

Attenuation of Proton Currents by Methanol in a Dioxolane-Linked Gramicidin A Channel in Different Lipid Bilayers

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ABSTRACT The mobility of protons in a dioxolane-linked gramicidin A channel (D_1) is comparable to the mobility of protons in aqueous solutions (Cukierman, S., E. P. Quigley, and D. S. Crumrine. 1997. *Biophys. J.* 73:2489–2502). Aliphatic alcohols decrease the mobility of H^+ in aqueous solutions. In this study, the effects of methanol on proton conduction through D_1 channels were investigated in different lipid bilayers and at different HCl concentrations. Methanol attenuated H^+ currents in a voltage-independent manner. Attenuation of proton currents was also independent of H^+ concentrations in solution. In phospholipid bilayers, methanol decreased the single channel conductance to protons without affecting the binding affinity of protons to bilayers. In glycerylmonooleate membranes, the attenuation of single channel proton conductances qualitatively resembled the decrease of conductivities of HCl solutions by methanol. However, in both types of lipid bilayers, single channel proton conductances through D_1 channels were considerably more attenuated than the conductivities of different HCl solutions. This suggests that methanol modulates single proton currents through D_1 channels. It is proposed that, on average, one methanol molecule binds to a D_1 channel, and attenuates H^+ conductance. The Gibbs free energy of this process (ΔG_0) is ~ 1.2 kcal/mol, which is comparable to the free energy of decrease of HCl conductivity in methanol solutions (1.6 kcal/mol). Apolar substances like urea and glucose that do not transport protons in HCl solutions and do not permeate D_1 channels decreased solution conductivity and single channel conductance by a considerably larger proportion than methanol. Cs^+ currents through D_1 channels were considerably less (fivefold) attenuated by methanol than proton currents. It is proposed that methanol partitions inside the pore of gramicidin channels and delays the transfer of protons between water and methanol molecules, causing a significant attenuation of the single channel proton conductance. Gramicidin channels offer an interesting experimental model to study proton hopping along a single chain of water molecules interrupted by a single methanol molecule.

INTRODUCTION

Gramicidin A (gA) is a highly hydrophobic pentadecapeptide secreted by *Bacillus brevis*. gA molecules partition in lipid bilayers and form ion channels selective for monovalent cations only. Intermolecular hydrogen bonds stabilize the association of two gA monomers across a lipid bilayer, resulting in the formation of an ion channel. Dissociation of gA monomers in the plane of the bilayer destabilizes the ion channel pore, and interrupts the flow of monovalent cations across the membrane (see reviews by Busath, 1993; Koeppe and Andersen, 1996; Wallace, 1990). It has been shown that two gA molecules can be covalently linked with a malonyl group (Bamberg and Janko, 1977; Urry et al., 1971). The resulting gA dimer formed ion channels in lipid bilayers with properties similar to the channel resulting from monomer-monomer association via H-bonds. However, and as predicted (Urry, 1971), the covalently linked gA dimer had an open time considerably longer than gA channels formed by the monomer-monomer association via H-bonds (Urry, 1971). More recently, Stankovic et al. (1989) linked two gA monomers with a dioxolane link. The rationale for using this

molecular linker was that it allows a continuous and constrained transition between the amino termini of two gA monomers, thus preserving the β -helicity of the gA channel. Another significant advantage of using the dioxolane as the linker between two gA monomers is that it can accept different chemical groups. This experimental strategy will hopefully prove itself a valuable model in the study of structure-function relationships in ion channels (Cukierman et al., 1997; Stankovic et al., 1990).

In our laboratories, different dioxolane-linked gA dimers are being synthesized and the biophysical properties of their ion channel pores are being studied in different lipid bilayers. In our initial study, the conduction of protons through a dioxolane linked gA dimer ($gA \sim D_1 \sim gA$, which for the sake of simplicity will be referred to as D_1 in this paper) was studied in different lipid bilayers, and their properties compared to those of natural gA channels (Cukierman et al., 1997). A significant conclusion of that study was that over a wide range of proton concentrations, proton mobility (μ_H) in D_1 was comparable to μ_H in aqueous solutions.

Classical hydrodynamic diffusion models cannot account for the high mobility of protons in aqueous solutions. The Stokes equivalent radius for H^+ calculated from μ_H in water is ~ 0.24 Å, which is not a realistic figure considering, for example, the relatively large number of water molecules solvating the proton. The physicochemical literature dealing with proton solvation and mobility in water is rich, complex, and cannot be easily summarized here. Therefore, and

Received for publication 27 May 1998 and in final form 4 September 1998.

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0006-3495/98/12/2811/10 \$2.00

with the risk of oversimplifying such a complex process, a few simple concepts regarding proton movement in aqueous and aqueous/methanol solutions will be introduced. Bernal and Fowler (1933) were the first to think of proton mobility in water as a two-step process consisting of 1) proton transfer between water molecules via tunneling (with consequent breaking of H-bonds, and formation of a new network of H-bonds), followed by 2) rotation of water molecules with reorganization of original hydrogen bond network. The transfer of a proton between two water molecules would occur with a suitable configuration of adjacent water molecules (Conway, 1964; Eigen, 1964). The rate-limiting step in proton transfer between water molecules would be the realignment of water molecules back to their original position, which only then can transfer a new proton. This mechanism of proton transfer in solution became known as the Grotthuss mechanism (Grotthuss, 1806). Proton tunneling between water molecules followed by reorientation of water molecules can certainly occur much faster than classical hydrodynamic diffusional processes (Nagle and Tristram-Nagle, 1983). Recently, Agmon (1995) suggested that the actual rate-limiting step in proton migration is the destabilization and cleavage of one hydrogen bond in the second solvation shell of $(\text{H}_3\text{O})^+$. In this model, proton jump is propelled by hydrogen bond cleavage taking place in front of the moving proton (Agmon, 1995). It should be mentioned that μ_{H} could be determined by the hydrodynamic flow of $(\text{H}_3\text{O})^+$, as in experimental conditions involving high pressure and/or high concentration of acids (Lown and Thirsk, 1971; see, however, Dippel and Kreuer, 1991 for a different interpretation), or at high concentrations of methanol (Conway et al., 1956).

Methanol has been used as a molecular probe to further our understanding of the causes underlying high mobility of protons in solution (Agmon et al., 1995; Conway, 1964; Conway et al., 1956; Erdey-Grúz, 1974). As with water, methanol conducts protons via a Grotthuss mechanism. The OH group of CH_3OH can H-bond with other methanol and/or water molecules. However, proton mobility in pure methanol or in water/methanol mixtures is considerably slower than in pure water. Several factors could account for such a decreased mobility: 1) $(\text{CH}_3\text{OH}_2)^+$ contains two transferable protons as opposed to three in $(\text{H}_3\text{O})^+$; by itself, this would reduce the probability of proton jumps by two-thirds; however, this is far from being the sole factor; 2) proton affinity of CH_3OH is considerably lower than H_2O . The equilibrium constant of the reaction



is 0.23 (Guss and Kolthoff, 1940), indicating a relatively large energy barrier (ΔG_0 is ~ 0.9 kcal/mol) for transferring H^+ from $(\text{H}_3\text{O})^+$ to CH_3OH ; 3) reorientation of methoxonium ions and rotation of CH_3OH molecules are slower than similar movements in H_2O molecules (Grunwald et al., 1962). Overall, the resultant effect is a significant reduction of μ_{H} in water/methanol solutions in relation to water

because protons would remain solvated by water most of the time. Therefore, in $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ mixtures, protons hop predominantly along chains of water molecules only. As the concentration of methanol increases, the probability of finding pure chains of water molecules decreases, thus substantially attenuating μ_{H} (Agmon et al., 1995).

Inspired by the physicochemical literature on proton transport in methanol/water solutions, we analyze, in this paper, the effects of methanol on proton transport in D_1 channels. Not only has this line of investigation not been attempted before, but we have reasoned that this experimental approach could add to our understanding of how protons move in solution as well as in ion channels.

MATERIALS AND METHODS

Lipid bilayers

Membranes were formed onto a 0.1-mm-diameter hole into a polystyrene cup (*cis*-side) nested inside a plastic chamber that formed the *trans*-side. Membranes had the following compositions: 1) PEPC 4:1 (60 mM in decane), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (PE), and 1-palmitoyl-2-oleoyl-phosphatidylcholine (PC). These synthetic lipids were obtained from Avanti Lipids (Alabaster, AL); 2) GMO, glycerylmonooleate (60 mM in decane) from Nu-Check (Elysian, MN). The rationale for using different membrane lipids was that we have previously found significant differences between proton conduction in D_1 channels in GMO and PEPC bilayers (Cukierman et al., 1997). Therefore, it was of interest to investigate the effects of methanol on D_1 channels reconstituted in these different membranes.

Solutions

HCl solutions at different concentrations were prepared by diluting a concentrated stock solution of HCl. Methanol was added to HCl solutions at different concentrations ranging from 1.24 M (5% v/v) to 5.56 M (22.5% v/v). Methanol and HCl were obtained from Fisher Scientific (Chicago, IL). D_1 was stored in methanol at $\sim 10^{-10}$ M, and in the experiments, 5 μl from this stock solution was added to only one side of the bilayer (bath concentration of ~ 0.5 pM). Experiments were performed at room temperature (21 – 22°C).

Single channel current measurements

Both sides of the membrane were connected to an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) via Ag/AgCl wires immersed in solutions. In PEPC bilayers, voltage clamp ramps from 0 to 380 mV were generated in ~ 7.5 s using Clampex (Axons Instruments). Because GMO bilayers are far less stable at high voltages than PEPC bilayers (see Cukierman et al., 1997), voltage ramps from 0 to ~ 200 – 250 mV were used in the former. The sampling frequency was 2–4 kHz for both bilayers, and single channel recordings were filtered using a low-pass Bessel filter at different frequencies (1–5 kHz). Single D_1 channel current-voltage relationships were always subtracted from current-voltage relationships of the bilayer without D_1 channels.

Measurement of solution conductivity

The conductivities of solutions used in this study were measured using a YSI-3200 conductivity meter with a conductivity cell $K = 10.00/\text{cm}$ (YSI3440, Yellow Spring Instruments, Yellow Springs, OH). Conductivity measurements were done at room temperature (21 – 22°C). Appropriate calibrating solutions were used before each set of measurements. Our

conductivity measurements of solutions with different HCl concentrations are in excellent agreement with those reported in the literature (Weast, 1989).

Proton concentrations at membrane/solution interface in GMO and PEPC bilayers

In the experimental conditions of this study (very low pH), PE and PC are protonated. Consequently, PEPC bilayers are positively charged. However, GMO membranes are neutral. To compare proton concentrations in different GMO or PEPC bilayers as seen by the channel openings, the concentration of protons at the membrane/solution interface ($[H^+]_{x=0}$) was calculated using a model based on the Gouy-Chapman-Stern model. Unless indicated otherwise, these concentrations will be used throughout this paper. Fig. 1 shows the relationship between proton concentrations at the membrane/solution interface ($[H^+]_{x=0}$) as a function of bulk proton concentration. Details of calculations and model can be found in previous publications and references therein (Cukierman, 1991; Cukierman et al., 1997). It is reasonable to assume that the actual proton concentration effectively sensed by openings of the D_1 channel is $[H^+]_{x=0}$.

RESULTS

Fig. 2 shows superimposed representative recordings of single channel proton currents in D_1 channels in response to different voltage clamp ramps in control experiments (no methanol) and in experiments in which 3.71 M methanol was present in both sides of the bilayer. In *A*, recordings were obtained in a PEPC bilayer with $[H^+]_{x=0} = 20$ mM (corresponding to a bulk concentration of protons of 125 mM, see Fig. 1) with no methanol (*top recording*), and with methanol (*bottom recording*). Methanol clearly attenuated proton currents through a single dioxolane-linked gramicidin A dimer (D_1). In *B*, recordings were obtained in a GMO membrane with $[H^+]_{x=0} = 1,250$ mM (same concentration

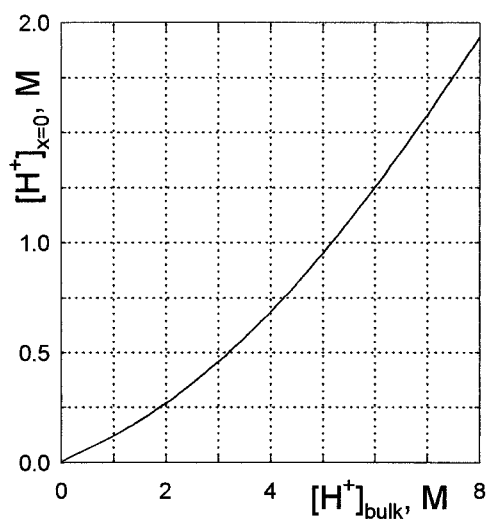


FIGURE 1 The relationship between proton concentration at the membrane/solution interface ($[H^+]_{x=0}$), and proton concentration in bulk solution in PEPC bilayers. The results in this figure reflect the application of a combination of Grahame, Langmuir, and Boltzmann equations to protonable phospholipids (for details on model and calculations see Cukierman, 1991; Cukierman et al., 1997; and references therein).

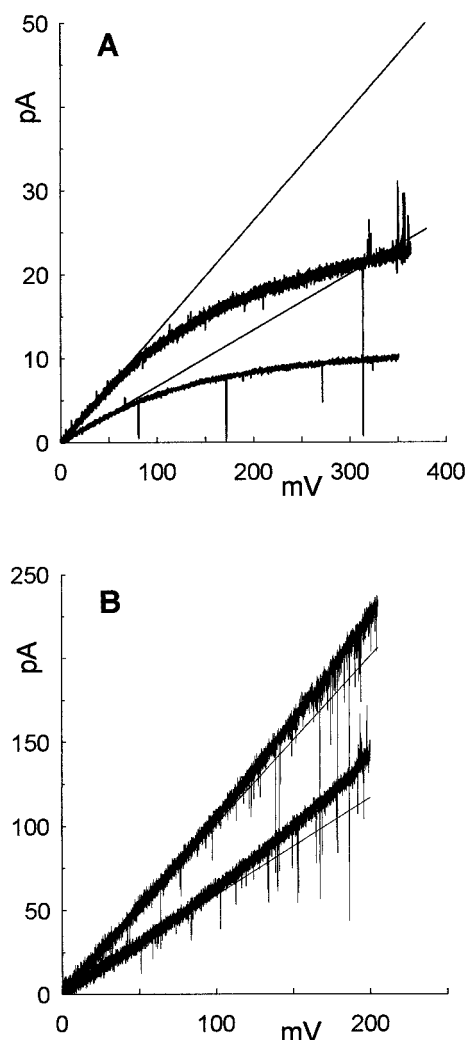


FIGURE 2 Superimposed proton current recordings through single D_1 channels in a PEPC bilayer at 20 mM $[H^+]_{x=0}$ (*A*) and in a bilayer made of GMO at $[H^+]_{x=0} = 1,250$ mM (*B*). Voltage clamp ramps from 0 to ~ 360 mV over a 7.5 s period were applied in *A* while smaller ramps to ~ 200 mV were applied in *B*. Sampling rate was 2 kHz, and proton currents were low-pass Bessel-filtered at 1 kHz. In each panel two different recordings are shown. The larger conductance recording was obtained in the absence of methanol, and the smaller conductance was obtained in the presence of 3.71 M methanol in both sides of the D_1 channel. Slopes of straight lines are 132 and 74 pS (*A*), and 1052 and 615 pS (*B*).

as in bulk solution) and, as in PEPC bilayers, single proton currents were attenuated by approximately the same proportion in methanol containing solutions. Fig. 2 also illustrates that current-voltage relationships in both types of membrane were not linear (Cukierman et al., 1997). Although in PEPC there is a marked sublinearity of the single channel current-voltage relationship, in GMO there is a slight supralinearity. Sub or supralinear behavior depend on the concentration of protons in solution as shown before (Cukierman et al., 1997). The departures from linearity of these current-voltage relationships will not be studied here. Both panels in Fig. 2 show superimposed straight lines that were fitted to the initial portion (usually 0–50 mV) of single

channel current-voltage relationships. In *A*, straight lines had slopes of 132 and 74 pS, and those in *B* had slopes of 1,052 and 615 pS. The linear portions of the current-voltage relationships will be used in other graphs in this study. The attenuation of proton currents in D_1 by methanol is not a voltage-dependent process. Single channel currents are attenuated by approximately the same proportion at different membrane voltages. This attenuation of single proton currents is not affected by the concentration of protons in solution (see below).

Fig. 3 summarizes our results on the effects of methanol on single channel proton conductances in different bilayers at different $[H^+]_{x=0}$. As shown before (Cukierman et al., 1997) the relationship between proton conductance in D_1 and $[H^+]_{x=0}$ in PEPC bilayers (*A*) follows an adsorption isotherm, while in GMO bilayers these parameters follow a linear relationship in the range of 0–1.5 M $[H^+]_{x=0}$ before saturating at higher proton concentrations (*B*, see also Fig. 9 in Cukierman et al., 1997). These qualitative characteristics are not affected by methanol. In *A*, the experimental points were fitted to the equation

$$g = g^{\max} \cdot [H^+]_{x=0} / ([H^+]_{x=0} + K_D) \quad (1)$$

with the following parameters: $g^{\max} = 1,080$ pS (control) and 692 pS (with methanol), and $K_D = 127$ mM (control) and 133 mM (methanol). Notice that K_D values were not significantly affected by 3.71 M methanol, although single channel proton conductances at different $[H^+]_{x=0}$ were markedly attenuated. In *B*, the initial portion of the conductance concentration relationships in control and methanol were approximated by straight lines with slopes of 858 pS/mol (control), and 543 pS/mol (with methanol). Notice that attenuations of proton conductances by methanol in GMO and PEPC were essentially the same (37%).

Fig. 4 shows measurements of solution conductivities (λ , mS/cm, *A*) and equivalent conductivities (λ_{eq} , mS/cm/mol, *B*) as a function of different HCl concentrations in the absence (*filled triangles*) and presence (*open triangles*) of 3.71 M methanol. Measurements were made in the same solutions used in bilayer studies. Methanol decreased the conductivity of different HCl solutions over a wide range of concentrations. Panel *A* resembles on a qualitative basis the behavior of single channel proton conductances in GMO bilayers (see Fig. 3 *B*): a linear dependence of λ is observed at relatively low $[HCl]$, before solution conductivity saturates at higher acid concentrations. However, and as will be demonstrated below, there are marked quantitative differences between attenuation of solution conductivities and single channel proton conductances in D_1 in GMO or PEPC bilayers. Notice that while Fig. 3 shows the quantitative effects of methanol on *proton* conductance in D_1 channels, Fig. 4 displays data on the total conductivity of HCl solutions that is the sum of individual contributions of *proton* and *chloride* conductivities. It is possible that methanol preferentially affects one component of the solution conductivity, and this would not allow a comparison between

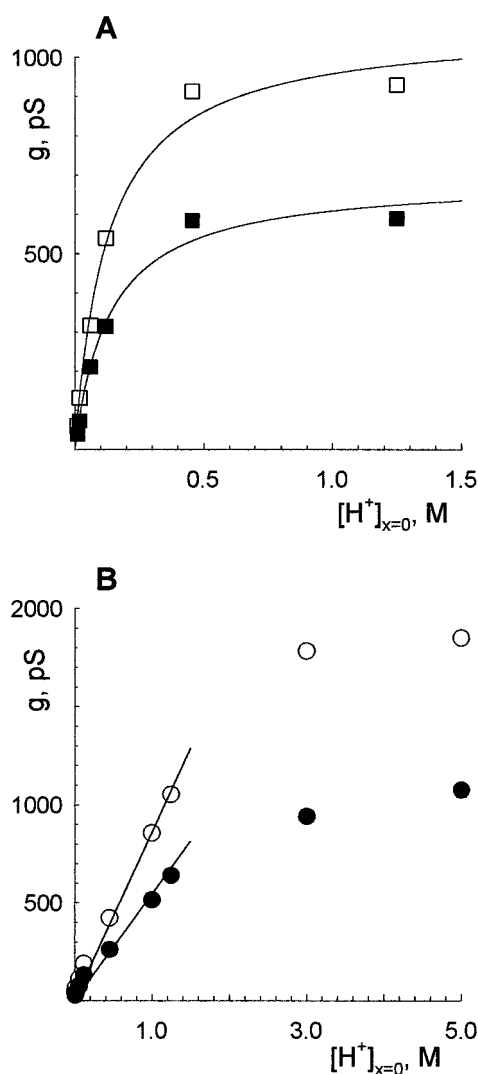


FIGURE 3 Single channel proton conductance versus proton concentration at the membrane/solution interface ($[H^+]_{x=0}$). Each point is the average of 3–7 different single channel measurements. Error bars were smaller than the size of symbols. (*A*) PEPC bilayers. Open squares were obtained in control conditions, and filled squares in the presence of 3.71 M methanol. Curves are best fittings of points to equation $g = g^{\max} \cdot [H^+]_{x=0} / ([H^+]_{x=0} + K_D)$. $g^{\max} = 1080$ pS (control) and 692 pS (with methanol); $K_D = 127$ mM (control) and 133 mM (methanol); (*B*) GMO bilayers. Open circles were obtained in control conditions, and filled circles with 3.71 M methanol on both sides of the bilayer. In contrast to graphs in *A*, notice the linear dependence between g and $[H^+]_{x=0}$ in the concentration range of 0–1.5 M. Slopes of straight lines are 858 pS/mol (control), and 543 pS/mol (with methanol).

results shown in Figs. 3 and 4. Fortunately, it has been shown that the transference number of protons (t_H) is constant in the $[HCl]$ range of ~0–5 M ($t_H = 0.84$, Lengyel et al., 1962). In addition, it was demonstrated that at relatively low concentrations (including ours in this study), methanol does not modify t_H appreciably (Erdey-Grúz, 1974). Therefore, data in Fig. 4 suggest that changes in HCl solution conductivity should reflect identical or very similar proportional changes in proton conductivity in different HCl solutions.

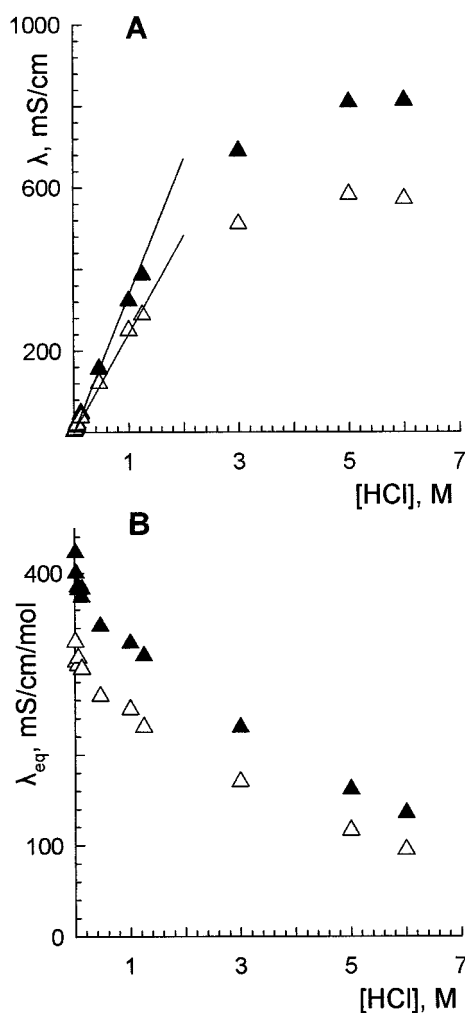


FIGURE 4 Conductivities (λ , A), and equivalent (or molar) conductivities (λ_{eq} , B) of HCl solutions (filled triangles) and with 3.71 M methanol (open triangles). Filled symbols are in excellent agreement with measurements previously reported (see Erdey-Grúz, 1974; Weast, 1989). Measurements were obtained at 23°C.

In Fig. 5, the ratios of single channel proton conductance in D_1 in methanol to control (circles in GMO bilayers, and squares in PEPC), and the ratios of solution conductivity in methanol to control (triangles) are being plotted against various proton concentrations. Full lines represent the average of single channel measurements in PEPC and GMO, and the average conductivity measurements. Dotted lines represent one SD of all measurements. The average rate of attenuation of solution conductivity was 76%, while the average attenuation of single channel conductance was 62%. This graph suggests that methanol is interacting with D_1 channels, and causing a larger current attenuation than the one observed in solution only (see Appendix).

To determine the characteristics of attenuation of proton conductances, the concentration-dependent effects of methanol on D_1 currents were studied. Fig. 6 shows the effects of different concentrations of methanol (0, 2.47, and 5.56 M methanol from larger to smaller current ramps) on single

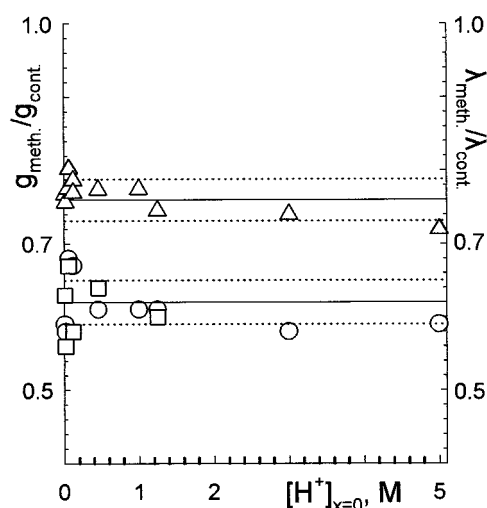


FIGURE 5 Relationship between the ratios of single channel proton conductances in methanol and control (g_{meth}/g_{cont} , squares in PEPC bilayers, circles in GMO bilayers), ratio of solution conductivities in methanol and control ($\lambda_{meth}/\lambda_{cont}$, triangles) versus proton concentration at the membrane/solution interface ($[H^+]_{x=0}$, in the case of D_1 single channel currents) or versus HCl concentrations (with conductivity measurements). Full lines show averages for ratios of single channel measurements (0.62) and for ratios of solution conductivities (0.76). Dotted lines represent ± 1 SD from average.

channel current voltage relationships in D_1 channels. PEPC membranes and D_1 channels were stable in methanol concentrations up to 5.56 M. GMO membranes, however, were not stable at methanol concentrations >3.71 M (results not shown). In Fig. 7, the single channel conductance measurements performed in PEPC bilayers (122 mM $[H^+]_{x=0}$, squares) and in GMO bilayers (1000 mM, circles) as a function of different methanol concentrations are shown. Also shown are the measurements of solution conductivities

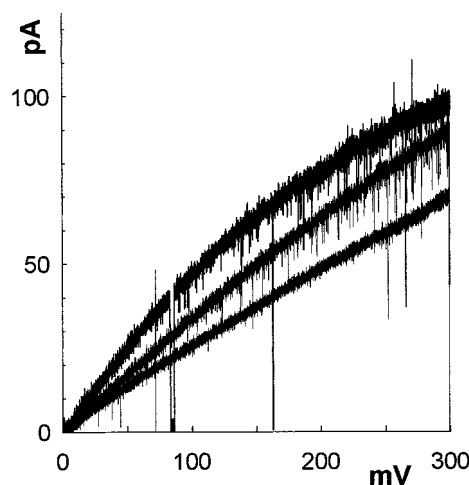


FIGURE 6 Single D_1 channel proton current-voltage relationships in different symmetrical concentrations of methanol. All curves were obtained in PEPC bilayers (122 mM) at methanol concentrations (from top to bottom) of 0, 2.47, and 5.56 M. Sampling rate was 2 kHz and recordings were low-pass Bessel filtered at 1 kHz.

at different methanol concentrations in 1000 mM HCl (relative attenuation of solution conductivity by methanol was not significantly dependent on hydrochloric acid concentration; see Fig. 3). Notice that, as in Fig. 5, attenuation of single channel proton conductances in D_1 was always larger than attenuation of solution conductivities at a given methanol concentration. In *B*, the data points in *A* were normalized to control (0 M methanol).

Assume that n methanol molecules cause attenuation of H^+ currents. In Eq. 2 below, the "O" state represents the fully conductive D_1 channel in the absence of methanol, and "B" represents the same channel with reduced g_H .



It can be demonstrated that in the above model, the relative conductance of D_1 to protons in methanol in relation to control conditions ($g_{\text{meth}}/g_{\text{cont}}$) is given by

$$g_{\text{meth}}/g_{\text{cont}} = (1 + [M]^n/K_D)^{-1} \quad (3)$$

where K_D is the dissociation constant of methanol from the channel.

Nonlinear regression analysis was applied to data points in GMO and in PEPC bilayers in Fig. 7 *B*. The curve (full line close to circles and squares in Fig. 7 *B*) that could best fit the experimental points had a K_D of 7.9 M, and $n = 1.3$ molecules of methanol. Dotted, and dotted-dashed lines (Fig. 7 *B*) were the best fits to experimental points in GMO and in PEPC, assuming $n = 1$ or 2, respectively. Fig. 7 *B* suggests that on average, ~ 1 molecule of methanol binds to D_1 and attenuates proton currents.

The same formal analysis used for attenuation of g_H by methanol was also applied to attenuation of λ_{HCl} . In this case,

$$\lambda_{\text{meth}}/\lambda_{\text{cont}} = (1 + [M]^m/K_x)^{-1} \quad (4)$$

The attenuation of the relative solution conductivity as a function of methanol (*triangles*) concentration was fitted to Eq. 4 (continuous line with open triangles in Fig. 7 *B*). Interestingly, the molecularity of λ_{HCl} attenuation by methanol was essentially the same as for single channel current attenuation ($x = 1.2$), and the dissociation constant ($K_x = 16.2$ M) was evidently larger than K_D .

The concentrations of methanol that attenuated single channel proton conductances in D_1 channels in HCl solutions were relatively high. It is of interest to compare the effects of apolar substances that do not conduct protons in solution and do not permeate D_1 channels to methanol effects on solution conductivity and single channel conductance. Urea and glucose decreased g_H and λ_{HCl} by a considerably larger proportion than methanol. Table 1 shows that 3.7 M methanol attenuated single channel proton conductances and 1 M HCl conductivity by 42% and 23%, respectively. At this concentration, urea attenuated single channel proton conductance and solution conductivity by 85% and 68%, respectively. Notice that the osmolarities of 3.7 M urea and methanol solutions are comparable, and that

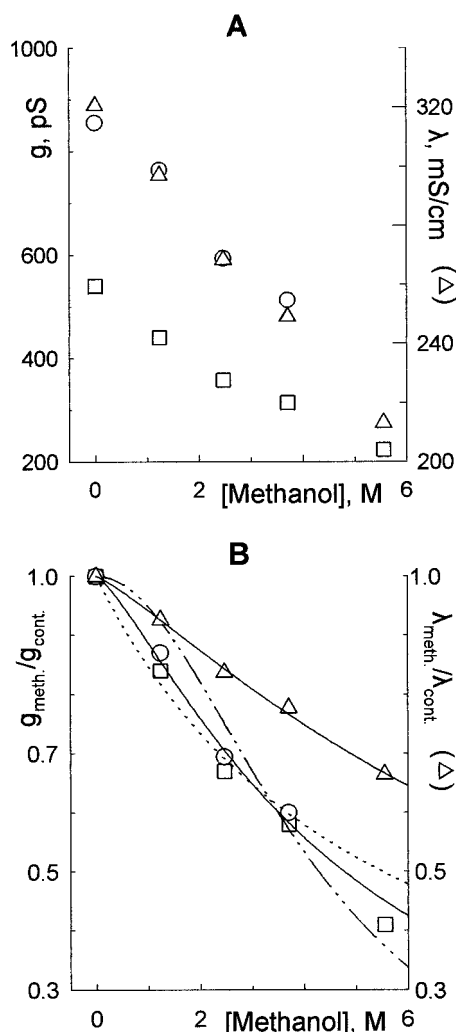


FIGURE 7 (*A*) Squares are the averages of single channel proton conductances in 122 mM $[H^+]_{x=0}$ in PEPC bilayers, circles are the averages of single channel proton conductances in 1 M $[H^+]_{x=0}$ in GMO bilayers, and triangles represent average measurements of solution conductivities (λ , mS/cm). These symbols (average of 3–5 different single channel measurements) are being plotted against different concentrations of methanol. Standard error bars were smaller than the size of symbols; (*B*) shows the same plot normalized for the conductances or conductivities in control conditions (absence of methanol). The lines in this graph represent different models of methanol binding to D_1 channels (see discussion of model and meaning of theoretical curves in the text).

the viscosity of the urea solution is actually lower than methanol solutions; 1 M glucose attenuated both single channel conductances and solution conductivity by 27% in relation to 1 M HCl. Notice that while the viscosity of 1 M glucose solution is larger than in 3.7 M methanol, λ values in these solutions are comparable (250 and 235 mS/cm), and the single channel conductance in 1 M glucose is larger than in methanol solutions. The comparison between 1 M solutions of urea and glucose is also of interest. The 1 M urea solution has a 40% lower viscosity, and approximately the same osmolarity and λ_{HCl} as 1 M urea solution. Nevertheless, the single channel proton conductance is larger in 1 M glucose by 42%.

TABLE 1 Methanol, urea, and glucose and some physicochemical properties of solutions and single D₁ channels

Experimental conditions	Conductance (pS)	Conductivity (mS/cm)	Osmolarity (Os/kg)	$\eta_{rel}^{\#}$
1 M HCl*	540 ± 10.7 (5)	323	2.28	1.05
+3.7 M methanol	315 ± 4.2 (7)	250	6.66	1.39
+3.7 M urea	81 ± 1.8 (5)	104	6.26	1.19
+1.24 M methanol	441 ± 1.9 (5)	297	3.55	1.13
+1 M urea	271 ± 4.0 (4)	211	3.29	1.04
+1 M glucose	387 ± 6.0 (5)	235	3.46	1.69
2 M CsCl	29.2 ± 0.5 (4)	209	>2.42	0.92
+3.7 M methanol	26.5 ± 1.0 (5)	159	>6.80	1.39

*Single channel conductances in D₁ were measured in PEPC bilayers at bulk concentrations of ions indicated. Single channel conductance is expressed as mean ± SE (number of measurements).

[#]Values for osmolarities and η_{rel} (relative viscosity of solution in relation to water viscosity at 20°C) were taken from Weast (1989). Total osmolarities of methanol, urea, or glucose solutions in 1 M HCl or 2 M CsCl were obtained by assuming additive osmotic properties of methanol (or urea, or glucose) and 1 M HCl or 2 M CsCl. η_{rel} values are from methanol and urea aqueous solutions without HCl or CsCl.

The effect of methanol on the single channel conductance of D₁ channels to Cs⁺ (g_{Cs}) was studied. The two bottom lines in Table 1 show that 3.7 M methanol attenuated g_{Cs} (in a 2 M CsCl solution) by ~9%, which is almost fivefold less than attenuation of single channel proton conductance. Notice that the single channel conductance with Cs⁺ is considerably smaller than with H⁺, and the attenuation of g_{Cs} by methanol is only a few pS, which is within experimental error of determination. This experimental result argues that attenuation of single channel proton conductance by methanol is not a consequence of alteration of some basic physicochemical property of membrane and/or channel that would affect channel conductance in general. Methanol seems to be a very potent and a relatively selective blocker of proton currents.

In summary, Table 1 shows 1) λ_{HCl} and g_H are not uniquely determined by solution viscosity; 2) osmolarity affects λ_{HCl} and g_H ; however, the nature of the osmoticant is decisive in determining these parameters; 3) clearly, at a given concentration, methanol attenuated both λ_{HCl} and g_H considerably less than urea or glucose; 4) single channel conductance to Cs⁺ is far less affected by methanol than proton conductance.

DISCUSSION

The novel and major experimental findings in this paper were 1) methanol attenuated single channel proton currents through a dioxolane-linked gramicidin A dimer (D₁ channel); 2) said attenuation is independent of proton concentration in the range of 10–5000 mM ($[H^+]_{bulk}$). It also is independent of membrane potential, and about the same in different GMO and PEPC bilayers, which in many aspects make D₁ channels behave differently in relation to proton conduction (Cukierman et al., 1997; see Figs. 2 and 3); 3) apolar substances that cannot transport protons in solution and do not fit inside the pore of D₁ channels decreased single channel proton conductances and solution conductivity by a larger proportion than methanol; 4) methanol decreased H⁺ currents by a considerably larger proportion than Cs⁺ currents.

Single channel proton currents through D₁ are determined by the resistivities of solutions on both sides of the channel, or more specifically, access resistances of the pore (R_a), and by the channel's intrinsic resistance to proton flow (R_c , see Cukierman et al., 1997 for discussion and references therein). The total resistance to proton flow in D₁ channels is given by ($2R_a + R_c$). Attenuation of proton currents in D₁ channels can occur as a consequence of an increased R_a or/and R_c . This study suggests that a decrease in both solution conductivity and channel conductance to proton flow contribute to attenuation of single channel proton conductance by methanol. Identification of the relative contribution of each of these resistances to total proton current flow in D₁ channels is an extremely complex problem that depends on the theoretical model used (see Appendix). Moreover, and from the practical point of view, identification of the relative contribution of each of these resistances to proton flow has been very difficult, if not impossible, to dissect experimentally (see Akesson and Deamer, 1991; Andersen, 1983; Table 1). Reduction of proton mobility and conductivity by methanol in aqueous solutions has been known (Agmon et al., 1995; Conway, 1964; Conway et al., 1956; Erdey-Grúz, 1974, and references therein). A full physical picture of this phenomenon has not yet emerged, but several factors that could account for this effect were mentioned in the Introduction. Fig. 5 shows that while the conductivities of HCl + 3.71 M methanol solutions were reduced, on average, to 76% of the conductivities in HCl solutions, the overall single channel proton conductance in D₁ in the presence of 3.71 M methanol was 62% of control condition (see also Fig. 7). This ~20% reduction (in relation to the decrease in solution conductivity) in proton conductance in D₁ must be sought to reside in interactions between methanol and the D₁ channel (see Appendix).

Molecular processes by which methanol could be attenuating H⁺ currents in D₁ channels

The gramicidin A channel has a narrow (~4 Å) pore that cannot accommodate more than one ion and/or water molecule at the same time in a given cross section of the pore.

Thus, transport of ions and water through gramicidin A channels must occur via a single file or no-pass transport mechanism (Finkelstein and Andersen, 1981; Levitt, 1984). The high mobility of protons through gramicidin A channels (Cukierman et al., 1997) suggests that protons hop along a chain of water molecules inside the pore (Levitt et al., 1978). Consequently, any process that modifies the dynamics or geometrical arrangement of water molecules inside the pore of the channel could affect single channel proton currents. One mechanism by which methanol could attenuate single proton currents in D_1 channels is by partitioning inside the pore and retarding the transfer of protons from $(H_3O)^+$ to (CH_3OH) . Indeed, proton solvation by water is highly favored by the presence of methanol. The equilibrium constant of reaction $(H_3O)^+ + CH_3OH \rightleftharpoons (CH_3OH_2)^+ + H_2O$ is 0.23 (Guss and Kolthoff, 1940), indicating a low transfer of protons from $(H_3O)^+$ to CH_3OH . The energetic cost of transferring one H^+ from $(H_3O)^+$ to CH_3OH in bulk water in equilibrium conditions is ~ 0.9 kcal/mol. The Gibbs free energy of g_H attenuation by methanol in D_1 channels is ~ 1.2 kcal/mol. This free energy must reflect at least three distinct physical processes which, unfortunately, cannot be separated at present: 1) partition of methanol in the channel, 2) methanol protonation, and 3) reorientation of water and methoxonium ions inside the channel. By analogy with H^+ transfer in water, processes 2 and 3 are necessary for proton transfer inside the channel. Interestingly, the ΔG_0 for attenuation of single channel conductance is comparable to the free energy of attenuation of λ_{HCl} in solution (1.6 kcal/mol, see Fig. 7). Thus, it is possible that methanol partitions inside the pore of the channel (see below), and delays the transfer of protons throughout the chain of water molecules with the consequent reduction in single channel proton conductance. If this mechanism is correct, then D_1 , as well as other gA channels, offers a unique model by which the flow of protons along a single chain of water molecules can be studied in the presence of approximately one methanol interspersed between water molecules.

Preliminary results (Quigley et al., unpublished observations) have shown that the methanol permeability of PEPC bilayers containing gramicidin A channels was consistently and considerably larger than in plain PEPC bilayers. Although the precise determination of the permeability of a single D_1 channel to methanol must await further work, these preliminary results support the hypothesis that methanol partitions inside the pore of D_1 channels and, therefore, conduct protons. It is not surprising that methanol permeates D_1 channels. Organic cations larger than methanol (methylammonium, hydrazinium, formamidinium) permeate gramicidin A channels (Eisenman et al., 1976; Seoh and Busath, 1993).

In the process of diffusing from bulk solution to inside the channel's pore, an ion must cross an interface between these compartments. The physicochemical properties of this interface are not well known. This adds considerably to the uncertainty in dissecting the properties of access resistance

of the channel from the intrinsic channel resistance itself. One mechanism by which methanol could attenuate single channel proton currents by a larger proportion than in bulk solution would be related to changes in the structure of water in the membrane-channel/solution interface. Methanol would adsorb to the membrane and its OH group would alter the structure of H-bonds in the membrane/solution interface. Proton transfer at the membrane/solution interface could be affected differently from that in bulk solution.

Methanol, urea, glucose, and single channel H^+ and Cs^+ conductances

The effects of urea and glucose, two apolar substances that do not conduct H^+ in solution, and do not permeate gramicidin A channels (Rosenberg and Finkelstein, 1978) were compared to the effects of methanol. At 3.7 M, urea decreased both g_H and solution conductivity more markedly than methanol. At this concentration, the urea solution has a similar osmolarity but a lower viscosity than methanol solutions (Table 1). Nevertheless, the single channel proton conductance in urea is only 25% of the conductance in methanol solutions. Also, the conductivity of urea solutions is $\sim 40\%$ of the conductivity of HCl in methanol solutions. Proton tunneling is strongly influenced by the geometry and distances between water molecules (Conway, 1964). High concentrations of solutes that do not participate in H^+ transfer act as molecular spacers, disrupting the organization of water molecules and blocking proton transfer. Qualitatively, this explains the larger decrease in λ_{HCl} caused by urea in relation to methanol. It is even possible that under these conditions, a significant fraction of current is carried by the hydrodynamic flow of $(H_3O)^+$ as it was proposed for very high concentrations of methanol in water (Conway et al., 1956). However, urea does not permeate gramicidin A channels (Rosenberg and Finkelstein, 1978), and the high osmolarity of urea solutions exerts osmotic stress on the channel's pore and could remove water (see Parsegian et al., 1986). This would hamper proton transfer inside the channel's pore. Consequently, the significantly larger attenuation of proton currents in D_1 channels in HCl solutions with urea in relation to methanol could be explained by the combination of these two different effects on solution conductivity, and on the channel's pore.

A similar line of reasoning can also be applied to results obtained in 1 M glucose solutions. In this case, λ_{HCl} is about the same as in 3.7 M methanol solution (250 vs. 235 mS/cm). The 1 M glucose solution has a larger viscosity than methanol solutions. However, proton transfer in solution is not determined by macroscopic viscosity (see above for results with urea, and Erdey-Grúz, 1974). The single channel conductance in 1 M glucose solutions is $\sim 23\%$ larger than in methanol solutions, but still significantly attenuated ($\sim 30\%$) in relation to 1 M HCl solution. Notice that 1.24 M methanol attenuated g_H by 16% (Fig. 7), while glucose, at a lower concentration of 1 M, attenuated g_H by almost twice as much (Table 1).

An interesting point arises when the effects of 1 M urea or glucose solutions are compared in relation to single channel proton conductances. Inasmuch as 1 M glucose solutions have a larger viscosity than 1 M urea, the single channel proton conductance is considerably more attenuated in the latter (notice that λ_{HCl} values and osmolarities are similar in both solutions). It is clear that Table 1 does not provide all the conceptual information necessary for a precise characterization of effects of apolar substances on single channel proton conductances in D_1 channels (see Andersen, 1983 for discussion on this problem). Nevertheless, it is reasonable to propose that substances that do not partition inside the D_1 's pore and do not participate in the Grotthuss mechanism attenuate single channel proton currents by a larger magnitude than methanol.

The value of g_{CS} was considerably less attenuated by methanol than g_{H} (9% vs. 42%). This information is consistent with the hypothesis presented in this study, and seems to discard the possibility that methanol could have the same effect on all ionic conductances via a general mechanism of action on the bilayer or D_1 channel. Moreover, alcohols with a carbon chain longer than methanol (ethanol and propanol) also attenuated proton currents (Cukierman, unpublished results). However, at a given concentration, ethanol or propanol attenuated g_{H} by a considerably smaller amount than methanol. For example, ethanol at a concentration of 3.7 M attenuated g_{H} by $\sim 10\%$ only, which is considerably less than the effect of methanol. These quantitative differences may well be explained by the presence of methanol (but not ethanol or propanol) inside the pore, thus causing an extra attenuation of proton currents in relation to alcohols with longer chains. Future work will directly address this question.

Finally, it should be mentioned that each of the apolar substances tested in this study has a propensity to partition in the lipid bilayer, and is likely to modify the dipole potential at the membrane-solution interface. The extent of this effect on single channel conductances in D_1 , as well as in other channels, is not known. Notice, however, that methanol did not attenuate g_{CS} by the same proportion as g_{H} , and ethanol caused a significantly less attenuation of g_{H} than methanol (Cukierman, unpublished observations).

Comparison with previous results

The single channel proton conductances reported for D_1 channels at low $[\text{H}^+]_{\text{bulk}}$ (<1.5 M) in control conditions in this study were, on average, 15% larger than in our previous study (Cukierman et al., 1997). In this study we have applied a voltage-clamp ramp protocol to quantify I-V relationships more accurately. In our previous study, single channel currents were measured at relatively few discrete potentials. At low proton concentrations there is a marked sublinearity of the I-V plots starting at relatively low voltages (Cukierman et al., 1997; see also Fig. 2 A). Consequently, it is likely that single channel proton conductances

were slightly underestimated in our previous study after linearizing the lower portion of I-V plots. At relatively high $[\text{H}^+]_{\text{bulk}}$, however, there is an agreement between g_{H} measurements in this and in our previous studies (Cukierman et al., 1997). The most important consequence of underestimating the single channel conductance at low $[\text{H}^+]_{\text{bulk}}$ is the overestimation of K_{D} in the conductance-concentration relationships, as shown in Fig. 3 A. We have previously found a K_{D} of ~ 300 mM for protons in PEPC bilayers, although in this study the value is ~ 130 mM. The previous underestimation of g_{H} at low $[\text{H}^+]_{\text{bulk}}$ does not challenge the main conclusions of that study (Cukierman et al., 1997).

Methanol effects on g_{H} in GMO and PEPC bilayers

The flow of protons in D_1 and g_{A} channels is certainly influenced by the lipid environment surrounding the channel (Cukierman et al., 1997; present results). Methanol attenuated proton currents in D_1 channels by the same proportion in GMO or PEPC bilayers (Fig. 5) demonstrating that methanol interaction with D_1 does not depend on the lipid environment. An interesting observation was the strong qualitative resemblance between concentration-dependent proton current attenuation in GMO membranes (Fig. 3 B), and the attenuation of λ in HCl solutions (Fig. 4 A). It suggests that proton currents in D_1 channels in GMO membranes are a scaled version of proton currents in aqueous solution (see also Fig. 7 A).

One major difference between proton conductances in GMO versus PEPC bilayers is that considerably larger proton conductances are present in the latter in the $[\text{H}^+]_{\text{x}=0}$ range of 0–1.5 M (Cukierman et al., 1997; see Fig. 3). The flow of protons through g_{A} channels is comparable to proton flow in solutions. Consequently, proton flow through D_1 can be limited by diffusion (Decker and Levitt, 1988; Levitt and Decker, 1988). One hypothesis to explain the difference between g_{H} in GMO and PEPC bilayers in the $[\text{H}^+]_{\text{x}=0}$ range of 0–1.5 M was that deprotonation of PE and PC close to the openings of the D_1 channels may serve as an extra (in relation to bulk solution) source of H^+ that can be transferred through the channel (Cukierman et al., 1997). Indeed, Cukierman et al. (1997) showed that the K_{D} of $g_{\text{H}}-[\text{H}^+]_{\text{x}=0}$ relationship in PEPC bilayers is within the range of K_{D} values measured for protonation of phospholipids (Marsh, 1990). It is interesting that methanol attenuates proton currents through D_1 channels in PEPC bilayers without modifying the K_{D} of this process (Fig. 3 B). This suggests that protonation or deprotonation of membrane phospholipids were not affected by methanol.

APPENDIX

The total resistance (R_{T}) to proton flow in D_1 channel is given by (see Discussion)

$$R_{\text{T}} = 2R_{\text{a}} + R_{\text{c}} \quad (\text{A1})$$

$$R'_T = 2R'_a + R'_c \quad (\text{A2})$$

where R_a and R_c are the proton access resistance and channel's intrinsic resistance to proton flow in D_1 channels, respectively. Primed symbols refer to resistances in presence of methanol.

The total access resistance of a small circular pore inside a membrane immersed in a conducting medium can be approximated by Hall (1975) and Levadny et al. (1998):

$$R_a = [\lambda_H(4a)]^{-1} \quad (\text{A3})$$

where a is the radius of D_1 channel (2.10^{-8} cm). Assume that methanol effects on single channel proton currents are limited to changes in R_a , i.e., $R'_c = R'_c$. After making the proper substitutions, and subtracting (A1) from (A2)

$$R'_a/R_a = \lambda_H/\lambda'_H = 1.32 \quad (\text{A4})$$

$$(R'_T - R_T) = 0.64[\lambda_H(4a)]^{-1} \quad (\text{A5})$$

Relationship (A5) was not experimentally observed in either PEPC or GMO bilayers, and over a wide range of proton concentrations. ($R'_T - R_T$) was always significantly and considerably (15–50-fold) larger than $0.64[\lambda_H(4a)]^{-1}$, suggesting that methanol interacts with D_1 channels and increases R_c . The effects of methanol on R_c could be on the pore itself or in the solution/channel interface. Because the organization of water molecules in the membrane/solution interface is likely to be different from that in bulk solution, the effects of methanol in the (membrane + channel)/solution interface must be quite different from those in bulk solutions where solution conductivities are actually measured.

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