Bile Acids Stimulate Cholangiocyte Fluid Secretion by Activation of Transmembrane Member 16A Cl\textsuperscript{−} Channels

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Bile acids stimulate a bicarbonate-rich choleresis, in part, through effects on cholangiocytes. Because Cl\textsuperscript{−} channels in the apical membrane of cholangiocytes provide the driving force for secretion and transmembrane member 16A (TMEM16A) has been identified as the Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel in the apical membrane of cholangiocytes, the aim of the present study was to determine whether TMEM16A is the target of bile-acid–stimulated Cl\textsuperscript{−} secretion and to identify the regulatory pathway involved. In these studies of mouse, rat, and human biliary epithelium exposure to ursodeoxycholic acid (UDCA) or tauroursodeoxycholic acid (TUDCA) rapidly increased the rate of exocytosis, ATP release, [Ca\textsuperscript{2+}], membrane Cl\textsuperscript{−} permeability, and transepithelial secretion. Bile-acid–stimulated Cl\textsuperscript{−} currents demonstrated biophysical properties consistent with TMEM16A and were inhibited by pharmacological or molecular (small-interfering RNA; siRNA) inhibition of TMEM16A. Bile acid–stimulated Cl\textsuperscript{−} currents were not observed in the presence of apyrase, suramin, or 2-aminoethoxydiphenyl borate (2-APB), demonstrating that current activation requires extracellular ATP, P2Y, and inositol 1,4,5-trisphosphate (IP3) receptors. TUDCA did not activate Cl\textsuperscript{−} currents during pharmacologic inhibition of the apical Na\textsuperscript{+}-dependent bile acid transporter (ASBT), but direct intracellular delivery of TUDCA rapidly activated Cl\textsuperscript{−} currents.

Conclusion: Bile acids stimulate Cl\textsuperscript{−} secretion in mouse and human biliary cells through activation of membrane TMEM16A channels in a process regulated by extracellular ATP and [Ca\textsuperscript{2+}]. These studies suggest that TMEM16A channels may be targets to increase bile flow during cholestasis. (HEPATOLOGY 2018;68:187-199).
and transport them across the basolateral membrane where they return to the hepatocyte in a process known as cholehepatic shunting.\(^5\) The local recycling of bile acids leads to an increase in Cl\(^{-}\) and HCO\(_3\)\(^{-}\) efflux into the duct lumen. This is important because studies from our laboratory as well as others have demonstrated that Cl\(^{-}\) channels in the apical membrane of cholangiocytes provide the driving force for biliary secretion.\(^6\)\(^7\)

Two Cl\(^{-}\) channels have been identified on a molecular basis in cholangiocytes, (1) the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated Cl\(^{-}\) channel,\(^10\) and (2) TMEM16A, also known as anoctamin or Ano-1, a Ca\(^{2+}\)-activated Cl\(^{-}\) channel.\(^11\) Importantly, in isolated cells and intact biliary epithelial monolayers, increases in [Ca\(^{2+}\)]\(_i\) increase Cl\(^{-}\) current density and transepithelial secretion, respectively, to values 3- to 5-fold greater than cAMP.\(^8\)\(^,\)\(^12\) Thus, these findings indicate that TMEM16A, not CFTR, may represent the dominant Cl\(^{-}\) channel regulating anion efflux, and Ca\(^{2+}\)-dependent signaling may represent the dominant pathway for modulating the anion permeability of the apical cholangiocyte membrane.

Over 20 years ago, Shimokura et al. demonstrated that UDCA directly stimulates Ca\(^{2+}\)-activated Cl\(^{-}\) channels in duct cells\(^13\); however, the molecular identification and regulatory mechanism(s) have remained unknown. Herein, we identify TMEM16A as the target of bile acid–induced cholangiocyte Cl\(^{-}\) secretion, utilizing mouse, rat, and human models, and describe the regulatory pathways involved.

Materials and Methods

CELL MODELS

Studies were performed in (1) mouse cholangiocytes isolated from medium- to large-sized intrahepatic ducts of C57BL/6 mice as described\(^14\)\(^,\)\(^15\); (2) mouse large cholangiocytes (MLCs) isolated from normal mice (BALB/c) and immortalized by transfection with the SV40 large-T antigen gene as described\(^16\); (3) normal rat cholangiocyte (NRC) monolayers;\(^17\) and (4) human Mz-Cha-1 cells derived from a human biliary adenocarcinoma of the gallbladder.\(^18\) For studies of apical surface liquid (ASL) and ATP release, MLC or NRC cells were cultured on collagen-coated semi-permeable transwell supports for 7-10 days, permitting the development of highly polarized cells with a high transepithelial resistance (R\(_t\) > 1,000 \(\Omega\) cm\(^2\)). Animal work described in this article has been approved and conducted under the oversight of the UT Southwestern Institutional Animal Care and Use Committee.

TMEM16A EXPRESSION

TMEM16A protein was detected by western blotting utilizing mouse monoclonal (C-5: sc-377115) or rabbit polyclonal TMEM16A antibody (H-41: sc-135235; Santa Cruz Biotechnology, Santa Cruz, CA). Signals were visualized using enhanced chemiluminescence detection (Pierce Biotechnology, Inc., Rockford, IL); specific signals were captured by x-ray and band-intensity quantified using ImageJ software (NIH, Bethesda, MD).

TMEM16A SILENCING

TMEM16A expression was inhibited using specific TMEM16A siRNA (TMEM16A-HSS123904). In brief, 25-nucleotide siRNAs were designed and synthesized (Supporting Methods) and transfected using Lipofectamine 2000 or Lipofectamine RNAi Max. Noncoding Stealth RNAi (medium guanine cytosine duplex; Invitrogen) was utilized in control (mock) transfections. Block-it Fluorescent Oligo (Invitrogen) was used to optimize transfection conditions and for selection of transfected cells for whole-cell patch-
clamp current recording. Experiments were performed 48-72 hours after transfection, and degree of TMEM16A silencing was evaluated by western blotting analysis.

**MEASUREMENT OF \([\text{Ca}^{2+}]_i\)**

MLCs were cultured for 2 days in glass coverslips, loaded with 2.5 \(\mu\text{g/mL}\) of Fura-2 AM (TEF Laboratories, Austin, TX) in isotonic buffer, and imaged on the stage of an inverted fluorescence microscope (Nikon TE2000). Changes of \([\text{Ca}^{2+}]\), were measured at an excitation wavelength of 340 nm for calcium-bound Fura-2 and 380 nm for calcium-free Fura-2 and an emission wavelength of 510 nm as described.

**MEASUREMENT OF EXOCYTOSIS**

Exocytosis was assessed by real-time imaging using the fluorescent dye, FM1-43 (Molecular Probes, Inc., Eugene, OR), as described. FM1-43 is weakly fluorescent in aqueous solution, but its fluorescence increases >300-fold when it binds plasma membrane; thus, it is a useful dye for the measurement of increases in plasma membrane attributed to fusion of vesicle membrane with the plasma membrane during exocytosis. Changes of FM1-43 fluorescence were measured at excitation wavelength 480 nm and emission wavelength at 535 nm and reported as % change in fluorescence.

**MEASUREMENT OF ATP RELEASE**

Bulk ATP release was studied from confluent cells using the luciferin-luciferase assay as described. Bioluminescence, reflecting ATP release, was quantified as arbitrary light units.

**MEASUREMENT of Cl\(^{-}\) CURRENTS**

Membrane currents were measured by whole-cell patch-clamp techniques with a standard extracellular and intracellular (pipette) solution (Supporting Methods). Recordings were made with an Axopatch ID amplifier (Axon Instruments, Foster City, CA) and digitized and analyzed using pCLAMP version 10 as described. Three voltage protocols were utilized: (1) holding potential of –40 mV, with 100-ms steps to 0 mV and –80 mV at 10-second intervals; (2) holding potential –40 mV, with 100-ms steps to 0 mV and +60 mV at 10-second intervals; and (3) holding potential –40 mV, with 450-ms steps from –100 mV to +100 mV in 20-mV increments. Protocols 1 and 2 were utilized for real-time tracings and protocol 3 for generation of current-voltage (I-V) plots. Results are reported as current density (pA/pF) to normalize for differences in cell size. Details of the buffer solutions, voltage protocols, and data acquisition are provided in the Supporting Information.

**MEASUREMENT OF ASL HEIGHT**

The height of the fluid level at the apical membrane of confluent MLC or NRC monolayers was performed as described. Confluent cells on 24-mm collagen-coated transwell permeable membranes were labeled with Calcein-AM and 80 \(\mu\text{L}\) of Dextran-Red (Molecular Probes) in phosphate-buffered saline was added to the apical chamber. Transwells were mounted on a Zeiss confocal microscope and images acquired using a Zeiss 40\(\times\)/0.8 water immersion lens. All images were analyzed with ImageJ (NIH, Bethesda, MD), as described.

**STATISTICAL ANALYSIS**

Detailed descriptions of the reagents, buffer solutions, experimental protocols, and statistical analysis are provided in Supporting Materials.

**Results**

**BILE ACIDS INCREASE INTRACELLULAR CALCIUM AND STIMULATE OPENING OF MEMBRANE \(\text{Ca}^{2+}\)-ACTIVATED \(\text{Cl}^{-}\) CHANNELS**

Exposure of cholangiocytes to either UDCA or TUDCA rapidly increased \([\text{Ca}^{2+}]_i\) (Fig. 1A,B). Two patterns were observed: a rapid peak with a short bile acid exposure and an oscillatory, but sustained peak with continued bile acid exposure (Fig. 1A and Supporting Fig. S1). Of note, the bile acids, cholic acid (CA) and taurocholic acid (TCA), did not increase \([\text{Ca}^{2+}]_i\). In parallel studies, exposure to UDCA or TUDCA rapidly activated membrane \(\text{Cl}^{-}\) currents. Under basal conditions with standard intra- and extracellular buffers, \(I_{\text{Cl}^{-}}\) was small. Exposure to either UDCA or TUDCA resulted in activation of currents within 2 minutes (representative traces shown in Fig. 1B). Bile-acid–stimulated \(\text{Cl}^{-}\) currents exhibited
characteristic biophysical features of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels with reversal at 0 mV (E\textsubscript{Cl–}), time-dependent activation, and outward rectification; identical properties to those previously described for TMEM16A.\textsuperscript{(11,22)} Currents were sustained for the duration of bile acid exposure, followed by gradual return to basal levels within 5-10 minutes following removal of bile acids from the extracellular buffer. Currents were not observed when Cl\textsuperscript{-} was removed from the recording solutions, when intracellular Ca\textsuperscript{2+} was chelated (10 mM of ethylene glycol tetraacetic acid [EGTA] in pipette), or with the bile acids, CA or TCA (Fig. 1D,E and Supporting Fig. S2). The magnitude of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} current density was...
proportional to the concentration of UDCA with a $K_1/2$-maximal concentration at 50 $\mu$M (Fig. 1D). Similar results were obtained in MLC cells and Mz-Cha-1 cells, and no differences in the biophysical properties of $[Ca^{2+}]_i$; responses or Cl$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$-
the specific channel antagonist, TMEM16Ainh-A01, or with the bile acids, CA or TCA (Fig. 5). The increase in ASL by UDCA and TUDCA was similar to that observed previously when ATP was applied apically to hydrolyze ATP. In the presence of the CFTR inhibitor, CFTR-inh172, UDCA failed to increase ASL. However, the inhibition was overcome by exogenous addition of ATP, suggesting that CFTR plays a role in facilitating ATP release. Together, these studies...
in confluent monolayers demonstrate that bile acids stimulate an integrated secretory response, increasing fluid volume at the apical membrane. Bile-acid–mediated secretion requires CFTR-dependent ATP release and activation of TMEM16A Cl\(_{\text{2}}\) channels.

BILE ACIDS ACTIVATE MEMBRANE Cl\(^{-}\) CURRENTS BY AN INTRACELLULAR MECHANISM

It has previously been shown that UDCA works intracellularly to activate Ca\(^{2+}\)-activated Cl\(^{-}\) currents.\(^{13}\) To determine whether the observed activation of membrane Cl\(^{-}\) channels by TUDCA also occurs intracellularly, additional whole-cell patch-clamp studies were performed utilizing intracellular dialysis. This technique allows delivery of individual bile acids to the cell interior by inclusion in the patch pipette and permits simultaneous measurement of whole-cell currents.\(^{25}\) For these studies, Na\(^{+}\) and K\(^{+}\) ions were replaced by Tris\(^{+}\) to minimize potential effects of bile acids on cation conductance. Intracellular delivery of TUDCA significantly increased whole-cell Cl\(^{-}\) currents. With high calcium buffering capacity (10 mM of EGTA in pipette), the same concentration of TUDCA had no effect on Cl\(^{-}\).
Additionally, when TUDCA was delivered intracellularly in the presence of apyrase in the bath solution, currents were significantly inhibited. Together, these studies indicate that TUDCA activates Cl\textsuperscript{-} currents through an intracellular mechanism that requires extracellular ATP and intracellular Ca\textsuperscript{2+}.

**TUDCA-STIMULATED INCREASES IN \([\text{Ca}^{2+}]_i\), Cl\textsuperscript{-} CURRENTS, AND FLUID SECRETION REQUIRE ACTIVITY OF THE APICAL ASBT**

Because uptake of TUDCA at the apical cholangiocyte membrane requires transport by ASBT\cite{26} the role of ASBT in bile-acid-stimulated \([\text{Ca}^{2+}]_i\), Cl\textsuperscript{-} currents, and ASL were assessed in separate studies. To determine whether TUDCA-stimulated TMEM16A Cl\textsuperscript{-} currents are dependent on ASBT, \([\text{Ca}^{2+}]_i\), and whole-cell Cl\textsuperscript{-} currents were measured in the presence or absence of the ASBT inhibitor, GSK2330672\cite{27}. Both the TUDCA-stimulated increase in \([\text{Ca}^{2+}]_i\), and whole-cell Cl\textsuperscript{-} currents were inhibited in the presence of GSK2330672 (Fig. 7A,B). Likewise, in polarized MLC or NRC monolayers, inhibition of ASBT by glycogen synthase kinase (GSK) prevented the normal bile-acid-stimulated increase in ASL (Fig. 7C). Together, these studies demonstrate that functional ASBT protein in the cholangiocyte membrane is required for TUDCA-stimulated increases in \([\text{Ca}^{2+}]_i\), Cl\textsuperscript{-} currents, and transepithelial fluid secretion.

**Discussion**

In these studies of human, rat, and mouse biliary epithelium, we have identified TMEM16A as the \textit{Ca}^{2+}-activated Cl\textsuperscript{-} channel that is activated by the uptake of bile acids at the apical cholangiocyte membrane and which contributes to ductular secretion. This is based on the following findings: (1) both UDCA and TUDCA increase exocytosis, ATP release, and \([\text{Ca}^{2+}]_i\) through a pathway involving P2Y and IP3 receptors, the same operative pathway involved in TMEM16A activation\cite{8,11}; (2) bile-acid-stimulated Cl\textsuperscript{-} currents have biophysical properties consistent with TMEM16A channels\cite{22}; (3) both TMEM16A siRNA and pharmacological antagonists of TMEM16A, and are unaffected by antagonists of CFTR; and (3) in polarized cholangiocyte monolayers, the increase in ASL volume observed with apical application of bile acids is abolished by...

\[\text{FIG. 4.} \text{ Bile acid increase exocytosis and ATP release in MLC cells.} (A) MLC cells on coverglass were loaded with FM1-43 and exposed to extracellular buffer (control), UDCA (250 \text{ \mu M}, -o-), TUDCA (250 \text{ \mu M}, -r-), or UDCA with NEM (100 \text{ \mu M}, -D-) and TUDCA with NEM (100 \text{ \mu M}, -D-). The values on the y-axis represent % increase in membrane fluorescence. (B) Cumulative data demonstrating maximum % increase in exocytosis (measured as membrane fluorescence) in response to UDCA (250 \text{ \mu M}), TUDCA (250 \text{ \mu M}), or UDCA or TUDCA after incubation with NEM (100 \text{ \mu M}). *P < 0.05 bile acid versus basal, n = 30; **P < 0.05 control versus NEM, n = 25. (C) Bile-acid-stimulated ATP release. ATP in the extracellular media was detected using the luciferin-luciferase assay and quantified as arbitrary light units (ALU). Values represent maximum ATP concentration within 15 minutes of bile acid exposure (TCA, n = 6; CA, n = 6; TUDCA, n = 12; UDCA, n = 12), mean \pm SEM; *P < 0.05 versus basal; **P < 0.01 UDCA with NEM versus UDCA alone (n = 6); #not significant.\]
TMEM16A inhibition. Additionally, we demonstrated that activation of TMEM16A by the conjugated bile acid, TUDCA, requires bile acid uptake because (1) Cl \textsuperscript{−} currents were not observed when the uptake of TUDCA was prevented by specific pharmacological inhibition of ASBT, and (2) Cl \textsuperscript{−} currents were activated when bile acids were delivered solely intracellularly by intracellular dialysis. Thus, TMEM16A represents a link between bile-acid–dependent and ductular bile formation and thus may contribute to biliary secretion and hypercholeresis associated with the cholehepatic shunting of bile acids.

It should be highlighted that activation of TMEM16A by bile acids occurs through a process involving the purinergic signaling complex in which ATP is released, binds P2Y receptors, and increases [Ca\textsuperscript{2+}], through IP3 receptor–dependent release from intracellular stores. Our previous studies in polarized normal rat cholangiocyte (NRC) and MLC monolayers demonstrate that this signaling pathway occurs at the apical membrane.\textsuperscript{58} This is in contrast to the activation of the cAMP-regulated Cl\textsuperscript{−} channel, CFTR, which is regulated by binding of the hormone secretin to receptors on the basolateral membrane.\textsuperscript{28} Thus, bile acid activation of TMEM16A represents an example of hepatobiliary coupling, in which bile acids released into bile by hepatocytes can signal to downstream cholangiocytes and modulate ductular Cl\textsuperscript{−} transport. Together with our findings, as well as previous

FIG. 5. ASL height in mouse cholangiocyte monolayers. (A) Representative X-Z confocal images of confluent polarized mouse cholangiocyte monolayers (green) after apical addition of Dextran-Red (red) to the apical compartment. Representative monolayers shown during basal conditions (top), after addition of UDCA (100 μM, middle), UDCA + TMEM16A-inhA01 (10 μM, middle), and UDCA + apyrase (5 U/mL, bottom). Bar shown in bottom panel represents 10 μm. (B) Cumulative data demonstrating effect of UDCA (n = 8) or TUDCA (n = 6) on ASL in presence or absence of TMEM16Ainh-A01 (n = 6) or apyrase (n = 4; *P < 0.05 vs. basal; **P < 0.05 vs. maximal bile-acid–stimulated ASL). (C) Cumulative data demonstrating effect of CA (100 μM) or TCA (100 μM) on ASL in the presence or absence of ATP (100 μM); n = 6-8 each; *P < 0.05 ATP versus bile acid alone; n.s. (not significant). (D) Cumulative data demonstrating effect of UDCA (100 μM, n = 10) on ASL in presence or absence of CFTR-inh172 (10 μM, n = 6) or CFTR-inh172 + ATP (100 μM, n = 5; *P < 0.05 versus basal; **P < 0.05 CFTR-inh + ATP versus CFTR-inh alone; n.s. versus basal.
findings, demonstrating that bile acids are potent stimulators of ATP release into bile, a model is demonstrated in which increases in the concentration of bile acids in the intrahepatic bile duct lumen is transmitted to ATP release, autocrine/paracrine P2 receptor binding, and TMEM16A-mediated increases in cholangiocyte membrane Cl– permeability (Fig. 8).

In describing the regulation of TMEM16A-mediated Cl– channel activity by bile acids, several points are in order. First, although these studies demonstrate a predominant role of TMEM16A in bile-acid–stimulated Cl– secretion, we cannot exclude contributions from other Cl– channels or TMEM16 isoforms. TMEM16A/ANO-1 is a 114-kDa membrane protein with 960 amino acids and eight transmembrane domains and is one of the ten members of the TMEM16/ANO family of proteins. Cholangiocytes express several isoforms, including a, f, j, and k, but of these, only TMEM16A (ANO1) is associated with Ca2+-activated Cl– currents. Additionally, CFTR does not appear to play a direct role in mediating the Cl– efflux in response to bile acids given that the biophysical properties of the bile-acid–stimulated Cl– currents are distinct from CFTR, which exhibits a linear current-voltage relation and no time dependence. Furthermore, pharmacological inhibition of CFTR neither affected the activation nor the magnitude of bile-acid–stimulated currents and it has been previously shown that UDCA does not increase cAMP (the second messenger required for CFTR activation) levels in cholangiocytes.
FIG. 8. Proposed model of bile-acid–stimulated TMEM16A Cl– channel activation in biliary epithelium. Uptake of bile acids at the apical cholangiocyte membrane results in ATP release. TUDCA uptake requires ASBT. Uptake of bile acids stimulates ATP release, which may involve exocytosis of ATP-enriched vesicles and is facilitated by CFTR. Released ATP binds P2Y receptors in an autocrine or paracrine manner, resulting in an increase in intracellular Ca2+ through IP3 receptor-mediated release from intracellular stores. The increase in [Ca2+]i activates TMEM16A Cl– channels, which provide the driving force for ductular secretion.
Cl\(^-\) currents demonstrated outward rectification and time-dependent activation at depolarizing potentials above +60 mV, in some cells the bile-acid–stimulated Cl\(^-\) currents demonstrated slightly different biophysical properties (time-dependent inactivation), and which were unaffected by TMEM16A silencing (Supporting Fig. S4). These biophysical properties are consistent with volume-activated Cl\(^-\) currents described in biliary epithelial cells,\(^ {31} \) the molecular identity of which have not been determined.

Second, these studies evaluated UDCA, TUDCA, CA, and TCA, finding that only UDCA and TUDCA increased TMEM16A activity. It is acknowledged that other bile acids may have distinct effects on the TMEM16A-mediated secretory pathway described here. In all our studies, ATP release and P2 receptor binding was required for bile-acid–stimulated TMEM16A activation. Previously, it has been shown that different bile acids have variable effects on ATP release into bile, with UDCA and TUDCA having the greatest effect.\(^ {29} \) Interestingly, the effects on ATP release parallel the known choleric effects of these bile acids,\(^ {3} \) suggesting that bile-acid–stimulated ATP release and activation of TMEM16A Cl\(^-\) channels may be the final common pathway responsible for bile-acid–stimulated ductular secretion.

Third, the mechanism by which bile acids interact with cholangiocytes to release ATP and initiate purinergic signaling and TMEM16A activation requires further study. It has previously been shown that UDCA-stimulated release of ATP into bile requires CFTR\(^ {15} \) and that cAMP-activation of CFTR can stimulate ATP release.\(^ {32} \) Thus, whereas our studies demonstrate that TMEM16A is the “downstream” Cl\(^-\) channel responsible for ATP-stimulated Cl\(^-\) efflux, CFTR appears to be the “upstream” target involved in bile-acid–stimulated ATP release. The mechanism by which CFTR facilitates cellular ATP release is unknown, but perhaps plays a role in tethering regulatory proteins at the apical membrane\(^ {33,34} \) or in the maturation, trafficking, or fusion of ATP-enriched vesicles, known to be involved in cholangiocyte ATP release.\(^ {23} \)

Fourth, although our studies demonstrate that bile acids work intracellularly and uptake of bile acids by cholangiocytes is necessary for TMEM16A activation, the intracellular targets for bile acids in the secretory process are unknown. Interestingly, we have previously shown that TMEM16A is positively regulated by protein kinase C alpha,\(^ {25} \) which is known to translocate to the plasma membrane in response to UDCA.\(^ {15} \)

Thus, bile acids may have important effects on secondary messengers or other signaling molecules involved in TMEM16A regulation. The intracellular target(s) of bile acids that are responsible for initiating the TMEM16A-mediated secretory pathway will be an important area for future investigations.

In summary, these studies have identified TMEM16A as the Ca\(^{2+}\)-activated Cl\(^-\) channel responsible for bile-acid–stimulated Cl\(^-\) secretion in biliary epithelium. Furthermore, these studies provide a molecular mechanism by which bile acids may stimulate hypercholeresis during choledochal shunting by directly activating the P2Y-IP3R-TMEM16A secretory complex. The identification of TMEM16A as the Cl\(^-\) channel responsible for cholangiocyte Cl\(^-\) efflux in response to bile acids may provide a target to modulate bile flow for the management of cholestatic liver diseases.

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