The Water Channel Aquaporin-8 Is Mainly Intracellular in Rat Hepatocytes, and Its Plasma Membrane Insertion Is Stimulated by Cyclic AMP*

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Fabiana García‡, Arlinet Kierbel‡, M. Cecilia Larocca‡, Sergio A. Gradilone§, Patrick Splinter§, Nicholas F. LaRussos§, and Raúl A. Marinellis‡

From the §Instituto de Fisiología Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Rosario, Rosario, Santa Fe, Argentina 2000 and the ¶Center for Basic Research in Digestive Diseases, Departments of Internal Medicine and Biochemistry and Molecular Biology, Mayo Medical School, Clinic, and Foundation, Rochester, Minnesota 55905

We previously found that water transport across hepatocyte plasma membranes occurs mainly via a non-channel mediated pathway. Recently, it has been reported that mRNA for the water channel, aquaporin-8 (AQP8), is present in hepatocytes. To further explore this issue, we studied protein expression, subcellular localization, and regulation of AQP8 in rat hepatocytes. By subcellular fractionation and immunoblot analysis, we detected an N-glycosylated band of ~34 kDa corresponding to AQP8 in hepatocyte plasma and intracellular microsomal membranes. Confocal immunofluorescence microscopy for AQP8 in cultured hepatocytes showed a predominant intracellular vesicular localization. Dibutyryl cAMP (Bt2cAMP) stimulated the redistribution of AQP8 to plasma membranes. Bt2cAMP also significantly increased hepatocyte membrane water permeability, an effect that was prevented by the water channel blocker dimethyl sulfoxide. The microtubule blocker colchicine but not its inactive analog lumicolchicine inhibited the Bt2cAMP effect on both AQP8 redistribution to cell surface and hepatocyte membrane water permeability. Our data suggest that in rat hepatocytes AQP8 is localized largely in intracellular vesicles and can be redistributed to plasma membranes via a microtubule-depending, cAMP-stimulated mechanism. These studies also suggest that aquaporins contribute to water transport in cAMP-stimulated hepatocytes, a process that could be relevant to regulated hepatocyte bile secretion.

Bile is formed primarily by hepatocytes and subsequently delivered to the bile ducts where it is modified by cholangio-cytes (i.e., the epithelial cells that line the bile ducts). Bile secretion by hepatocytes involves the active transport of solutes followed by the passive movement of water into the bile canaliculus in response to osmotic gradients created by these solutes (1, 2). Although a substantial amount of data have been published about the molecular identification of solute transporters and the mechanisms regulating solute transport by hepatocytes (3), little attention has been focused on the mechanistic and regulatory aspects involved in hepatocyte water transport.

Water can cross cellular plasma membranes through the lipid portion of the bilayer by a diffusion mechanism or through aquaporin water channels. Aquaporins, a family of recently identified integral membrane proteins, increase cell membrane water permeability facilitating rapid movement of water in response to osmotic gradients (4, 5).

We previously found based on biophysical and molecular biology studies that water transport across hepatocyte plasma membranes occurs mainly via a non-channel mediated pathway (6). As this observation seems to be in contradiction with the recent identification of transcript for the water channel aquaporin-8 (AQP8) in hepatocytes (7–9), we further explored this issue by studying the protein expression, subcellular localization, and possible regulation of AQP8 water channels in isolated rat hepatocytes.

MATERIALS AND METHODS

Isolation and Incubation of Hepatocytes—Hepatocytes were isolated from livers of male Wistar rats by collagenase perfusion and mechanical disruption as described previously (10). Hepatocytes suspended in Krebs-Ringer-Hepes buffer, pH 7.4, were incubated at 37 °C according to the protocols: (a) for 10 min in the presence of 0 or 100 μM dibutyryl cAMP (Bt2cAMP; Sigma); (b) for 1 h in the presence of 50 μM colchicine or lumicolchicine (Sigma) and then for an additional 10 min in the presence of 0 or 100 μM Bt2cAMP. Subcellular fractionation and water transport studies were carried out after these treatments. Cell viability (assessed by trypan blue exclusion) was always >85% and not affected by the treatments.

Short-term Culture of Hepatocytes—Freshly isolated hepatocytes were suspended in Leibovitz 15 medium (L-15; Life Technologies, Inc.), cultured on collagen-coated coverslips, and maintained at 37 °C for 4 h in an air atmosphere. Incubations were performed according to these protocols: (a) for 10 min in the presence of 0 or 100 μM Bt2cAMP, (b) for 1 h in the presence of 50 μM colchicine or lumicolchicine and then for an additional 10 min in the presence of 0 or 100 μM Bt2cAMP. Confocal immunofluorescence microscopy was carried out after these treatments.

Preparation of Subcellular Membrane Fractions—Membrane fractions enriched in plasma or intracellular microsomal membranes were prepared from hepatocytes by differential centrifugation as previously described by us (11). Briefly, cells were washed and sonicated in 0.3 m sucrose containing 0.1 m phenylmethylsulfonyl fluoride and 0.1 m leupeptin (Sigma). The plasma membrane fraction was obtained by centrifugation at 200,000 × g for 60 min on a discontinuous 1.3 m
Hepatocyte Aquaporin-8 Water Channels

**Fig. 1. Expression and N-glycosylation of AQP8 protein in hepatocytes.** A, anti-AQP8 immunoblot of total membranes from hepatocytes or indicated tissues. Lanes were loaded with 30 μg of protein for hepatocyte and liver and 100 μg of protein for colon, kidney, and lung (see “Materials and Methods” for details). B, anti-AQP8 immunoblot of total hepatocyte membranes (30 μg protein/lane) before (−) or after (+) digestion with peptide/N-glycosidase F.

**Fig. 2. Localization of AQP8 in hepatocytes by subcellular fractionation and immunoblotting: effect of Bt2cAMP.** Hepatocytes were incubated in the absence (controls) or presence of 100 μM Bt2cAMP (DBcAMP) for 10 min at 37 °C, and subcellular fractionation was performed as described under “Materials and Methods.” A, anti-AQP8 immunoblot of plasma and intracellular microsomal membranes (20 μg total protein/lane). B, densitometric analysis of three separate experiments in each group (control and Bt2cAMP (DBcAMP)). Data are expressed in percent values as mean ± S.E. *p < 0.05 for Bt2cAMP effect (Student’s t-test).

Sucrose gradient. After removing the plasma membrane band, the sucrose gradient was sonicated, diluted to 0.3 M, and centrifuged at 17,000 × g for 30 min. The resulting supernatant was centrifuged at 200,000 × g for 60 min to yield the intracellular microsomal membrane fraction. Fractions enriched in plasma or intracellular microsomal membranes were also prepared from homogenates of colon mucosa and total liver following the same procedure. For experiments in Fig. 1, hepatocytes as well as total liver, colon mucosa, kidney, and lung tissues from normal rats were homogenized in 0.3 M sucrose containing protease inhibitors, subjected to low-speed centrifugation to obtain post nuclear supernatants, and then subjected to centrifugation at 200,000 × g for 60 min to yield “total membranes.” Proteins in membrane fractions were assayed according to Lowry et al. (12) using bovine serum albumin as a standard. Alkaline phosphatase activity (a plasma membrane marker) was assessed using a commercially available enzyme kit (Sigma).

**Immunoblotting—Solubilized membrane fractions were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (PerkinElmer Life Sciences). After blocking, blots were incubated overnight at 4 °C with rabbit affinity-purified antibodies against AQP8 (1 μg/ml, Alpha Diagnostics International, San Antonio, TX). The blots were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Promega), and bands were detected by enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech). Autoradiographs were obtained by exposing polyvinyl difluoride membranes to Kodak XAR film, and the bands were quantitated by densitometry (Hunerius Scan 500, Dallas, TX). To control for nonspecific reactions, incubation experiments were performed by using an antibody preabsorbed with a 10-fold molar excess of AQP8 immunizing peptide (Alpha Diagnostics International). Selected samples were digested with 10 μg/ml peptide/N-glycosidase F (PNGase F; New England Biolabs).

**Immunofluorescence and Confocal Microscopy—**After culturing and treatment (see above), hepatocytes were fixed with 2% paraformaldehyde for 10 min at room temperature, permeabilized with 0.2% Triton X-100 for 2 min, and incubated overnight at 4 °C with rabbit affinity-purified AQP8 antibodies (10 μg/ml; Alpha Diagnostics International). After washing, coverslips were incubated with Texas Red-conjugated goat anti-rabbit secondary antibody (Molecular Probes) for an additional hour and mounted with ProLong (Molecular Probes). Fluorescence localization was detected by confocal microscopy with a laser scanning microscope (Carl Zeiss LSM-510). Images were collected with the same confocal settings in a particular set of experiments. With these settings no autofluorescence was detected. Controls using omission of primary or secondary antibodies revealed no labeling. Images were processed using Adobe Photoshop software.

**Water Transport Studies—**Following incubation as described above, hepatocytes were suspended in Heps (25 mM)-buffered sucrose (300 mosm, pH 7.4), cooled at 10 °C, and subjected to hypotonic challenge (100 mosm). The size of hepatocytes was determined by quantitative phase contrast microscopy, a methodology previously validated by us (6). The osmotic membrane water permeability (Pf) was calculated from the initial rate of cell swelling as described previously (6). In some experiments, before assessing water permeability, hepatocytes were incubated for 5 min with 500 mM dimethyl sulfoxide (Me2SO; negative controls for rat AQP8 expression) (7, 8). The 34 kDa band was absent when AQP8 antibody was preabsorbed with a 100-fold molar excess of the immunizing peptide (not shown). After incubation with N-glycosidase, the 34 kDa band shifted to the predicted 28 kDa molecular mass (Fig. 1B) indicating the presence of N-glycosylation in the AQP8 molecule.

**RESULTS**

**Aquaporin-8 Protein Expression in Hepatocytes**

As shown in Fig. 1A, immunoblotting analysis for AQP8 detected a band of 34 kDa in total liver and hepatocyte membranes. An identical band in location was obtained for colon mucosa (another rat AQP8 message-expression tissue) (7, 8), whereas no band was detected for kidney or lung tissues (negative controls for rat AQP8 expression) (7, 8). The 34 kDa band was absent when AQP8 antibody was preabsorbed with a molar excess of the immunizing peptide (not shown). After incubation with N-glycosidase, the 34 kDa band shifted to the predicted 28 kDa molecular mass (Fig. 1B) indicating the presence of N-glycosylation in the AQP8 molecule.

**Subcellular Localization of Aquaporin-8 in Hepatocytes**

**Effect of Bt2cAMP—**Subcellular fractionation followed by immunoblot analysis showed the AQP8 band in hepatocyte...
plasma and intracellular microsomal membranes (Fig. 2). Similar results were obtained with total liver, whereas in colon mucosa the immunoreactive band was only present in plasma membranes (not shown). Exposure of cells to Bt2cAMP resulted in an increase of AQP8 in hepatocyte plasma membranes (125%, $p < 0.05$) and a simultaneous decrease of AQP8 in microsomal membranes (58%, $p < 0.05$) (Fig. 2). Based on these data and the total proteins in each hepatocyte membrane fraction, we estimated that under basal (non-stimulated) conditions most of the hepatocyte AQP8 (75%) would reside in intracellular microsomal membranes; 25% would be in plasma membranes. After Bt2cAMP treatment, AQP8 became predominant in plasma membranes (i.e., 60% of total). Bt2cAMP did not alter the yields of total membrane proteins, the specific activity of plasma membrane alkaline phosphatase in hepatocyte membrane fractions, or the amount of AQP8 in total hepatocyte membranes (data not shown). Confocal immunofluorescence microscopy in cultured hepatocytes was in agreement with the quantitative immunoblot analysis. Thus, AQP8 labeling was observed mostly in intracellular vesicular structures throughout the cytosol; plasma membranes exhibited very low labeling (Fig. 3A). Bt2cAMP caused a decrease in AQP8 intracellular labeling and a simultaneous increase in plasma membrane. (Fig. 3B).

Together, these data are consistent with a predominant intracellular vesicular location of AQP8 in hepatocytes, and a cAMP-induced translocation of the water channel to the cell surface.

**Effect of cAMP on the Osmotic Water Transport in Hepatocytes**—The time course of relative hepatocyte volume in response to an inwardly directed sucrose gradient is shown in Fig. 4A. The osmotic gradient caused water influx and cell swelling. The rate of the swelling response was significantly increased by Bt2cAMP. The water channel blocker Me$_2$SO inhibited Bt2cAMP-induced increases in hepatocyte water transport. Corresponding $P_f$ values are shown in Fig. 4B. Average hepatocyte volume (in isotonic media) was not affected by Bt2cAMP.

These data support the notion that cAMP promotes osmotic

![Fig. 3. Localization of AQP8 in hepatocytes by confocal immunofluorescence: effect of Bt2cAMP.](image)

![Fig. 4. Effect of Bt2cAMP on the osmotic water transport in hepatocytes.](image)
of three separate experiments expressed as percent change induced by Hepatocytes were incubated at 37 °C for 1 h in the presence of 50 μm colchicine or 50 μm lumicolchicine and then for an additional 10 min in the absence (−) or presence (+) of 100 μm Bt2cAMP (DBcAMP). Subcellular fractionation was performed as described under “Materials and Methods.” A, anti-AQP8 immunoblot of plasma and intracellular microsomal membranes (20 μg total protein/lane). B, densitometric analysis of three separate experiments expressed as percent change induced by Bt2cAMP (mean ± S.E.). * p < 0.05 compared with lumicolchicine/Bt2cAMP (Student’s t test).

membrane water transport in hepatocytes via insertion of aquaporin water channels.

Effect of Colchicine on cAMP-induced Redistribution of AQP8—Colchicine but not its inactive analog lumicolchicine markedly inhibited the Bt2cAMP action on AQP8 redistribution to cell surface (Figs. 5 and 6) or hepatocyte P1 (Fig. 7).

Together, these data suggest that the cAMP-dependent redistribution of AQP8 from intracellular vesicles to the cell surface depends on microtubules.

DISCUSSION

To our knowledge, this is the first study on the functional expression and regulation of AQP8 protein in mammalian cells. The major findings reported here relate to mechanistic and regulatory aspects involved in hepatocyte water transport. Our data, using isolated rat hepatocytes, suggest that the water channel AQP8 is localized largely in intracellular vesicles and can be redistributed to plasma membrane via a microtubule-dependent, cAMP-stimulated mechanism. Thus, hepatocytes would be able to regulate their membrane water permeability.

AQP8 has been identified recently (7–9), and its transcript has been found to be present in rat hepatocytes; nevertheless, studies on protein expression were not undertaken (7). In the present study, we consistently found AQP8 protein expression in rat hepatocytes. Digestion with N-glycosidase indicated that AQP8 molecule is N-glycosylated in agreement with the predicted consensus site for N-linked glycosylation in the AQP8 deduced amino acid sequence (8).

Our biochemical and immunofluorescence studies indicate that hepatocyte AQP8 is primarily located within the cell, presumably in a vesicular compartment. AQP8 expression on cell surface is very low under basal (non-stimulated) conditions, suggesting a small contribution of this aquaporin to total membrane water permeability. This agrees with our previous observation that, in the basale state, water transport across hepatocyte plasma membranes occurs mainly via a non-channel-mediated pathway because AQP8 is sequestered within an intracellular compartment (6). Upon Bt2cAMP stimulation, intracellular AQP8 is re-localized to plasma membrane, thereby raising the cell surface water permeability and facilitating osmotic water transport.

Bt2cAMP is well known to activate protein kinase A. The AQP8 molecule lacks consensus protein kinase A phosphorylation sites (8), which suggests that the effect of cAMP is not directed toward AQP8 protein itself but rather to protein mediators involved in vesicle trafficking. Moreover, Bt2cAMP has also been found to stimulate the vesicle trafficking to the hepatocyte plasma membrane of several other transporters, i.e. Cl−/HCO3− exchanger (15), canalicular bile acid transporter (16), organic anion transporter, mrp2 (17, 18), multidrug resistance protein transporter (19), and basolateral Na+/taurocholate cotransporter (20). Our experiments also show that Bt2cAMP-induced AQP8 vesicle trafficking seems to be dependent on the integrity of microtubules as observed for some of the mentioned solute transporters (15–18). Thus, whether AQP8 is packaged along with solute transporters in the same population of vesicles as has been observed for aquaporin-1 water channels and cystic fibrosis transmembrane regulator CI− channels in cholangiocytes (21) or are in separate vesicles that respond to the same signal needs to be determined.

Cyclic AMP stimulates microtubule assembly by phosphorylation of microtubule-associated proteins via cAMP-dependent kinases (22). Thus, the effect of Bt2cAMP on AQP8 trafficking may be mediated by stimulation of microtubule-associated proteins.

Cyclic AMP is also able to induce synthesis of transporters in hepatocytes (23). Nevertheless, new aquaporin synthesis is unlikely to significantly contribute to cAMP-induced increase of plasma membrane AQP8 because total hepatocyte AQP8 was unaltered by Bt2cAMP. In addition, the stimulatory effect of cAMP was seen within a 10-min period; protein synthesis usually requires a longer time.

The fact that in colon mucosa AQP8 is not present in intracellular membranes suggests that this aquaporin is constitutively expressed in colon epithelia and that the subcellular distribution and regulation of AQP8 is tissue specific.

Bile duct epithelial cells or cholangiocytes express at least two other members of the aquaporin family of proteins, i.e. aquaporin-1, located mainly in the apical (luminal) membrane domain and in intracellular vesicles (11, 24, 25), and aquaporin-4, present only in the basolateral membrane domain (26). The cAMP-dependent hormone secretin regulates the subcellular distribution of aquaporin-1 by stimulating its exocytic insertion exclusively in the apical plasma membrane domain of cholangiocytes (25), a mechanism that seems to be involved in ductal bile formation. In contrast, aquaporin-4 in cholangiocytes is constitutively expressed in the basolateral plasma membrane domain (26). In hepatocytes, cyclic AMP-regulated targeting of membrane transporters has been reported to in-
volve both the apical as well as the basolateral plasma membrane domains (15–20). Additional studies with different experimental approaches will be required to determine the polarized distribution and regulated vectorial trafficking of AQP8 in hepatocytes.

Rat hepatocytes also express another aquaporin water channel, i.e. aquaporin-9 (27). Whether this channel shares the subcellular location and regulation with AQP8 is a matter of further studies.

Immunohistochemistry studies for AQP8 in intact rat liver 2 as well as membrane fractionation and immunoblotting of total liver homogenate showed that AQP8 associated mainly to an intracellular membrane compartment. These observations suggest that our findings in isolated cells are relevant to the in vivo situation. As mentioned, hepatic bile is thought to be formed by the passive movement of water from plasma to bile canaliculus in response to osmotic gradients established by the active secretion of solutes such as bile acids and glutathione (1, 2). Interestingly, hepatocyte bile secretion can be increased by cAMP and by hormones acting through the cAMP cascade, phenomena that are also inhibited by colchicine (28, 29). Thus, it is tempting to speculate that AQP8 could facilitate the osmotically driven water transport during cAMP-stimulated hepatocyte bile formation.

In conclusion, our data support the concept that rat hepatocytes are able to modulate water permeability by inducing the microtubule-dependent targeting of vesicles containing AQP8 water channels to the plasma membrane, a process that could be relevant to regulated hepatocyte bile secretion.

2 P. Splinter and N. F. LaRusso, unpublished data.
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