of a negative ionic defect.

Taking into account the results of the present study, it can be proposed that the process of proton transport through gramicidin channels actually includes voltage-induced transmembrane movement of negatively charged species (a negative ionic defect, i.e., deficient proton on a group) (Nagle and Nagle, 1983) as a rate-limiting step. The scheme of hydrogen-bonded chain rearrangement is practically equivalent if either protons or negative defects are translocated across the membrane (Fig. 6). We propose that proton permeation across the membrane via the migration of a hydrogen bond defect involves transient localization of a charge inside the gramicidin channel. The main difference between the two variants of the model shown in Fig. 6 consists in the sign of the localized charge being negative to account for the dipole potential effect on the proton conduction.

It should be noted that in contrast to modulation of the membrane dipole potential, varying the surface potential of

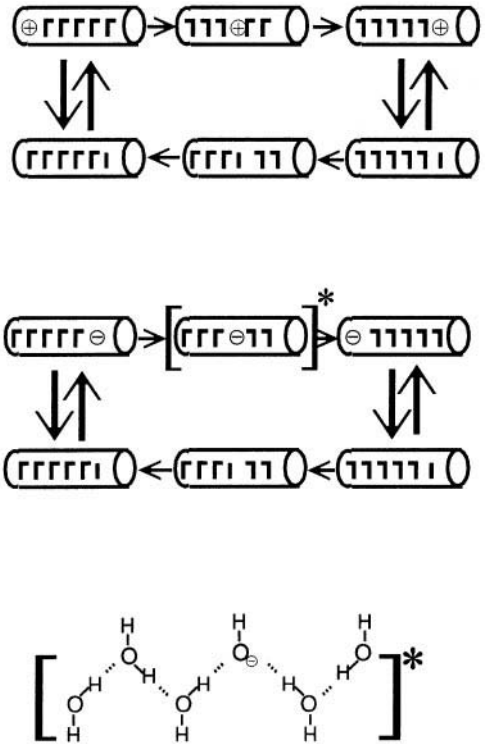

FIGURE 6 Schemes of proton conduction mechanisms. Designations are taken from Schumaker et al. (2001). Pore water molecules are depicted as angles with oxygen at the vertex. The top segment shows the permeation of an excess proton from the left side to the right and the relaxation of the defect (the vertical bar) in the hydrogen-bonding structure so that water molecules are realigned to accept another proton. Below is the modified scheme where proton is conducted via displacement of proton vacancies in the water chain (see the supplementary diagram in the square brackets for the intermediate state of the vacancy translocation).

BLMs produced qualitatively similar effects on the proton and the alkali metal conductance of gramicidin channels; in particular, creation of the negative surface potential upon admixing DPhPG to BLM led to the increase in the gramicidin proton conductance in our experiments, similarly to the earlier data on the influence of negatively charged lipids on alkali metal cation fluxes through gA channels (Apell et al., 1979; Rahmann et al., 1992; Mittler-Neher and Knoll, 1993; Rostovtseva et al., 1998). These results imply that movement of protons outside gA channels is necessarily involved in the electric current flowing across BLMs, and the translocation of negative defects inside the channels does not solely determine the total rate of proton transport.

In our experiments, the gA proton conductance was characterized by $[H^+]^{0.5}$ dependence in the range of [HCl] from 5 mM to 500 mM (or $[H^+]^{0.65}$ dependence in the presence of 6-ketocholestanol) (Fig. 3). According to the conclusions made recently by De Godoy and Cukierman (2001), the deviation of the relationship between g_H and $[H^+]$ from the direct proportionality may be regarded as an inherent property of the proton transfer along the water wire inside the gramicidin channel. As shown in Cukierman (2000) and De Godoy and Cukierman (2001), the exact value of the slope of the $\log(g_H) - \log[H^+]$ dependence can be calculated if surface charge effects in the phospholipids are taken into account. The reduced slope of the current-concentration relationship may be also associated with its being measured in a rather narrow range of $[H^+]$ including a shoulder region between 0.01 and 0.1 M observed by Eisenman et al. (1980) for GMO membranes. This shoulder is suggested to correspond to a transition between regimes of proton transfer differing in the rate-limiting steps (Eisenman et al., 1980; Phillips et al., 1999). The fact that 6-ketocholestanol induced a noticeable change in the slope of the $\log(g_H) - \log[H^+]$ dependence also favors a complicated mechanism of proton permeation through gramicidin channels, which might include several rate-limiting steps with contributions varying with H^+ concentration. Nevertheless, it is clear that the mechanism of proton permeation through gramicidin channels is qualitatively different from that for other cations. The results of the present work indicate that a voltage-induced displacement of negative defects along the water wire inside the channels may represent an essential step of transmembrane proton conduction by gramicidin.

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