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Reconstitution of Functional Water Channels in Liposomes Containing Purified Red Cell CHIP28 Protein†

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ABSTRACT: Water rapidly crosses the plasma membranes of red blood cells (RBCs) and renal tubules through highly specialized channels. CHIP28 is an abundant integral membrane protein in RBCs and renal tubules, and *Xenopus laevis* oocytes injected with CHIP28 RNA exhibit high osmotic water permeability, P_f [Preston et al. (1992) *Science 256*, 385–387]. Purified CHIP28 from human RBCs was reconstituted into proteoliposomes in order to establish if CHIP28 is itself the functional unit of water channels and to characterize its physiological behavior. CHIP28 proteoliposomes exhibit P_f which is up to 50-fold above that of control liposomes, but permeability to urea and protons is not increased. Like intact RBC, the P_f of CHIP28 proteoliposomes is reversibly inhibited by mercurial sulfhydryl reagents and exhibits a low Arrhenius activation energy. The magnitude of CHIP28-mediated water flux (11.7×10^{-14} cm^3/s per CHIP28) corresponds to the known P_f of intact RBCs. These results demonstrate that CHIP28 protein functions as a molecular water channel and also indicate that CHIP28 is responsible for most transmembrane water movement in RBCs.

Water slowly crosses the lipid bilayer of most cell membranes; however, in certain cell types large water flux occurs through specialized channels. These channels permit rapid RBC swelling and shrinking in response to small changes in extracellular osmolality. Reabsorption of water in mammalian renal-collecting ducts is mediated by antidiuretic hormone (ADH) responsive water channels and in mammalian proximal tubule epithelia by constitutively active water channels. Extensive biophysical studies have established that these water channels are inhibited by organic mercurial sulfhydryl reagents such as *p*-(chloromercuri)benzenesulfonate (pCMBS) and $HgCl_2$. Each of these water channels is hydrophilic, exhibiting low activation energies ($E_a < 5$ kcal/mol), a value similar to that of isotopic water diffusing through aqueous solutions (Finkelstein, 1986; Macey, 1984; Harris et al., 1991). Some transmembrane water movement occurs through glucose transporters (Fischbarg et al., 1990). However, the glucose transporter of the RBC, Glut1, cannot account for the magnitude of RBC transmembrane water flux and does not exhibit the low E_a and mercurial sensitivity that is characteristic of the hydrophilic water channels discussed above (Zhang et al., 1991; Dempster et al., 1991; Zeidel et al., 1992).

CHIP28 is an abundant channel-forming integral membrane protein of $M_r = 28\,500$ from RBC and proximal renal tubules (Denker et al., 1988; Smith & Agre, 1991); several lines of evidence indicate that CHIP28 accounts for most water flow in these tissues. The total number of CHIP28 monomers in a human RBC, 2×10^5 (Denker et al., 1988), is similar to the number of water channels in RBCs estimated by biophysical analyses, 2.7×10^5 (Solomon et al., 1983). The 28.5-kDa molecular size of the CHIP28 monomer (Preston & Agre, 1991) is similar to the 30-kDa functional unit of water channels revealed by radiation inactivation studies of proximal renal tubules (van Hoek et al., 1991). Most importantly, microinjection of *Xenopus laevis* oocytes with 0.1–10 ng of in vitro transcribed CHIP28 RNA proportionally increased the osmotic water permeability coefficient (P_f) and the amount of immunoreactive CHIP28 protein expressed; this increase in P_f was sensitive to $HgCl_2$ and exhibited an activation energy <3 kcal/mol (Preston et al., 1992). Nevertheless, it remains possible that CHIP28 induces water channel expression or regulates water channel function in oocytes. Therefore, CHIP28 may be a necessary component of the water channel but, by itself, may not be sufficient for transmembrane water movement. Purified CHIP28 was therefore reconstituted into liposomes of defined lipid composition to establish directly the role of CHIP28 in water channel function.

MATERIALS AND METHODS

Materials. Octyl glucoside (octyl β-D-glucopyranoside) was obtained from Calbiochem. Triton X-100, dithiothreitol, PMSF (phenylmethanesulfonyl fluoride), and *N*-lauroylsarcosine were from Sigma. Carboxyfluorescein was from Mo-

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lecular Probes, Junction City, OR. Anti-fluorescein antibody was prepared as described (Harris et al., 1990). Crude *Escherichia coli* lipid was from Avanti Polar Lipids, Inc., and neutral lipid was removed by acetone/ether wash (Ambudkar & Maloney, 1986a). The purified *E. coli* bulk phospholipid is composed of phosphatidylethanolamine (70%), phosphatidylglycerol (15%), and cardiolipin (15%) (Chen & Wilson, 1984). Anticoagulated normal human blood was obtained from the American Red Cross or from a volunteer.

Purification of CHIP28 Protein. Methods were adapted from Denker et al. (1988) and Smith and Agre (1991). Large-scale purification began with 100 mL of packed RBCs from a blood bank unit. RBC membranes and KI-extracted membrane vesicles were prepared as described (Bennett, 1983). The vesicles were extracted in 200 mL with 1% (w/v) *N*-lauroylsarcosine, 1 mM NH_4HCO_3, 1 mM NaN_3, 1 mM dithiothreitol, and 0.5 mM PMSF by shaking for 1 h at room temperature and pelleted by centrifugation at 31000*g* in a Beckman JA-14 rotor for 4 h at 4 °C. The pellet was washed once in 600 mL of 7.5 mM sodium phosphate, pH 7.4, and centrifuged overnight at 15000*g* at 4 °C. The pellet was solubilized by shaking for 1 h at room temperature in 300 mL of chromatography buffer (20 mM Tris-HCl, pH 7.8, 1 mM NaN_3, 1 mM dithiothreitol) also containing 3% (v/v) Triton X-100 and was spun at 30000*g* for 4 h. The supernatant was filtered through a 0.22-μm Millex GV membrane (Millipore). The material was separated into five equivalent fractions which were separately loaded onto a 4.6 × 50 mm POROS Q/H anion-exchange column (PerSeptive Biosystems, Cambridge, MA) equilibrated with chromatography buffer containing 0.1% (v/v) Triton X-100 running at 3 mL/min while attached to a Pharmacia FPLC apparatus. The column was eluted with a 12-mL gradient of 0.2–0.6 M NaCl in the same buffer while the optical absorbance was monitored at 280 nm. The major protein peaks eluted at 0.25–0.30 M NaCl, and the peaks from five separate runs were combined, diluted with 5 volumes of chromatography buffer containing 1.2% (w/v) octyl glucoside, and readsorbed to the column which was washed until the baseline absorbance was stable (approximately 25 mL). The column was eluted with a 6-mL gradient of 0–0.6 M NaCl at the same rate while 0.5-mL fractions were collected. The major peak appeared in two fractions and was comprised of CHIP28 (the 28-kDa protein itself and the 40–60-kDa glycosylated protein) when analyzed on SDS–polyacrylamide electrophoresis gels (Laemmli, 1970) stained with silver. Purified CHIP28 was stored overnight at 0 °C or frozen for several days at −80 °C prior to reconstitution.

Small-scale purifications were performed using 24 mL of packed RBCs from whole blood freshly drawn into ACD solution. Centrifugations were performed at 44000*g* for 90 min in a Beckman JA-20 rotor, but the scaled-down methods were otherwise similar to the large preparations. The *N*-lauroylsarcosine extracted, washed pellet was directly solubilized in 72 mL of chromatography buffer containing 3% (w/v) octyl glucoside by shaking for 1 h at room temperature. This was pelleted by centrifugation, filtered, and loaded onto the POROS Q/H column equilibrated with chromatography buffer containing 1.2% (w/v) octyl glucoside running at 3 mL/min. The peak from a 12-mL 0.2–0.6 M NaCl gradient was diluted into 15 mL in the same buffer (without NaCl), reapplied onto the column, and eluted with a 4-mL gradient of 0–0.6 M NaCl while 0.1-mL fractions were collected. The major peak was contained in 0.3 mL and was comprised of CHIP28.

Reconstitution into Proteoliposomes. Reconstitution was carried out in a final volume of 1 mL containing 60–90 μg of purified CHIP28 protein in chromatography buffer, 9 mg of bath-sonicated *E. coli* phospholipid, 1.25% (w/v) octyl glucoside, and 50 mM Tris-HCl (pH 7.5). The mixture was briefly blended on a vortex mixer and incubated for 20 min on ice. Proteoliposomes (or liposomes prepared without protein) were formed at room temperature by rapidly injecting the mixture into 25 mL of buffer A [50 mM MOPS, pH 7.5, 150 mM *N*-methyl-D-glucamine (NMDG) chloride also containing 10–15 mM carboxyfluorescein, 1 mM dithiothreitol, and 0.5 mM PMSF]. For the measurement of proton permeability, proteoliposomes and liposomes were loaded with buffer B (5 mM MOPS, pH 7.5, 150 mM KCl, 0.5 mM carboxyfluorescein, 1 mM dithiothreitol, and 0.5 mM PMSF). The suspension was incubated for 20 min at room temperature. Proteoliposomes or liposomes were collected by centrifugation for 1 h at 123000*g* in a Beckman Type 42.1 rotor at 4 °C. The pellet was resuspended in 8 mL of buffer A or B and then centrifuged for 1 h at 152000*g* in a Beckman Type 50 Ti rotor. Proteoliposomes or liposomes were resuspended in 300 μL of buffer A or B and stored at 4 °C for 16–24 h. Typically 50% (54 ± 6%, n = 5) of the CHIP28 protein and 70% of the phospholipid were recovered in proteoliposomes. The lipid to protein ratio was in the range of 120:1 to 219:1. These proteoliposomes have internal volumes of 1 μL/mg of phospholipid (Ambudkar & Maloney, 1986b). Protein and phospholipid were measured as described (Ambudkar & Maloney, 1986b).

Membrane Permeability Measurements. P_f was measured by exposing CHIP28 proteoliposomes or control liposomes to an osmotic gradient by rapid mixing with an equal volume of another solution containing buffer A plus sufficient sucrose to increase osmolality in the mixed solutions to a value 120% of that in the original solution. This was performed with a stopped-flow fluorometer (SF.17MV, Applied Photophysics, Leatherhead, U.K.) with a measured dead time of 0.7 ms. The excitation wavelength was set at 490 ± 1.5 nm using a 150-W mercury–xenon arc lamp and monochromator (f/3.4 grating monochromator, both from Applied Photophysics); the emission wavelength was >515 nm using a cut-on filter (Oriel Corp., Stratford, CT). Extravesicular carboxyfluorescein fluorescence was completely quenched using anti-fluorescein antibody (Harris et al., 1990). Within the range of osmolalities used in our experiments, the vesicles acted as perfect osmometers, and relative volume (absolute volume divided by initial volume) was linearly related to relative fluorescence (absolute fluorescence divided by initial fluorescence). The data obtained from 8 to 16 determinations was averaged and fit to single-exponential curves using software provided by Applied Photophysics (Zeidel et al., 1992). The fitting parameters were then used to determine P_f by first applying the linear conversion from relative fluorescence to relative volume and then iteratively solving the water permeability equation using MathCAD software (MathSoft, Cambridge, MA):

$$dV(t)/dt = (P_f)(SAV)(MVW)[(C_{in}/V(t)) - C_{out}] \quad (1)$$

where $V(t)$ is relative intravesicular volume as a function of time, P_f is osmotic water permeability in centimeters per second, SAV is the vesicle surface area to volume ratio, MVW is the molar volume of water (18 cm^3/mol), and C_{in} and C_{out} are the initial concentrations of total solute inside and outside the vesicle, respectively (Zeidel et al., 1992).

Measured radii of control liposomes and CHIP28 proteoliposomes were determined by electron microscopy and averaged 7.0 ± 0.7 × 10^{-6} cm. The number of CHIP28 molecules per milliliter of suspension was calculated from the amount of protein per milliliter in each preparation and the molecular weight of CHIP28 (28 500). The measured total entrapped volume per milligram of phospholipid, the calculated volume of each liposome, and the total number of liposomes per milliliter of suspension were calculated from the total phospholipid content. Dividing the number of protein molecules per milliliter of suspension by the number of proteoliposomes per milliliter of suspension gave the number of protein molecules per proteoliposome. Note that if the CHIP28 functional unit is a dimer or tetramer (Smith & Agre, 1991), the permeability of each functional unit (p_f) is double or quadruple the value calculated below. Correspondingly, the number of functional CHIP28 units/RBC is reduced by one-half or one-quarter, respectively. These changes do not alter the P_f of the RBC predicted if CHIP28 is the water channel.

Urea permeability was determined by monitoring the relative fluorescence of entrapped carboxyfluorescein during the urea efflux. Solutions inside and outside the vesicle were of equal osmolality but differed in the concentrations of permeant and impermeant solutes (Grossman et al., 1992). Thus, internal urea concentration was 374 mM and external urea concentration was 187 mM with the remaining osmolality balanced by 187 mM sucrose. Under these conditions, the permeant solute effluxes down its concentration gradient from inside to outside the vesicles; this flux creates an osmotic gradient, leading to efflux of water, increased carboxyfluorescein concentration within the vesicles, and additional carboxyfluorescein self-quenching. The general formula defining solute exit from a vesicle is

$$J_{urea} = d(urea)/dt = P_{urea}(SA)\Delta C \quad (2)$$

where J_{urea} is urea flux, P_{urea} is the permeability coefficient for urea, SA is the surface area of the vesicle, and ΔC is the difference in urea concentration between the inside and outside of the vesicle, which diminishes as the system reaches equilibrium. If

$$V_{rel} = V(t)/V_0 \quad (3)$$

where V_0 is the initial volume of the vesicle and $V(t)$ is the absolute volume at time t, then, under our conditions, it can be shown that

$$dV_{rel}/dt = P_{urea}[SA/V_0][0.00134][(1122/V_{rel}) - 1496] \quad (4)$$

Using parameters from the single-exponential curve fit to the data and the relationship between relative volume and relative fluorescence, eq 4 was solved for P_{urea} using MathCAD.

Proton permeability was measured in vesicles containing 150 mM KCl and 5 mM MOPS, incorporating 1 μM valinomycin to prevent accumulation of protons from generating a positive intravesicular voltage. Vesicles at pH 7.40 were abruptly exposed to a solution of pH 6.80, and the time course of the fall in intravesicular pH was followed on the stopped-flow device by monitoring the quenching by protons of 0.5 mM entrapped carboxyfluorescein. Over the pH range employed, fluorescence was linearly related to pH; extravesicular fluorescence was quenched with anti-fluorescein antibody. To ensure that increases in P_{H^+} caused by CHIP28 could have been detected, 1 μM gramicidin A was added and increased P_{H^+} by over 50-fold in each experiment. The ethanol vehicle used for valinomycin and gramicidin did not alter the high P_f of CHIP28 proteoliposomes (Harris et al., 1990).

RESULTS AND DISCUSSION

E. coli bulk phospholipid was employed since it has previously been used for functional reconstitution of several transport proteins including the glucose transporter of RBCs (Maloney & Ambudkar, 1989; Chen et al., 1986). Highly purified CHIP28 protein was incorporated into proteoliposomes by detergent dilution; control liposomes were formed in an identical manner without CHIP28 protein. Analysis of the CHIP28 proteoliposomes in silver-stained SDS–polyacrylamide gels demonstrated the 28-kDa CHIP28 and 40–60-kDa N-glycosylated CHIP to be approximately 98% pure; a minor contaminant of 24 kDa is a CHIP28 degradation product (Figure 1A).

The coefficient of osmotic water permeability, P_f, was determined by rapidly increasing the extravesicular osmolality in a stopped-flow fluorometer. Water efflux from the vesicles reduced the intravesicular volume, increasing the self-quenching of the carboxyfluorescein (see Materials and Methods). The P_f was markedly increased in CHIP28 proteoliposomes [P_f = 0.054 ± 0.008 (SD) cm/s at 37 °C] as compared with control liposomes (0.0097 ± 0.004 cm/s; Figure 1B,C). Similar results were obtained from all three proteoliposome preparations, despite differences in RBC source and purification procedures; protein and phospholipid determinations revealed that two independent preparations contained approximately 220 CHIP28 monomers per proteoliposome (211 and 233 on two measurements; see Materials and Methods). Therefore, the osmotic water permeability of each protein molecule (p_f) was 11.7 ± 1.8 × 10^{-14} cm^3/s. This value exceeds the p_f values obtained for the gramicidin water and proton channel [(3–6) × 10^{-14} cm^3/s; Finkelstein, 1986]. On the basis of the calculated CHIP28 p_f, the CHIP28-mediated osmotic water permeability of a human RBC was estimated [0.017 cm/s = [(11.7 × 10^{-14} cm^3/s per CHIP28) × (2 × 10^5 CHIP28 monomers/RBC)]/(1.4 × 10^{-6} cm^2/RBC, the surface area of the human RBC)]. This value is in agreement with the observed P_f of human RBCs (0.02 cm/s; Finkelstein, 1986; Macey, 1984), suggesting that nearly all CHIP28 proteins in the proteoliposomes are functional, regardless of their topological orientation. In addition, the ability of CHIP28 to conduct water does not appear to require the specialized lipid composition or bilayer structure of the RBC.

When P_f measurements in CHIP28 proteoliposomes or control liposomes were performed at varying temperatures, Arrhenius activation energies (E_a) of 3.1 ± 0.5 and 16.0 ± 1.1 kcal/mol, respectively, were obtained (Figure 2B). The low E_a exhibited by CHIP28 proteoliposomes agrees well with values reported for intact RBC and RBC membranes (3.2–4.1 kcal/mol; Finkelstein, 1986; Macey, 1984; Zeidel et al., 1992). Both 1 mM pCMBS, the organic mercurial, and 1 mM $HgCl_2$ markedly inhibited P_f in CHIP28 proteoliposomes, reducing these values to 0.009 ± 0.005 cm/s for pCMBS and 0.007 ± 0.003 cm/s for $HgCl_2$. These values are similar to those obtained in control liposomes (Figure 2A). Inhibition by pCMBS was partial at 5 and 10 min and complete at 20–30 min at 37 °C. By contrast, $HgCl_2$ inhibition was complete within 5 min. Neither pCMBS nor $HgCl_2$ had any effect on the P_f of control liposomes. Inhibition by both mercurial reagents was completely reversed by treatment with the sulfhydryl reagent, β-mercaptoethanol (5 mM). The time courses for inhibition of P_f and reversal of inhibition in CHIP28 proteoliposomes both resemble the kinetics for intact RBC (Macey, 1984; Zeidel et al., 1992).

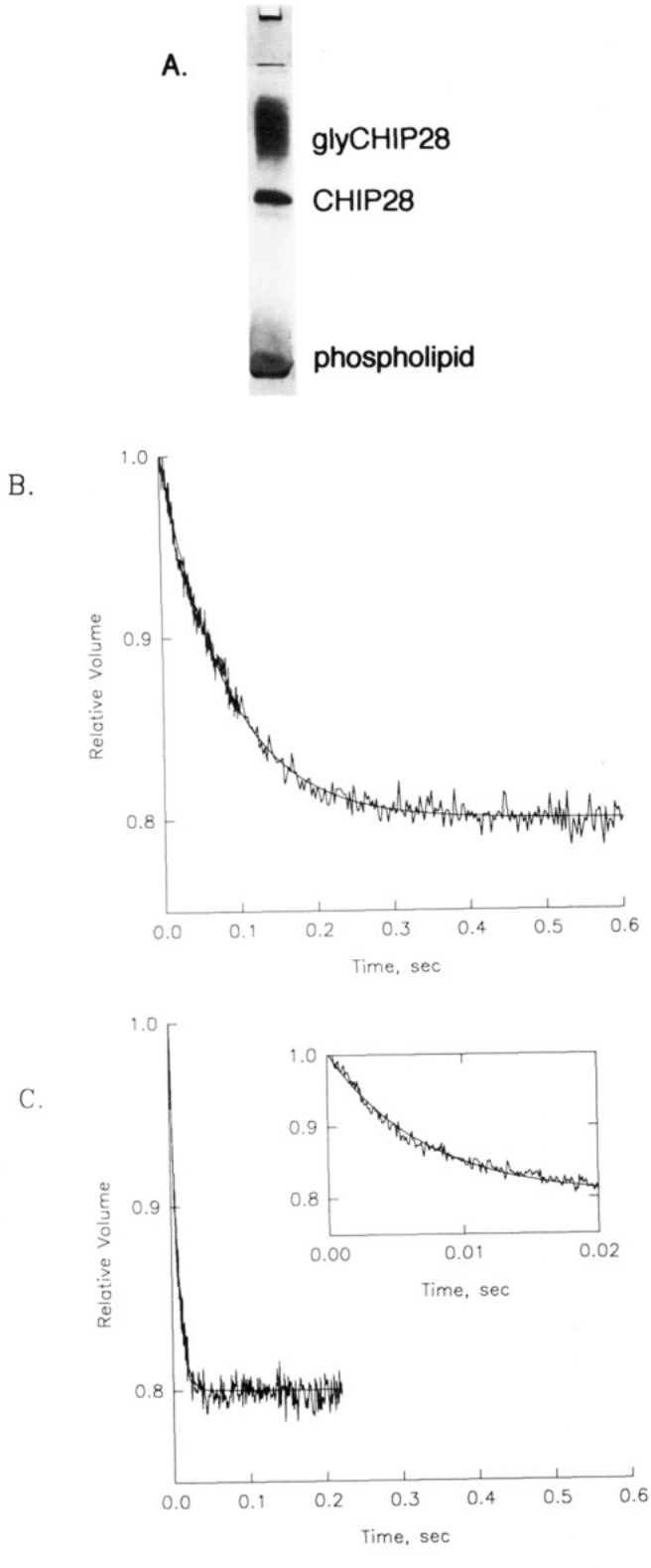

FIGURE 1: Panel A: Silver-stained 12% SDS–polyacrylamide gel electrophoresis slab (Laemmli, 1970) of proteoliposomes containing approximately 1 μg of purified CHIP28 protein. Panel B: Water flux in control liposomes. Liposomes loaded with carboxyfluorescein (CF) were abruptly exposed to a 20% increase in extravesicular osmolality in a stopped-flow device, and the resulting increase in quenching of fluorescence was monitored. Relative volume was calculated from relative fluorescence and is shown here as a function of time. Data and fitted single exponentials are shown. Curves shown in all figures are representative of similar results obtained in three separate CHIP28 proteoliposome and control liposome preparations. Panel C: Water flux in CHIP28 proteoliposomes containing, on average, 211–233 protein molecules per proteoliposome. The inset shows an expanded initial time scale.

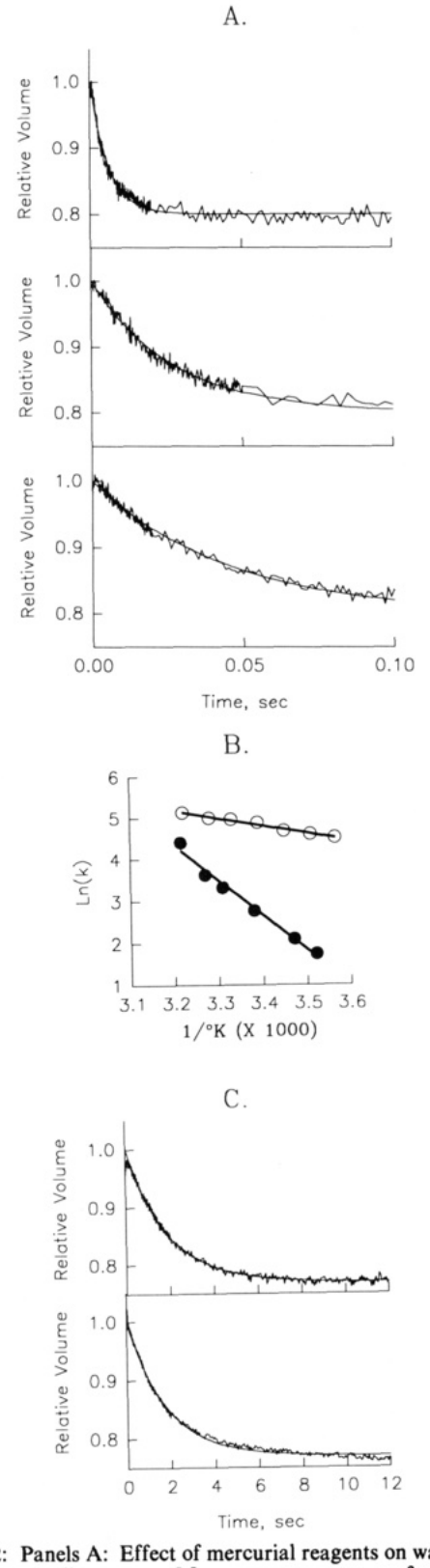

FIGURE 2: Panels A: Effect of mercurial reagents on water flux in CHIP28 proteoliposomes. Measurements were performed as in Figure 1. Top panel: Untreated CHIP28 proteoliposomes. Middle panel: Effect of 1 mM pCMBS for 25 min at 37 °C. Bottom panel: Effect of 1 mM $HgCl_2$ for 3 min at 37 °C. Panel B: Activation energies of water flow in control liposomes (filled circles) and CHIP28 proteoliposomes (open circles). Panels C: Urea fluxes in control liposomes (top panel) and CHIP28 proteoliposomes (bottom panel).

Early studies of water and small nonelectrolyte permeabilities of RBC suggested that small nonelectrolytes may cross the membrane via the water channel (Goldstein & Solomon, 1960). Phloretin, however, inhibits urea flux in human RBC

without altering water flux. Moreover, water and urea transport can be dissociated in phylogeny. For example, duck RBCs exhibit high water and low urea permeabilities, whereas amphiuma RBCs exhibit low water and high urea permeabilities (Finkelstein, 1986; Macey, 1984). To determine the selectivity of the CHIP28 water channel, vesicles were loaded with urea at a level similar to the measured K_m for urea transport in RBCs and were abruptly mixed with solutions of identical osmolality containing half the urea concentration (Grossman et al., 1992). As the urea diffused down its concentration gradient, water efflux occurred, leading to further self-quenching of CF fluorescence. Unlike water permeability, the presence of functional CHIP28 did not augment urea permeability (Figure 2C). Thus, the CHIP28 water channel excludes urea.

Although the gramicidin channel conducts water and protons rapidly, inhibitor studies in intact RBC suggest that the RBC water channel does not conduct protons (Finkelstein, 1986; Macey, 1984). To test this in CHIP28 proteoliposomes, we abruptly lowered extravesicular pH from 7.42 to 6.80 and monitored the rate of proton flux (Harris et al., 1990). The proton permeability, P_{H^+}, was comparable in CHIP28 proteoliposomes (0.0068 ± 0.0003 cm/s at 37 °C) and control liposomes (0.0063 ± 0.0004 cm/s), indicating that the CHIP28 channel does not conduct protons.

Despite an abundance of biophysical data, the constitutive water channels of RBC and renal proximal tubule as well as the ADH-responsive water channels of distal nephron and toad urinary bladder have remained poorly characterized at molecular levels. The CHIP28 protein was shown to have a tetrameric structure similar to that of multisubunit membrane channels (Smith & Agre, 1991), and water channels were found in *X. laevis* oocytes after injection with CHIP28 RNA (Preston et al., 1992). This report describes the first successful reconstitution of functional water channels, by incorporation of purified CHIP28 protein into synthetic lipid bilayers of defined composition. Proteoliposomes containing pure CHIP28 exhibit osmotic water permeability which is nearly identical to that of water channels in native RBC membranes, including the magnitude of P_f, the selectivity for water molecules, the sensitivity to inhibitors, and the Arrhenius activation energy. The fact that this protein can itself reconstitute water channel function indicates that the functional component of the water channel in intact cells is likely composed only of CHIP28 molecules and that other proteins are not necessary for water channel function. Taken together, all information indicates that CHIP28 functions as the predominant water channel of RBCs and proximal renal tubules.

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