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Water and Ion Permeation of Aquaporin-1 in Planar Lipid Bilayers

MAJOR DIFFERENCES IN STRUCTURAL DETERMINANTS AND STOICHIOMETRY*

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The aquaporin-1 (AQP1) water channel protein is known to facilitate the rapid movement of water across cell membranes, but a proposed secondary role as an ion channel is still unsettled. Here we describe a method to simultaneously measure water permeability and ion conductance of purified human AQP1 after reconstitution into planar lipid bilayers. Water permeability was determined by measuring Na^+ concentrations adjacent to the membrane. Comparisons with the known single channel water permeability of AQP1 indicate that the planar lipid bilayers contain from 10^6 to 10^7 water channels. Addition of cGMP induced ion conductance in planar bilayers containing AQP1, whereas cAMP was without effect. The number of water channels exceeded the number of active ion channels by approximately 1 million-fold, yet p-chloromethylbenzenesulfonate inhibited the water permeability but not ion conductance. Identical ion channel parameters were achieved with AQP1 purified from human red blood cells or AQP1 heterologously expressed in *Saccharomyces cerevisae* and affinity purified with either N- or C-terminal poly-histidine tags. Rp-8-Br-cGMP inhibited all of the observed conductance levels of the cation selective channel (2, 6, and 10 pS in 100 mM Na^+ or K^+). Deletion of the putative cGMP binding motif at the C terminus by introduction of a stop codon at position 237 yielded a truncated AQP1 protein that was still permeated by water but not by ions. Our studies demonstrate a method for simultaneously measuring water permeability and ion conductance of AQP1 reconstituted into planar lipid bilayers. The ion conductance occurs (i) through a pathway distinct from the aqueous pathway, (ii) when stimulated directly by cGMP, and (iii) in only an exceedingly small fraction of AQP1 molecules.

The balance of water is central to the adaptation of every living organism to its environment (1). Water diffusion occurs through the lipid matrix (2) across transient defects arising from density fluctuations in the bilayers (3, 4) and through specialized aqueous pores (5). The discovery of aquaporin channel proteins provided the first molecular insight into how water may cross the plasma membrane (6).

After a century of investigation, it is still not entirely resolved whether transepithelial water flow is mediated by local osmosis (7), or secondary active transport is induced by protein cotransporters (8, 9). The former theory is supported by the observation that aquaporin-1 (AQP1)[1] is required for NaCl-driven water transport across descending thin limbs of the kidney (10) and by the finding that local osmotic gradients also drive the water flux associated with sodium/glucose cotransport in oocytes expressing the high affinity intestinal and renal sodium/glucose cotransporter (11). In the absence of an osmotic gradient across an epithelium (12), water movement may be mediated by a local tonicity gradient in the immediate vicinity of the membrane. An investigation of coupling between aquaporin-mediated water fluxes with solute transport is important for understanding epithelial fluid transport.

There has long been a general agreement that water moves through water channels in a single file fashion, so that the water molecules cannot pass each other within the channel (13). This has recently been confirmed by high resolution structural studies of AQP1 (14) as well as the related glycerol transport protein GlpF (15). Whereas most aquaporins are not believed to conduct ions, some exceptions have been reported. Anion currents are found in *Xenopus laevis* oocytes expressing AQP6 after activation with micromolar concentrations of Hg^{2+} or low pH (16). Forskolin was reported to stimulate membrane water and cation permeability of *X. laevis* oocytes expressing AQP1 (17), however this was not confirmed by other laboratories (18). It was subsequently reported that AQP1 functions as a cGMP-gated ion channel when expressed in *X. laevis* oocytes (19). This study was undertaken to investigate whether water permeation of AQP1 can be measured when the protein is reconstituted into planar lipid bilayers and to determine whether cGMP-activated ion conductance can be detected.

MATERIALS AND METHODS

Purification of AQP1 from Human Red Cells—AQP1 was purified from red cells as described previously (20) using reagents, except where noted, from Sigma. 400 ml of washed human red cells were hypotonically lysed and washed until the membranes were white in ice-cold 7.5 mM sodium phosphate, pH 7.5, containing EDTA, phenylmethylsulfonyl fluoride, and leupeptin. Peripheral proteins were extracted with 1 M potassium iodide, and other integral proteins were solubilized in 1% (w/v) *N*-lauroylsarcosine. The membrane vesicles containing AQP1 were solubilized in 4% (v/v) Triton X-100, adsorbed, and eluted from a POROS Q anion exchanger column, and AQP1 was adsorbed onto a 10 ml butyl-Sepharose column (Amersham Pharmacia Biotech) and eluted

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[1] The abbreviations used are: AQP1, aquaporin-1; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; pCMBS, p-chloromethylbenzenesulfonate; pS, picosiemens.

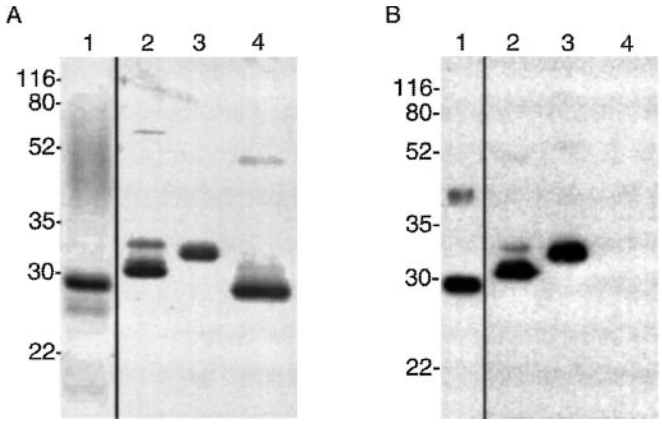

FIG. 1. **SDS-polyacrylamide gel electrophoresis analysis of purified AQP1 proteins is shown.** *A,* silver-stained 14% acrylamide slab containing ~1 μg of protein/lane. *Lane 1*, native AQP1 purified from human red cells; *lane 2*, C-terminal 6-histidine-tagged AQP1 heterologously expressed in *S. cerevisae* and purified by nickel-nitrilotriacetic acid affinity chromatography; *lane 3*, N-terminal 10-histidine-tagged AQP1 processed similarly; *lane 4,* N-terminal 10-His-tagged AQP1 truncated at residue 237. *B,* immunoblot of duplicate slab containing ~0.1 μg of protein/lane and probed with antibody specific for the C terminus of AQP1.

with an ammonium sulfate gradient (80% descending to 0% saturation). Protein purity was established by SDS-polyacrylamide gel electrophoresis (Fig. 1).

Heterologous Expression and Purification of AQP1 from Yeast—Protease-deficient (*pep4*) *S. cerevisiae* were transformed with the pYES2 plasmid (Invitrogen) in which DNA encoding human AQP1 with a C-terminal 6-histidine fusion was inserted downstream of the *GAL1* promoter. Human AQP1 containing an N-terminal 10-histidine sequence (residues 2–3 were replaced with GHHHHHHHHHHSSGHIWGRH) was similarly inserted into the pYES2 plasmid, and the C-truncated construct was produced by chameleon double-stranded site-directed mutagenesis (Stratagene) of residue 237 to a stop codon.

The recombinant yeast were cultured in 4 liters of Ura$^-$ minimal media and transferred during the log phase to galactose-rich media overnight to induce protein expression. Cells were disrupted by three cycles of French press at 18,000 p.s.i. Intact cells and nuclear debris were cleared by centrifugation at 2000 × *g*, and the membrane fraction was harvested by centrifugation at 300,000 × *g*. The membrane fraction was solubilized in 200 ml of buffer A (100 mM K_2HPO_4, 200 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 2% octylglucoside (*N*-octyl-β-D-glucopyranoside, Calbiochem)) containing 20 mM imidazole loaded onto a 1-ml nickel-nitrilotriacetic acid affinity chromatography column (Qiagen), washed with 100 volumes of buffer A containing 50 mM imidazole, and eluted with four 500-μl volumes of buffer A containing 1 M imidazole. Elution fractions 2 and 3 each contained ~1 mg of protein as determined by the Schaffner-Weissmann filter assay (21). Protein purity was established by SDS-polyacrylamide gel electrophoresis (Fig. 1).

AQP1 Reconstitution into Proteoliposomes—Purified AQP1 proteins were reconstituted into proteoliposomes by dialysis. The reconstitution mixture was prepared at room temperature by sequentially adding 100 mM MOPS-Na, pH 7.5 (Fluka, Buchs, Switzerland), 1.25% (w/v) octylglucoside, purified AQP1 (a final concentration of 0.5–1 mg/ml), and 20–50 mg/ml of preformed *Escherichia coli* polar phospholipid vesicles. To prepare these vesicles, a chloroform solution of *E. coli* total lipid extract (acetone/ether preparation, Avanti Polar Lipids, Alabaster, AL) was added to a round bottom flask, and the solvent was removed by evaporation. The thin lipid film was vortex mixed for 3 min with 2 mM β-mercaptoethanol, 100 mM MOPS-Na, pH 7.5. Large unilamellar vesicles were prepared by extrusion using the small volume apparatus LiposoFast (Avestin, Inc., Ottawa, Canada) with filters of 100 nm pore diameter (22). The reconstitution mixture was loaded into SPECTRA/POR 2.1 dialysis tubing, molecular mass cut-off 15,000 (Spectrum Laboratories, Laguna Hills, CA), and dialyzed against 100 volumes of assay buffer (50 mM MOPS, 100 mM NaCl, 0.3 μM $CaCl_2$ (Merck), adjusted to pH 7.5 with HCl) for 48 h at room temperature. Proteoliposomes were harvested by centrifugation (60 min at 100,000 × *g*) and were resuspended into assay buffer at a concentration of 5–10 mg/ml.

Planar Lipid Bilayers—Employing liposomes or AQP1 proteoliposomes, planar lipid bilayers were prepared without or with AQP1 protein. The technique has recently been used for the reconstitution of aquaporin Z, the water channel protein from *E. coli* (23). It is based on spontaneous monolayer formation at the air-water interface of vesicle suspensions (24, 25). Two such monolayers were combined in the 150-μm-diameter aperture in a 10-μm-thick polytetrafluoroethylene septum separating the two aqueous phases of the chamber. The septum was pretreated with a hexadecane-hexane mixture (volume ratio of 1:200). By raising the water levels in both compartments above the aperture, the two monolayers covered the polytetrafluoroethylene septum forming a bilayer within the aperture (26), similar to the bilayer formation from solvent-spread monolayers (27). The bathing assay buffer was agitated by magnetic stirrer bars. For experiments at an acidic pH, 50 mM MES was added.

Membrane Water Permeability—Transmembrane water flux leads to solute concentration changes in the immediate vicinity of the membrane. Water passing through the membrane dilutes the solution it enters and concentrates the solution it leaves (1). The thickness δ of the stagnant water layer responsible for the polarization effect is defined in terms of the concentration gradient at the membrane water interface (28) in Equation 1

$$\frac{|C_s - C_b|}{\delta} = \left.\frac{\partial C_b}{\partial x}\right|_{x=0} \qquad \text{(Eq. 1)}$$

where x is the distance from the membrane. C_b and C_s denote the solute concentrations in the bulk and at the interface, respectively. Within the unstirred layer adjacent to the membrane ($-\delta < x < \delta$), the solute concentration C is an exponential function of the distance x to the membrane (29) as seen in Equation 2

$$C(x) = C_s \exp\left(\frac{-vx}{D} + \frac{ax^3}{3D}\right) \qquad \text{(Eq. 2)}$$

where D, v, and a are the diffusion coefficient, the linear velocity of the osmotic volume flow, and the stirring parameter, respectively. With the knowledge of v, the transmembrane water permeability P_f can be calculated as shown in Equation 3

$$P_f = \frac{v}{C_{osm} V_W} \qquad \text{(Eq. 3)}$$

where V_w is the partial molar volume of water, and C_{osm} is the near-membrane concentration of the solute used to establish the transmembrane osmotic pressure difference. If the polarization of membrane-impermeable sodium ions is used to determine water flux, the Equation 4 can be used to correct the urea bulk concentration, $C_{b:urea}$, for the dilution by water flux (*i.e.* to obtain C_{osm}) because the diffusion coefficients of the osmolyte urea and sodium are very close (30).

$$C_{osm}/C_{b:urea} = C_s/C_b \qquad \text{(Eq. 4)}$$

The Na^+-sensitive electrodes were made of glass capillaries containing mixture A of Sodium Ionophore II (Fluka) according to the procedure described by Amman (31). Their tips had a diameter of approximately 1–2 μm. Electrodes with a 90% rise time below 0.6 s were selected. Artifacts attributed to very slow electrode movements are improbable, yet the possible effects of time resolution or the distortion of the unstirred layer were tested by taking measurements while moving the microelectrode toward and away from the bilayer. Because no hysteresis was found, it can be assumed that an electrode of appropriate time resolution was driven without any effect on the unstirred layer. The osmotic gradient was induced by urea (Laborchemie Apolda, Apolda, Germany) added to the *trans*-side of the membrane only.

The experimental arrangement was similar to the one described previously (32, 33). Voltage sampling was performed by an electrometer (Model 617, Keithley Instruments, Inc., Cleveland, Ohio) connected to a personal computer. Continuous motion of the microelectrode perpendicular to the surface of the lipid bilayer was handled by a hydraulic microdrive manipulator (Narishige, Tokyo, Japan). Touching the membrane was indicated by a steep potential change. Because the velocity of the electrode motion was known (1 μm s^{-1}), the position of the Na^+ sensor relative to the membrane could be determined at any instant of the experiment. The accuracy of the distance measurements was estimated to be ± 5 μm.

AQP1 Ion Conductance—Under voltage clamp conditions, the transmembrane current was measured by a patch clamp amplifier (model EPC9, HEKA, Germany). To monitor the current across single ion channels, the sampling frequency of the patch clamp amplifier was fixed at 0.5 kHz. The recording filter was a 4-pole Bessel with a 3-db

corner frequency of 0.1 kHz. The acquired raw data were analyzed with the help of the TAC software package (Bruxton Corporation, Seattle, WA). Gaussian filters between 7 and 37 Hz were applied to reduce noise.

RESULTS

Water and Ion Permeability of Human Red Cell AQP1—Planar lipid bilayers were formed from protein-free liposomes or from proteoliposomes containing purified red cell AQP1. These membranes were stable for hours permitting steady state water permeability measurements in the presence of an osmotic gradient. The determination of Na^+ concentration profiles (Fig. 2*A*) revealed that diffusion polarization adjacent to planar lipid bilayers is increased when AQP1 is present. In a representative experiment (Fig. 2*A*), the coefficient of osmotic water permeability (P_f) rose from 23 μm/s at a base line to 50 μm/s with AQP1. This permitted calculation of the number of water channels (n) from the measured membrane water permeability (P_f) and the known single channel water permeability ($p_f = 6–11 \times 10^{-14}$ cm^3/subunit/s (34, 35)).

$$n = P_f A/p_f = 0.0035 \text{ cm/s} \times (0.0075 \text{ cm})^2/(6 \times 10^{-14} \text{ cm}^3/\text{s}) = 10^7 \quad \text{(Eq. 5)}$$

A confirmation that the increase in water permeability occurs through AQP1 was achieved with p-chloromethylbenzenesulfonate (pCMBS), a known inhibitor of water channel proteins (36), which reduced the membrane water permeability to base line.

The planar bilayers containing AQP1 exhibited no detectable membrane currents, however, the incubation with cGMP for at least 5 min induced discrete ion conductances (Fig. 2*B*). Similar conductivities were induced by the addition of 8-Br-cGMP (Fig. 2*B*), whereas the addition of cAMP was without effect (data not shown). The number of ion channels was apparent from the number of steps in the record of the transmembrane current. Only one ion channel was usually open at a time, approximately 1 million-fold below the number of water channels that are constitutively open. The impact of the rare ion channel opening on water permeability was demonstrated to be negligible (data not shown). Moreover, although water permeability was fully inhibited by pCMBS, ion conductance was not inhibited (Fig. 2*B*). Thus, the permeation pathways for water and ions are not identical.

Three different single channel conducting levels (2, 6, and 10 pS) were distinguishable in 100 mM NaCl (Fig. 3). Because the conductivity sublevels of cyclic nucleotide-gated ion channels are believed to represent differences in the occupancy of four identical cGMP binding sites of the channel (37), it may be inferred that the ion-conducting unit is the AQP1 tetramer. Despite the huge number of functionally active AQP1 water channels in the planar bilayers, the base-line current across the membrane did not exceed one pA, even when the membrane was clamped at 100 mV and the single channel currents were linearly related to the voltage applied.

Water and Ion Permeability of Heterologously Expressed Human AQP1—The discrepancy between the tiny number of ion channels and the huge number of water channels raised the question of whether ion conductance was mediated by AQP1 itself or by a contaminant, which remained after purification from red cells. To address this question, poly-histidine-tagged AQP1 was expressed in yeast. Upon reconstitution into planar bilayers, recombinant human AQP1 with the poly-histidine tag at either the N terminus (data not shown) or the C terminus facilitated water transport as efficiently as the native water channel protein (Fig. 4*A*). When incubated with cGMP for 5 min, planar bilayers containing poly-histidine-tagged AQP1 exhibited ion conductances that were indistinguishable from the preparations containing the native AQP1 protein. Moreover, the cGMP-mediated ion channel activation is reversible, because competitive dose-dependent inhibition was achieved with Rp-8-Br-cGMP (Fig. 4*B*). Low concentrations of the cyclic nucleotide inhibitor dramatically reduced the probability that the channel is in the open state. High concentrations of the agent completely inhibited the channel activity.

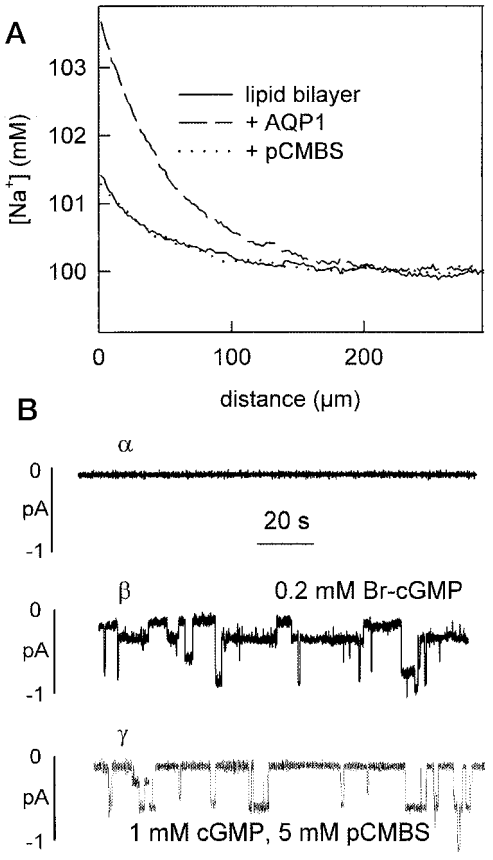

FIG. 2. **Water permeability and ion conductivities of planar bilayers reconstituted with purified human red cell AQP1.** *A,* cation polarization was measured by Na^+-sensitive electrodes at the indicated distances from planar lipid bilayers. Osmotic water permeability was determined in response to a 1-M urea gradient at pH 7.5, plain lipid bilayers (P_f = 23 μm/s), bilayers reconstituted with AQP1 without treatment (P_f = 50 μm/s), bilayers reconstituted with AQP1 and treated with 1 mM pCMBS (P_f = 23 μm/s). *B,* ion conductance measurements were performed on planar bilayers containing AQP1. Without the addition of nucleotide, the membrane was electrically silent (α), whereas ion conductances were observed after the addition of 0.2 mM 8-Br-cGMP (β) or 1 mM cGMP followed by 5 mM pCMBS (γ). The membranes were clamped at −60 mV. To reduce noise, a Gaussian filter of 24 Hz was applied.

Ion Selectivity and Mechanism of Activation—The conductances observed with AQP1 were compared with conductances of known cGMP-gated channels. cGMP activated K^+ and Na^+ conductance equivalently in planar bilayers containing AQP1. The open time of the channel increases with the cGMP concentration and the incubation time. Within minutes of the addition of 10 μM cGMP, the channel opened for short intervals (milliseconds to a few seconds), whereas the addition of 100 μM cGMP increased the open time to nearly 1 min (Fig. 5, *top*). After incubation for 20–30 min, channel lifetimes of several minutes were observed (data not shown). Moreover, in 100 mM KCl, all three conducting states were observed, and their conductances were equivalent to those measured in 100 mM NaCl (compared with Fig. 3).

Cation selectivity of the channel was derived from the reversal potential, −29 mV in the applied 4-fold KCl gradient (Fig. 5, *bottom*). Potentials are given with regard to the hyperos-

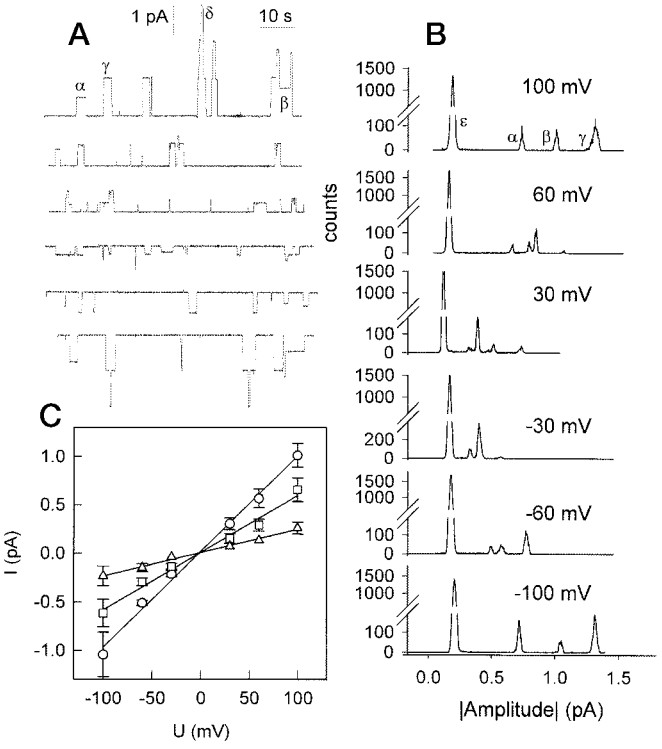

FIG. 3. **Ion conductivities induced by 1 mM cGMP in planar bilayers containing AQP1.** The membranes are confirmed to have increased water permeability (P_f = 55 μm/s, data not shown). *A,* representative serial experimental records are shown with the current across planar membranes clamped at 100, 60, 30, −30, −60, and −100 mV (from *top* to *bottom*). Although more than one channel was seldom open, an instance where three channels were simultaneously open is indicated by the letter δ. *B,* histograms from nine independent experiments reveal current voltage characteristics of three different single channel conductance levels (marked as α, β, and γ). The conductivity measured between the openings is identical to the conductivity of the lipid bilayer (ε). *C,* the amplitudes of each of the three conductance states follow a linear relationship with the applied voltage from −100 to +100 mV. To reduce noise, a Gaussian filter of 7 Hz was applied.

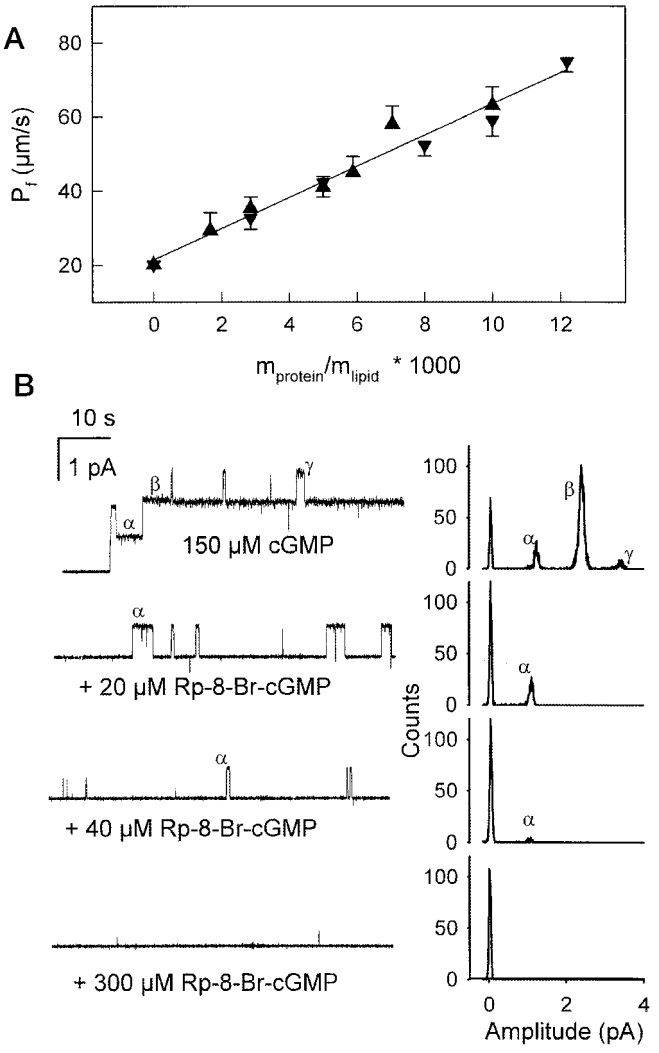

FIG. 4. **Water permeability and ion conductivities of purified recombinant human AQP1 expressed in yeast.** *A,* the membrane water permeability of planar bilayers containing recombinant human AQP1 with C-terminal 6-histidine tag (▼) was identical to bilayers containing AQP1 purified from human red blood cells (▲). *B,* planar lipid bilayers containing recombinant human AQP1 with N-terminal 10-histidine tag exhibited ion channel activity when incubated for 20 min in 150 μM cGMP. Up to three channels (α, β, γ) opened simultaneously. Subsequent incubation in the indicated concentrations of Rp-8-Br-cGMP caused a reduction in the number of active ion channels, channel lifetime, and open probability until full inhibition was achieved. The current *tracings* are shown on the *left*, and *histograms* are shown on the *right*. To reduce noise, a Gaussian filter of 17 Hz was applied.

motic solution kept electrically at ground. Permeability ratios (P_{K^+}/P_{Cl^-}) can be calculated by fitting the reversal potentials to the Goldman-Hodgkin-Katz Equation (38)

$$\frac{P_{K^+}}{P_{Cl^-}} = \frac{[C_{Cl^-}]_v - [C_{Cl^-}]_0 \exp(\varphi_{rev}F/RT)}{[C_{K^+}]_v \exp(\varphi_{rev}F/RT) - [C_{K^+}]_0} \quad \text{(Eq. 6)}$$

where C denotes concentrations on the applied potential (v) and ground (0) sides of the membrane, F is Faraday's constant, and RT is thermal energy. The conductivity ratio P_{K^+}/P_{Cl^-} for all three conductance levels of planar bilayers containing AQP1 was ~12, demonstrating cation selectivity.

Cyclic nucleotide-gated channels are usually activated by ligand binding to a domain at the C terminus. A sequence with similarities to the cGMP binding motif was described in the C terminus of AQP1 (19), so a recombinant AQP1 protein lacking the C terminus was engineered by introducing a stop codon at position 237 (39). When reconstituted into planar lipid bilayers, the truncated protein increased the water permeability of planar membranes to 56 μm/s, equivalent to that achieved with full-length AQP1 at the same protein-to-lipid ratio (Fig. 6*A*). In contrast, bilayers containing the truncated protein did not exhibit detectable cGMP-induced ion channel activity (Fig. 6*B*). Thus, cGMP-induced ion conductance was eliminated without affecting water permeability.

DISCUSSION

Our studies demonstrate that the osmotic water permeability of AQP1 can be measured when the protein is reconstituted into planar lipid bilayers. Using this technique (40, 41), it was possible to quantify water transport, because it alters the Na^+ concentration in the immediate vicinity of the lipid bilayer (Fig. 2). Moreover, this process is inhibited by pCMBS, a known inhibitor of AQP1 and other aquaporin water channel proteins (36) that interacts with a specific cysteine in a pore-lining domain (42). Using the known single AQP1 subunit water permeability (34, 35), it was possible to estimate that from 10^6 to 10^7 functional AQP1 proteins were reconstituted into the planar membrane of 150-μm diameter. Although this is a very large number of individual molecules, the well understood behavior of AQP1 protein isolated from human red blood cells (43) or expressed and purified from yeast (44) has permitted detailed biophysical studies of the water permeability (34) and the atomic structure of the molecule (14). Moreover, the density of AQP1 in the aperture, up to 125 AQP1 tetramers/μm^2, is

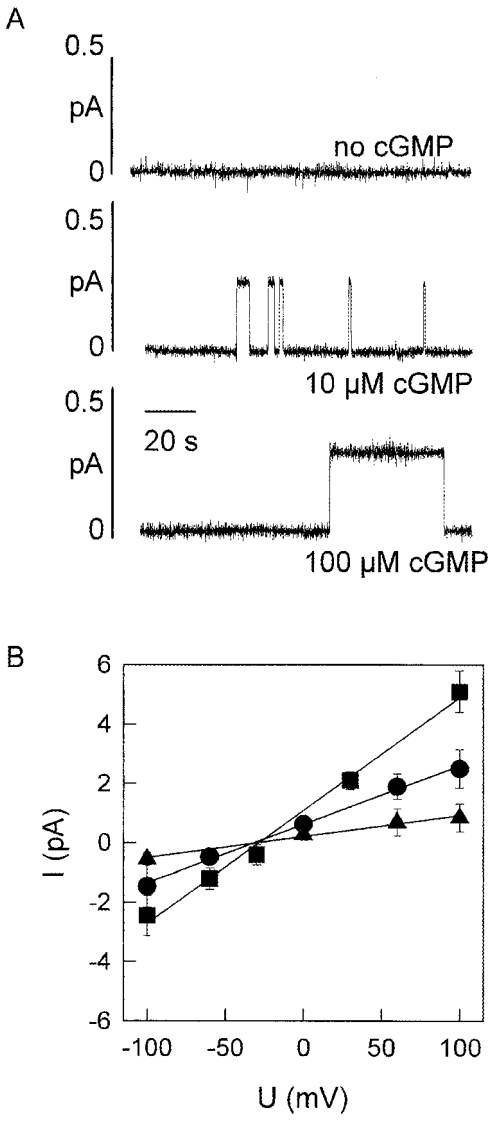

FIG. 5. **K^+ conductance of a planar lipid bilayer containing purified recombinant human AQP1 with C-terminal 6-histidine tag.** *A,* ion channel activity was not detected (*top*) until a single channel was activated with 10 μM cGMP (*middle*) and the open state was prolonged with 100 μM cGMP (*bottom*). Single channel K^+ conductivity of all sublevels did not differ from measurements with Na^+. *B,* to measure cation selectivity, a transmembrane KCl concentration gradient of 400–100 mM yielded a reversal potential (−29 mV) corresponding to a 12-fold preference of cations over anions. Potentials are given for the hyperosmotic solution; the hypoosmotic was kept electrically at ground. To reduce noise, a Gaussian filter of 17 Hz was applied.

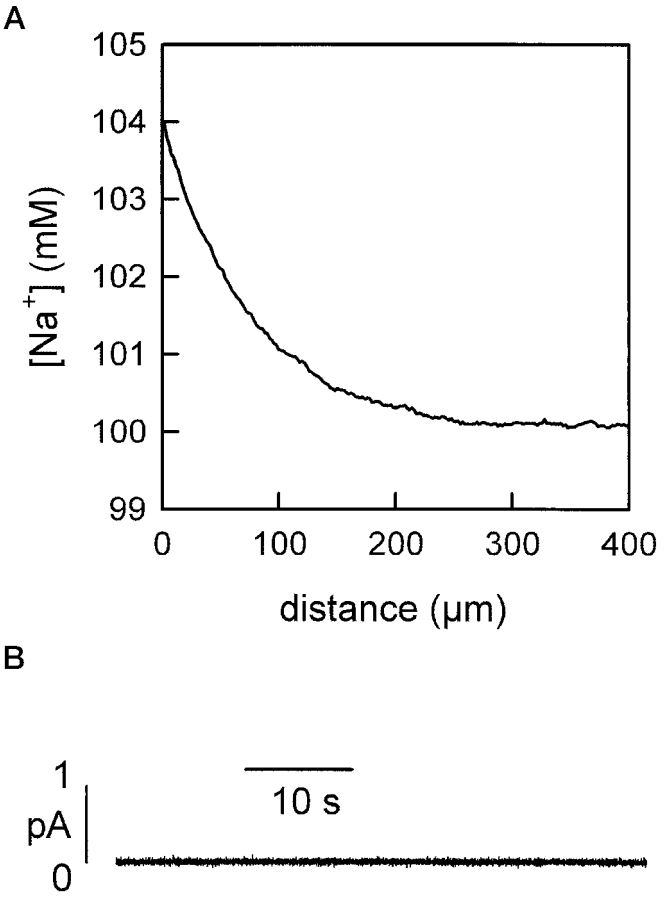

FIG. 6. **Truncated recombinant AQP1 lacking the C terminus retains water permeability but not ion conductivity.** *A,* the membrane water permeability (56 μm/s) calculated from the Na^+ concentration profile is equivalent to that of AQP1 purified from human red cells measured at the same protein-to-lipid ratio (1:100). *B,* ion conductivity could not be induced with 120 μM cGMP.

below the density of the protein in native red cell membranes, ~325 AGP1 tetramers/μm^2 (45), and is far below the maximum density achieved during preparation of AQP1 membrane crystals, $\sim 2 \times 10^4$ AQP1 tetramers/μm^2 (46).

Measuring the single channel water permeability of AQP1 proteins reconstituted into planar lipid bilayers is relatively labor-intensive and only semi-quantitative, but it permits the simultaneous measurement of any membrane currents. This approach provided an opportunity to evaluate the report that AQP1 expressed in *X. laevis* oocytes will conduct cations when activated by cGMP (19). Our studies have qualitatively confirmed this finding and the possibility that this represents an extraneous molecule that seems improbable, because heterologously expressed human AQP1 containing either N-terminal or C-terminal poly-histidine tags yielded identical behavior. Moreover, the ion conductances were induced by cGMP, and the stable analog 8-Br-cGMP but not cAMP, and the protein failed to respond when the putative cGMP binding motif in the C terminus was deleted (Fig. 6).

Several differences were noted between the ion conductances studied by patch clamp recordings of AQP1 expressed in oocytes (19) and our studies of AQP1 in planar bilayers. The cross-sectional areas of conductance differ by an order of magnitude, 150 pS in oocytes (in 150 mM NaCl) *versus* 10 pS in planar bilayers (in 100 mM NaCl). The oocytes exhibited a single conductance level, whereas AQP1 in planar bilayers exhibited three conductance states, a characteristic of cyclic nucleotide-gated channels (37). The qualitative differences have also been reported for other proteins such as Na^+ channels when studies of oocyte membranes were compared with planar bilayers. For example, some of the differences in Na^+ channel behavior in oocytes and planar bilayers has been ascribed to direct associations with short actin filaments (47), but this is unlikely to be the case for AQP1, because AQP1 was not found to be associated with the red cell membrane skeleton when analyzed biochemically (43) or by the biophysical measurements of redistribution within native membranes (48). Most striking is the astonishingly low number of ion channels compared with water channels. The previous report (19) describing cGMP-induced ion conductances of AQP1 in oocytes did not attempt to quantify the number of channels in the membrane, and it has been shown with epitope-tagged epithelial sodium channels that the open probability may range from only 0.004 to 0.014 (49), however to our knowledge, 1 million-fold discrepancy is unprecedented and may represent improper folding or degradation of a rare AQP1 protein.

The atomic structure of human AQP1 has been determined by electron crystallography (14), and the structure of the *E. coli* protein GlpF, a glycerol-transporting member of the aquaporin family, has been established by x-ray crystallography (15). The structures of AQP1 and GlpF are highly related, causing the proteins to be referred to as "fraternal twins" (50), and the transmembrane pores within AQP1 and GlpF both reside in the center of the individual subunits. The site where four subunits come together always causes a finite gap among the molecules.[2] Interestingly, the high resolution structural studies of GlpF revealed an unsuspected structure resembling an ion channel at the 4-fold axis of symmetry (15). The studies of AQP1 have not yet provided the resolution necessary to discern the structure at the 4-fold axis, however, the recent success in preparing three-dimensional crystals of AQP1 may permit this resolution (51). The 4-fold axis may provide an explanation for our studies, which indicate that the water permeability and the ion conductances do not occur through the same pathway in AQP1. Although the cGMP-induced ion conductance by AQP1 is an interesting biophysical exercise, this does not clarify the importance of the finding since the number of ion channels is more than 1 million-fold lower than the number of water channels. Even in an AQP1-rich tissue such as red cells, which contain up to 5×10^4 tetramers/cell, this translates to less than one channel/cell. Thus, whereas only an extremely small subpopulation of AQP1 molecules may behave as ion channels, this contrasts with a far more durable property of AQP1, the ability to serve as water-selective channels.

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