Water Transporting Properties of Hepatocyte Basolateral and Canalicular Plasma Membrane Domains*

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Previous work from our laboratory supports an important role for aquaporins (AQP), a family of water channel proteins, in bile secretion by hepatocytes. To further define the pathways and molecular mechanisms for water movement across hepatocytes, we directly assessed osmotic water permeability ($P_w$) and activation energy (E$_a$) in highly purified, rat hepatocytes basolateral membrane vesicles (BLMV) and canalicular membrane (CMV) vesicles by measuring scattered light intensity using stopped-flow spectrophotometry. The time course of scattered light for BLMV and CMV fit well to a single-exponential function. In BLMV, E$_a$ was $108 \pm 4$ micros$^{-1}$ ($25^\circ$C) with an E$_s$ of 7.7 kcal/mol; in CMV, E$_a$ was $86 \pm 5$ micros$^{-1}$ ($25^\circ$C) with an E$_s$ of 8.0 kcal/mol. The AQP blocker, dimethyl sulfoxide, significantly inhibited the $P_w$ of both basolateral (81 $\pm 4$ micros$^{-1}$; $25^\circ$C) and canalicular (59 $\pm 4$ micros$^{-1}$; $30^\circ$C) membrane vesicles. When CMV were isolated from hepatocytes treated with dibutyryl cAMP, a double-exponential fit was needed, implying two functionally different vesicle populations; one population had $P_w$ and E$_s$ values similar to those of CMV from untreated hepatocytes, but the other population had a very high $P_w$ ($655 \pm 135$ micros$^{-1}$, $25^\circ$C) and very low E$_s$ (2.8 kcal/mol). Dimethyl sulfoxide completely inhibited the high $P_w$ value in this second vesicle population. In contrast, $P_w$ and E$_s$ of BLMV were unaltered by cAMP treatment of hepatocytes. Our results are consistent with the presence of both lipid- and AQP-mediated pathways for basolateral and canalicular water movement across the hepatocyte plasma membrane barrier. Our data also suggest that the hepatocyte canalicular membrane domain is rate-limiting for transcellular water transport and that this domain becomes more permeable to water when hepatocytes are exposed to a choleretic agonist, presumably by insertion of AQP molecules. These data suggest a molecular mechanism for the efficient coupling of osmotically active solutes and water transport during canalicular bile formation.

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Water can cross cellular plasma membranes through the lipid portion of the bilayer by a solubility diffusion mechanism or through aquaporins (AQP) water channels. Aquaporins are a family of integral membrane proteins that increase cell membrane water permeability facilitating the rapid movement of water in response to osmotic gradients (1–3). We and others recently reported that rat hepatocytes express three AQP's: AQP8, AQP9, and AQP0 (4–11). AQP8, which is the most abundant AQP in hepatocytes, is predominantly localized to intracellular vesicles, and its translocation to the canalicular plasma membrane is under hormonal regulation (6, 11, 12). AQP9, an aquaglyceroporin, resides exclusively on the basolateral plasma membrane of hepatocytes and may facilitate the movement of water and certain small solutes (Ref. 5 and see also Ref. 25). AQP0 is localized intracellularly, and its significance in hepatocytes is currently unclear (11). Our previous studies using isolated hepatocytes suggest that under basal (non-stimulated) conditions, water transport across hepatocyte plasma membranes occurs mainly via a non-AQP-mediated, diffusional pathway (5, 11, 13). This situation is due to the very low canalicular membrane expression of AQPs in resting, non-stimulated hepatocytes. However, upon stimulation of hepatocytes with a secretagogue (e.g. cAMP), intracellular AQP8 is redistributed to the canalicular plasma membrane, thereby increasing the cell surface water permeability (6, 11) of the secretory pole of the hepatocyte and presumably facilitating canalicular bile secretion (11). To directly define the pathways and molecular mechanisms for osmotic water transport in hepatocytes, we examined the water transport properties of the individual hepatocyte canalicular and basolateral plasma membrane domains using highly purified hepatocyte membrane vesicles. Membrane water permeability coefficients and activation energies were determined using stopped-flow spectrophotometry, a well established method that makes use of the dependence of scattered light on vesicle volume to quantitate the time course of net water flow in response to transmembrane osmotic gradients (14).

MATERIALS AND METHODS

Isolation and Treatment of Rat Hepatocytes—Highly purified (>98%) rat hepatocytes were isolated from livers of male Fisher rats as described (15). Briefly, livers were perfused with oxygenated Hepes buffer solution containing 0.02% EGTA (Sigma) to remove blood cells and then transferred to a temperature-controlled chamber at 37°C and perfused with an Hepes buffer solution containing 0.45 mg/ml Collagenase D (Roche Applied Science). Following perfusion, hepatocytes were gently removed from the biliary tree by mechanical disruption and filtered.

4 The abbreviations used are: AQP, aquaporin; AQP8, aquaporin-8 water channels; DBcAMP, dibutyryl cAMP; BLMV, basolateral membrane vesicles; CMV, canalicular membrane vesicles.
twice through 40-μm nylon mesh. Hepatocytes were purified further by isopycnic centrifugation through a discontinuous Percoll gradient (Amersham Biosciences). Viability was greater than 90% as assessed by trypan blue exclusion. Cells were suspended in Krebs-Ringer-HEPES buffer, pH 7.4, in the presence of 0 or 100 μM dibutyryl cAMP (DBcAMP) for 10 min at 37 °C, washed, and frozen until the preparation of plasma membrane fractions.

**Isolation of Canalicular and Basolateral Hepatocyte Plasma Membranes**—Canalicular and basolateral plasma membranes were prepared from freshly isolated hepatocytes as described previously by us (16, 17). Briefly, hepatocytes were washed and sonicated in 0.3 M sucrose containing 0.01% soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM leupeptin (Sigma). The plasma membrane fraction was obtained by centrifugation at 200,000 × g for 60 min on a discontinuous 1.3 M sucrose gradient. This membrane fraction was further subfractionated by high speed centrifugation through discontinuous sucrose gradients to obtain two subfractions enriched in either canalicular or basolateral hepatocyte plasma membrane domains. Hepatocytes exposed to DBcAMP and corresponding controls were processed in parallel; the protein membrane yields did not differ between groups. The purity and cross-contamination of the membranes, as assessed using canalicular and basolateral marker enzymes, were similar to those observed previously by us (11). Protein concentration was determined by the fluorescamine method using bovine serum albumin as standard (18).

**Vesicle Measurements**—To determine the size of vesicles, electron micrographs were taken on a Jeol electron microscope. Pellets of membrane vesicles were fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2), treated with 1% OsO4, and washed with the same phosphate buffer. After dehydration and embedding in Epox, thin sections were stained with uranyl and lead citrate. Size measurements were made on micrographs at ×30,000 magnification using the software Image-Pro Plus (Media Cybernetics). The software was set to select objects (i.e. vesicular structures) in the range of 50–500 nm.

**Stopped-Flow Measurements**—The time course of vesicular volume was followed from changes in intensity of scattered light at the wavelength of 450 nm using an Applied Photophysics SX.15MV stopped-flow reaction analyzer which has a 1.5-ms dead time and 99% mixing efficiency in <1 ms. The sample temperature was controlled by a circulating water bath. To perform the experiments, 50 μl of a concentrated vesicle suspension was diluted into 450 μl of 50 mM sucrose and 5 mM HEPES-Tris, pH 7.4 (buffer A). One of the syringes of the stopped-flow apparatus was filled with the membrane suspension, whereas the other was filled with the same buffer containing increasing amounts of sucrose to establish a hypertonic gradient upon mixing. The final protein concentration after mixing was 150 μg/ml. Immediately after applying a hypertonic gradient, water outflow occurs, and the vesicles shrink, causing an increase in scattered light intensity. The data were fitted to either single or double exponential functions. The osmotic water permeability coefficient ($P_{o}$) was calculated as described (19, 20), using the equation: $P_{o} = \frac{V_{o}}{C_{o}} \cdot \frac{\Delta C}{\Delta V_{o}}$, where $K_{exp}$ is the fitted exponential rate constant, $V_{o}$ is the initial mean vesicle volume, $A_{s}$ is the mean vesicle surface, $V_{m}$ is the molar volume of water, and $\Delta C$ is the osmotic gradient.

The medium osmolarity was measured by freezing point depression, using an Advanced Micro Osmometer 3300 (Advanced Instruments, Inc.). In some experiments, before the stopped-flow experiments, vesicles were incubated for 5 min with 500 mM dimethyl sulfoxide (Me2SO), an established aquaporin water channel blocker (6, 11, 21–23).

**RESULTS**

**Water Permeability of Hepatocyte Basolateral and Canalicular Membrane Vesicles**—Fig. 1 shows the electron micrographs of hepatocyte basolateral and canalicular membrane vesicles. The vesicles had a similar appearance, displaying somewhat irregular shapes and showing a lack of contamination by other hepatocyte organelles. Mean basolateral vesicle diameter was 166 ± 2 (S.E.) nm (n = 1664), and mean canalicular vesicle diameter was 159 ± 1 (S.E.) nm (n = 1757). Osmotic water permeability was measured by a stopped-flow method in which vesicles were subjected rapidly (<1 ms) to a hypertonic osmotic gradient. The time course of vesicle volume was followed from the change in scattered light. Fig. 2 shows the time course of osmotic water transport in hepatocyte basolateral and canalicular plasma membrane vesicles in response to hypertonic osmotic gradients obtained by adding sucrose to the external buffer. No change in scattered light was observed when vesicles were mixed with isosmotic buffer, showing an absence of mixing artifacts. There was a time-dependent increase in the amplitude and rate constant of the scattered light with increasing osmotic gradients (Fig. 2). Data fit well to a single exponential function for all gradients, indicating the presence of functionally homogeneous populations of basolateral and canalicular vesicles. $P_{o}$ values at 25 °C, calculated from the kinetic data and the size of the vesicles, were 115 μm s$^{-1}$ for the basolateral vesicles and 90 μm s$^{-1}$ for the canalicular vesicles. $P_{o}$ values were independent of the size of the osmotic gradient except at very high osmotic gradients (400 mosm) when there was a slight decrease in basolateral and canalicular $P_{o}$ (about 13%), possibly because of mechanical restrictions to vesicle shrinkage.

**Effect of Temperature on Water Permeability of Hepatocyte Basolateral and Canalicular Membrane Vesicles**—To examine further the mechanism of basolateral and canalicular water permeability, the activation energy ($E_{a}$) was determined from temperature dependence data. Fig. 3 shows a marked increase in the rate of osmotic water transport when vesicles were subjected to the same osmotic gradient at increasing temperatures. Fig. 3 (bottom panel) summarizes these data in the form of an Arrhenius plot; Table I shows the calculated $P_{o}$ values at different temperatures and the corresponding $E_{a}$.

**Water Permeability of Hepatocyte Basolateral and Canalicular Vesicles from DBcAMP-treated Hepatocytes**—Fig. 4 shows the typical tracings of a time course of scattered light intensity...
FIG. 2. Time course of osmotic water transport in hepatocyte basolateral and canalicular plasma membrane vesicles at 25 °C. Vesicles in buffer A (see “Materials and Methods”) were mixed with an equal volume of the same buffer containing increasing amounts of sucrose to give the indicated hypertonic gradients. Individual curves from triplicate measurements along with single exponential fits are given.

**TABLE I**

Temperature dependence of osmotic water permeability in hepatocyte basolateral and canalicular plasma membrane vesicles

| Temperature (°C) | Basolateral $P_f$ ($\mu$mol$^{-1}$s$^{-1}$) | Canalicular $P_f$ ($\mu$mol$^{-1}$s$^{-1}$) |
|------------------|---------------------------------------------|------------------------------------------|
| 37               | 179                                         | 151                                      |
| 25               | 115                                         | 91                                       |
| 12               | 67                                          | 57                                       |
| 5                | 46                                          | 41                                       |

| Activation energy (kcal/mol) |
|-----------------------------|
| 7.7                         |
| 8.0                         |

(water transport) in response to a 200 mosmol hypertonic sucrose gradient in basolateral and canalicular vesicles from hepatocytes in the basal state or treated previously with DBcAMP. The scattered light from BLMV ± exposure to DBcAMP and untreated CMV increased progressively and reached a plateau representing data that fit to a single-exponential curve function and support a rather homogeneous vesicle population. Data for basolateral vesicles showed a slow exponential increase in osmotic water transport with a $P_f$ value of $71 \pm 10 \mu$mol$^{-1}$s$^{-1}$, whereas DBcAMP treatment did not modify basolateral $P_f$. A similar $P_f$ was observed for canalicular vesicles from untreated hepatocytes ($86 \pm 5 \mu$mol$^{-1}$s$^{-1}$) (Fig. 4). In contrast, the course of scattered light from CMV + DBcAMP showed a markedly faster rise in slope and increase in initial intensity and fit to a double-exponential curve function representative of two functionally non-homogeneous vesicle populations. Indeed, when we performed calculations based only on the early portion of the time course (0–100 ms), we found a 6-fold higher $P_f$ value of $491 \pm 81 \mu$mol$^{-1}$s$^{-1}$. Together, analysis of the kinetics of water transport indicated that the fractional DBcAMP-dependent rapid component was 0.25; i.e., roughly 25% of canalicular vesicles have an 8-fold higher $P_f$ value than the rest of the vesicles. A weighted $P_f$ (Fig. 4) was calculated using the low and high $P_f$ values and their percentages of contribution to the total, i.e., weighted $P_f$ = (low $P_f \times 75 +$ high $P_f \times 25$)/100. Also, pretreatment of hepatocytes with DBcAMP markedly reduced the canalicular $E_a$ from 8.0 to 2.8 kcal/mol but had no effect on $E_a$ for basolateral vesicles. Together, these data suggest a DBcAMP-induced specific alteration in hepatocyte canalicular membranes, which, based on our previous work (11), reflects the isolation of two functionally different subpopulations of vesicles derived from the canalicular membrane, likely reflecting one with and one without newly inserted AQP8 molecules.

Effect of Me$_2$SO on the Water Permeability of Hepatocyte Basolateral and Canalicul plasma Membrane Vesicles—Fig. 5 shows the effect of the aquaporin blocker, Me$_2$SO, on the time course of osmotic water transport of hepatocyte basolateral and canalicular vesicles and the corresponding calculated $P_f$, respectively. The weighted $P_f$ was used for the DBcAMP-treated canalicular vesicles. Basolateral and canalicular $P_f$ values were inhibited by 25 and 30%, respectively, whereas DBcAMP-induced canalicular $P_f$ was completely blocked.

**DISCUSSION**

The major findings in this study relate to the properties of hepatocyte plasma membrane domains influencing transcellular water transport and the molecular mechanisms of canicular bile secretion. Using an established technique to assess biophysical properties of membranes, we found that: (i) hepatocyte canalicular and basolateral plasma membrane domains differ in their permeability coefficients but not their activation energies; (ii) an inhibition of AQPs diminished the permeability coefficients of both membrane domains; and (iii) exposure of isolated hepatocytes to a choleretic agonist (i.e., DBcAMP) increased the $P_f$ and lowered the $E_a$ of canalicular but not basolateral membrane vesicles. These data extend our previous observations that AQP8 is redistributed to the canalicular hepatocyte membrane in response to a secretagogue and demonstrate directly an agonist-induced selective increase in the $P_f$ of the canalicular membrane domain. The data support the importance of both diffusion and channel-mediated water transport in canalicular bile secretion.

To our knowledge, this is the first study to measure $P_f$ and $E_a$ in isolated hepatocyte basolateral and canalicular plasma membrane domains. Although our previous studies in single hepatocytes and in polarized hepatocyte couplets have yielded
valuable information about the mechanisms of hepatocyte water transport in these cells (6, 11), pitfalls in studying water flow across intact cells are unstirred layers, time resolution of flow measurements, and membrane area amplification. These problems can be circumvented when osmotic flows are measured across isolated membrane vesicles with stopped-flow spectrophotometry because volume flows are monitored continuously with time resolution <1 ms, and osmotic flows are related to real membrane area without interference of unstirred layers. The disadvantage of this approach is that vesicle populations may be non-homogeneous in size and composition. In this study, the size analysis based on electron microscopy and image processing showed reasonably homogeneous vesicle preparations with respect to size. Moreover, the optical signal from basolateral and canalicular vesicles fit well with one exponential function, indicating homogeneity in size and in water permeability.

Hepatocyte basolateral and canalicular \( P_f \) values were higher than those of a tissue thought to have a predominant lipid solubility diffusion mechanism for water permeation (i.e. small intestine with \( P_f \) values about 60 \( \mu \text{m} \text{s}^{-1} \)) (19), but lower

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**FIG. 3.** Temperature dependence of hepatocyte basolateral and canalicular plasma membrane osmotic water transport. The time course of scattered light intensity was measured for a 200 mosM hypertonic sucrose gradient at the indicated temperatures. Top panels, individual curves along with single exponential fits. In the bottom panel, data were plotted in the form of an Arrhenius plot. Each point is mean ± S.E. for triplicate measurements.
than a tissue with very high flow, AQP-mediated water transport (i.e. kidney cortex with $P_f$ values of 300–600 μm/s) (19, 24). The $E_a$ is generally $>10$ kcal/mol for water movement by a lipid solubility diffusion mechanism, e.g. $E_a$ for small intestine is 13.3 kcal/mol (19), whereas $E_a$ is $<6$ kcal/mol for water movement through aqueous channels, e.g. $E_a$ for kidney cortex is 1.0 kcal/mol (19). The high $E_a$ for water movement through lipids is thought to be related to the formation and breaking of hydrogen bonds as water moves from the aqueous to lipid phases and back to the aqueous phase. Relatively high $E_a$ values like those for hepatocyte membrane water permeation (i.e. $~8$ kcal/mol) may be due to a relatively low number of channels present
in the lipid matrix or to water movement through narrow channels in which significant interactions can occur. This is consistent with the recently reported very low single water permeability of basolateral AQP9 water channels (25) and with the low surface expression of canaliculal AQP8 (6) in hepatocytes. Thus, the $P_f$ and $E_a$ values obtained for hepatocyte basolateral and canalicular membranes suggest the presence of both lipid- and AQP-mediated pathways for water movement.

The sensitivity of basolateral and canalicular water transport to the AQP inhibitor Me$_2$SO also supports the contribution of AQP water channels to hepatic water movement. Although the exact mechanism by which Me$_2$SO exerts its action has not yet been determined, at a concentration of 500 mM, Me$_2$SO blocks AQP-mediated water transport in kidney brush border membrane vesicles (21) without affecting water transport by lipid solubility diffusion (21). In addition, we have successfully used Me$_2$SO to block aquaporin-mediated water transport in hepatocytes (6, 11, 25) where Me$_2$SO was also found to specifically inhibit the aquaporin-associated increase in osmotic membrane water permeability of aquaporin-transfected cells (22). In our previous studies, we found that mercuric chloride, another potent inhibitor of AQPs, was toxic to hepatocytes at doses required to inhibit AQPs; since the mechanism of toxicity is unknown, and to avoid any possibility of mercuric chloride damage to hepatocytes vesicles, we chose Me$_2$SO as an alternative.

After treatment of hepatocytes with DBcAMP, the osmotic water transport for canalicular vesicles showed a bi-exponential time course consistent with a functionally heterogeneous vesicle population. A double-exponential fitting requires the presence of at least two distinct populations of vesicles, whereas multiple parallel pathways for water transport in one vesicle type would give a single additive water permeability response (20). Thus, a portion of canalicular vesicles from DBcAMP-treated hepatocytes showed a fast exponential increase with a very high $P_f$ (655 μm/s), most likely reflecting a population of vesicles derived from the canalicular membrane that contain the agonist-induced increase in AQP8 molecules. AQP8-expressing hepatocytes are able to redistribute the AQP water channel to the canalicular plasma membrane (11). In agreement with this finding, the $E_a$ for water permeation was markedly lower (2.8 kcal/mol) after DBcAMP treatment. These data agree well with our previous results that choleretic secretagogues such as DBcAMP induce the redistribution of AQP8 in hepatocytes from intracellular vesicles to the canalicular plasma membrane (11).

Another interesting finding of this study is that in the basal or non-stimulated state, the canalicular plasma membranes had a $P_f$ value ~20% lower than that of basolateral membranes. In addition to a differential membrane domain expression of AQPs, this finding may reflect the higher cholesterol content of canalicular membranes (26), which is known to reduce water permeability in liposome systems (27). The lower canalicular $P_f$ value suggests that this membrane domain is the rate-limiting step for basal, diffusional transcellular water transport in hepatocytes. Moreover, the fact that cAMP-stimulated canalicular targeting of AQP8 markedly increases the water permeability of the canalicular membrane suggests that this membrane is rate-limiting for stimulated, channel-mediated, water transport as well.

We recently reported that AQP blockers completely abolished osmotic water transport in isolated polarized rat hepatocyte couplets (11) consistent with the importance of transcellular osmotic water flow in canalicular bile secretion. Transcellular $P_f$ can be calculated from the basolateral and apical $P_f$ values after correction for membrane folding factors (24) as has been done for kidney proximal tubular epithelia in which basolateral and apical membrane folding factors have been estimated to be 36 and 20, respectively (24). Assuming similar membrane area amplifications for hepatocyte membrane $P_f$ value of 0.22 and 0.31 cm$^2$/s for resting and DBcAMP-treated hepatocytes, respectively. Corresponding estimates for rat kidney proximal tubule, in which water flow seems to be entirely transcellular (24, 28), is of the same order of magnitude, being reported to be 0.38 cm$^2$/s (24). Thus, bearing in mind both the assumptions and the approximate nature of this calculation, our results support a major role for a transcellular pathway for hepatocyte water transport (i.e. canalicular bile secretion) under stimulated conditions.

In conclusion, we observed the presence of both lipid- and AQP-mediated pathways for basolateral and canalicular osmotic water movement. They also suggest that the canalicular plasma membrane domain is rate-limiting for transcellular water transport in hepatocytes and that under cAMP-mediated stimulation, this domain becomes highly permeable to water, facilitating transcellular osmotic water transport. This mechanism may be relevant to efficient coupling between osmotically active solute and water transport during canalicular bile formation.

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