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Research article

Expression and subcellular localization of the AQP8 and AQP1 water channels in the mouse gall-bladder epithelium

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Background information. Transepithelial transport of water is one of the most distinctive functions by which the gall-bladder rearranges its bile content. Water is reabsorbed from the gall-bladder lumen during fasting, whereas it is secreted into the lumen following meal ingestion. Nevertheless, the molecular mechanism by which water is transported across the gall-bladder epithelium remains mostly unclear.

Results. In the present study, we investigate the presence and subcellular localization of AQP (aquaporin) water channels in the mouse gall-bladder epithelium. Considerable AQP8 mRNA was detected in the gall-bladder epithelium of mouse, calf, rabbit, guinea pig and man. Studies of subcellular localization were then addressed to the mouse gall-bladder where the transcript of a second AQP, AQP1, was also detected. Immunoblotting experiments confirmed the presence of AQP8 and AQP1 at a protein level. Immunohistochemistry showed intense expression of AQP8 and AQP1 in the gall-bladder epithelial cells where AQP8 was localized in the apical membrane, whereas AQP1 was seen both in the apical and basolateral membranes, and in vesicles located in the subapical cytoplasm.

Conclusions. The pattern of subcellular distribution of AQP8 and AQP1 strongly corroborates the hypothesis of a transcellular route for the movement of water across the gall-bladder epithelium. Osmotic water would cross the apical membrane through AQP8 and AQP1, although AQP1 would be the facilitated pathway for the movement of water across the basolateral membrane. The presence of two distinct AQPs in the apical membrane is an unusual finding and may relate to the membrane's ability both to absorb and secrete fluid. It is tempting to hypothesize that AQP1 is hormonally translocated to the gall-bladder apical membrane to secrete water as in the bile duct epithelium, a functional homologue of the gall-bladder epithelium, whereas apical AQP8 may account for the absorption of water from gall-bladder bile.

Introduction

Gall-bladder is the storage organ for bile. The principal transport function of the gall-bladder epithelium is the absorption of NaCl and water in near-isosmotic proportions leading to concentration of the impermeant components of bile in the lumen of the gall-bladder (Reuss, 1991). This involves apical membrane entry of Na^+ and Cl^- and basolateral extrusion of both ions (Reuss et al., 1991; Cremaschi and Porta, 1992) with the consequent parallel movement of water. Gall-bladder fluid absorption is under hormonal control since the vasoactive intestinal polypeptide and serotonin inhibit the net movement of electrolyte and water across the gall-bladder

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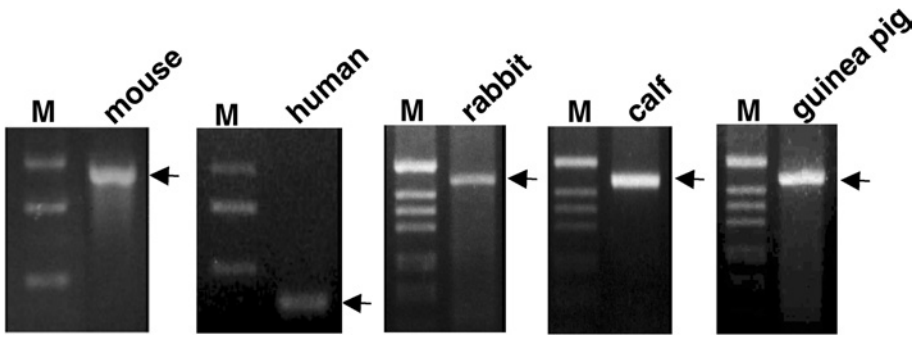
Key words: aquaporin, cystic bile, gall-bladder apical membrane, hepatobiliary tract, water transport.
Abbreviations used: AQP, aquaporin; CaM, Ca^{2+}-calmodulin; 2-ME, 2-mercaptoethanol; ORF, open reading frame; PAP, peroxidase–antiperoxidase; RT, reverse transcriptase.

Figure 1 | RT–PCR analysis of AQP8 expression in mouse, human, rabbit, calf and guinea pig gall-bladders

RT–PCR of total RNA samples from the scraped epithelial layer of these gall-bladders using a primer pair for a highly conserved region of the known AQP8 ORFs (mouse, rabbit, calf and guinea pig AQP8, band of 434 bp; human AQP8, band of 201 bp). M, DNA mass marker (1 kb DNA ladder).



epithelial barrier by converting absorption to secretion (Moseley, 1999). On the other hand, increase in intracellular cAMP levels have been shown to inhibit apical-membrane Na^+/H^+ and Cl^-/HCO_3^- exchangers and activate apical-membrane Cl^- and K^+ conductances with consequent inhibition of net NaCl and water absorption (Reuss et al., 1991). Similar to the bile-duct epithelium, secretion of fluid by the gall-bladder epithelium also occurs under the stimulation of the secretagogue hormone secretin (Igimi et al., 1992). The general assumption is that fluid is absorbed from the gall-bladder lumen during the interdigestive phase, whereas water and solutes are secreted into the lumen during digestion (Glickerman et al., 1997; Kulaksiz et al., 2004).

Major advances have been achieved during recent years in many aspects regarding the molecular identity, physiology and regulation of the solute transporters involved in gall-bladder fluid balance (Meyer et al., 2000, 2002; Narins et al., 2004). Although the movement of bile water across the gall-bladder mucosa is believed to be of central importance both physiologically and pathophysiologically (Masyuk et al., 2002; van Erpecum, 2005), the mechanisms and the molecular systems that directly sustain water transport across the gall-bladder epithelium have not yet been described. Important clues were recently provided by the recognition of a member of the AQP (aquaporin) family of water channels (King et al., 2004), AQP1, expressed in the epithelial cells of human gall-bladder (Nielsen et al., 1993). Besides being consistent with a transcellular route for the transepithelial movement of water, this finding suggested that other AQP isoforms might be expressed in the gall-bladder epithelium since this tissue is capable of both absorbing and secreting water.

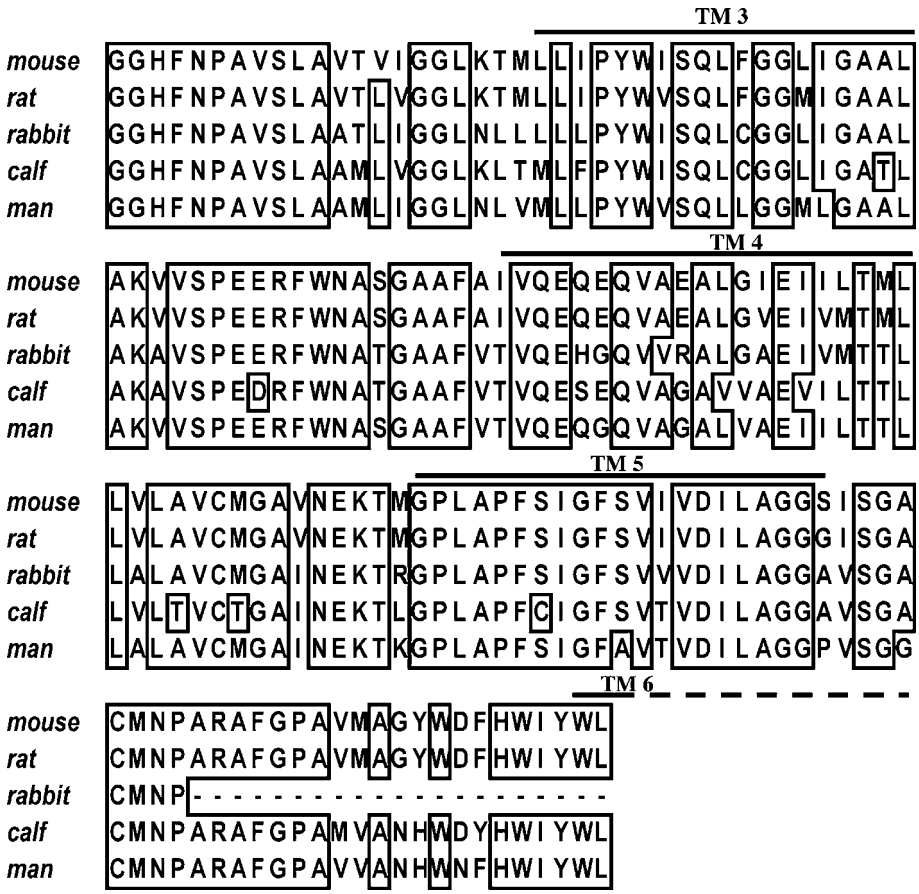
The present study was undertaken to confirm the presence of AQP1 in animal gall-bladders and investigate the expression of additional AQPs in the epithelium of mouse gall-bladder. Also, the presence of AQPs was investigated in the human gall-bladder known to be uninflamed and with a 'normal' bile composition: i.e. tissues from patients with pigment stones. Besides confirming the presence of AQP1 in animal gall-bladders, the results reported here show the presence of a second AQP, AQP8, in both human and animal gall-bladders. The subcellular distribution of AQP8 and AQP1 in mouse gall-bladder is also underscored and roles for water channels in the absorptive/secretory function of gall-bladder are suggested.

Results

AQP8 mRNA is expressed in animal and human gall-bladders

RT (Reverse transcriptase)–PCR was performed to evaluate the expression of AQP8 mRNA in animal (mouse, calf, rabbit and guinea pig) and human gall-bladders. The specificity of the cDNAs amplified by RT–PCR was confirmed by cloning and sequencing the PCR fragments. Abundant AQP8 transcript was found in all specimens analysed (Figure 1), suggesting ubiquitous expression of this AQP in the mammalian

Figure 2 | Comparative alignment of deduced amino acid sequences of animal and human AQP8
Deduced amino acid sequence alignments were performed by PILEUP program analysis of fragments from mouse, rat, rabbit, calf and human AQP8. Single boldface lines indicate four of the six transmembrane domains (TM 3–TM 6) that correspond to an internal portion of the molecule not including the N- and C- termini. Positions with fully conserved residues are enclosed.



gall-bladder. Amino acid sequence alignment of the translated cDNA fragments showed a fair conservation of AQP8 among the mammalian species investigated (Figure 2). This was in line with a previous study showing divergence among the different AQP8 sequences only in the intracytoplasmic N- and C-terminus regions (Koyama et al., 1998). The cloned cDNA fragments of calf and rabbit AQP8 were registered in the GenBank® database with the accession numbers AY743596 and AY743597 respectively.

Other RT–PCR experiments with samples of total RNA extracted from scraped gall-bladder epithelia showed the presence of the AQP1 transcript in the mouse gall-bladder, whereas no other known mammalian AQPs (AQP0, AQP2-7 and AQP9) other than AQP1 and AQP8 were found (results not shown).

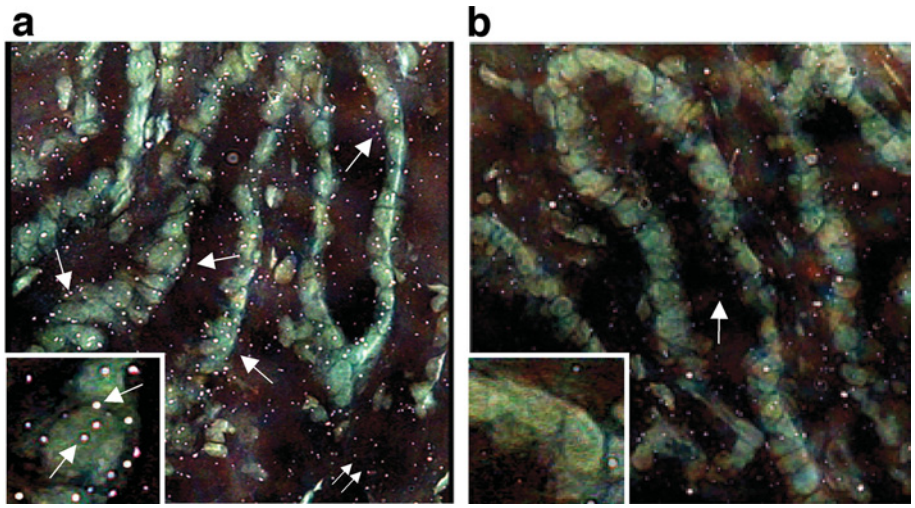
After demonstrating the systematic presence of the AQP8 transcript in human and the above-mentioned animal gall-bladders, studies aimed to define the subcellular distribution of AQP8 and AQP1 were restricted to the mouse gall-bladder.

In situ hybridization localization of AQP8 in mouse gall-bladder

Studies of *in situ* hybridization were performed to localize the AQP8 transcript in the mouse gall-bladder. Confirming the RT–PCR studies, the gall-bladder sections hybridized with the radioactive antisense probe showed intense AQP8 expression in the epithelial layer (Figure 3a). Weak radioactivity was detected in the muscular layer of the mouse gall-bladder (Figure 3a). Proving the specificity of the

Figure 3 | *In situ* hybridization analysis of AQP8 in mouse gall-bladder

(**a**) Mouse gall-bladder hybridized with the AQP8 antisense probe. Arrows show intense staining in the epithelial cells. Weak staining is observed in the muscle layer (double arrows). (**b**) Mouse gall-bladder hybridized with the sense probe. Some unspecific staining is randomly distributed throughout the gall-bladder section (arrow). Original magnifications: (**a**, **b**) × 400; insets, × 800.



antisense probe, no significant signals were observed in gall-bladders incubated with the sense probe (Figure 3b).

Immunohistochemical distribution of AQP8 and AQP1 in mouse gall-bladder

The cellular and subcellular distribution of AQP8 in mouse gall-bladder was assessed by immunohistoperoxidase analysis, using rabbit affinity-purified AQP8 antibodies directed against the N-terminus of the rat AQP8 polypeptide and known to cross-react with mouse AQP8 (Ferri et al., 2003). Confirming the *in situ* hybridization study, clear immunostaining was observed over the apical membrane of the epithelial cells lining both the neck and corpus regions (Figures 4a and 4b). Weak AQP8 immunoreactivity was observed in the intracellular compartment of the epithelial cells and, occasionally, in the intracellular compartment of the muscular layer of the corpus portion (results not shown). No immunostaining was observed in control gall-bladders where the AQP8 antibodies were omitted (Figure 4c). Additional immunohistochemistry experiments were performed to verify whether another AQP already recognized in the human gall-bladder epithelium, AQP1 (Nielsen et al., 1993), was also expressed in the murine counterpart. Suggesting systematic expression of AQP1 in the mammalian gall-bladder epithelium, strong immunolabelling was seen over the apical and lateral membranes as well as in subapical vesicles of the epithelial cells (Figures 5a and 5b). As expected (Ma et al., 2001), specific AQP1 immunolabelling was seen in the endothelial cells lining the submucosal microvessels (Figure 5b). No immunoreactivity was seen in the muscular layer (results not shown).

Immunoblotting analysis of AQP8 and AQP1 in mouse gall-bladder

Immunoblotting studies were performed to understand better the subcellular distribution of AQP8 in fractions of mouse gall-bladder membranes prepared at different gravitational forces. Bands of 28 and 34 kDa, most probably corresponding to the core and glycosylated forms of AQP8 respectively, were seen in the plasma membrane-enriched fraction (17 000 *g*) (Figure 6a). Weak reactivity was often encountered in the 1000 *g* pellet (Figure 6a), a mitochondria-enriched fraction. This latter observation is consistent with our previous study describing the multiple subcellular localizations featured by AQP8 in hepatocyte mitochondria (Ferri et al., 2003). Immunoblotting of the plasma membrane-enriched fraction of mouse gall-bladder incubated with the AQP1 antibodies showed reactive bands at 28 and 34–38 kDa, most probably corresponding to the core and glycosylated forms of AQP1 (Figure 6b) respectively.

Figure 4 | Immunohistochemical distribution of AQP8 in mouse gall-bladder

(**a**) Neck region. Intense immunostaining is observed in the apical membrane of the epithelial cells (arrows). (**b**) Corpus region. As in the neck region, AQP8 reactivity is clearly seen over the apical membrane of the epithelial cells (arrows, main panel and inset). (**c**) Negative control. No immunostaining is observed in control gall-bladders omitting the AQP8 antibody. Original magnifications: (**a**, **b** and **c**) × 400; inset, × 1000.

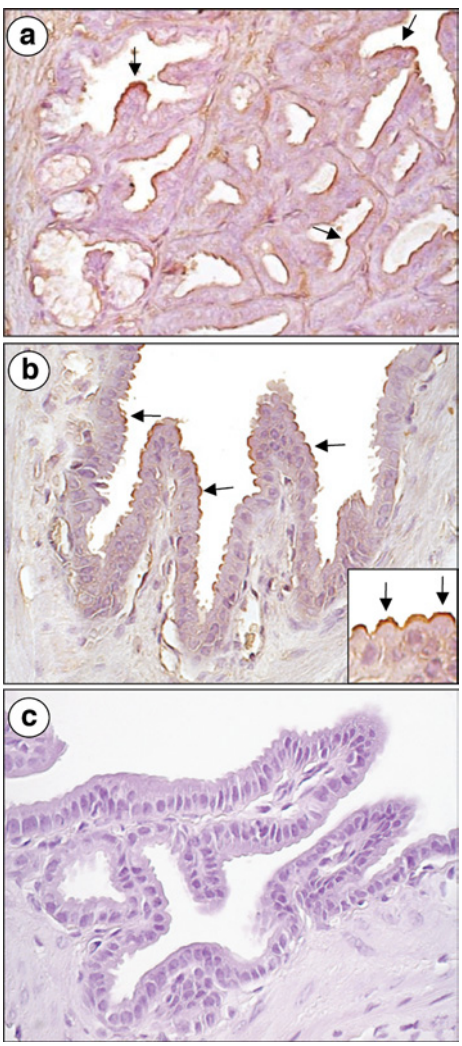
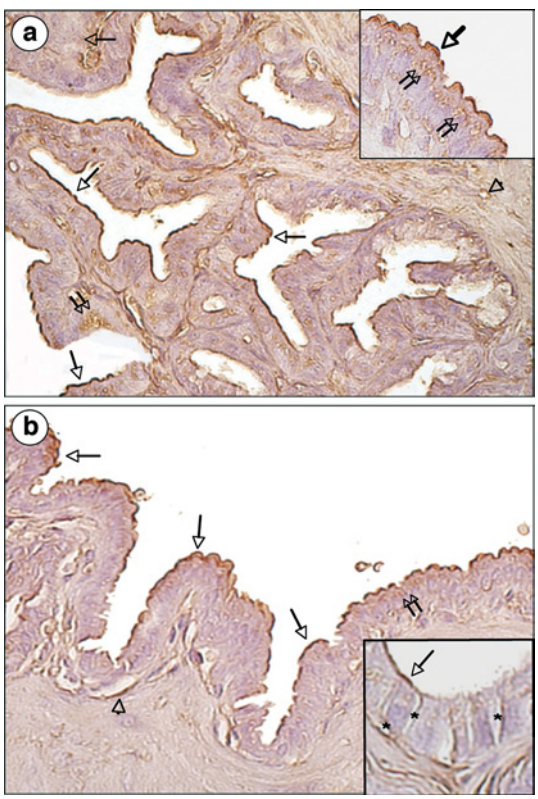


Figure 5 | Immunohistochemical localization of AQP1 in mouse gall-bladder

(**a**) Neck region. Strong immunoreactivity is observed in the apical membrane of the epithelial cells (arrows, main panel and inset) as well as in intracellular vesicles located at the subapical pole (double arrows, main panel and inset). (**b**) Corpus region. Intense AQP1 staining is seen in the apical membrane of the epithelial cells (arrows, main panel and inset) and in subapical vesicles. Labelling is observed over the plasma membrane of the endothelial cells lining the submucosal microvessels (arrowhead). High magnification micrographs show dilation of the lateral space between adjacent epithelial cells (inset, *). Original magnifications: (**a**, **b**) × 400; insets, × 1000.

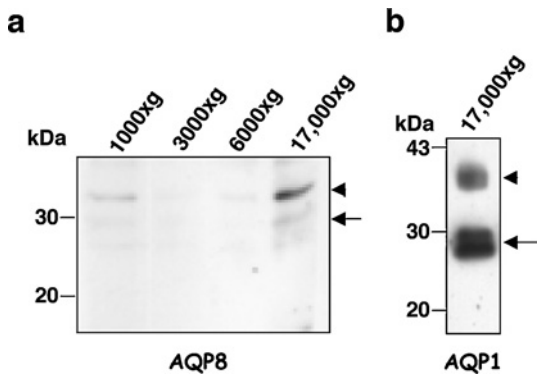


Discussion

The recent demonstration of AQP water channels in the hepatobiliary tract is highly instructive, and provides important insights into the understanding of the molecular mechanisms underlying the movement of water across the hepatobiliary epithelia in bile formation and bile flow (Masyuk et al., 2002; Portincasa et al., 2003). In the present study, we report the expression at a cellular and a subcellular level of the AQP1 and AQP8 water channels in mouse gall-bladder epithelium. AQP8 is also found in the calf, rabbit and guinea pig. A novel and pathophysiologically relevant finding in the present study is that AQP8 is also expressed in human gall-bladder from patients with small pigment stones without inflammation and a bile composition similar to that of healthy subjects, in terms of biliary lipids (Portincasa et al., 1996). Taken together, our results suggest

Figure 6 | Immunoblotting analysis of AQP8 and AQP1 in mouse gall-bladder epithelium

(**a**) Blot incubated with the polyclonal AQP8 antibody. Reactive bands of 28 (arrow) and 34 (arrowhead) kDa are observed in the plasma membrane-enriched fraction (17 000 ***g***) of the mouse gall-bladder epithelium. Although of much weaker intensity, a similar profile is seen in the mitochondria-enriched fraction (1000 ***g***). (**b**) Plasma membrane-enriched fraction of the gall-bladder epithelium incubated with the polyclonal AQP1 antibody. Two bands of strong intensity are seen at molecular masses of 28 (arrow) and 34–38 (arrowhead) kDa respectively.



that AQP8 and AQP1 account for the osmotically driven absorption and secretion of gall-bladder bile water.

A first indication arising from this work is the fact that expression of AQP8 is a common feature of many (if not every) mammalian gall-bladders; indeed, different characteristics of the gall-bladders studied here range from absorption to secretion (Cremaschi and Porta, 1992; Bazzini et al., 2001; Cuthbert, 2001). A similar situation appears to characterize AQP1, which is also found in the animal and human gall-bladders. The presence of two distinct AQPs in the gall-bladder epithelium strongly corroborates the hypothesis that transepithelial water transport across the gall-bladder mucosa occurs principally through water channels and not through a paracellular pathway (Spring, 1998).

An intriguing finding is that AQP1 and AQP8 are co-expressed at the apical membrane of the mouse gall-bladder epithelium. Redundancy in the apical membrane of epithelial cells is a not a usual situation, since comparable patterns of expression have been described only in the intercalated cells of human pancreas (Burghardt et al., 2003). Thus it is conceivable to speculate that the presence of AQP1 and AQP8 in the apical membrane of the gall-bladder epithelium accounts for the lack of any obvious defect in gall-bladder function in AQP1 null humans (Preston et al., 1994) or mice (Ma et al., 2001). Because the gall-bladder epithelium has been described to possess both absorptive and secretory functions (Igimi et al., 1992), a tempting hypothesis is that apical AQP8 may account for the absorption of water from gall-bladder bile, whereas apical AQP1 may mediate the hormonally regulated secretion of water into the gall-bladder lumen. Because AQP1 is also expressed in the basolateral membrane where no other AQPs are found, it is probable that basolateral AQP1 mediates both the uptake and efflux of water into and out of gall-bladder epithelial cells respectively. The possibility of AQP1 acting as the hormonally regulated water channel underlying the secretion of water into the gall-bladder lumen is corroborated by the morphological evidence of AQP1-immunoreactive vesicles located in the subapical region of gall-bladder epithelial cells (Figure 5). Such a pattern strongly resembles the one described for AQP1 in cholangiocytes, the epithelial cells that line the bile ducts, where AQP1 is known to be regulated by secretin (Marinelli et al., 1997). Marinelli and co-workers have suggested that cholangiocyte AQP1 is translocated from subapical vesicles to the apical plasma membrane in response to secretin stimulation (Marinelli et al., 1997, 1999) to underlie the considerable secretion of water, which characterizes ductal bile formation (Raeder, 1995). Supporting a role for AQP1 in gall-bladder fluid secretion, secretin has been shown to cause direct secretion of fluid from gall-bladder epithelial cells (Igimi et al., 1992). Cholangiocytes have been reported to express the mRNA of additional AQPs including AQP8 (Masyuk et al., 2002). Unlike AQP1, AQP8 might mediate absorption of bile water from the gall-bladder lumen. A role for AQP8 in mediating transepithelial absorption of water has been already described by us and others in rat digestive epithelia (Calamita et al., 2001; Matzusaki et al., 2004). Involvement of AQP1 and AQP8 in gall-bladder fluid balance is a matter of remarkable interest in understanding the molecular pathogenesis of gallstone disease. A number of studies clearly show that major changes in mucosal transport pathways are observed during the process of cholesterol rather than pigment stone formation (Kuver

and Lee, 2004). Augmented gall-bladder absorption of water and electrolytes is observed during the early stages of cholesterol gallstone formation and this phenomenon leads to increased solute concentration and cholesterol nucleation in bile (Conter et al., 1986; Lee and Choi, 2004). Later, gall-bladder fluid absorption decreases during chronic cholecystitis observed in cholesterol gallstone formation (Jacyna et al., 1988). Also, CaM (Ca^{2+}-calmodulin) tonically inhibits gall-bladder electrolyte absorption in animals fed with a non-lithogenic diet, and CaM-mediated inhibition of NaCl transport is lowered in animals fed with cholesterol (Moser et al., 2000). Thus dysfunctional CaM regulation, or generally a failure of the regulatory process, may be a stimulus to increase gall-bladder absorption and to promote cholesterol crystallization, crystal growth and precipitation/agglomeration. In line with these facts, administration of amiloride, an inhibitor of Na^+ absorption, has resulted in a significant decrease in the incidence of cholesterol gallstones in the animal models fed a cholesterol-enriched lithogenic diet (Strichartz et al., 1989). Confirming the pathophysiological relevance of the AQP water channels in gallstone diseases, we already have evidence of dysregulated AQP8 and AQP1 expressions in the gall-bladder epithelium of mice with cholesterol gallstone disease (K.J. van Erpecum, D.Q.-H. Wang, D. Ferri, M. Svelto, P. Portincasa, J.-J. Hendrickx, M.I. Schipper and G. Calamita, unpublished work). Emerging evidence documenting the pathophysiological correlations between biliary lipids, AQP water channels and cholesterol gallstone is discussed in this issue of Biol. Cell (van Erpecum, 2005). Further studies from this laboratory are focusing on the functional involvement of AQP1 and AQP8 in healthy mouse gall-bladder as well as changes in AQP expression during early and late stages of cholesterol gallstone formation, when excess cholesterol is present in bile.

In conclusion, this study documents the expression and subcellular distribution of two AQP water channels, AQP1 and AQP8, in the gall-bladder epithelium. Besides corroborating the hypothesis of a channel-mediated pathway for the movement of water across the gall-bladder epithelium, the results reported here suggest that AQP8 and AQP1 play significant roles in the transepithelial fluid transport by which gall-bladder rearranges its bile content depending on the digestion state.

Materials and methods

Animals

Adult male BALB/c mice, and adult guinea pigs were from Harlan Italy (San Pietro al Natisone, Italy) and B.M.G. (Cividate al Piano, Italy) respectively. Mice and guinea pigs were fed with a standard diet and watered *ad lib*. For all the experiments, animals were decapitated after anaesthesia and the gall-bladders were collected as described below. Gall-bladders from male calves weighing about 200 kg were collected directly at the public slaughterhouse. The organs were opened, everted and washed clean of bile at room temperature ($20 \pm 2^{\circ}C$) and transported to the laboratory in Krebs–Henseleit solution bubbled with 95% O_2 and 5% CO_2, with the following composition (mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 24.9 $NaHCO_3$ (pH 7.4). Similarly, rabbit gall-bladders were collected from approx. 3 kg adult New Zealand rabbits at a private slaughterhouse (property of Bernasconi, Bizarrone, Italy) and epithelial cells were obtained by scraping the mucosal surface with a glass coverslip; scraped tissues were frozen in liquid nitrogen and transported to the laboratory. Fresh gall-bladder tissues were obtained from patients with small pigment gallstones undergoing elective laparoscopic cholecystectomy. In all cases, signs and symptoms of haemolytic disorders were excluded and subjects were otherwise healthy. At gross examination, the gall-bladder mucosa and wall showed no sign of inflammation, as also confirmed at histology (results not shown). Immediately after collection, small parts of tissues were fixed in 4% (w/v) paraformaldehyde for histological examination or frozen in liquid nitrogen and then at $-80^{\circ}C$ for later analysis.

RT–PCR

Mice gall-bladders were removed from the killed animals and frozen in liquid nitrogen. Calf, guinea pig and rabbit gall-bladder epithelial cells were collected by scraping the mucosal layer of the everted gall-bladder using a glass coverslip. Total RNAs were isolated using the TRIzol® extraction kit (Invitrogen, San Diego, CA, U.S.A.) by following the manufacturer's instructions. The samples of total RNA from mouse, calf, rabbit and guinea pig were submitted to RT–PCR as described previously (Calamita et al., 2001) by using the primers AQP8-30*cons* (5′-GGTGGACACTTCAACCCTGC-3′) and AQP8-31*cons* (5′-CCCAGCCAGTAGATCCAATG-3′) which relates to highly conserved regions of the AQP8 ORF (open reading frame) leading to a cDNA fragment of 434 bp. The human AQP8 primers hAQP8-3 (5′-GTCCTGAGGAGAGGTTCTGG-3′) and hAQP8-4 (5′-GGATATCCACGGTGACGGCA-3′) were used to amplify a 201 bp fragment of the human AQP8 cDNA. The cDNAs amplified were then cloned into the pCR2.1 vector (TA cloning kit, Invitrogen, San Diego, CA, U.S.A.) and the identity of the inserted DNA fragments was verified by sequencing.

In situ hybridization analysis

In situ hybridization was performed as described previously by Wilcox (1993). Briefly, ^{35}S-labelled antisense and sense RNA probes were synthesized with T7 and SP6 RNA polymerase using a linearized cDNA template encoding the entire mouse AQP8 coding region. Cryostat sections (10 μm thickness) of mouse gall-bladder were fixed in 4% paraformaldehyde in PBS and treated with 5 μg/ml proteinase K (Promega, WI, U.S.A.) in

500 mM NaCl, 10 mM Tris/HCl (pH 8.0), for 10 min at room temperature and hybridized with each probe (1×10^6 c.p.m./section) overnight at 55°C. After washing in 2 × SSC 1 mM EDTA and 10 mM 2-ME (2-mercaptoethanol) at room temperature, the sections were treated with 20 μg/ml ribonuclease A in 500 mM NaCl and 10 mM Tris/HCl, pH 8.0, for 30 min at room temperature, followed by washing in 0.1 × SSC (1 mM EDTA and 10 mM 2-ME) at 55°C for 4 h and washing in 0.5 × SSC at room temperature. After dehydration, the sections were exposed to photographic emulsion for 5 days at 4°C, developed and counterstained with haematoxylin for use with a bright or dark field microscope.

Subcellular membrane fractionation

Mice were killed after anaesthesia and gall-bladders were quickly removed and processed extemporaneously. For the isolation of subcellular membrane fractions, 20 gall-bladders were homogenized with a Potter–Elvehjem homogenizer (4 strokes in 1 min at 500 rev./min) in an isolation medium consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris/HCl, 1 mM EDTA and 5 mM EGTA (pH 7.4). The homogenate was centrifuged at 500 *g* for 10 min at 4°C and the pellet consisting of nuclei and unbroken cells was discarded; the resulting supernatant was centrifuged at 1000 *g* for 10 min at 4°C and the related pellet (1000 *g* membrane fraction) was washed twice before being resuspended in isolation medium containing a cocktail of proteinase inhibitors (1 mM PMSF, 1 mM leupeptin and 1 mM pepstatin A). The 1000 *g* supernatant was collected, centrifuged at 3000 *g* for 10 min at 4°C and the resulting pellet was washed twice leading to the 3000 *g* membrane fraction. A similar procedure was used to prepare the 6000 and 17 000 *g* fractions of subcellular membranes. All chemicals used for the preparations except digitonin were from Sigma (St. Louis, MO, U.S.A.).

Antibodies

Affinity-purified antibodies directed against an N-terminal peptide of rat AQP8 were described in our previous work (Ferri et al., 2003), whereas antibodies to the C-terminal portion of rat AQP1 were from Sigma.

Immunoblotting analysis

Aliquots (60 μg of proteins) of subcellular fractions prepared as above were heated at 90°C and electrophoresed in an SDS/13% acrylamide gel (Mighty Small II, Amersham Biosciences, CA, U.S.A.) using a low molecular mass protein ladder (Amersham Biosciences, Buckinghamshire, U.K.). The resolved proteins were transferred electrophoretically on to nylon membranes that were blocked in 5% (w/v) low fat milk in blocking buffer [20 mM Tris/HCl, 0.15 M NaCl and 1% (w/v) Triton X-100, pH 7.5] for 1 h, and further incubated with the AQP8 antibodies at a final concentration of 1 μg/ml blocking solution. Horseradish peroxidase anti-rabbit IgG-treated membranes (anti-rabbit IgG peroxidase antibody, Sigma) were developed by luminal-chemiluminescence (ECL® Plus, Amersham Biosciences, Buckinghamshire, U.K.).

Immunohistochemical experiments

Mice were killed after ether anaesthesia and their gall-bladders were quickly removed. The samples were fixed in 10% (w/v) formalin, dehydrated through graded ethanols and embedded in paraffin wax. Serial sections of 4 μm thickness were cut. Before staining, rehydrated sections were incubated for 5 min at 37°C in 0.01% trypsin and 0.1% $CaCl_2$ (pH 7.8). AQP8 and AQP1 were localized by the PAP (peroxidase–antiperoxidase) method. Endogenous peroxidase was blocked by 1% (w/v) H_2O_2 for 10 min at room temperature. Sections were then incubated for 5 h at 37°C, with AQP8 (Ferri et al., 2003) or AQP1 (Sigma) affinity-purified antibodies at a concentration of 5 μg/ml in blocking buffer (1% normal goat serum in PBS). Successively, sections were treated for 1 h at 37°C with goat anti-rabbit IgG (Sigma) diluted 1:100 in blocking buffer and then incubated with PAP (Sigma) at dilution 1:100, for 1 h at 37°C. The immunolabelling was visualized by incubation with 3-3′-diaminobenzidine-H_2O_2 medium for 10 min at room temperature. Finally, the sections were dehydrated, cleared and mounted with DPX. Controls were performed by omitting the primary antibodies. Images were captured using an E 600 photomicroscope equipped with a digital camera DMX1200 (Nikon, Kawasaki, Japan).

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