Aquaporins (AQPs) facilitate the transepithelial water flow involved in epithelial fluid secretion in numerous tissues; however, their function in the pancreas is less characterized. Acute pancreatitis (AP) is a serious disorder in which specific treatment is still not possible. Accumulating evidence indicate that decreased pancreatic ductal fluid secretion plays an essential role in AP; therefore, the aim of this study was to investigate the physiological and pathophysiological role of AQPs in the pancreas. Expression and localization of AQPs were investigated by real-time PCR and immunocytochemistry, whereas osmotic transmembrane water permeability was estimated by the dye dilution technique, in Capan-1 cells. The presence of AQP1 and CFTR Cl\(^{-}\) channel. In contrast, the expression of AQP1 is mainly localized to the apical membrane of ductal cells in the human pancreas. Bile acid treatment dose- and time-dependently decreased mRNA and protein expression of AQP1 and reduced expression of this channel was also demonstrated in patients suffering from acute and chronic pancreatitis. HCO\(_3^{-}\) and fluid secretion significantly decreased in AQP1 KO versus WT mice and the absence of NOH.
INTRODUCTION

The pancreas secretes 1.5–2 l of fluid per day (Hegyi et al., 2011a; Pallagi et al., 2015). This fluid contains the inactive form of digestive enzymes and is prominently rich in HCO$_3^-$ produced by the ductal cells (Pallagi et al., 2015). The main functions of this HCO$_3^-$-rich fluid are (i) to prevent the premature activation ofzymogens (Pallagi et al., 2011), (ii) to wash out the toxic factors (such as bile acids) from the ductal tree (Venglovecz et al., 2008), and (iii) to provide an alkaline environment in the duodenum for the optimal function of digestive enzymes (Steward et al., 2005). In the last few years, the importance of ion transport proteins has been highlighted in the course of pancreatitis; therefore intensive research has been conducted in order to characterize their pathological roles (Hegyi and Rakonczay, 2015; Pallagi et al., 2015). These clinical and experimental studies indicate that impaired ductal HCO$_3^-$ secretion makes the pancreas more susceptible to inflammatory diseases such as acute or chronic pancreatitis (CP) (Hegyi and Rakonczay, 2010; Hegyi et al., 2011b; Takacs et al., 2013; Pallagi et al., 2014; Maleth et al., 2015). In contrast, much less is known about the water transport processes, despite the fact that movement of electrolytes is osmotically coupled to water flow.

Aquaporins (AQPs) are small membrane proteins that primarily mediate the transport of water molecules and recent research also emphasize their importance in certain regulatory processes (Rodriguez et al., 2011; Ribatti et al., 2014; Madeira et al., 2015). In mammals, 13 AQP isoforms have been identified so far and a few of them show a species-specific expression pattern in the pancreas. In humans, AQP1, -5, -8, and -12 are present in the pancreas (Hurley et al., 2001; Tani et al., 2001; Furuya et al., 2002; Burghardt et al., 2003; Itoh et al., 2005). AQP1 is the first AQP which has been described and is exclusively permeable to water (Preston et al., 1992). The presence of this channel has been shown in the apical and lateral plasma membrane of centroacinar cells, and in the apical and basolateral membranes of intercalated and intralobular ducts (Furuya et al., 2002; Burghardt et al., 2003). AQP5 is an aquaglyceroporin which mediates the transport of glycerol, urea and other small solutes beside water. Expression of AQP5 has only been detected in the apical plasma membrane of intercalated ducts but not in the centroacinar cells (Burghardt et al., 2003). AQP1 and -5 are colocalized with the cystic fibrosis transmembrane conductance regulator (CFTR) Cl$^-$ channel at the apical membrane of the ductal cells which indicates that these channels influence each other's function (Burghardt et al., 2003). AQP8 and -12 are exclusively localized to centroacinar cells. AQP8 is expressed on the apical plasma membrane (Hurley et al., 2001; Tani et al., 2001), whereas AQP12, a relatively new member of the AQP family, is an intracellular water channel (Itoh et al., 2005). Several studies suggest that altered expression or functions of AQPs are often associated with different diseases affecting the kidney, colon, lacrimal, or salivary glands (Steinfeld et al., 2001; Tsubota et al., 2001; Bedford et al., 2003; Zhu et al., 2016). However, only scarce information is available regarding the role of AQPs in pancreatitis. Ohta et al. (2009) have shown that in the absence of AQP12 the course of cerulein-induced pancreatitis is much worse, probably due to the defect in the secretion of zymogens. In addition, decreased expression of AQP1 and -8 has been found in this pancreatitis model that may also affects the outcome of pancreatitis (Kitami et al., 2007). The aim of this study is to provide the first detailed characterization regarding the role of AQPs in the pancreas, both under physiological and pathophysiological conditions and to study its relationship to the CFTR Cl$^-$ channel. We have chosen to focus on AQP1, since this isoform is abundantly expressed in both acinar and ductal cells of mouse and human pancreas and the role of this AQP has not been evaluated in pancreatitis yet. Using in vitro models, human tissues and transgenic mouse, we have shown that AQP1 plays essential role in ductal fluid and HCO$_3^-$ secretion, lack of CFTR decreases its expression and deletion of AQP1 is strongly associated with increased susceptibility of the gland to pancreatitis.

MATERIALS AND METHODS

Ethical Approval

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (United States, Department of Health and Human Services). In addition, the experimental protocol was approved by the local Ethical Board of the University of Szeged, Hungary and by the National Scientific Ethical Committee on Animal Experimentation (Budapest, Hungary). The use of human tissue was approved by the local ethical committee (University of Szeged, Hungary) and written informed consent was obtained from the patients.

Transgenic Mice

AQP1 knock out (KO) mice were a kind gift from Dr. Alan Verkman (University of California, CA, United States) and Dr. Alastair Poole (University of Bristol, United Kingdom). CFTR KO mice were kindly supplied by Dr. Ursula Seidler (Hannover Medical School, Hannover, Germany). Animals were kept in standard plastic cages on 12:12 h light-dark cycle at room temperature (23 ± 1°C) and had free access to standard or CFTR...
specific laboratory chow and drinking solutions. Functional experiments were performed on litter-matched (age 12–16 weeks, both sexes) wild-type (WT) and AQP1 KO mice. All mice were genotyped prior to the experiments. For genotyping, genomic DNA from the tail was isolated and amplified by traditional PCR.

**Human Pancreatic Tissue Samples**

Human pancreatic tissue samples were obtained from autopsy and from surgical resections. Control tissue ($n = 5$) were collected from the tumor-free region of the pancreas of patients with neuroendocrine tumors. Tissue samples from patients with acute necrotizing pancreatitis (ANP; $n = 5$) or CP ($n = 5$) were from autopsy and from surgical resections. The average age of ANP patients was 56 ± 2.8 years, and the male/female ratio was 1.5:1. The average age of CP patients was 56.8 ± 2.8 years, and the male/female ratio was 4:1.

**Cell Cultures and Treatments**

Capan-1, Panc-1, and Miapaca-2 cells were obtained from the American Type Culture Collection (Manassas, VA, United States). Capan-1 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 Medium supplemented with 15% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine and 1% (v/v) Penicillin–Streptomycin (PS). Panc-1 and Miapaca-2 were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) high glucose Medium supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine, 2.5% (v/v) horse serum and 1% (v/v) PS. All three cell lines were kept in a humidified incubator at 37°C. Cells from passage numbers 20–60 were used in this study. In case of PCR experiments 10⁶, whereas in the case of immunostaining 10⁴ cells were seeded into 75 cm² tissue culture flasks or glass bottom petri dishes, respectively, and incubated for 24 h at 37°C. After the incubation, cells were treated with chenodeoxycholic acid (CDCA; 100, 300, and 500 µM) for 30–60 min, at room temperature. After the incubation, ducts were attached to a cover glass, which formed the base of a perfusion chamber, mounted on the stage of an IX71 live cell imaging fluorescence microscope (Olympus, Budapest, Hungary) and excited at 440 and 490 nm. Emissions were monitored at 530 nm. Five to seven region of interests (ROIs) were examined in each experiment, and one measurement per second was obtained. The 490/440 fluorescence ratio was calibrated to pH₇ using the high K⁺-nigericin technique, as previously described (Thomas et al., 1979; Hegyi et al., 2004).

**Measurement of HCO₃⁻ Secretion**

In order to estimate HCO₃⁻ efflux, the activity of the Cl⁻/HCO₃⁻ exchanger was measured by the Cl⁻ withdrawal technique. Removal of luminal Cl⁻ from the standard HCO₃⁻/CO₂-buffered solution induced an alkalization in the cells due to the reverse mode of the exchanger. Re-addition of Cl⁻ induces HCO₃⁻ secretion via the Cl⁻/HCO₃⁻ exchanger. Under these conditions the initial rate of acidification reflects the activity of the Cl⁻/HCO₃⁻ exchanger. In order to evaluate base efflux [$−(\Delta pH/Δt)$] the following equation was used: $−(\Delta pH/Δt) = ΔpH/Δt × 𝜋_{total}$, where $ΔpH/Δt$ is the rate of acidification measured over the first 60 s and $𝜋_{total}$ is the total buffering capacity of the cell.

**Measurement of in Vitro Ductal Fluid Secretion**

Fluid secretion of intra/interlobular pancreatic ducts was measured using a swelling method, as described previously (Fernandez-Salazar et al., 2004). Briefly, isolated pancreatic ducts were attached to a cover glass which formed the base of a perfusion chamber and mounted on the stage of an IX71 live cell imaging fluorescence microscope. Low magnification, bright-field images were acquired at 1-min intervals using a CCD camera (Hamamatsu ORCA-ER, Olympus, Budapest, Hungary). At the end of each experiment, ducts were perfused with hypotonic solution in order to check the integrity of the duct wall. Ducts that do not respond to the hypotonic challenge were excluded from the analysis. Changes in relative luminal volume was analyzed by Scion Image software (Scion Corporation, Frederick, MD, United States) (Fernandez-Salazar et al., 2004; Pascua et al., 2009).
Measurement of in Vivo Pancreatic Fluid Secretion

In order to measure pancreatic fluid secretion in vivo, magnetic resonance imaging (MRI) was performed on WT and AQP1 KO mice (Maleth et al., 2015). Animals were allowed free access to pineapple juice 12 h before the MRI examination. MRI was performed in a 7.1 Tesla animal scanner (Bruker, Ettlingen, Germany). Strong T2-weighted series of the complete abdomen were acquired before and after retroorbital injection of 10 IU units/kg body weight (b.w.) secretin (ChiroStim, ChiRhoClin, Burtonville, MD, United States). The time between injection and MRI was 6 min. The sequences were acquired using the image parameters: TR/TE 4400/83 ms; flip angle: 180°; matrix 256 × 256; field of view 40 × 40 mm; bandwidth 315 Hz/pixel; slice thickness 1 mm; 20 slices. All image analyses were performed using Osirix (version 5; Pixameo, Bernex, Switzerland). In order to exclude effects of the basal secretion, MRI datasets after and before secretion were subtracted. The created images show the total excretion after secretin stimulation. Excreted fluid is defined as high signal intensity in created images. The fluid excretion into the small intestine was segmented in each slice. The software calculated the volume of the segmented areas. This volume represents the total excreted volume (TEV). In order to minimize artifacts, image noise was reduced.

Measurement of Osmotic Transepithelial Water Permeability

The osmotic transcellular water movement (Pt) was estimated using the cell-impermeant dye, Texas Red™ Dextran as previously described (Levin et al., 2006). Briefly, cells (5 × 10⁵) were grown on a polyester permeable support (Transwell, 12 mm diameter and 0.4 µm pore size). Monolayer confluence was checked by measuring the transepithelial electrical resistance (RT) using an EVOM-G Volt/Ohm Meter (World Precision Instruments, Sarasota, FL, United States). Cells were washed with isoosmolar phosphate-buffered saline (PBS) from the basolateral surface and hyperosmolar PBS (complemented with 300 mM D-mannitol and 0.25 µg/mL Texas Red Dextran) from the luminal surface. In some experiments, different concentrations of bile acids were added to the apical solution. Transwells were then placed into a CO₂ incubator and 5 µL samples were collected from the apical solution at specified time points. Water moves along the osmotic gradient that causes the dilution of the fluorescent dye, in the apical solution. Fluorescence was measured at 595 nm excitation and 615 nm emission, using a Fluoro Max-4 spectrofluorometer (Horiba Scientific, Tokyo, Japan). For the calculation of Pt, the following formula was used: dV(0)/dt = PtSvw*(Φ₁−Φ₂), where S is the tissue surface area, vw is the partial molar volume of water and Φ₁−Φ₂ is the transepithelial osmotic gradient.

Real-Time PCR

Total RNA was purified from individual cell culture samples using the RNA isolation kit of Macherey-Nagel (Macherey-Nagel, Düren, Germany). All the preparation steps were carried out according to the manufacturer’s instructions. RNA samples were stored at −80°C in the presence of 30 U of Prime RNase inhibitor (Fermentas, Lithuania) for further analysis. The quantity of isolated RNA samples was checked by spectrophotometry (NanoDrop 3.1.0, Rockland, DE, United States). In order to monitor gene expression, QRT-PCR was performed on a RotorGene 3000 instrument (Corbett Research, Sydney, NSW, Australia) using the TaqMan probe sets of specific aquaporin genes (Applied Biosystems, Foster City, CA, United States). Information about the genes and the TaqMan assays is collected in Table 1. 3 µg of total RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's instructions in final volume of 30 µL. The temperature profile of the reverse transcription was the following: 10 min at room temperature, 2 h at 37°C, 5 min on ice and finally 10 min at 75°C for enzyme inactivation. These steps were carried out in a Thermal Cycler machine (MJ Research, Waltham, MA, United States). After dilution with 30 µL of water, 1 µL of the diluted reaction mix was used as template in the QRT-PCR. For all the reactions TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, United States) were used according to the manufacturer's instructions. Each reaction mixture (final volume 20 µL) contained 1 µL of primer-TaqMan probe mix. The QRT-PCR reactions were carried out under the following conditions: 15 min at 95°C and 45 cycles of 95°C for 15 s, 60°C for 1 min. Fluorescein dye (FAM) intensity was detected after each cycle. Non-template control sample was used for each PCR run to check the primer-dimer formation. Relative gene expression ratios were calculated as ∆Ct values (Ct values of gene of interest versus Ct values of human hypoxanthine phosphoribosyltransferase gene). In the case of treatments, relative changes in gene expression were determined using the ∆∆Ct method as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). A ∆ΔCt was calculated using the following formula: ∆ΔCt = ∆Ct of treated cells – ∆Ct of control, nontreated cells. The N-fold differential expression in the target gene was expressed as 2−∆ΔΔCt. Genes with expression values less than or equal to 0.5 were considered to be down-regulated, whereas values higher than or equal to 2 were considered to be up-regulated. Values ranging from 0.51 to 1.99 were not considered to be significant.

Immunocytochemistry

Capan-1 cells were washed with PBS twice and fixed in paraformaldehyde (4% in PBS) for 30 min at room temperature (RT). In order to avoid non-specific antibody binding cells were incubated with 10% donkey serum and 1% BSA for further 30 min. After blocking, cells were incubated with primary AQP7 human, polyclonal antibodies (1:100 dilutions; Abcam, Cambridge, United Kingdom) at 4°C overnight. Petri dishes were then washed with PBS and incubated with FITC-conjugated AffiniPure Donkey Anti-Rabbit IgG secondary antibody (1:400 dilutions; DAKO, Milan, Italy) for 60 min at RT. Nuclei were counterstained with Dapi. Dishes were then mounted and observed by a
Fluowview 10i-W confocal microscopy (Olympus, Budapest, Hungary).

**Immunohistochemistry**

Paraffin-embedded, 3- to 4-µm-thick sections of surgically removed resection specimens and autopsy tissue samples were used for immunohistochemistry. After deparaffinization of tissue samples with EZ Prep Concentrate 10X (Ventana Medical Systems, Tucson, AZ, United States), endogenous peroxidase blocking and antigen retrieval (CC1; Ventana Medical Systems), pancreas sections were incubated with polyclonal AQP1 antibody (1:100 dilution; Alomone Labs, Jerusalem, Israel) overnight at 4°C. Immunohistochemical staining was performed with horseradish peroxidase multimer-based, biotin-free detection technique according to the protocol of the automated Ventana system (Ventana Benchmark XT; Ventana Medical Systems). For visualization, the UltraView Universal diaminobenzidine (DAB) Detection Kit (Ventana Medical Systems) was applied. Sections from human pancreas were used as positive controls. For negative control, primary antibodies were substituted with antibody diluent (Ventana Medical Systems). The stained slides were digitized with Mirax Pannoramic MIDI1 and Mirax Pannoramic SCAN digital slide scanners (3DHistech Ltd., Budapest, Hungary).

Cryosections from WT, AQP1, and CFTR KO mice pancreas were fixed in 2% paraformaldehyde, permeabilized in 10% Tween 20-sodium citrate, blocked with 5% goat serum followed by immunofluorescent double staining for AQP1 mouse monoclonal antibody (1:500 dilutions; Thermo Fisher, Rockford, IL, United States) and CFTR rabbit polyclonal antibody (1:100 dilutions; Alomone Labs, Jerusalem, Israel) at 4°C, overnight. Following washing, sections were incubated with secondary antibodies goat-anti-mouse (Alexa fluor 488, Thermo Fisher, Rockford, IL, United States) and goat-anti-rabbit (Alexa fluor 568, Thermo Fisher, Rockford, IL, United States) for 2 h at room temperature in the dark. Nuclei were counterstained with Dapi. Sections were then mounted and analyzed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft., Budaörs, Hungary).

**Quantification of the Immunostainings**

In order to quantify AQP1 or CFTR positively stained area, 10–12 representative, digital images were taken from the human (normal pancreas, AP and CP) and mouse (WT, AQP1 KO and CFTR KO) pancreas sections and from Capan-1 cells. Pictures were then converted to gray scale (16-bit) and thresholded in order to select the positively stained area, using the ImageJ software. The intensity of DAB (human pancreas) or fluorescence signal (mice pancreas and Capan-1 cells) varied on an arbitrary scale from 0 to 255, where 0 is the negative staining (white pixels) and 255 is the maximal staining (black pixels). In the human samples, the mean integrated density of the positively stained area was normalized to the mean integrated density of the total image and converted into percentage. In the cell line, the mean integrated density of the positively stained area was normalized to the total cell number and expressed in arbitrary units. In the mice samples, the ductal area was selected and the total fluorescence intensity was calculated and summarized which was than normalized to the ductal area (µm²) and expressed in arbitrary units/µm². Comparison of each groups were calculated by one-way ANOVA, followed by the Holm-Sidak method. Statistical significance was defined as $p \leq 0.05$. Data are expressed as means ± SEM.

**Induction of Acute Pancreatitis in Mice**

Acute pancreatitis (AP) was induced in mice by hourly (10 times) intraperitoneal (i.p.) injections of cerulein (50 µg/kg) (Niederer et al., 1985; Ding et al., 2003; Pallagi et al., 2014) following anesthesia with i.p. 85 mg/bwkg pentobarbital (Bimeda MTC, Cambridge, ON, Canada). The control animals received the same amount of saline. Two hours after the final injection, mice were euthanized by pentobarbital overdose (200 mg/bwkg i.p.). Animals were exsanguinated through the cardiac puncture and the pancreata were immediately removed. The collected blood was centrifuged at 4°C with 2500 RCF for 15 min and the sera were stored at −20°C until use. The pancreas was trimmed from fat and lymphatic tissue, put into 6% neutral formaldehyde solution and stored at −80°C until use. Serum amylase activity was measured with a commercial colorimetric kit.
Aquaporins are involved in transepithelial water transport in numerous tissues (Tradtrantip et al., 2009). In the next step, we investigated the role of this protein in pancreatic ductal fluid secretion using gene modified mice. Using traditional PCR we have shown the presence of AQPI both in the isolated, intra/interlobular pancreatic ducts and the total pancreas (Figure 2A). Slight expression of AQP5 was also detected in the pancreatic ducts, whereas the mRNA presence of AQP8 and -12 was only observed in the total pancreas (data not shown). We decided to characterize the role of AQPI, since this isoform is constitutively expressed in both the ductal and acinar cells and also can be found in the mouse and human pancreas (Table 2). In contrast to AQPI, AQP3 is not expressed in the mouse pancreas and this isoform is either involved in tumor progression than pancreatitis (Direito et al., 2017; Huang et al., 2017). Although AQP5 is present in the intra/interlobular pancreatic ducts of mice and human, and probably plays role in ductal fluid secretion (Burghardt et al., 2003), KO animal for this isoform was not available to us. Table 2 summarizes the presence of AQP isosforms in mice and human.

The rate of fluid secretion was measured over 30–40 min using sealed ducts and the swelling technique. Initially, ducts were perfused with the standard HEPES-buffered solution and then perfusion was switched to HCO$_3^-$/CO$_2$-buffered solution to initiate HCO$_3^-$-dependent secretion. As shown in Figure 2B, the presence of HCO$_3^-$ induced a dynamic swelling of the WT ducts as a result of fluid secretion into the closed luminal space. In contrast, ducts from AQPI KO mice showed no or only a slight response to HCO$_3^-$ . Under stimulated conditions (forskolin; 10 µM), the rate of fluid secretion further increased in the WT ducts, whereas in the case of KO ducts, no response was detected. Figure 2C summarizes the relative luminal volume changes in WT and KO ducts after forskolin stimulation. As shown in Figure 2C, the relative luminal volume increase reduced by 89.9 ± 6.2% in the absence of AQPI1, indicating that this AQPI isoform plays an essential role in fluid secretion.

We also investigated the rate of pancreatic fluid secretion in vivo by MRI cholangiopancreatography in anesthetized mice. Fluid secretion was stimulated by retroorbital administration of 10 U/kg b.w. secretin, and then TEV was measured in WT and KO animals. Similarly, to the in vitro measurements, the pancreatic fluid secretion significantly reduced in AQPI KO (0.0041 TEV/cm$^3$/min) vs. WT (0.023 TEV/cm$^3$/min) mice (Figures 2D,E).

Next, we were curious whether the decreased ductal fluid secretion is also associated with impaired HCO$_3^-$ efflux in AQPI KO animals. For the measurement of ductal HCO$_3^-$ secretion, intra/interlobular pancreatic ducts were isolated from the pancreas of WT and AQPI KO mice. Ducts were perfused both from the luminal and basolateral membrane with HCO$_3^-$/CO$_2$-buffered solution. Removal of Cl$^-$ from the apical solution induced an alkalisation inside the cells, due to the reverse mode of the Cl$^-$/HCO$_3^-$ exchanger. Addition of Cl$^-$ back to the external solution decreased the pH, indicating HCO$_3^-$ efflux through the exchanger. Under these conditions, the initial rate of recovery from alkalosis reflects the activity of the Cl$^-$/HCO$_3^-$ exchanger. Using this technique, we showed that the rate of acidification significantly reduced in AQPI KO (42 ± 3.2%) vs. WT ducts indicating that pancreatic ductal HCO$_3^-$ secretion is impaired in the absence of AQPI1 (Figures 2F,G).
AQP1 Expression Decreased in CFTR KO Mice

The decreased HCO$_3^-$ secretion in AQP1 KO mice indicates that beside the transport of water, AQP1 interacts with one or more ion transporters which are involved in HCO$_3^-$ secretion. The CFTR Cl$^-$ channel plays essential role in ductal HCO$_3^-$ secretion by maintaining a luminal [Cl$^-$] which is necessary for HCO$_3^-$ efflux through the Cl$^-$/HCO$_3^-$ exchanger. Several studies presume a physical interaction between the CFTR Cl$^-$ channel and certain AQP isoforms (Schreiber et al., 1999; Cheung et al., 2003; Jesus et al., 2014a,b). Colocalization of this two channel has also been found in the human pancreas (Burghardt et al., 2003). In the following step, we performed immunostaining on the pancreas of AQP1 and CFTR KO mice in order to characterize the possible relation between the two channels. In WT mice, AQP1 expression was detected throughout the whole plasma membrane, whereas expression of CFTR exclusively localized to the apical membrane of the ducts (Figure 3A). The absence of AQP1 caused a slight but not significant decrease in the expression of CFTR, indicating that the impaired HCO$_3^-$ secretion in the AQP1 KO mice is not due to the decreased expression of CFTR (Figure 3A, middle line). Interestingly we have found that expression of AQP1 dramatically decreased in the intra/interlobular ducts of CFTR KO mice, especially at the apical membrane (Figure 3A, bottom line and Figure 3B). We have also found that the absence of CFTR did not affect the expression of AQP1 in the blood vessels indicating that some kind of interaction may exist between these two channels in the pancreatic ductal cells. Although further investigations are needed to clarify whether the two channels are able to regulate each others function, expression or trafficking.

Expression and Function of AQPs Significantly Decreased After Bile Acid Treatment

In the next step, we studied the effect of pancreatitis-inducing factors on the expression of AQP1. Gallstone obstruction and heavy alcohol consumption are the two major causes of pancreatitis. Capan-1 cells were treated with CDCA and GCDCA (100, 300, and 500 µM) (Venglovecz et al., 2008, 2011; Muili et al., 2013), EtOH (1, 10, and 100 mM), POA and POAEE (10, 100, and 200 µM) (Criddle et al., 2006; Judak et al., 2014; Maleth et al., 2015) for 6, 12, 24, and 48 h and the mRNA expressions of AQP1 were analyzed by RT-PCR. Among the investigated agents, CDCA had the most marked effect, it dose- and time-dependently decreased the expression of AQP1 (Figure 4A). GCDCA, POA, and POAEE caused a significant decrease at 12 and 24 h, primarily at higher doses, which partially regenerated after 48 h in the continuous presence of the agents (Supplementary Figures 1A–C). In contrast to bile acids, EtOH initially (24 h) increased the expression of AQP1 that was followed by a decrease (Supplementary Figure 1D). In order to decide whether the downregulating effect of CDCA can also be observed at protein level, we performed immunostaining on the CDCA-treated cells, using specific antibodies against AQP1. CDCA dose- and time-dependently
decreased the protein expression of AQP1 was consistent with the PCR data (Figure 4B). Representative ICC pictures show that incubation with 500 µM CDCA time-dependently decreased the AQP expression in the cells (Figure 4C). The effect of CDCA on the activity of AQP1 was also investigated. As shown in Figure 4D, 100 and 300 µM CDCA had no effect on the transepithelial water movement, however, at 500 µM P₁ was significantly impaired.

TABLE 2 | mRNA expression of AQP isoforms in mice and human.

| Known isoforms | Isoforms in mice pancreas | Isoforms in mice pancreatic ducts | Isoforms in human pancreas | Isoforms in human pancreatic ducts | Available KO mice |
|----------------|---------------------------|----------------------------------|---------------------------|-----------------------------------|-------------------|
| AQP0           | –                         | –                                | –                         | –                                 | –                 |
| AQP1           | √                         | –                                | √                         | √                                 | √                 |
| AQP2           | –                         | –                                | –                         | –                                 | –                 |
| AQP3           | –                         | –                                | –                         | –                                 | –                 |
| AQP4           | –                         | –                                | ⟨⟩                      | –                                 | –                 |
| AQP5           | √                         | –                                | √                         | –                                 | –                 |
| AQP6           | –                         | –                                | –                         | –                                 | –                 |
| AQP7           | –                         | –                                | –                         | –                                 | –                 |
| AQP8           | √                         | –                                | –                         | –                                 | –                 |
| AQP9           | –                         | –                                | –                         | –                                 | –                 |
| AQP10          | –                         | –                                | –                         | –                                 | –                 |
| AQP11          | –                         | –                                | –                         | –                                 | –                 |
| AQP12          | √                         | –                                | √                         | –                                 | –                 |
Acute Pancreatitis Is Aggravated in AQP1 KO Mice

In order to test the hypothesis that AQP1 may be involved in the pathomechanism of pancreatitis, we investigated the severity of cerulein-induced AP in WT and AQP1 KO mice. WT and AQP1 KO mice were given 10 hourly injections of either physiological saline (control) or supramaximal doses of cerulein (50 μg/kg per injection) i.p. to induce AP. After saline injection, the pancreas had normal histology in both WT and KO animals. In contrast, i.p. injections of cerulein caused extensive cell damage both in the WT and KO animals. The extension of pancreatic necrosis were markedly higher in the AQP1 KO (25 ± 2.8%) vs. WT mice (12.1 ± 3.2%), whereas no differences were observed in the extent of edema and in the infiltration of inflammatory cells (Figures 5A–C). Serum amylase activities were significantly higher in KO (1605 ± 6 U/l) vs. WT mice (1285 ± 51 U/l) after the induction of AP (Figure 5D). As shown on the representative histological images (Figure 5E), the rate of pancreatic necrosis was more extensive in AQP1 KO mice. Overall, these results indicate that in the absence of AQP1 the course of pancreatitis is more severe.

Expression of AQP1 Is Decreased in Acute and Chronic Pancreatitis

Since we found that the lack of AQP1 exacerbates the course of cerulein-induced pancreatitis in mice, we tested whether this water channel is also involved in the pathomechanism of pancreatitis in humans. Therefore, in the next step we investigated the expression of AQP1 in pancreatic tissues samples obtained from five patients with ANP and five patients with CP. Control pancreatic tissue were obtained from tumor-free tissue surrounding neuroendocrine pancreatic tumors. In order to localize AQP1, IHC was performed. In the normal pancreas, strong AQP1 immunoreactivity was detected in the intra/interlobular and intercalated ducts, the acinar and centroacinar cells (Figures 6A–C). The staining in acinar cells and smaller ducts is mainly localized to the lateral and apical surface of the cells (Figures 6B,C). In the interlobular ducts, the apical plasma membrane was positive to AQP1 with some cytoplasmic staining, whereas Langerhans islets were completely negative for AQP1. These results are consistent with previous observations (Burghardt et al., 2003). In the ANP and CP pancreatic tissue sections the expression of AQP1 strongly reduced in the interlobular ducts, whereas intralobular and intercalated ducts still exhibited weak to moderate AQP1 immunoreactivity (Figures 6D–G). In case of acinar cells, the expression of AQP1 slightly decreased in the inflamed pancreas, especially in ANP. Quantification of DAB intensity showed that AQP1 staining was significantly higher in normal pancreas vs. the ANP or CP groups (Figure 6H).

DISCUSSION

Acute and chronic pancreatitis are serious disorders characterized by inflammation and injury of the gland.
FIGURE 4 | Effect of chenodeoxycholic acid on the expression and activity of AQP1. (A) Capan-1 cells were treated with chenodeoxycholic acid (CDCA) for 6, 12, 24, and 48 h and the relative gene expression of AQP1 was investigated by real-time PCR. Data represent mean ± SEM of three, independent experiments.
(B) Protein expression of AQP1 was investigated by immunocytochemistry in Capan-1 cells. Protein expression was measured from the mean fluorescence intensity normalized to the total cell number and expressed in arbitrary units. Data represent mean ± SEM of three, independent experiments. (C) Representative immunofluorescence staining of Capan-1 cells show the expression of AQP1 after the treatment with CDCA (500 µM) for 6, 12, 24, and 48 h. (D) Summary bar chart shows the changes in osmotic transcellular water movement (P_f) of Capan-1 cells in the presence of various concentrations of CDCA. Transwell without cells was used for absolute positive control. Data represent mean ± SEM of three, independent experiments. *p ≤ 0.05 vs. Control.

Gallstones and heavy alcohol consumption are responsible for approximately 80% of all AP patients (Parniczky et al., 2016), whereas alcohol abuse is the primary cause of CP (Szucs et al., 2017). Although the therapy improved a lot in the last few years the morbidity and mortality of pancreatitis is still excessively high (Parniczky et al., 2016). Defects in ductal fluid secretion have been proposed as an important factor in the pathomechanism of pancreatitis (Hegyi et al., 2011b). This fluid is high in HCO₃⁻ which prevents the premature activation of zymogens and provides an optimal environment in the duodenum for the action of digestive enzymes. The secreted fluid is also beneficial from the point of view that washing out the potential toxic factors such as activated digestive enzymes or bile acids from the ductal tree and therefore prevents the pancreas from their damaging effects (Venglovecz et al., 2008). AQP1 is a water channel that extensively expressed in the acinar and ductal cells where mediates pancreatic fluid production. Several studies indicate that altered expression or localization of AQP1s associates with different gastrointestinal disorders, such as gastritis or diarrhea; therefore, many studies have been conducted to identify the specific role of particular AQP isoforms. Although, AQP1 plays an essential role in pancreatic physiology its function under pathological conditions is not known.

In the present study, we showed that (i) bile acids strongly decrease the expression of AQP1 in pancreatic ductal cells, (ii) chronic or acute inflammation of the pancreas is associated with decreased expression of AQP1, (iii) the absence of AQP1 reduces ductal fluid and HCO₃⁻ secretion, (iv) and induces a more severe pancreatitis in a cerulein-induced pancreatitis model. Basically there are two ways for water transport. One is the paracellular way along the osmotic gradient generated by the ion transporters and the other one is the transcellular pathway through the AQP1s. In case of the pancreatic ducts, it is not clear which pathway is the dominant. The pancreatic ductal epithelium is a moderately leaky epithelium which favors the paracellular movement of water (Novak and Greger, 1988). Nevertheless, administration of luminal Hg²⁺, a non-specific inhibitor of AQP1s, decreased ductal fluid secretion by more than 90% in rat pancreatic ducts, which strengthen the importance of transcellular pathway (Ko et al., 2002). In the present study, we demonstrated that pancreatic ductal cells express functionally active AQP1s. We have identified the presence of AQP1, -3, and -5 on the ductal cells that are consistent with previous observations (Burghardt et al., 2003). Treatment of the cells with pancreatitis-inducing agents, mostly decreased the expression of AQP1s both at mRNA and protein levels. Among the investigated agents, CDCA had the biggest effect, which dose- and time-dependently decreased the expression of AQP1. These data are partly consistent with previous findings in the colon, where bile acid treatment reduced the protein expression of AQP3,
whereas increased the levels of AQP7 and -8 (Yde et al., 2016). In case of EtOH, the expression of AQP5 initially increased (presumably due to a compensatory mechanism), then a decrease was observed, similarly to the rat stomach, where intragastrical administration of 1 mM EtOH caused analogous changes in the expression of AQP5 (Bodis et al., 2001). High concentration of CDCA also decreased the activity of the water channels, which is somewhat related to our previous findings on guinea pig pancreatic ducts, where 1 mM CDCA strongly inhibited ductal HCO$_3^-$ and thus fluid secretion (Venglovecz et al., 2008). The involvement of AQP1 in epithelial water movements has been described in various tissues, although there is still no consensus among researchers regarding the functional importance of this water channel (Marples, 2000). In cholangiocytes, the absence of AQP1 does not affect the fluid secretion (Mennone et al., 2002), although inhibition of the secretin-induced translocation of AQP1 reduces bile flow more than half (Marinelli et al., 1997, 1999). It has been also demonstrated that inhibition of AQPs by HgCl$_2$ dose-dependently reduced the osmotically induced volume increase in these cells (Roberts et al., 1994).

In order to identify the mechanism by which AQP1 influences ductal HCO$_3^-$ secretion, we investigated the relation of this channel with the CFTR Cl$^-$ channel. CFTR is a cAMP-activated Cl$^-$ channel, which primarily located at the apical membrane of epithelial cells and plays a crucial role in the maintenance of fluid homeostasis. Growing number of studies indicate that a molecular interaction exists between CFTR and certain AQP isoforms. It has been demonstrated that activation of CFTR by cAMP, increases the water permeability of the cells through the activation of AQP3 (Schreiber et al., 1999; Jourdain et al., 2014).

In order to determine the role of AQP1 in pancreatic fluid secretion, we used AQP1 KO mice. No differences were observed in body weight and physical appearance between WT and KO mice, although the lifespan of AQP1 deficient mice was slightly lower. Using both in vitro and in vivo approaches, we found that fluid and HCO$_3^-$ secretion significantly reduced in the absence of AQP1. These data are partly in contrast with previous observations demonstrating that defect in AQP1 expression cause only a small, but not significant decrease in the rate of stimulated pancreatic fluid secretion (Ma et al., 2001). Although this discrepancy can be explained by the different methods used for the measurement of pancreatic fluid. Nevertheless, other studies have found that decreased expression or function of AQP1 dramatically reduce ductal fluid secretion (Ko et al., 2002; Gabbi et al., 2008). Ko et al. (2002) have shown that luminal or basolateral administration of HgCl$_2$ dose-dependently decrease the fluid secretory rate and osmotic water permeability of isolated pancreatic ducts. It has been also described that the defect in pancreatic fluid secretion in liver X receptor β-deficient mice is related to the decreased expression of AQP1 in the pancreatic ducts of these mice (Gabbi et al., 2008).

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The relation between AQPs and CFTR has been also confirmed in rat epididymis, where CFTR potentiates AQP9-mediated water permeability (Cheung et al., 2003). Moreover, a direct interaction between AQPs and CFTR has been also observed in Sertoli cells (Jesus et al., 2014a,b). Using double immunostaining we showed for the first time that AQP1 and CFTR are co-localized at the apical membrane of pancreatic ductal cells. Lack of CFTR significantly decreased the expression of AQP1, indicating that CFTR somehow controls the water permeability of ductal cells. Similar results have been found in respiratory epithelial cells, where in the presence of CFTR inhibitor or mutant CFTR the water permeability significantly decreased (Schreiber et al., 1999, 2000; Jourdain et al., 2014) which highlights the significance of this water channel in cystic fibrosis. In contrast, expression of CFTR does not depend on the presence of AQP1, since in the absence of this water channel, strong CFTR staining was detected. This result indicates that the decreased HCO$_3^-$ secretion in the AQP1 KO mice is not due to the impaired expression of CFTR. Nevertheless, we cannot exclude that although the expression of CFTR did not change but the channel does not work correctly, however, further functional experiments are needed to confirm this hypothesis.

There is more and more evidence that impaired pancreatic fluid secretion plays role in the pathomechanism of pancreatitis (Hegyi and Rakonczay, 2010; Pallagi et al., 2014; Maleth et al., 2015). So in the next step, we investigated whether the lack of AQP1 has any effects on the progression of pancreatitis. The loss of AQP1 itself does not damage the pancreas and does not cause pancreatitis in mice, which assuming the compensating effect of AQP5; however, induces a more severe disease progression. The involvement of AQP1 in the pathophysiology of pancreatitis has been already raised previously (Kitami et al., 2007). Kitami et al. (2007) have found that AQP1 expression decreased both in the ductal and acinar cells in a cerulein-induced pancreatitis model, which indicate that reduced levels of AQP1 may contribute to exocrine insufficiency. These findings are in accordance with our observation that expression of AQP1 decreased in the ductal and acinar cells of patients with AP or CP. The importance of AQP1 in the exocrine pancreas has been also confirmed by the fact that this water channel is abundantly expressed in the zymogen granules of acinar cells and plays an essential role in zymogen swelling and probably secretion (Choy et al., 2002). AQP12 is also expressed in the zymogen granule of acinar cells and huge amount of this isofrom is present in the rough endoplasmic reticulum (Ohta et al., 2009). In the absence of AQP12, high concentration of cholecystokinlin octapeptide makes the pancreas more susceptible to pancreatitis, presumably by the aberrant exocytosis of zymogen granules in these mice (Ohta et al., 2009). All of these previous observations indicate that AQP1 plays essential role both in ductal and acinar functions and we speculate that the absence of AQP1 makes the pancreas more sensitive in two ways: (1) ductal fluid secretion is not sufficient and (2) exocytosis of zymogen granules is impaired.

In this study, we provided an overview regarding the expression and role of AQP1 in the physiology and pathophysiology of the pancreas. Our data indicate that AQP1 interacts with the CFTR Cl$^-$ channel and takes part in the formation of pancreatic fluid. Moreover, we have found that...
AQP1 plays role in the pathology of pancreatitis. We hypothesize that absence of the channel makes the pancreas more sensitive to pancreatitis, probably due to the decreased pancreatic fluid and HCO$_3^-$ secretion. Our novel findings not only help to understand the pathomechanism of pancreatitis better, but open up new therapeutic opportunities in the treatment of the disease.

**AUTHOR CONTRIBUTIONS**

PP performed microfluorimetric and videomicroscopy experiments. LK, ÁZ, and LP were involved in molecular biology experiments. AB, MS, and J-PK performed MRI and JM and ML interpreted the MRI pictures. Genotyping and breeding of AQP mice were done by EB and EG. Pancreatitis was induced by ZB. KB and ET did the immunostainings on the human and mice pancreatic samples, respectively, and quantified fluorescence intensity. ZR was involved in data interpretation and edited the manuscript. VV was involved in all of the above mentioned experiments, analyzed the data, and drafted the manuscript. PH supervised the project and edited the manuscript. All authors approved the final version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00854/full#supplementary-material

**FIGURE S1** | Expression of AQP after various treatments. Capan-1 cells were treated with (A) glycochenodeoxycholic acid (GCDCA), (B) palmitoleic acid (POA), (C) palmitoleic acid ethyl ester and (D) ethanol (EtOH) for 6, 12, 24, and 48 h and the relative gene expression of AQP1 was investigated by real-time PCR. Data represent mean ± SEM of three, independent experiments. *p < 0.05 vs. Control.

**AUTHOR CONTRIBUTIONS**

This study was supported by the National Research, Development and Innovation Office (K109756 to VV, K116634 to PH, and K119938 to ZR), the Economic Development and Innovation Operative Programme Grants (GINOP-2.3.2-2016-00015 and GINOP-2.3.2-15-2016-00020 to PH), the National Research, Development and Innovation Office, by the Ministry of Human Capacities (EFOP 3.6.2-16-2017-00006 to ZR), by Bolyai Postdoctoral Fellowship of the Hungarian Academy of Sciences (HAS) to VV (00531/11/5), the HAS-USZ Momentum Grant to PH (LP2014-10/2017), the TÁMOP 4.2.4.A/2–11–1-2012-0001 of the National Excellence Program, the Deutsche Forschungsgemeinschaft (DFG MA 4115/1-2/3, DFG SE 2702/2-1, and GRK 1947:A3), the Federal Ministry of Education and Research (BMBF GANI-MED 03IS2061A and BMBF 0314107, 01ZZ9603, 01ZZ0103, 01ZZ0403, 03ZIK012) and the European Union (EU-FF-7: EPC-TM), V-630-S-150-2012/132/133 and PePPP center of excellence MV; ESF/14-BM-A55-0045/16.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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