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Research article

Proton exclusion by an aquaglyceroprotein: a voltage clamp study

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Background information. In silico both orthodox aquaporins and aquaglyceroporins are shown to exclude protons. Supporting experimental evidence is available only for orthodox aquaporins. In contrast, the subset of the aquaporin water channel family that is permeable to glycerol and certain small, uncharged solutes has not yet been shown to exclude protons. Moreover, different aquaglyceroporins have been reported to conduct ions when reconstituted in planar bilayers.

Results. To clarify these discrepancies, we have measured proton permeability through the purified *Escherichia coli* glycerol facilitator (GlpF). Functional reconstitution into planar lipid bilayers was demonstrated by imposing an osmotic gradient across the membrane and detecting the resulting small changes in ionic concentration close to the membrane surface. The osmotic water flow corresponds to a GlpF single channel water permeability of 0.7×10^{-14} cm$^3 \cdot$ subunit$^{-1} \cdot$ s^{-1}. Proton conductivity measurements carried out in the presence of a pH gradient (1 unit) revealed an upper limit of the H^+ (OH^-) to H_2O molecules transport stoichiometry of 2×10^{-9}. A significant GlpF-mediated ion conductivity was also not detectable.

Conclusions. The lack of a physiologically relevant GlpF-mediated proton conductivity agrees well with predictions made by molecular dynamics simulations.

Introduction

Epithelial water transport is primarily transcellular, mediated by aquaporins (Schnermann et al., 1998). Paracellular volume flow through tight junctions is of minor importance (Kovbasnjuk et al., 1998). Thus water flow through aquaporins must be sufficiently fast and selective at the same time. Homoeostasis of cells involved in water transport requires that movement of other solutes, ions, and even protons through aquaporins is blocked (Finkelstein, 1987). Equilibration of transmembrane proton gradients by aquaporins would lead to energy depletion, changes in transmembrane potential and hamper proton dependent steps in cellular signal transduction.

Proton exclusion has been shown in experiments in which lipid vesicles reconstituted with aquaporin-1 (AQP1) were exposed to rapidly changing external pH. The rate required for equilibration of internal pH was taken as a measure of proton permeability, P_H. P_H did not differ from those of bare liposomes (Zeidel et al., 1992). Under the same conditions, insertion of gramicidin channels produced a 50-fold increase in P_H (Zeidel et al., 1992). Provided that aquaporins are conducting protons with 100-fold lower efficiency than gramicidin, the incremental P_H should remain unnoticed. Nevertheless, such a P_H would be still physiologically relevant, since the proton turnover rate (M_H) of gramicidin channels exceeds that of typical proton channels by orders of magnitude (DeCoursey, 2003). M_H of the uncoupling protein-1

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Key words: diffusion, microelectrode, proton wire, unstirred layer, water permeability.
Abbreviations used: AQP, aquaporin, C_{osm}, transmembrane osmotic gradient; GlpF, bacterial glycerol facilitator; L, total number of lipid molecules; M_H, proton turnover rate; M_{ion}, upper ion turnover number; OG, n-octyl β-D-glucopyranoside; p_f, single channel water permeability; P_f, osmotic water permeability; P_H, proton permeability; UCP1, uncoupling protein-1; V_w, molecular volume of water.

(UCP1), for example, is 100-fold smaller (Urbankova et al., 2003).

To exclude the possibility that aquaporins conduct protons at comparable rates, voltage clamp experiments were performed in which the bacterial aquaporin-Z (AqpZ) was reconstituted into planar lipid bilayers. Water transport through a total amount of 10^7 AqpZ molecules per bilayer was found to be electrically silent. Even at pH 4.5, a measurable current through the orthodox aquaporin was not observable (Pohl et al., 2001). From the upper estimate of the AqpZ ion/water permeability ratio $R = 2 \times 10^{-10}$ and the single channel water permeability $p_f = 2 \times 10^{-14}$ cm$^3 \cdot$ s^{-1} (Pohl et al., 2001), the upper ion turnover number M_{ion} can be calculated to be 0.1 s^{-1} according to (Finkelstein and Andersen, 1981; Saparov and Pohl, 2004):

$$M_{ion} = M_w R = \frac{N_A p_f R}{V_w} \quad (1)$$

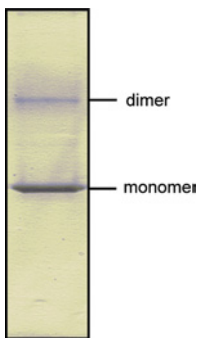
where N_A, M_w and V_w denote Avogadro's number, the water turnover number and the molecular volume of water respectively. Since M_H cannot exceed M_{ion}, M_H of AqpZ is at least 100- or 10 000-fold smaller than the respective values of UCP1 or gramicidin. This observation indicates that proton transport through AqpZ is physiologically meaningless.

Experimental evidence for proton exclusion by aquaglyceroproteins is much weaker. The plant aquaglyceroprotein, nodulin-26, was found to exhibit ion permeation when reconstituted into planar lipid bilayers (Weaver et al., 1994; Lee et al., 1995). Increased membrane conductance was not detected, however, when both proteins were expressed in *Xenopus laevis* oocyte membranes (Mulders et al., 1995; Rivers et al., 1997). Explicit measurements of proton conductivity have not been reported. The only indication for proton exclusion comes from molecular dynamics simulations. Based on the known structure, the bacterial glycerol facilitator, GlpF, was shown *in silico* to inhibit proton migration along the water wire within the channel (de Groot and Grubmuller, 2001; Tajkhorshid et al., 2002).

The present work aims to prove experimentally the predictions made by computer simulations. Therefore the highly purified GlpF-channel (Figure 1) is reconstituted into planar lipid bilayers. This pure system is exploited for simultaneous measurement of water and ion conductance (Pohl et al., 2001). The contribution of protons to the overall membrane conductivity is determined from the shift of the reversal potential that is induced by a transmembrane pH gradient.

Figure 1 | SDS/PAGE analysis of purified Glpf protein

13% acrylamide gel stained with Coomassie Brilliant Blue. ~1 μg of protein was loaded on the gel.



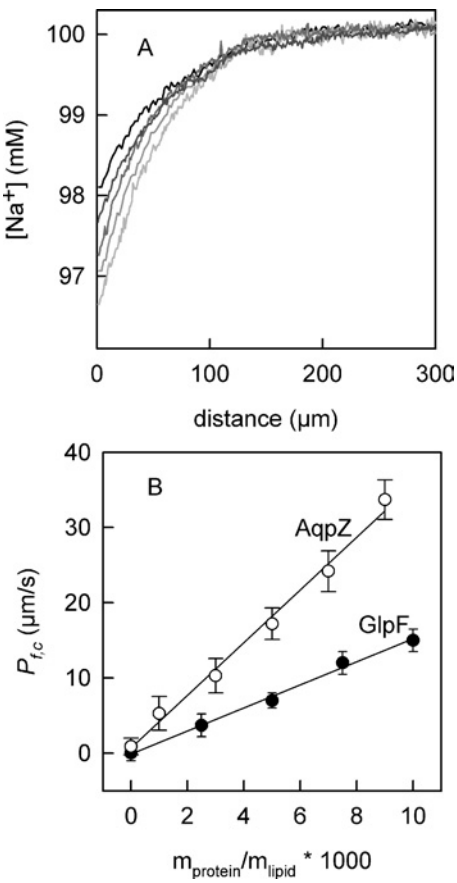
Results and discussion

GlpF protein was expressed in *Escherichia coli* and purified (Figure 1) according to Borgnia and Agre (2001). The solubilizing detergent was removed by dialysis and substituted with *E. coli* lipids (Avanti Polar Lipids, Alabaster, Alabama, U.S.A.). Planar membranes were formed from protein-free liposomes and GlpF proteoliposomes (see Pohl et al., 2001; Saparov et al., 2001). Both liposome populations were mixed in different ratios to vary the abundance of GlpF in the planar bilayers. A steady state water flux through the reconstituted protein was induced by a transmembrane concentration gradient of choline chloride. The resulting dilution of sodium ions in the immediate membrane vicinity was detected by scanning ion sensitive microelectrodes. The more GlpF was reconstituted, the more pronounced was the diffusion polarization observed. Representative experimental records are shown in Figure 2(A). With increasing protein lipid ratio, the osmotic water permeability $P_{f,c}$ introduced by the GlpF channels increased to 35 μm/s (23°C).

The hydraulic permeability coefficient of a single channel, p_f, is equal to the absolute hydraulic conductivity of all channels divided by the number of channels, n (Finkelstein, 1987). n is anticipated to be equal to the total number of lipid molecules, L, in the bilayer divided by the molar lipid to protein ratio, r, where L is derived from twice (for both leaflets) the membrane area, A, divided by the area, b,

Figure 2 | The incremental water flux through planar membranes induced by the reconstitution of GlpF

(**A**) At the indicated distances from the planar bilayer, the sodium concentration was measured. Solute dilution was due to an osmotic water flux induced by 0.5 M choline chloride. In the immediate membrane vicinity, the solute polarization increased with an increase in the protein lipid ratio. The membranes with the highest protein content are represented by the lighter traces. (**B**) The channel-mediated water permeability ($P_{f,c}$) is plotted against the protein lipid ratio in the membrane. It is compared with the $P_{f,c}$ of AqpZ measured by the same method. The AqpZ data are taken from Pohl et al. (2001), Copyright 2001 National Academy of Sciences, U.S.A. The buffer contained 10 mM Mes, Mops, 100 mM NaCl, 50 mM choline chloride. pH on the hypertonic and hypotonic sides were, respectively, 4.5 and 5.5.



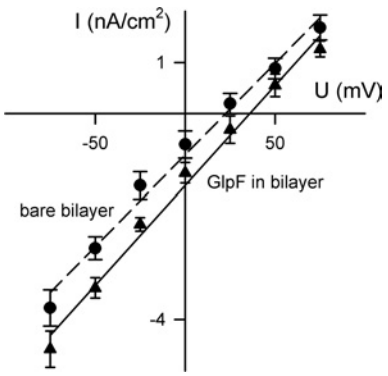
per lipid molecule:

$$p_f = P_{f,c}A/n = P_{f,c}Ar/2L = P_{f,c}br/2 \qquad (2)$$

$P_f r$ corresponds with the slope of the regression line (Figure 2B) corrected for the differences in molecular

Figure 3 | Volt-ampere characteristics of a GlpF-containing membrane

The conductivities of bilayers with reconstituted protein and protein-free membranes were 39 ± 18 nS/cm^2 and 34 ± 15 nS/cm^2 respectively. GlpF was reconstituted at a protein: lipid ratio of 1:100. The experimental conditions were similar to those in Figure 2.



masses of the protein (30 kDa) and the lipid (800 Da). For $b = 78A^2$ (Hsieh et al., 1997), p_f of GlpF is found to be 0.34×10^{-14} cm$^3 \cdot$ subunit $\cdot$ s. The result was obtained assuming that protein incorporation is 100% efficient. The real efficiency is probably less than this. Reconstitution of AQP0, for example, was approx. 50% efficient (Zampighi et al., 1985). If our value is in the same range, then p_f adopts a value of approx. 0.7×10^{-14} cm$^3 \cdot$ subunit$^{-1} \cdot$ s^{-1}.

p_f amounts to approx. 30% of the value found for AqpZ (Pohl et al., 2001). In stopped flow experiments, p_f of GlpF was estimated to be only approx. 17% of AqpZ. Given the accuracy at which the protein concentration in purified samples is usually assessed and possible differences in the efficiency of reconstitution, the agreement between both values is satisfactory.

The conductivity of GlpF for ions was tested by current measurements under voltage clamp conditions (Figure 3). With 39 ± 18 nS/cm^2, the total membrane conductance, G, of a GlpF containing membrane did not significantly differ from the conductivity of a bare bilayer, which amounted to 34 ± 15 nS/cm^2. To determine the contribution of H^+ or OH^- conductance, $G_{H/OH}$, the Nernst potential for protons was measured (Gutknecht, 1987). It is equal to the shift of the reversal potential, ψ_0, induced by a transmembrane pH gradient. For this

purpose a pair of well-buffered solutions was chosen having similar osmolarities, ionic strength and concentrations of all ions except H^+ and OH^- (Gutknecht, 1987; Fuks and Homble, 1994). The transference number of H^+/OH^- was to be:

$$T_{H/OH} = \psi_0/\psi_N \quad (3)$$

where ψ_N is the theoretical value of Nernst equilibrium potential (59 mV for a pH gradient of 1). The final proton conductivity $G_{H/OH}$ is calculated as:

$$G_{H/OH} = T_{H/OH} \cdot G \quad (4)$$

For bare and GlpF-containing membranes, $G_{H/OH}$ was equal to 14 ± 7 nS/cm^2 and 23 ± 9 nS/cm^2, respectively. Measured at the highest protein to lipid ratio of 1:100, the difference between the mean values is not significant. Nevertheless, assuming that the incremental $\Delta G_{H/OH} = 9$ nS/cm^2 were caused by GlpF, the water to proton transport ratio was calculated from the relationship (Hodgkin, 1951; Walter et al., 1982):

$$j_{H/OH} = \frac{RT}{z^2F^2}\Delta G_{H/OH} \quad (5)$$

where $j_{H/OH}$ denotes the H^+ (OH^-) flux density. *R, T, F* and *z* have their usual meanings. According to Equation 5 the maximum flux of H^+ (OH^-) in Figure 3 is approx. 2.4×10^{-15} mol $\cdot$ s^{-1} $\cdot$ cm^{-2}. The simultaneously measured water flux density, j_w:

$$j_w = P_f C_{osm} \quad (6)$$

(where C_{osm} is the transmembrane osmotic gradient) was equal to 1.5 μM $\cdot$ s^{-1} $\cdot$ cm^{-2} (Figure 2). Thus, the upper limit of the H^+ (or OH^-) to H_2O molecules transport stoichiometry is approx. 2×10^{-9} (at pH $\sim$5). According to Equation 1 this ratio corresponds with an upper estimate of the proton turnover number of 0.5 s^{-1}. A comparison with the turnover numbers of gramicidin and the proton channel of the 5-HT receptor, which at this pH are, respectively, 10^5- or 10^2-fold higher (DeCoursey, 2003), suggests that proton transport by GlpF is physiologically meaningless.

In silico studies of the glycerol facilitator GlpF have shown that the protein transports water and excludes protons (de Groot and Grubmuller, 2001; Tajkhorshid et al., 2002). The origin of the barrier for proton movement is under debate. According to molecular dynamics simulations, the barrier is hypothesized: (i) to be electrostatic in nature (de Groot et al., 2003; Jensen et al., 2003; Ilan et al., 2004), or (ii) to be associated with the change in proton solvation energy upon moving from the bulk solvent to the centre of the channel (Burykin and Warshel, 2004). According to (i), the positive electrostatical potential peaks in the region where two short α-helices entering the membrane from the extra- and intracellular surfaces meet with their N-termini. At that location both half helices contain an NPA (asparagine, proline, alanine) motif, which is conserved throughout the AQP superfamily. The electrostatic barrier amounts to 25–30 kJ $\cdot$ mol^{-1}, i.e. it matches the barrier height for the passage of protons across pure lipid bilayers (de Groot et al., 2003). The alternative hypothesis (ii) suggests that the barrier exists even when all the protein residual charges are set to zero (Burykin and Warshel, 2003). In these simulations the barrier has been associated with the change in proton solvation energy upon moving from the bulk solvent to the centre of the channel (Burykin and Warshel, 2004). Our experiments do not allow us to distinguish between both mechanisms of proton exclusion; however, we report the first simultaneous measurements of proton conductivity and water permeability of an aquaglyceroprotein. Our study confirms the existence of the barrier to proton movement. It is at least as high as the barrier imposed by the lipid bilayer. In GlpF no more than two in 10^9 transported molecules are H^+ (or OH^-). To determine the origin of the barrier, experiments are under way with (i) members of the aquaporin family that are able to conduct ions (see Hazama et al., 2002) or (ii) model channels that allow proton and water passage (see Saparov et al., 2000).

Materials and methods

Expression and purification of GlpF

The expression plasmid pTrc10HisGlpf (Borgnia and Agre, 2001) encoding the His-tagged Glpf was a gift from Dr Mario J. Borgnia and Dr Peter Agre. The expression vector was transformed into *E. coli* strain *XL1*-Blue (Stratagene), and was selected for by ampicillin resistance. The GlpF protein was expressed in *E. coli* and purified as described by Borgnia and Agre (2001). Briefly, cells were grown in Luria–Bertani media containing 50 μg/ml ampicillin at 37°C to a density of approx. 0.6 (*D* at 600 nm). The expression of the recombinant protein was induced by addition of 0.3 mM isopropyl β-D-thiogalactoside (IPTG) at 37°C for 14 h. Harvested cells were resuspended in buffer

(pH 7.0) containing 100 mM K_2HPO_4, 1 mM $MgSO_4$, 0.1 mg/ml DNase I and protein inhibitors, and were disrupted by three cycles through a French pressure cell at 1000 psig (pounds-force per square inch gauge). Undisrupted cells and debris were removed by centrifugation at 10 000 *g* for 30 min, and the membrane fraction was obtained by centrifugation at 140 000 *g* for 1 h.

The membrane fraction was solubilized in buffer containing 3% n-octyl β-D-glucopyranoside (OG), 100 mM K_2HPO_4, 200 mM NaCl, 10% (v/v) glycerol, 50 mM imidazole, and 5 mM β-mercaptoethanol at pH 8.0 on ice for 1 h. After removing insoluble membrane by centrifugation at 140 000 *g* for 1 h, soluble fraction was mixed with 1/10 volume of Ni-NTA agarose beads (Qiagen) and incubated with slow agitation at 4°C for 1 h. The beads were packed in a column and washed with 40× bead volume of wash buffer containing 0.25% OG, 100 mM K_2HPO_4, 200 mM NaCl, 10% (v/v) glycerol, 100 mM imidazole, and 5 mM β-mercaptoethanol at pH 7.0. His-tagged recombinant protein was eluted with 1 bed volume of buffer containing 1% OG, 100 mM K_2HPO_4, 200 mM NaCl, 10% (v/v) glycerol, 1 M imidazole, and 5 mM β-mercaptoethanol at pH 7.0. Protein concentration was estimated from the intensity of the band stained with Coomassie Brilliant Blue by SDS/PAGE. BSA was used as a standard.

Planar membranes

Planar bilayers were formed from proteoliposomes (Schindler, 1989; Pohl et al., 2001; Saparov et al., 2001). The technique is based on the finding that monolayers spontaneously form at the air/water interface of any vesicle suspension. Two such monolayers were combined in an aperture (approx. 150 µm in diameter) of a 25 µm thick polytetrafluoroethylene (PTFE) septum separating the two aqueous phases of the chamber. The septum was pretreated with a hexadecane/hexane mixture (ratio by volume = 1:199). The membrane bathing buffer was agitated by magnetic stirrer bars.

Proton conductivity

Current-voltage relationships were measured by a patch-clamp amplifier (model EPC9; HEKA, Lambrecht, Germany). To determine the contribution of H^+ or OH^- conductance, $G_{H/OH}$, to the total membrane conductance, G, the shift of the reversal potential was measured that was induced by a transmembrane pH gradient (Gutknecht, 1987; Fuks and Homble, 1994).

Water flux

Water concentrates the solution it leaves and dilutes the solution it enters (Barry and Diamond, 1984). To monitor these concentration changes, which are restricted to stagnant water layers in the immediate membrane vicinity, Na^+-sensitive microelectrodes made of glass capillaries were used. Their tips (1–2 µm in diameter) were filled with cocktail A of Sodium Ionophore II (Fluka) according to the procedure described by Amman (Amman, 1986). Stepwise movement of the electrodes relative to the membrane was realized by a hydraulic stepdrive (Narishige, Japan). In the immediate membrane vicinity, the dependence of Na^+ concentration at the interface, C_s, from distance, x, to the membrane was fitted by the equation $C(x) = C_s\exp(-vx/D + bx^3/3D)$ to reveal the linear drift velocity of the osmotic volume flow, v, and the stirring parameter b (Pohl et al., 1997). D is the Na^+ diffusion coefficient. v is related to the osmotic water permeability P_f by $P_f = v/(C_{osm}\ V_w)$, where C_{osm} and V_w are the transmembrane osmotic gradient and the molecular volume of water (Finkelstein, 1987). $P_{f,c}$ is equal to P_f diminished by the water permeability of the bare lipid bilayer. C_{osm} has been obtained correcting the bulk choline chloride concentration for dilution in the immediate membrane vicinity (Pohl et al., 1998).

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