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Kinetic isotope effects of proton transfer in aqueous and methanol containing solutions, and in gramicidin A channels

Anatoly Chernyshev[a], Régis Pomès[b,c], Samuel Cukierman[a,*]

[a]*Department of Physiology Loyola University Medical Center, 2160 South First Ave, Maywood, IL 60153, USA*
[b]*Structural Biology and Biochemistry, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, M5G 1X8, Canada*
[c]*Department of Biochemistry, University of Toronto, Toronto, Canada*

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Abstract

The electrochemical conductivities of HCl and DCl were measured in: H_2O and D_2O; in methanol and fully deuterated methanol; and in water–methanol solutions. The single channel conductances to H^+ (g_H) and D^+ (g_D) in various gramicidin A (gA) ion channels incorporated in glycerylmonooleate planar bilayers were also measured. Kinetic isotope effects (KIE) were estimated from the ratio of conductivity measurements. In 1 and 5 M HCl aqueous solutions and in 1 M HCl + 3.7 M methanol, the KIE (≈ 1.35) is not different from values previously determined in dilute acid solutions. This suggests that the mobility of protons in those solutions is largely determined by proton transfer. In 10 M HCl, however, where the mobility of protons is likely to be determined by hydrodynamic diffusion, the measured KIE is considerably larger (1.47). Possible causes for this effect are discussed. The KIE of proton conductivities in 5 and 50 mM HCl in methanol and *d*-methanol is ≈ 1.15. This is considerably smaller than the ratio between conductivities of 5 mM KCl in methanol and *d*-methanol (1.24). The KIE values (1.22–1.37) for g_H in gA channels in 1 M HCl are significantly larger than for other monovalent cations and consistent with H^+ transfer. Methanol reduces g_H in gA channels. The KIE of this effect is not different from the one measured in the absence of methanol. Possible mechanisms for the methanol-induced block of H^+ conductivities in solution and gA channels are discussed.

Keywords: Water wire; Ionic permeability; Grotthuss mechanism; Single channel conductance; Proton transfer in deuterated methanol; Deuterium oxide

1. Introduction

The conductivity of protons in water is larger than of any other ion. This high conductivity cannot be explained by the hydrodynamic mobility of a solvated proton [$(H_3O)^+$ for example]. A proton transfer mechanism that became known as Grotthuss's could account for the relatively high mobility or conductivity of protons in water [1,2]. Consider a set of water molecules interconnected via hydrogen bonds (H-bonds). In a classical Grotthuss's mechanism, the mobility of protons would occur in two distinct (hop and turn) steps

*Corresponding author. Tel.: +1-708-216-9471; fax: +1-708-216-6308.
E-mail address: scukier@lumc.edu (S. Cukierman).

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[1–5]. The hopping step consists of a proton transfer between $(H_3O)^+$ and an adjacent H_2O. As the proton hops along water molecules, the dipole moments of waters rotate in approximately the same direction of proton hopping. If another proton is to be transferred in the same direction as the previous one, waters must rotate back (turn step) to their initial configuration [1,2,5]. Historically, the turn step (water rotation) has been considered the limiting step for proton mobility in bulk water. Agmon [6,7] has argued that the rate-limiting step in proton transfer between water molecules cannot be the rotation of bulk water molecules. Instead, it was proposed that the rate-limiting step of proton transfer in bulk water is the disruption of one H-bond between waters in the first and second solvation shells of $(H_3O)^+$ [6–9].

The production of ATP in all cells is ultimately driven by a translocation of protons through a membrane protein. Consequently, understanding the mechanisms by which protons are transferred inside proteins is a significant challenge in biology. In particular, it is of interest to elucidate the mechanisms by which protons are transferred along an approximately unidimensional chain of water molecules interconnected via H-bonds (water or proton wires) [3,4]. It has been demonstrated that water wires are present inside the cavities of various proteins involved in bioenergetic processes [10–13]. It is possible that proton transfer in water wires follows hop-and-turn steps similar to a Grotthuss mechanism in which the turn step appears to be rate limiting [3,4,14–16].

The structures of bioenergetic proteins are extremely complex, and the properties of proton transfer in these proteins cannot be directly measured at the single molecular level. On the other hand, it is possible to study proton transfer in a membrane protein (gramicidin A, gA) that forms ion channels in lipid bilayers. gA is a highly hydrophobic pentadecapeptide secreted by *Bacillus brevis* [17]. It consists of an alternating sequence of D- and L-amino acids [18] that defines a right-handed $\beta^{6.3}$ helix in lipid bilayers [19–21]. The side chain residues of the gA channel are in contact with the hydrophobic lipid environment while the carbonyls and amides face the hydrophilic pore of gA. Each gA molecule resides in one monolayer of a lipid bilayer. The association via six H-bonds between the amino termini of two gA's located in opposite monolayers forms an ion channel that is selectively permeable to monovalent cations. The functional gA channel is $\approx$25 Å long, and its hydrophilic pore ($\approx$4 Å in diameter) contains a single water wire comprised of seven to nine water molecules [22,23]. The lifetime of the gA channel is determined by the dissociation rate of gA monomers in bilayers.

In our laboratory, the amino termini of two gA molecules have been linked to a dioxolane group [24–26]. The presence of two chiral carbons in the dioxolane linker defines the SS and RR diastereoisomers of dioxolane-linked gA channels (for the sake of simplicity, these channels will be referred to as the SS and RR channels). There are significant differences between the proton transfer properties in native gA, SS and RR channels [24,25,27–30]. Those distinct properties make these molecules interesting models to probe the relationships between structure and function of proton transfer in proteins.

The conductivity of protons in some alcohols, and methanol in particular, is considerably larger than of other monovalent cations [31–33]. In analogy to what had been proposed for water, this 'extra' proton conductivity led to the suggestion that protons could also be transferred between methanol molecules by a Grotthuss-like mechanism [31,32]. Our particular interest on methanol effects on proton transfer relates to the fact that this molecule could fit inside the pore of gA channels. Thus, it was of interest to probe if and how methanol modulates H^+ transfer in gA channels. We have demonstrated that, indeed, methanol caused a significant attenuation of proton currents in SS channels [34]. These experimental results were consistent with a model in which one methanol molecule is present between water molecules in the water wire of the SS channel (and/or at the channel/solution interface), and somehow attenuates proton transfer through the channel. Quite interestingly, longer chain alcohols like ethanol or propanol, that are not likely to fit inside the pore of gA channels, do not attenuate proton currents in the various gA channels studied (Godoy and Cukierman unpublished) [34]. The mechanisms,

by which proton currents in the SS channel are attenuated by methanol are not known.

Consequently, our experimental proposals in this study were: (1) to measure the kinetic isotope effect (KIE) of proton transfer in native gA, SS and RR channels. Are these KIE consistent with a proton transfer mechanism in water? (2) To further our understanding of the mechanisms by which methanol could attenuate H^+ conductivity in water and in various gA channels, KIE values were also measured in HCl or DCl solutions in the presence of CH_3OH (or CD_3OD). Previous measurements of KIE for proton conductivities have apparently been limited to dilute aqueous solutions of HCl [35–39]. In order to properly evaluate the KIE for proton transfer in various gA channels under several of our experimental conditions, it became necessary to perform conductivity measurements in several aqueous and methanol solutions. To our knowledge, several electrical conductivity measurements and associated KIE values in various solutions are presented here for the first time.

2. Material and methods

2.1. Bilayers

Planar lipid bilayers were formed from a decane solution ($\approx$60 mg/ml) of glycerylmonooleate (GMO, NuCheck Co., Elysian, MN; Sigma, St. Louis, MO). Bilayers were formed across a 150-μm diameter hole on a polystyrene partition separating two aqueous compartments. The formation of the lipid bilayer was monitored by visual inspection and capacitance measurements. Experiments were performed at room temperature (23–25 °C).

2.2. Solutions

The values of single channel conductances to protons (g_H, measured in picosiemens, pS) were measured in 0.05, 1 and 5 M HCl (or DCl) in H_2O (or D_2O). Experiments were also performed in 1 M HCl (or DCl) solutions with 15% v/v of CH_3OH (or CD_3OD). The final concentration of methanol or fully deuterated methanol was $\approx$3.7 M. HCl and CH_3OH (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA). DCl (99.7% D) and D_2O (99.9% D) were purchased from Aldrich (Milwaukee, WI). CD_3OD (99.8% D) was purchased from Alfa Aesar (Ward Hill, MA).

2.3. Measurements of solution conductivities

Electrical conductivities of freshly prepared solutions were measured at 24 °C with a YSI-3200 conductivity meter (Yellow Spring Instruments, Yellow Springs, OH). Equivalent conductivities [Λ, mS/(cm M), where mS is milisiemens] are reported in this study.

2.4. gA channels

The synthesis, purification and characterization of dioxolane-linked gA channels were previously described [26,27]. The native gA channels used in this study were purchased from Fluka (Milwaukee, WI). gA channels were added from a methanol stock solution ($\approx 10^{-8}$ M) that was routinely stored at ≈ -15 °C.

2.5. Single channel current measurements

Proton currents through a single channel molecule were measured by voltage clamping the lipid bilayer using an Axopatch 200B (Axon Instruments, Union City, CA). For native gA channels, a constant 50 mV DC voltage step was applied across the membrane. For the covalently linked gA dimers, voltage clamp ramps from 0 to $\approx$100 mV were applied in $\approx$5 s. Values for g_H in picosiemens (pS) were measured by regression analysis of the linear portion (usually from 0 to $\approx$75 mV) of I–V plots. pClamp (Axon Instruments) was used for applying voltages and recording single channel currents. At least five distinct single channel measurements were obtained from at least two distinct lipid bilayers in each experimental condition. Experimental points in this study are shown as mean $\pm$ S.E.M. In most plots, the error bars of the experimental points are smaller than the size of the symbols.

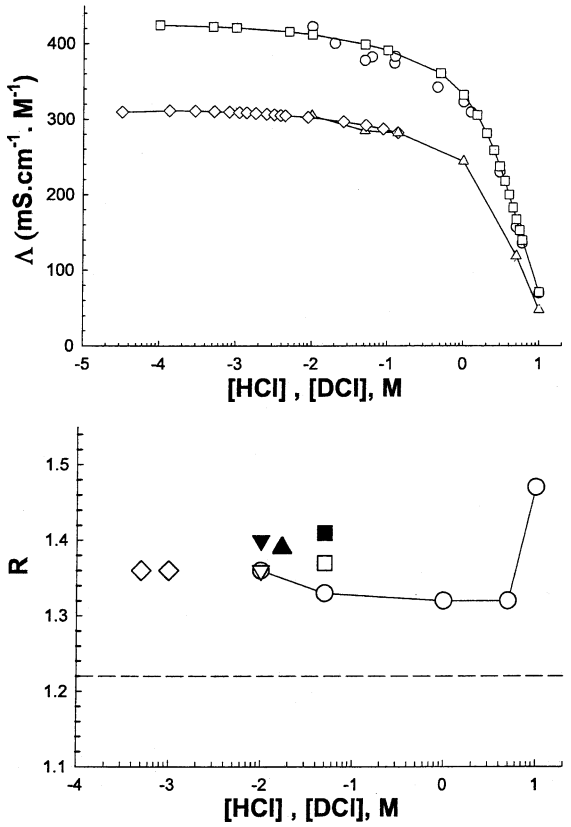

Fig. 1. Top panel: equivalent electrical conductivities (Λ) of HCl (circles and squares) or DCl (triangles and diamonds) in water. Squares and diamonds are from published data [35,36] while circles and triangles represent our measurements. Bottom panel: ratios (R) between Λ values of HCl and DCl solutions (open symbols); and Λ_H/Λ_D (filled symbols). See text for detailed description and sources of some of the experimental points.

3. Results and discussion

3.1. KIE in aqueous solutions

The upper panel in Fig. 1 shows the equivalent conductivities (Λ) of HCl and DCl solutions at various concentrations. The squares and diamonds were obtained from available data [35,36]. Circles and triangles represent our own measurements. The lower panel in Fig. 1 shows the ratios (R) $\Lambda_{HCl}/\Lambda_{DCl}$ (open symbols) and Λ_H/Λ_D (filled symbols). Diamonds were calculated from squares and triangles at a few selected concentrations of HCl and DCl (upper panel of Fig. 1). The open circles in the bottom panel of Fig. 1 are from our measurements (calculated from circles and triangles in the top panel of Fig. 1). The inverted open triangle is from measurements by Baker and La Mer [37]. The inverted filled triangle is the KIE for proton transfer after correction for Λ_{Cl}. This correction consisted in measuring the conductivity of a 10 mM KCl solutions and subtracting it from Λ_{HCl} of a 10 mM HCl solution [37]. In a similar way, the filled triangle is also the KIE for proton transfer after correction for Λ_{Cl} using 17 mM KCl [38]. The open square was measured in 50 mM HCl, and the corresponding filled square is the KIE for proton transfer after direct measurements of the transference numbers for H^+ and D^+ [39]. The dashed line in this graph represents the ratio between the shear viscosities of D_2O and H_2O at 25 °C [36,39,40]. Table 1 lists some of our measurements of Λ_{HCl} and Λ_{DCl} at various acid concentrations (see also Fig. 1). Also shown is the Λ_{KCl} in H_2O and D_2O (3 M KCl). The KIE values for diluted concentrations of HCl up to 5 M are within the range of 1.32–1.36. In 10 M HCl, however, the KIE increases to 1.47. The ratio between Λ_{KCl} in H_2O and D_2O is 1.17, and this value is consistent with the ratio between shear viscosities of deuterium oxide and water (Fig. 1).

The ratio between the electrical conductivities of dilute solutions of various alkalines in H_2O and D_2O is approximately 1.20 [38,41,42]. In concentrated 3 M KCl, this ratio is 1.17 (Table 1). These numbers are similar to the ratio between the viscosities of D_2O and H_2O at room temperature (1.22) [36,39,40], and in agreement with the idea that the mobility of these ions is determined in part by the frictional hindrance of the solvent. By contrast, the ratio between the electrical conductivities of HCl and DCl solutions is considerably larger ($\approx$1.35, Fig. 1). The significantly larger KIE for H^+ conductivity in water in relation to other ions suggests that proton mobility in a wide range of acid concentrations is not determined by the hydrodynamic diffusion of $(H_3O)^+$.

Apparently, measurements of KIE were previously limited to dilute solutions of HCl (see Fig. 1). In this study, KIE values for Λ_{HCl} in 1, 5, and 10 M acid solutions were determined. The ratios

Table 1
Equivalent conductivities of HCl, DCl and KCl solutions[a]

	0.05 M HCl or DCl	1 M HCl or DCl	5 M HCl or DCl	10 M HCl or DCl	3 M KCl
H_2O	377.6	322.8	157.0	69.74	85.37
D_2O	279.6	244.2	118.9	47.51	73.20
KIE	1.35	1.32	1.32	1.47	1.17

[a] Measurements performed at 24.0 °C (Λ in mS cm^{-1} M^{-1}).

between Λ_{HCl} and Λ_{DCl} at 1 and 5 M concentrations (1.32, Table 1) are not very different from measurements in dilute acid solutions (Fig. 1, bottom panel). Thus, it is possible that a substantial fraction of proton mobility in 5 M HCl and DCl is still likely to be determined by proton transfer. However, in 10 M HCl, the KIE value is considerably larger (1.47) than in dilute acid solutions and in 3 M KCl (1.17).

The transfer of protons between water molecules is obliterated as [HCl] increases [43,44]. It has been proposed that the high mobility (or conductivity) of H^+ in water is a consequence of an almost isoenergetic equilibrium between $(H_9O_4)^+$ and $(H_5O_2)^+$ [6–9,45–47]. Experimental conditions that disrupt this equilibrium would attenuate proton conductivity in water [6,7]. In particular, the structure of solvated protons in concentrated acid solutions is quite different from dilute solutions [48,49]. In 2 molal HCl for example, the ratio between the mobilities of H^+ and Cl^- is 6.5, and in 10 molal solutions, this ratio decreases to $\approx$2.5 [49]. This attenuation is caused by a significant reduction of H^+ mobility [43]. As [HCl] increases, the relative contribution of proton transfer to Λ_H will also decrease, and at very concentrated HCl solutions, it is likely that the mobility of protons is determined by the hydrodynamic diffusion of clusters of solvated protons [29,43,44,48,49]. Consequently, the expectation was that in 10 M HCl the KIE would be close to the ratios between the viscosities of deuterium oxide and water and/or between the Λ_{KCl} values in concentrated 3 M KCl in H_2O and D_2O. Instead, a KIE of 1.47, which is considerably larger than those ratios has been measured (Fig. 1 and Table 1). Assuming that Λ_H in 10 M HCl is determined essentially by the hydrodynamic diffusion of protonated water molecules, one explanation for the larger KIE in 10 M HCl is that the size or volume of protonated water(s) in a D_2O cluster is larger than in H_2O. Stronger H bonds between D_2O molecules compared to H_2O [6,48,49] could explain differences between the sizes of water clusters. In itself, stronger D-bonds between water molecules could also hamper the mobility of clusters of protonated water(s).

3.2. KIE in gA channels in aqueous solutions

In Fig. 2, representative single channel H^+ and D^+ currents vs. transmembrane voltage (I–V plots) for the SS and RR channels in 1 M HCl and DCl solutions are shown. The single channel conductances in this figure are: g_H (883 pS, SS; 376 pS, RR); g_D (697 pS, SS; 288 pS, RR). Fig. 3 shows single channel recordings of native gA channels at a transmembrane voltage of 50 mV. In this figure, g_H and g_D for single channel openings were $\approx$762 pS and $\approx$600 pS, respectively. A summary of measurements of g_H and g_D in 0.05, 1 and 5 M HCl and DCl solutions for the various gA channels is reported in Table 2.

The KIE values for H^+ transfer in various gA channels at several concentrations of HCl and DCl are shown in Table 3. The KIE values of H^+ transfer in the various gA channels varied from 1.22 in native gA channel at 50 mM HCl and DCl to 1.37 for the RR channel in 5 M HCl and DCl (overall average: 1.31 ± 0.02). These results are in agreement with determinations of KIE previously performed in native gA channels only [51].

The single channel conductances of native gA channels to alkaline metals in H_2O and D_2O were previously determined [42,51]. The ratios between these single channel conductances in H_2O and

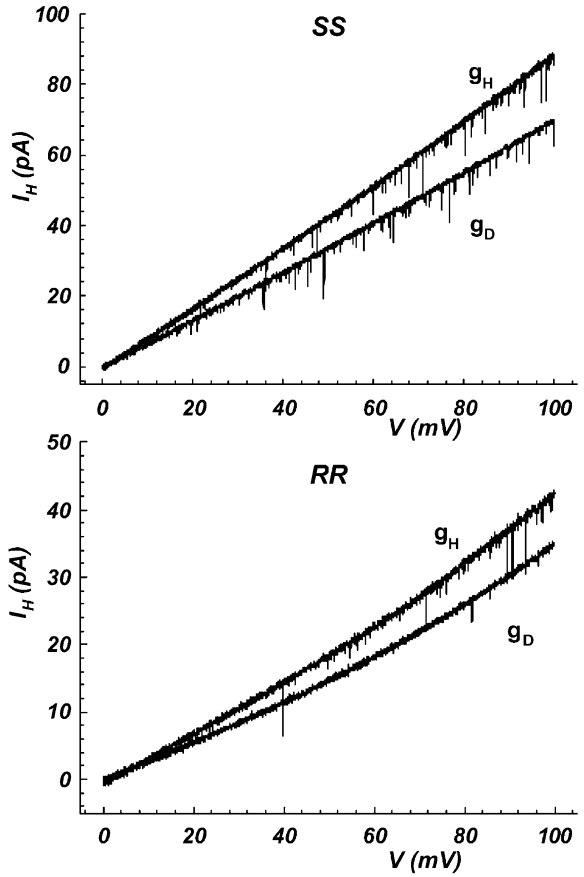

Fig. 2. I–V (picopamperes, pA vs. milivolt, mV) plots of H^+ or D^+ currents of single SS and RR channels. The single channel recordings were low-pass Bessel filtered at 0.5 kHz and digitized at 5 kHz. The small downward deflections in each recording are unresolved (due to filtering) channel closures.

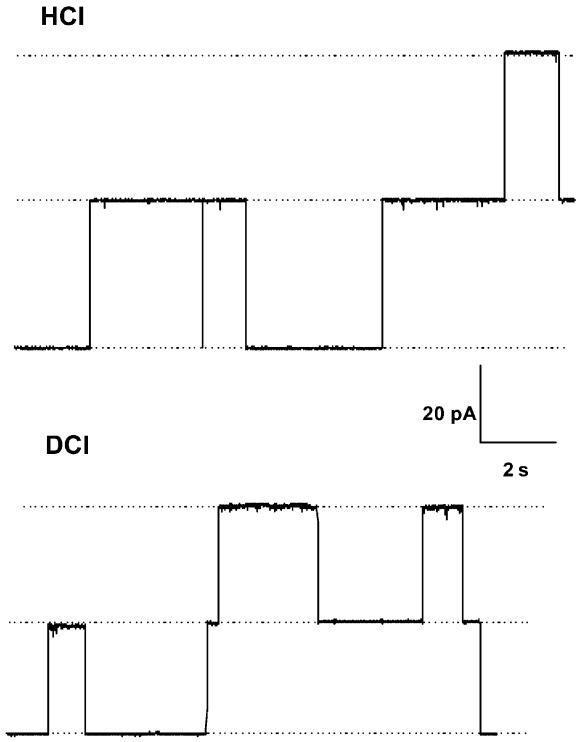

Fig. 3. H^+ or D^+ currents in native gA channels at an applied membrane potential of +50 mV. Channel recordings were low-pass Bessel filtered at 100 Hz and digitized at 1 kHz. Channel openings are represented by upward deflections of the current trace. The top and bottom recordings have three and four distinct channel openings, respectively.

D_2O were between 1.03 for Li^+ and 1.16 for Cs^+. For Na^+, whose permeation in native gA channels is limited by the permeability of water molecules in a single file diffusion mechanism [22], that ratio was 1.11. Notice that because in

Table 2
Single channel conductances (pS) to H^+ or D^+ of gA channels in various solutions (mean ± S.E.M., *n*)

	GA	SS	RR
50 mM HCl	53.1 ± 0.7 (27)	108.0 ± 3.2 (18)	–
1 M HCl	746.5 ± 4.7 (12)	891.3 ± 7.7 (7)	366.9 ± 7.6 (16)
5 M HCl	2612.3 ± 26.3 (19)	1849.7 ± 18.4 (4)	1559.1 ± 70.7 (10)
50 mM DCl	43.5 ± 0.6 (20)	79.7 ± 1.4 (26)	–
1 M DCl	588.7 ± 8.5 (12)	679.3 ± 4.7 (19)	278.3 ± 12.0 (11)
5 M DCl	1926.3 ± 26.2 (7)	1417.1 ± 25.1 (12)	1140.3 ± 37.9 (9)
1 M HCl + CH_3OH	515.9 ± 5.5 (9)	551.3 ± 11.2 (10)	264.2 ± 12.4 (12)
1 M DCl + CD_3OD	380.3 ± 4.0 (17)	422.3 ± 11.8 (9)	201.2 ± 5.0 (25)

Table 3
Kinetic isotope effects of solution conductivities and single channel conductances in HCl

	50 mM	1 M	5 M
Solutions	1.35	1.32	1.32
gA	1.22±0.01	1.27±0.01	1.36±0.02
SS	1.36±0.01	1.31±0.01	1.31±0.01
RR	–	1.32±0.03	1.37±0.02

the present experimental conditions gA channels are selective only to H^+ (Cl^- does not permeate the channel), the ratio between g_H values in HCl and DCl is indeed the real KIE for proton transfer between the Ag/AgCl electrodes located on different compartments across gA channels, and does not need to be corrected for the Cl^- conductivity (see Fig. 1 above and related text). That the KIE values for H^+ transfer in native gA, and in the SS and RR channels are significantly larger than the ratios for alkalines (especially the one measured for Na^+ [42,50,51]) suggests that it is likely that a proton transfer mechanism is operating inside these channels and not the hydrodynamic flow of $(H_3O)^+$. An alternative interpretation would be that the rate limiting step for proton transfer in gA channels is not inside the channel (or at membrane-channel/solution interface) but in bulk solution. However: (1) g_H is strongly modulated by the nature of the lipid bilayer [24,25,30,52,53,54]; (2) the activation energies for g_H in various gA channels (~28 kJ/mol) are significantly different from the activation energy of H^+ conductivity in bulk solution [52]; and (3) Native gA, SS and RR channels have different susceptibilities to methanol blockade of proton currents (see below). Taken together, these results indicate that the limiting step for H^+ transfer is in the gA channel or at the membrane-channel/solution interface rather than in bulk solution.

3.3. KIE in methanol and in methanol/water mixtures

Table 4 shows measurements of Λ in HCl, DCl and KCl solutions in methanol. The ratios between Λ values in methanol and *d*-methanol solutions (5 and 50 mM HCl) are ≈1.15. Interestingly, Table 4 also shows that the ratio between the conductivity measurements of 5 mM KCl in methanol and *d*-methanol (1.24) is larger than the ratios of Λ_{HCl} in methanol and *d*-methanol.

Two distinct experimental observations led to the proposal that protons could be transferred between methanol molecules: (1) in pure methanol (as in water) there is an extra conductivity of HCl in relation to LiCl, KCl and NaCl [31,33,53]. We have now confirmed this for KCl and extended the measurements to deuterated methanol solutions (Table 4). Notice that the difference between Λ_{HCl} and Λ_{KCl} [the 'extra' proton conductivity, ≈41 mS/(cm M)] is approximately the same in both CH_3OH and CD_3OD. (2) Λ_{HCl} has an anomalous mole fraction dependence on methanol in water/methanol solutions. Λ_{HCl} is attenuated as the methanol mole-fraction in aqueous solutions increases from 0 to 80%. However, above 80% there is a significant and continuous increase in Λ_{HCl} [31,32,53].

The ratio between Λ_{KCl} in methanol and *d*-

Table 4
Equivalent conductivities of HCl, DCl and KCl in methanol containing solutions[a]

	5 mM HCl or DCl	5 mM KCl	50 mM HCl or DCl	1 M HCl or DCl
CH_3OH[b]	129.74	87.82	103.78	
CD_3OD[b]	111.42	70.78	90.14	
KIE	1.16	1.24	1.15	
3.7 M CH_3OH				243.00
3.7 M CD_3OD				181.00
KIE				1.34

[a] Measurements performed at 24.0 °C (Λ in mS cm^{-1} M^{-1}).
[b] 100% methanol solutions.

methanol provides an indication of how the relative mobilities of K^+ and Cl^- are affected in these different solutions. The fact that: (1) in methanol and *d*-methanol solutions there is an 'extra' conductivity of HCl in relation to KCl solutions; and (2) the KIE of proton conductivity in methanol is considerably smaller than the ratio of Λ_{KCl} in methanol and *d*-methanol provides additional support for proton transfer between methanol molecules. Interestingly, H^+ transfer in methanol is considerably less sensitive to H/D substitution than in water. The reason for this effect is not known.

The ratio between the conductivities of 1 M HCl and DCl solutions in 3.7 M methanol (and *d*-methanol) is 1.34 (Table 4, last column). The equilibrium constant of the reaction $(H_3O)^+ + CH_3OH \leftrightarrow H_2O + (CH_3OH_2)^+$ is 0.23 [54]. Thus, CH_3OH is a poorer base than H_2O. Assuming that a given $(H_3O)^+$ has the same probability of being solvated by either H_2O or CH_3OH, the energetically favored pathway for H^+ transfer in methanol/water mixtures is between $(H_3O)^+$ and H_2O [31,32,55]. The KIE for H^+ transfer in 3.7 M methanol solutions (1.34) is close to that measured in its absence (1.32, Table 1 and Fig. 1) and considerably larger than in pure methanol solutions (see above). This suggests that in 3.7 M methanol, protons are transferred mainly along chains of molecules containing only water [55]. Because in methanol/water mixtures there is a reduction in the number of pathways containing water molecules only, proton conductivity in these solutions is significantly attenuated in relation to pure water.

3.4. KIE in gA channels in aqueous solutions containing methanol

Fig. 4 shows I–V plots for the SS and RR channels in 1 M HCl + 3.7 M CH_3OH (upper traces in both top and bottom panels), and in 1 M DCl + 3.7 M CD_3OD (lower traces in top and bottom panels). In Fig. 5, representative recordings of native single gA channels in solutions identified at the top of each panel are shown. The attenuation of H^+ or D^+ currents by methanol or *d*-methanol in gA channels has the following characteristics (Tables 2 and 5):

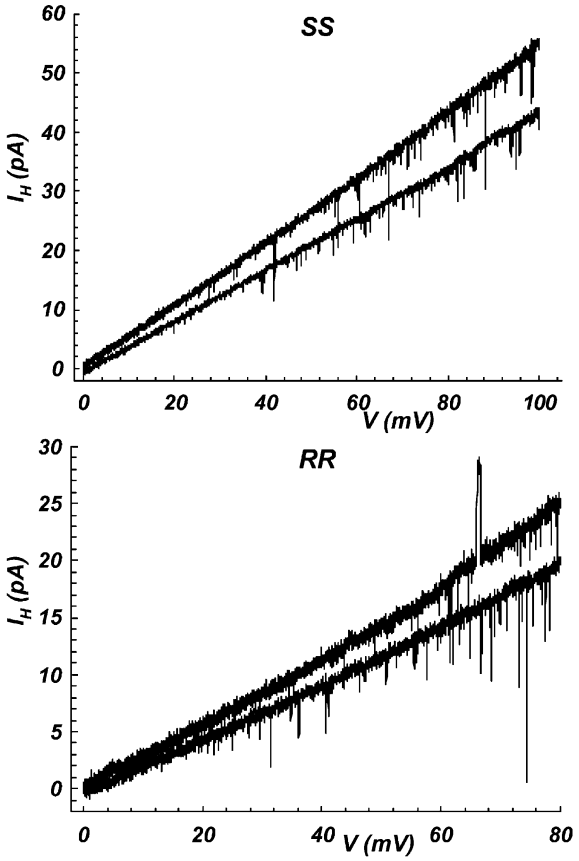

Fig. 4. I–V plots of H^+ and D^+ currents of single SS and RR channels in 1 M HCl + 3.7 M CH_3OH (top recordings for each panel) and in 1 M DCl + 3.7 M CD_3OD (bottom recordings for each panel). The single channel recordings were low-pass Bessel filtered at 0.5 kHz and digitized at 5 kHz.

1. Quigley et al. [34] reported that in 1 M HCl, 3.7 M CH_3OH attenuated g_H by ≈37% in the SS channel. This observation has now been independently confirmed for the SS channel (38% attenuation in g_H). Moreover, methanol also attenuated g_H in the RR and native gA channels by 28 and 31%, respectively (Table 5). Because attenuations of g_H are stronger than the attenuation of Λ_{HCl} in 1 M HCl solutions (Table 5), it is likely that methanol partitions in the pore of gA channels or at the membrane-channel/solution interfaces and causes an 'extra' reduction of H^+ currents than in bulk solution [34].

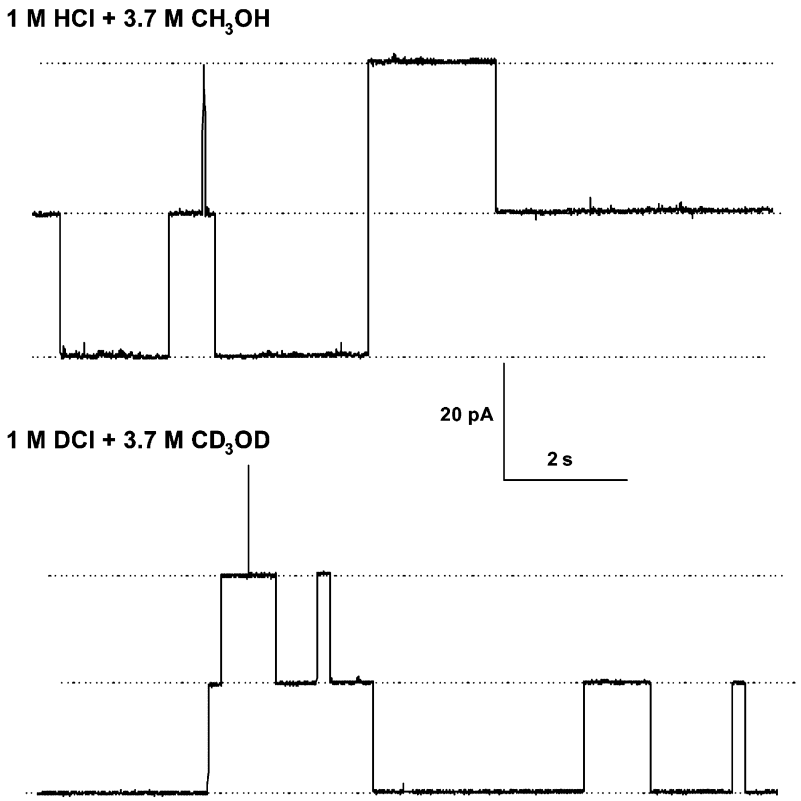

Fig. 5. H^+ or D^+ currents in native gA channels at an applied membrane potential of +50 mV. Channel recordings were low-pass Bessel filtered at 100 Hz and digitized at 1 kHz. Channel openings are represented by upward deflections of the current trace. Five and six distinct channel openings are seen in the top and bottom recordings, respectively.

2. D^+ currents in various gA channels were attenuated by CD_3OD by approximately the same ratio as H^+ currents by CH_3OH. *d*-Methanol attenuated g_D (1 M DCl) by 38, 28 and 35% in the SS, RR and native gA channels, respectively.
3. The attenuation of g_H (or g_D) by methanol is larger in the SS channel, followed by gA and RR. This strengthens the hypothesis [34] that the blockade of proton currents in gA channels is significant, and cannot be simply explained by the attenuation of proton conductivity in bulk solution. It is possible that these gA channels have distinct partition coefficients for methanol. Moreover, because the difference between the various gA channels relates to the absence of the dioxolane linker as in native gA, or to the different conformations of the linker in the SS and RR channels, it may well be that the

Table 5
Kinetic isotope effects of conductivities in solutions and in Gramicidin A channels in 1 M HCl + 3.7 M Methanol

	Solution	gA	SS	RR
$\Lambda_{HCl,methanol}/\Lambda_{HCl}$ or $g_{H,methanol}/g_H$	0.77	0.69 ± 0.01	0.62 ± 0.02	0.72 ± 0.03
$\Lambda_{DCl,D\text{-}methanol}/\Lambda_{DCl}$ or $g_{D,D\text{-}methanol}/g_D$	0.74	0.65 ± 0.02	0.62 ± 0.02	0.72 ± 0.02
$L_{HCl,methanol}/L_{HCl,D\text{-}methanol}$ or $g_{H,methanol}/g_{D,D\text{-}methanol}$	1.34	1.36 ± 0.01	1.31 ± 0.01	1.31 ± 0.04

residence time (or permeability) of methanol in the pore is modulated by interactions with the dioxolane.

4. The KIE's for proton transfer in methanol solutions are 1.36 (gA) and 1.31 (SS and RR channels). On average, these values are not different from proton transfer in water/methanol solutions (Table 3).

In principle, two distinct and non-mutually exclusive mechanisms could account for a decreased g_H in various gA channels in the presence of methanol [28,34]: (a) Assume that one methanol molecule resides inside the pore of the SS channel and shuttles protons between waters by a hop-turn mechanism. As a consequence, the transfer of protons in the channel occupied by a methanol molecule would occur with a decreased efficiency (smaller g_H). Distinct mechanisms could account for this effect. For example, if the protonation of a water molecule by the H^+ released from an adjacent methanol, and/or the protonation of methanol by a H^+ released from an adjacent water molecule is significantly slower than the H^+ transfer between two adjacent water molecules (see above), then g_H would be attenuated. Another possibility could be that the reorientation step of the methanol molecule inside the channel is significantly slower than the reorientation of water molecules. In these cases, the amplitude of single channel H^+ currents would dwell between an open state (no methanol inside the pore), a closed state (channel closure) and an intermediary level of current between the fully closed and open states (methanol is inside the pore and transfers H^+ with a decreased efficiency in relation to water); (b) A distinct possibility is that while methanol is inside the channel, H^+ transfer in the channel is completely blocked. In this case, the amplitude of the single channel proton current would dwell between an open state (no methanol inside the pore), a closed state (channel closure) and a fully blocked state (methanol inside the pore). The fully blocked and closed states cannot be discriminated in electrical recordings of single channel H^+ currents. The shortest time resolution of single channel H^+ currents in planar bilayers is of the order of tens of μs [28]. If it is assumed that either mechanism A (intermediary open current level between open and closed states caused by methanol in the pore) or B (complete blockade of the open state by methanol) occurs in a time scale considerably shorter than tens of microseconds, it will not be possible to detect these phenomena in the recordings of single channel H^+ currents. The measurements of KIE in gA channels in water–methanol solutions were undertaken with the aim of discriminating between possible blockade models of H^+ or D^+ currents by methanol. Even though the KIE for the attenuation of proton currents by methanol in gA channels did not permit a distinction between mechanisms A and B above, the possibility that the KIE for H^+ transfer with a methanol molecule in a single file of water molecules in the channel may have the same value as in its absence cannot be eliminated.

In summary, the novel results and conclusions in this study were: (1) in 10 M HCl the KIE for proton transfer is substantially larger than in more dilute acid solutions. It is possible that there are significant differences between the structures of solvated H^+ and D^+; (2) the KIE for proton conductivity in pure methanol and in mixtures of water/methanol solutions were measured. While in water/methanol mixtures the KIE values are consistent with proton transfer occurring between water molecules, the KIE values measured in pure methanol solutions suggest that a proton transfer mechanism does indeed occur between methanol molecules. (3) KIE values measured in various HCl solutions in several gA channels are similar to that measured in HCl solutions, and considerably larger than with other monovalent cations. This suggests that proton transfer occurs between water molecules in the various gA channels.

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