Rat Hepatocytes Transport Water Mainly via a Non-channel-mediated Pathway*

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During bile formation by the liver, large volumes of water are transported across two epithelial barriers consisting of hepatocytes and cholangiocytes (i.e. intrahepatic bile duct epithelial cells). We recently reported that a water channel, aquaporin-channel-forming integral protein of 28 kDa, is present in cholangiocytes and suggested that it plays a major role in water transport by these cells. Since the mechanisms of water transport across hepatocytes remain obscure, we performed physiological, molecular, and biochemical studies on hepatocytes to determine if they also contain water channels. Water permeability was studied by exposing isolated rat hepatocytes to buffers of different osmolarity and measuring cell volume by quantitative phase contrast, fluorescence and laser scanning confocal microscopy. Using this method, hepatocytes exposed to hypotonic buffers at 23 °C increased their cell volume in a time and osmolarity-dependent manner with an osmotic water permeability coefficient of $66.4 \times 10^{-4}$ cm/s. In studies done at 10 °C, the osmotic water permeability coefficient decreased by 55% ($p < 0.001$, at 23 °C; $t$ test). The derived activation energy from these studies was 12.8 kcal/mol. After incubation of hepatocytes with amphotericin B at 10 °C, the osmotic water permeability coefficient increased by 198% ($p < 0.001$) and the activation energy value decreased to 3.6 kcal/mol, consistent with the insertion of artificial water channels into the hepatocyte plasma membrane. Reverse transcriptase polymerase chain reaction with hepatocyte RNA as template did not produce cDNAs for three of the known water channels. Both the cholesterol content and the cholesterol/phospholipid ratio of hepatocyte plasma membranes were significantly ($p < 0.005$) less than those of cholangiocytes; membrane fluidity of hepatocytes estimated by measuring steady-state anisotropy was higher than that of cholangiocytes. Our data suggests that the osmotic flow of water across hepatocyte membranes occurs mainly by diffusion via the lipid bilayer (not by permeation through water channels as in cholangiocytes).

Bile formation by the liver involves two phases: secretion of primary bile by hepatocytes at the canalicular domain and delivery to a network of interconnecting ducts where bile is modified by cholangiocytes via the secretion of ions and water.

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The abbreviations used are: CHIP, channel-forming integral protein of 28 kD; AQP-CD, aquaporin water channel of the collecting duct; MIWC, mercurial-insensitive water channel; $P_o$, osmotic water permeability coefficient; $P_d$, diffusional water permeability coefficient; $E_A$, activation energy; RT, reverse transcriptase; PCR, polymerase chain reaction.

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MATERIALS AND METHODS

Preparation of Hepatic Epithelial Cells

Hepatic epithelial cells were isolated from male Fisher 344 rats (250–350 g) obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Rats were provided with standard laboratory diet and water ad libitum and anesthetized with pentobarbital (50 mg/kg, intraperitoneal).

Hepatocytes—Pure (>98%) preparations of hepatocytes were isolated from rat livers by collagenase perfusion, mechanical dissociation, and iso-osmotic centrifugation through a discontinuous Percoll gradient as described previously (14).

Cholangiocytes—Pure (>95%) preparations of cholangiocytes were isolated from rat livers using an immunofluorescence technique previously described by us (15). Cell viability as assessed by trypan blue exclusion was always greater than 90% for both cell preparations.

Osmotic Water Permeability Studies

Cell Volume Measurements—The size of cells in isotonic and hypotonic extracellular buffers was assessed by quantitative phase contrast microscopy as described previously by us (13). Briefly, freshly isolated cells were attached to 22-mm square glass coverslips with Cell Tak (Collaborative Biomedical Products, Bedford, MA) and mounted on the stage of an Olympus CK2 inverted phase contrast microscope (Leeds Precision Instruments, Minneapolis, MN) equipped with an Olympus SC-2A camera. Cells were bathed in isotonic (300 mosM) Hepes-buffered saline containing (in mM): 140 NaCl; 5.4 KCl; 0.8 NaHPO4; 25 Na-Hepes; 0.8 MgSO4; pH 7.4, 23°C. Studies were performed by exposing cells at time 0 to extracellular buffers of different osmolarity (range: 30–300 mosM); buffers were prepared by diluting Hepes-buffered saline with distilled water and measuring osmolarity with an Osmette S (Precision Instruments Inc., Natick, MA). Total membrane phospholipid was measured with a commercial kit (Boehringer Mannheim). Total membrane phospholipid was measured with a commercial kit (Wako Chemicals U. S. A., Inc., Richmond, VA) using the protocol supplied by the manufacturer.

Membrane Lipid Composition and Fluidity of Hepatic Epithelia

Membrane vesicles were prepared from purified cholangioocyte and hepatocyte plasma membranes as described previously by us (25) and others (26, 27). Total membrane cholesterol was measured spectrophotometrically using a commercially available kit (Boehringer Mannheim). Total membrane phospholipid was measured with a commercially available kit (Wako Chemicals U. S. A., Inc., Richmond, VA) using the protocol supplied by the manufacturer.

Membrane fluidity was estimated by measuring steady-state anisotropy by fluorescence polarization as described previously (28). Briefly, 100 μg of membrane protein were added to 2.0 ml of 250 nm sucrose buffer containing 5 μl of 1:10,000,000,1,3,5-trihexylxaniline (Molecular Probes) and allowed to equilibrate for 1 h. Steady-state anisotropy was measured in an SLM 4800 spectrofluorometer (SLM, Urbana, IL) with polarization filters parallel and perpendicular to the excitation beam. Measurements were made at 25°C using an excitation wavelength of 362 nm and emission wavelength of 420 nm.

RESULTS

Osmotic Water Permeability Studies—As shown in Fig. 1, hepatocytes rapidly and significantly increased in cell size within the first 60 s of exposure to hypotonic buffers. By quantitative phase contrast microscopy, individual hepatocytes expanded by 50% within 60 s of exposure to 30 mosM sucrose (18). These results were confirmed by sequential phase-contrast and fluorescence microscopy of calcein-loaded hepatocytes exposed to hypotonic Hepes-buffered saline or sucrose buffers for up to 1 min (Table I). These data indicate that substitution with sucrose of permeant ions involved in volume regulation produced no significant differences in hepatocyte osmotic response, suggesting that cell volume regulation did not influence substantially to the response over the time of our experiments.

Hepatocyte volume changes in hypotonic Hepes-buffered saline buffer (100 mosM) was also analyzed by laser scanning confocal microscopy followed by three dimensional reconstruction. Relative cell volume values obtained using this methology (155.2 ± 14.0, n = 7) agree with the corresponding values shown in Fig. 1B using quantitative phase-contrast microscopy. Importantly, the viability of hepatocytes (as assessed by trypan blue exclusion) was unchanged after exposure to hypotonic buffers.
Using the initial slope of the curves generated in Fig. 1B, the calculated Pf value for isolated rat hepatocytes was $66.4 \pm 10^{-2}$ cm/s at 23 °C. The effect of extracellular buffer temperature on the volume of hepatocytes in hypotonic buffer is shown in Fig. 2A. Temperature had a significant (p < 0.0001, analysis of variance) effect on the time-dependent increase in hepatocyte volume in hypotonic (30 mosM) buffer; the magnitude of the increase in hepatocyte volume increasing with increasing buffer temperature. Indeed, when comparing studies done at 23°C and 10°C, the osmotic water permeability coefficient decreased by 55% (p < 0.001 at 23°C, t test). In contrast, the magnitude of the time-dependent increase in cholangiocyte volume in hypotonic (30 mosM) buffer was not significantly different between studies at 10, 23, or 30 °C (data not shown).

TABLE I

| Buffers                  | Change in Cell Volume |
|--------------------------|----------------------|
|                          | Phase-contrast       |
|                          | Fluorescence         |
| Hepes-buffered saline    | 228.3 ± 22.5          |
| Sucrose-Hepes            | 233.3 ± 12.6          |

Using the initial slope of the curves generated in Fig. 1B, the calculated $P_f$ value for isolated rat hepatocytes was $66.4 \times 10^{-4}$ cm/s at 23 °C. The effect of extracellular buffer temperature on the volume of hepatocytes in hypotonic buffer is shown in Fig. 2A. Temperature had a significant (p < 0.0001, analysis of variance) effect on the time-dependent increase in hepatocyte volume in hypotonic (30 mosM) buffer; the magnitude of the increase in hepatocyte volume increasing with increasing buffer temperature. Indeed, when comparing studies done at 23°C and 10°C, the osmotic water permeability coefficient decreased by 55% (p < 0.001 at 23°C, t test). In contrast, the magnitude of the time-dependent increase in cholangiocyte volume in hypotonic (30 mosM) buffer was not significantly different between studies at 10, 23, or 30 °C (data not shown).

From these data, we determined the Arrhenius relationship between the logarithm of $P_f$ and the reciprocal value of absolute temperature for both hepatocytes and cholangiocytes as shown in Fig. 2B. Based on these data, the $E_a$ value for hepatocytes was 12.8 kcal/mol.

Preincubation of hepatocytes with amphotericin B significantly (p < 0.0001) increased both the rapidity and the magnitude of the increase in hepatocyte volume following exposure to hypotonic (100 mosM) buffer (Fig. 3A) at 10 °C. Moreover, the effect of buffer temperature on osmotic-induced water movement by hepatocytes was significantly reduced following pretreatment with amphotericin B (Fig. 3B). Accordingly, the $E_a$ value for hepatocytes in the presence of amphotericin B was 3.6 kcal/mol, a value similar to cholangiocytes and compatible with channel-mediated water movement. Of note, the viability of hepatocytes in the presence of amphotericin B was >90% indicating that the stimulatory effect of amphotericin B on hepatocyte water movement was not due to cell toxicity.

Gene Expression of Water Channels—Shown in Fig. 4A are the results from a gel electrophoresis of products obtained by RT-PCR using specific oligonucleotides for rat MIWC. The gel shows a band at 500 bp in the lane where RNA isolated from rat kidney was used as a template, our positive control. No band, however, was detected in the lane where an equal amount of RNA from purified rat hepatocytes was used as template, nor in the lane containing water as template, our negative control. By DNA sequencing, the band obtained using rat kidney RNA was greater than 95% homologous to the rat brain MIWC.
sequence (11). Similarly, Fig. 4B shows the results from a gel electrophoresis of products obtained by RT-PCR using specific DNA primers for rat AQP-CD. As expected, the gel shows a band at 504 bp in the lane where RNA isolated from rat kidney was used as template, our positive control; by DNA sequencing, the band obtained using rat kidney RNA was greater than 95% homologous to the rat kidney AQP-CD sequence. Note however, that no band was detected using an equal amount of RNA from purified rat hepatocytes as template, nor when using water, our negative control. Finally, as previously reported by us (13), no band was detected on gel electrophoresis of products obtained by RT-PCR using specific primers for aquaporin-CHIP and hepatocyte total RNA as template (13).

Membrane Lipid Composition and Fluidity—Table II shows the results of membrane lipid analysis and steady-state anisotropy for hepatocytes and cholangiocytes. The cholesterol content and cholesterol/phospholipid ratio of hepatocyte membranes were both significantly (p < 0.005) less than those of cholangiocyte membranes. In particular, the cholesterol/phospholipid ratio of hepatocyte membranes was approximately 8-fold less than that of cholangiocytes. It is well documented that the cholesterol/phospholipid ratio is one of the important parameters affecting membrane fluidity. Thus, as expected, steady-state anisotropy measurements of hepatocyte membranes yielded significantly (p < 0.005) lower values than that obtained from cholangiocyte membranes.

**DISCUSSION**

The major findings of the study relate first to functional and molecular studies of water transport in isolated hepatocytes and second to a comparative analysis of the membrane lipid compositions of rat hepatocytes and cholangiocytes, two hepatic epithelia which transport water via different mechanisms. Our data suggest that the osmotic flow of water across the hepatocyte membranes occurs mainly by diffusion across the lipid bilayer rather than by permeation through water channels, a process we have previously described in cholangiocytes (13).

We observed no hepatocyte volume regulation over the time of our experiments. This finding agrees with recent reports showing that hepatocytes exposed to hypotonic stress display no significant volume regulatory decrease over 1 min (18, 29). However, since the changes in cell volume at 1 min of exposure to hypotonic solutions were smaller than might be expected for an osmometric cell response, the possibility of a very rapid cell volume regulation response during the swelling phase cannot be completely excluded. Nevertheless, since such responses are likely to have a finite threshold, their contribution to the initial change in cell volume (from which P, was estimated) would likely be small. Several lines of evidence indicate that P, was not substantially restricted by non-membrane barriers, such as external or cytoplasmic unstirred layers (30): (a) there was no lag in hepatocyte swelling after a change in buffer osmolarity; (b) E was 12.8 kcal/mol, much higher than that predicted if P, were limited by an unstirred layer (~5 kcal/mol); (c) P, increased with insertion of amphotericin B water channels; and (d) the initial rate of cell swelling was proportional to osmotic gradient size (data not shown). The calculated P, and E, values, together with the fact that amphotericin B-induced membrane channels increased P, and lowered E, are consistent with water movement through the lipid portion of the hepatocyte.
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plasma membrane rather than through protein channels. Having generated biophysical data consistent with the notion that the principal mechanism regulating osmotic water movement by hepatocytes is diffusion via the lipid bilayer, we next explored this concept at a molecular level by determining whether hepatocytes express the transcript for three of the previously described water channels. Using RT-PCR and oligonucleotides based on reported DNA sequences, we demonstrated that hepatocytes do not express the transcript for any of the three known water channels, aquaporin-CHIP, AQP-CD, and MIWC. Thus, both biophysical and molecular data strongly suggest that the principal mechanism of water movement by hepatocytes is not channel-mediated, but rather is diffusional.

As recently described by us (13), cholangiocytes transport water via a water channel, that is likely, aquaporin-CHIP. In spite of the presence of water channels, cholangiocytes have a relatively low $P_f$ value of $5 \times 10^{-4}$ cm/s. Although our data suggest that water movement by hepatocytes is due to diffusion via lipid bilayer, the $P_f$ value for hepatocytes is higher than that of cholangiocytes. Furthermore, cholangiocytes have a markedly lower diffusional water permeability coefficient ($P_d$), $<5 \times 10^{-4}$ cm/s, compared to that of hepatocytes (31). Indeed, to our knowledge, this $P_d$ value for cholangiocytes represents the lowest $P_d$ value reported thus far (5). These important differences in the mechanisms by which water traverses hepatocytes and cholangiocytes suggested that there might be major differences in the biochemical properties of the plasma membranes of these two epithelia. Nevertheless, results of such comparisons must be interpreted with caution because both $P_f$ and $P_d$ values are dependent on cell surface area, and these two cell types could differ with respect to their surface areas.

As predicted, we found the membrane lipid composition of hepatocytes to be markedly different from that of cholangiocytes. Both the cholesterol content and the cholesterol/phospholipid ratio of hepatocyte plasma membranes were significantly less than those of cholangiocyte membranes. This difference reflects the unusually high cholesterol content of cholangiocyte plasma membranes, an observation previously made by us on plasma membranes derived from cholangiocytes after bile duct ligation (25). As expected from the differences in lipid composition, the membrane fluidity of hepatocytes, estimated by measuring steady-state anisotropy, was higher than that of cholangiocytes. Membrane fluidity is recognized to influence transmembrane transport processes, including water movement (32). Thus, these differences in membrane lipid composition and fluidity between hepatocytes and cholangiocytes may help to provide a biophysical explanation for their different mechanisms of water transport; i.e. water can easily diffuse across the highly fluid hepatocyte plasma membrane but requires a channel to traverse the stiff cholangiocyte plasma membrane.

Historically, the paracellular pathway has been considered by some to be the principal route of water movement across hepatocytes during primary bile formation, involving passive movement of water from blood to bile between hepatocytes in response to osmotic gradients established largely by the active movement of bile acids into the canaliculus. This concept stems from inferences made from ultrastructural studies of hepatocytes demonstrating: (i) enhanced tight junction penetration of electron-dense substances under cholestatic conditions (33, 34) and (ii) balloon-like projections in the basolateral (sinusoidal) membrane adjacent to tight junctions in association with bile acid-stimulated cholestasis (34). Furthermore, the demonstration that hepatocyte couples have low electrical resistance (35) is consistent with hepatocytes being "leaky" epithelium through which paracellular movement of water may occur. Nevertheless, while current opinion appears to favor a paracellular pathway of water movement across hepatocytes, the studies on which this premise is based are limited and largely indirect. Although our data suggest that osmotic-induced transmembrane water movement by hepatocytes does not occur via a channel-mediated mechanism, the $P_f$ value for hepatocytes is higher than the value for cholangiocytes which have aquaporin-CHIP water channels in their plasma membranes. Thus, it seems plausible that diffusional water movement across hepatocytes in response to osmotic gradients may play an important role in primary bile formation at bile canaliculi. Indeed, we obtained $P_f$ values in isolated hepatocytes which rapidly lose their polarity; thus, these values do not necessarily reflect the osmotic permeability of the canalicular membrane. Of course, this concept does not exclude a paracellular pathway in water movement across hepatocytes nor does it exclude the possibility of other nonselective, membrane channels such as glucose transporters from playing a contributory role in water movement (36). Unfortunately, it is currently difficult to directly distinguish transcellular from paracellular water movement in vivo, because of the lack of a suitable experimental model. Additional studies requiring new experimental approaches will be required to determine the quantitative contribution of a transcellular versus a paracellular pathway in primary bile formation by hepatocytes. Nevertheless, the work described here excludes selective water channels as important conduits for transcellular water movement across hepatocyte plasma membranes. Moreover, the biophysical properties of hepatocyte plasma membranes characterized by us clearly indicate that a diffusional mechanism for transcellular water movement could be very important in primary bile formation.

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