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Bicarbonate Secretion of Mouse Cholangiocytes Involves Na\(^+\)-HCO\(_3\)- Cotransport in Addition to Na\(^+\)-Independent Cl\(^-\)/HCO\(_3\)- Exchange

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Bicarbonate secretion from cholangiocytes is required for appropriate adjustment of primary canicular bile along the biliary tract. In human and rat cholangiocytes, bicarbonate secretion is mediated by anion exchanger (AE) 2, an electroneutral Na\(^+\)-independent Cl\(^-\)/HCO\(_3\)- AE also involved in intracellular pH (pH\(_i\)) regulation. In Ae2a,b-deficient mice, pH\(_i\) is increased in lymphocytes and fibroblasts, whereas it is surprisingly normal in cholangiocytes. Here, we analyze the mechanisms for HCO\(_3\)- secretion in cultured Ae2a,b\(^{+/+}\) and Ae2a,b\(^{-/-}\) mouse cholangiocytes by microfluorimetric measurement of pH\(_i\) changes upon established perfusion maneuvers. Cl\(^-\) withdrawal by isethionate-based perfusions showed that Ae2a,b\(^{+/+}\) but not Ae2a,b\(^{-/-}\) mouse cholangiocytes can display Cl\(^-\)/HCO\(_3\)- exchange, which is therefore entirely mediated by Ae2. Nevertheless, simultaneous withdrawal of Cl\(^-\) and Na\(^+\) revealed that mouse cholangiocytes possess an additional transport activity for HCO\(_3\)- secretion not observed in control rat cholangiocytes. Propionate-based maneuvers indicated that this supplemental Na\(^+\)-driven HCO\(_3\)- secreting activity is Cl\(^-\)-independent, consistent with a Na\(^+\)-HCO\(_3\)- cotransport (NBC). NBC activity is greater in Ae2a,b\(^{-/-}\) than Ae2a,b\(^{+/+}\) mouse cholangiocytes, and membrane-depolarization experiments showed that it is electrogenic. Consistent with the potential role of Slc4a4/Nbc1 as the involved transporter, Ae2a,b\(^{-/-}\) mouse cholangiocytes exhibit up-regulated expression of this electrogenic NBC carrier. Whereas Ae2-mediated Cl\(^-\)/HCO\(_3\)- exchange in Ae2a,b\(^{+/+}\) mouse cholangiocytes is stimulated by cyclic adenosine monophosphate (cAMP) and acetylcholine, the NBC activity is down-regulated by cAMP and adenosine triphosphate (ATP) in Ae2a,b\(^{-/-}\) mouse cholangiocytes. Polarized Ae2a,b\(^{-/-}\) mouse cholangiocytes placed in Ussing chambers show decreased (but not abolished) cAMP-dependent Cl\(^-\) current and increased ATP-dependent/Ca\(^{2+}\)-activated Cl\(^-\) secretion, which run in parallel with decreased cystic fibrosis transmembrane conductance regulator messenger RNA expression and increased intracellular Ca\(^{2+}\) levels. Conclusion: Bicarbonate secretion in mouse cholangiocytes involves two differentially regulated activities: Ae2-mediated Cl\(^-\)/HCO\(_3\)- exchange and Na\(^+\)-HCO\(_3\)- cotransport.

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Abbreviations: ACh, acetylcholine; AE, anion exchanger; ATP, adenosine triphosphate; AUC, area under the curve; cAMP, cyclic adenosine monophosphate; cAMP/SM, cAMP-stimulation mixture; CFTR, cystic fibrosis transmembrane conductance regulator; Isc, short-circuit current; KRB, Krebs-Ringer bicarbonate; mRNA, messenger RNA; NBC, Na\(^+\)-HCO\(_3\)- cotransport; NRC, normal rat cholangiocyte; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; pHi, intracellular pH.

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Bile flow is produced through the coordinated secretory function of hepatocytes and cholangiocytes. These polarized cells possess different carriers in their apical and/or basolateral plasma membrane that provide vectorial transport of biliary solutes. Primary bile generated by hepatocytes is fluidized and alkalized by cholangiocytes along the biliary tract. Bicarbonate secretion from cholangiocytes to the duct lumen requires intracellular accumulation of $\mathrm{HCO}_3^-$, which is partially achieved through catalyzed hydration of $\mathrm{CO}_2$ and subsequent $\mathrm{H}^+$ extrusion through Na$^+$/H$^+$ exchange. Moreover, $\mathrm{HCO}_3^-$ loading can be assisted by additional transport activities that may vary between animal species. In rat cholangiocytes, for instance, there is a basolateral $\mathrm{HCO}_3^-$ influx mediated by Na$^+$/H$^+$ co-transport (NBC). In human cholangiocytes, however, basolateral influx of $\mathrm{HCO}_3^-$ occurs mainly through Na$^+$-dependent Cl$^-$/HCO$^-$ exchange.

In both human and rat cholangiocytes, the secretion of $\mathrm{HCO}_3^-$ to bile occurs through a Na$^+$-independent electroneutral Cl$^-$/HCO$^-$ exchange mediated by the anion exchanger (AE) 2 (AE2/SLC4A2), which is an acid loader of the SLC4/AE family generally involved in intracellular pH (pHi) regulation. Secretin-stimulated bicarbonate-rich hydrocholeresis has been postulated to involve the synchronized action of AE2 and two additional transport proteins, the cyclic adenosine monophosphate (cAMP)-responsive Cl$^-$ channel cystic fibrosis transmembrane conductance regulator (CFTR) and the water channel aquaporin-1. Upon secretin/receptor interaction and subsequent increase in cAMP levels and protein kinase A activation, intracellular vesicles with all three flux proteins coredirected to the luminal membrane of cholangiocytes. Accompanying phosphorylation and activation of CFTR lead to apical efflux of Cl$^-$, which is ultimately exchanged with $\mathrm{HCO}_3^-$ through AE2. Resultant secretion of $\mathrm{HCO}_3^-$ is followed by osmotically driven efflux of water through aquaporin-1, ending in typical bicarbonate-rich hydrocholeresis induced by secretin. The effect of this mechanism may be reinforced by the recently described CFTR-associated release of adenosine triphosphate (ATP) from cholangiocytes to bile. Autocrine/paracrine stimulation of apical purinergic receptors by ATP is then followed by increased intracellular Ca$^{2+}$, activation of apical Ca$^{2+}$-dependent Cl$^-$ channels, and efflux of Cl$^-$, which is likewise exchanged with $\mathrm{HCO}_3^-$ through AE2. Bicarbonate-enriching processes elicited by secretin might also involve CFTR-independent effects of cAMP on the AE2-mediated Cl$^-$/HCO$^-$ exchange, as observed in vitro in human cholangiocytes in the presence of CFTR inhibitors and in hepatocyte-derived cells that lack CFTR. Acetylcholine (ACh) may further assist biliary $\mathrm{HCO}_3^-$ secretion through Ca$^{2+}$-dependent Cl$^-$ efflux from cholangiocytes and by potentiating the effect of secretin on both intracellular cAMP levels and Cl$^-$/$\mathrm{HCO}_3^-$ exchange in a calcineurin-dependent manner.

We previously hypothesized that abnormalities of AE2 in primary biliary cirrhosis (PBC) could play a pathogenic role in the disease and generated a mouse model targeted for the three major Ae2a, Ae2b1, and Ae2b2 isoforms (Ae2a,b$^{-/-}$ mice). These mice showed biochemical, serologic, histopathologic, and immunologic features compatible with PBC. pH assessment in lymphocytes and fibroblasts of Ae2a,b$^{-/-}$ mice confirmed the expected intracellular alkalinization, while cholangiocytes isolated from these mice were found to have normal pH values. Therefore, we decided to analyze the mechanisms involved in $\mathrm{HCO}_3^-$ secretion in cultured Ae2a,b$^{-/-}$ and Ae2a,b$^{-/-}$ mouse cholangiocytes. Our present data show that wild-type but not Ae2a,b$^{-/-}$ mouse cholangiocytes exhibit Cl$^-$/$\mathrm{HCO}_3^-$ anion exchange (AE) activity. This indicates that AE activity in normal mouse cholangiocytes is only mediated by AE2, in agreement with previous data in human and rat cholangiocytes. Characteristically, however, mouse cholangiocytes can display an additional transport activity for $\mathrm{HCO}_3^-$ secretion that is more pronounced in Ae2a,b$^{-/-}$-deficient cholangiocytes. This additional activity is Cl$^-$-independent and Na$^+$-dependent (consistent with NBC activity), electrogentic, and responds differentially to agonists such as cAMP, ATP, and ACh.

**Material and Methods**

**Cultured Cholangiocytes from Normal and Ae2a,b$^{-/-}$ Mice.** Cultured cholangiocytes were obtained from male Ae2a,b$^{-/-}$ and Ae2a,b$^{-/-}$ mice, starting with isolation of intrahepatic bile duct units, followed by their seeding and culture on collagen monolayer (see Supporting Information). Normal rat cholangiocytes (NRCs) were similarly obtained from male Wistar rats. Animal interventions were approved by the Instrumental Animal Care and Use Committee.

**Determination of $\mathrm{HCO}_3^-$ Transport Activities.** Cultured cholangiocytes were assessed for $\mathrm{HCO}_3^-$ transport activities by microfluorimetry (see Supporting Information). Cells initially perfused with Krebs-Ringer bicarbonate (KRB) medium were monitored for pH$^+$ variations upon two types of perfusion maneuvers (see detailed buffers in Supplementary Table 1). First, to test Cl$^-$/$\mathrm{HCO}_3^-$ exchange and Na$^+$ dependency, KRB was changed to KRB without Cl$^-$ (substituted with isethionate) or without both Cl$^-$ and Na$^+$ (substituted with N-methyl-D-glucamine). These Cl$^-$-free based maneuvers are designed to elicit efflux of intracellular Cl$^-$ in exchange with extracellular $\mathrm{HCO}_3^-$, increasing pH$^+$. In the case of AE2,
which physiologically functions as a HCO$_3^-$-exchanger (i.e., acid-loader), Cl$^-$/HCO$_3^-$ exchange activity is measured in a reverse mode. The exchange activity (expressed as transmembrane base fluxes $J_{\text{OH}^-}$) is calculated from the tangent of the experimental plot of the alkalinization induced by Cl$^-$ removal. A second type of maneuver starting with propionate-KRB perfusion$^{5,18,22}$ was aimed to analyze HCO$_3^-$ secretion mechanisms in a more physiological manner for AE2. Here, intracellular alkalinization induced by propionate removal may activate acid-loading mechanisms to extrude HCO$_3^-$ and recover resting pH$_t$. In this case, HCO$_3^-$ secretion rates ($J_{\text{OH}^-}$) are calculated from the tangent of the experimental plot of the recovery toward resting pH$_t$. The stimulatory effect of either a cAMP/stimulation mixture (cAMP/SM) (100 µM N$^6$-2-O-dibutryl-cAMP, 3 µM forskolin, and 100 µM 3-isobutyl-1-methyl-xanthine)$^{5,18,22}$ 100 µM ACh$^{13}$ or 10 µM ATP$^{18,23}$ and the inhibitory effect of 500 µM DIDS (all from Sigma) on HCO$_3^-$ secretion could be assessed by application of these compounds 5 minutes before and along the perfusion maneuver.

**Voltage Clamp Measurement of Short-Circuit Current on Polarized Mouse Cholangiocytes.** Short-circuit current ($I_{sc}$) was monitored in confluent polarized mouse cholangiocytes as described for NRCs$^{24}$ (see Supporting Information for details). Briefly, when cells cultured on collagen-coated inserts increased the transepithelial electrical resistance $\geq 1,000 \, \Omega \cdot \text{cm}^2$, inserts were mounted in U2500 Dual-Channel Self-Contained Ussing Chambers (Warner Instruments, Hamden, CT). Each half-chamber (filled with a NaCl-rich solution at 37°C and continuously bubbled with 5% CO$_2$/95% O$_2$) was connected to a microcomputer-controlled current/voltage clamp amplifier, and $I_{sc}$ (µA/cm$^2$) was determined when transepithelial voltage was clamped to 0 mV. The system was monitored along the experiments in response to current pulses every 6 seconds. Under these conditions, changes in $I_{sc}$ were recorded following simultaneous apical and basolateral stimulation with a particular cAMP-SM (500 µM 8-cpt-cAMP plus 450 µM 3-isobutyl-1-methyl-xanthine) or with 100 µM ATP (all from Sigma), essentially as described.$^{24}$

**Determination of the Intracellular Levels of Ca$^{2+}$ and cAMP, Immunoblotting, and Real-Time Polymerase Chain Reaction.** These procedures are described in the Supporting Information.

**Statistical Analysis.** Data are expressed as the mean ± standard error of the mean. Two-group comparisons were performed by using adequate nonparametric (Mann-Whitney) or parametric t tests. Two-tailed $P$ values $<0.05$ were considered statistically significant.

### Results

**Normal Mouse Cholangiocytes Display Ae2 Activity.** Ae2 gene silencing in human$^4$ and rat$^5$ cholangiocytes have indicated that HCO$_3^-$ secretion is mediated by AE2. In mouse cholangiocytes, however, the mechanisms for HCO$_3^-$ secretion remain to be fully elucidated. Thus, we assessed HCO$_3^-$ secretion activities in cultured $Ae2a,b^{+/+}$ and $Ae2a,b^{-/-}$ mouse cholangiocytes, using normal rat cholangiocytes as a control. As shown in Fig. 1A, selective withdrawal of Cl$^-$ elicited intracellular alkalinization in control NRCs, indicating the occurrence of Cl$^-$/HCO$_3^-$ exchange ($J_{\text{OH}^-} = 20.97 \pm 0.72 \text{ mmol/L/minute}$). Likewise, Cl$^-$ withdrawal induced intracellular alkalinization in normal ($Ae2a,b^{+/+}$) mouse cholangiocytes, which reveals that these cells also display Cl$^-$/HCO$_3^-$ exchange activity ($J_{\text{OH}^-} = 10.63 \pm 0.95 \text{ mmol/L/minute}$) (Fig. 1B). In $Ae2a,b^{-/-}$ mouse cholangiocytes, however, no intracellular alkalinization was observed after Cl$^-$ withdrawal (Fig. 1C), demonstrating that the Cl$^-$/HCO$_3^-$ exchange activity displayed by normal mouse cholangiocytes is entirely mediated by Ae2.

**Mouse but not Rat Cholangiocytes Possess an Additional Cl$^-$-Independent Na$^+$-Driven HCO$_3^-$ Secretory Activity that May Maintain Bicarbonate Secretion in the Absence of Ae2.** As mentioned previously, the activity displayed by AE carriers (AE2 activity included) is independent of Na$^+$. Indeed, in the case of control NRCs, we could confirm that the Cl$^-$/HCO$_3^-$ exchange activity elicited by simultaneous withdrawal of Cl$^-$ and Na$^+$ ($J_{\text{OH}^-} = 20.80 \pm 0.97 \text{ mmol/L/minute}$) was similar to that observed in the presence of Na$^+$ (Fig. 1A). In mouse cholangiocytes, however, assessment of Na$^+$ dependency uncovered a surprising effect in these cells. Unlike NRCs, both $Ae2a,b^{+/+}$ and $Ae2a,b^{-/-}$ mouse cholangiocytes showed intracellular acidification after simultaneous removal of Cl$^-$ and Na$^+$ from the media (Fig. 1B,C), indicating the presence of a Na$^+$-driven acidification activity that involves HCO$_3^-$ secretion. This Na$^+$-driven activity was more pronounced in $Ae2a,b^{-/-}$ than normal mouse cholangiocytes ($J_{\text{OH}^-} = 10.92 \pm 2.02$ versus 1.82 ± 0.95 mmol/L/minute; $P < 0.001$), which might be partially due to the counterbalancing Ae2-mediated alkalinization that takes place in normal but not $Ae2a,b^{-/-}$ mouse cholangiocytes (see Fig. 1B,C for the first maneuver of Cl$^-$ withdrawal).

To further ascertain that the Na$^+$-driven acidification activity selectively observed in mouse cholangiocytes involves secretion of HCO$_3^-$ and is Cl$^-$-independent, we turned to propionate-based maneuvers in the presence and absence of Cl$^-$ (see Material and Methods). As expected, control NRCs showed a final step of acidification
change in these cells. By contrast, \( \text{Ae2}_{a,b}^{+/+} \) mouse cholangiocytes showed no inhibition of the \( \text{HCO}_3^- \) extrusion rate despite \( \text{Cl}^- \) withdrawal (\( \text{JOH}^- = 2.87 \pm 0.33 \) mmol/L/minute in the presence of \( \text{Cl}^- \) versus 2.83 \pm 0.29 mmol/L/minute in the absence of this anion) (Fig. 2B), thus unmasking a \( \text{Cl}^- \)-independent \( \text{HCO}_3^- \) extrusion activity. This observation was even clearer in \( \text{Ae2}_{a,b}^{-/-} \) mouse cholangiocytes. In spite of their \( \text{Ae2} \) deficiency, these cells were able to display \( \text{HCO}_3^- \) extrusion activity regardless of the presence or absence of extracellular \( \text{Cl}^- \) (\( \text{JOH}^- = 4.91 \pm 1.12 \) versus 5.89 \pm 0.80 mmol/L/minute, respectively; Fig. 2C). In fact, \( \text{HCO}_3^- \) extrusion rate values were significantly higher in \( \text{Ae2}_{a,b}^{-/-} \) than \( \text{Ae2}_{a,b}^{+/+} \) mouse cholangiocytes (\( P < 0.05 \) in the presence of \( \text{Cl}^- \) and \( P < 0.01 \) in its absence). This \( \text{Cl}^- \)-independent and \( \text{Na}^+ \)-driven activity to secrete \( \text{HCO}_3^- \) observed in mouse cholangiocytes is by definition a \( \text{Na}^+/-\text{HCO}_3^- \) cotransport (i.e., an NBC activity).25,26 As shown in Fig. 2D, additional experiments indicated that this activity is sensitive to DIDS (a nonspecific inhibitor of \( \text{HCO}_3^- \) transporters)25 in both \( \text{Ae2}_{a,b}^{+/+} \) (\( \text{JOH}^- = 2.81 \pm 0.23 \) versus 1.03 \pm 0.44 mmol/L/minute in the presence of DIDS; \( P < 0.001 \)) and \( \text{Ae2}_{a,b}^{-/-} \) (\( \text{JOH}^- = 6.15 \pm 0.86 \) versus 3.05 \pm 0.63 mmol/L/minute; \( P < 0.05 \)) mouse cholangiocytes.

\( \text{Na}^+/-\text{Driven Cl}^-/-\text{Independent HCO}_3^- \) Secretion

Displayed by Mouse Cholangiocytes Is Electrogenic.

Electrogenicity of the NBC activity in mouse cholangiocytes was analyzed using propionate-based maneuvers under depolarizing conditions with 10-fold the physiological extracellular concentration of \( \text{K}^+ \).27 Upon membrane depolarization, an electrogenic-driven \( \text{HCO}_3^- \) extrusion would be diminished, whereas an electroneutral transport would remain unchanged.27 Whereas \( \text{HCO}_3^- \) secretion activity in NRCs was maintained (consistent with its complete mediation by electroneutral \( \text{Ae2} \) activity) (Fig. 3A), the secretion rate was diminished in \( \text{Ae2}_{a,b}^{+/+} \) mouse cholangiocytes (\( \text{JOH}^- = 3.09 \pm 0.19 \) versus 1.54 \pm 0.16 mmol/L/minute; \( P < 0.001 \)) (Fig. 3B) and totally abolished in \( \text{Ae2}_{a,b}^{-/-} \) mouse cholangiocytes (\( \text{JOH}^- = 4.83 \pm 0.46 \) versus 0 mmol/L/minute; \( P < 0.001 \)) (Fig. 3C), indicating that the \( \text{Na}^+/-\text{HCO}_3^- \) cotransport displayed by mouse cholangiocytes is electrogenic.

Slc4a4 Expression Is Up-Regulated in \( \text{Ae2}_{a,b}^{-/-} \) Mouse Cholangiocytes.

Among NBC carriers, \( \text{Slc4a4} \) and \( \text{Slc4a5} \) have been reported to be electrogenic,26,28 whereas \( \text{Slc4a7} \) is electroneutral.27,29 We therefore analyzed the messenger RNA (mRNA) expression of these cotransporters as well as the \( \text{Na}^+/-\text{dependent Cl}^-/-\text{HCO}_3^- \) exchanger \( \text{Slc4a8} \) in mouse cholangiocytes. Both \( \text{Ae2}_{a,b}^{+/+} \) and \( \text{Ae2}_{a,b}^{-/-} \) mouse cholangiocytes showed expression of \( \text{Slc4a4} \) mRNA, which was 1.8-fold up-regulated following Cl- / HCO3- removal in these cells.
ulated in \(\text{Ae2}_{a,b}^{-/-}\) versus \(\text{Ae2}_{a,b}^{+/+}\) mouse cholangiocytes \((P < 0.01)\) (Fig. 3D). Slc4a7 mRNA was similarly detected in mouse cholangiocytes of the two genotypes (Fig. 3D), whereas no mRNA expression of Slc4a5 and Slc4a8 was found in any genotype (not shown). Western blotting confirmed a four-fold increased expression of Slc4a4 at the protein level in the \(\text{Ae2}_{a,b}^{+/+}\) mouse cholangiocytes (Fig. 3E).

**HCO\(_3^-\)** Secretion Is Stimulated by cAMP in \(\text{Ae2}_{a,b}^{+/+}\) Cholangiocytes but Inhibited in \(\text{Ae2}_{a,b}^{-/-}\) Cholangiocytes.** Rise of intracellular cAMP levels is known to stimulate Ae2 activity in human and rat cholangiocytes, resulting in an increase of HCO\(_3^-\) secretion to bile.\(^1\) As shown in Fig. 4A, application of a cAMP/SM to \(\text{Ae2}_{a,b}^{+/+}\) mouse cholangiocytes increased the HCO\(_3^-\) extrusion rate \((J_{\text{OH-}} = 2.81 \pm 0.23 \text{ versus } 4.10 \pm 0.48 \text{ mmol/L/minute}; P < 0.05)\), whereas the same application to \(\text{Ae2}_{a,b}^{-/-}\) mouse cholangiocytes diminished HCO\(_3^-\) extrusion \((J_{\text{OH-}} = 6.15 \pm 0.86 \text{ versus } 3.64 \pm 0.45 \text{ mmol/L/minute}; P < 0.001)\) (Fig. 4B). On the other hand, analysis of intracellular cAMP levels (both baseline and following cAMP stimuli) indicated that \(\text{Ae2}_{a,b}^{-/-}\) mouse cholangiocytes have raised levels compared with \(\text{Ae2}_{a,b}^{+/+}\) mouse cholangiocytes (Fig. 4C).

**Acetylcholine Stimulates HCO\(_3^-\)** Secretion in \(\text{Ae2}_{a,b}^{+/+}\) but not in \(\text{Ae2}_{a,b}^{-/-}\) Mouse Cholangiocytes.** ACh is also known to regulate HCO\(_3^-\) secretion in the biliary epithelium.\(^{13-15}\) As shown in Fig. 5A, the presence of ACh increased HCO\(_3^-\) secretion in \(\text{Ae2}_{a,b}^{+/+}\) mouse cholangiocytes \((J_{\text{OH-}} = 3.63 \pm 0.48 \text{ versus } 6.79 \pm 0.56 \text{ mmol/L/minute}; P < 0.001)\). However, the presence of ACh did not affect HCO\(_3^-\) secretion in \(\text{Ae2}_{a,b}^{-/-}\) mouse cholangiocytes (Fig. 5B).

**ATP Inhibits HCO\(_3^-\)** Secretion in \(\text{Ae2}_{a,b}^{+/+}\) Mouse Cholangiocytes. ATP has also been reported to play an indirect role in HCO\(_3^-\) secretion in the biliary epithelium.\(^{13,30}\) In our experimental setting of perfused cells, however, the presence of 10 \(\mu\)M ATP did not modify the HCO\(_3^-\) secretion rate in \(\text{Ae2}_{a,b}^{+/+}\) mouse cholangiocytes \((J_{\text{OH-}} = 5.53 \pm 0.66 \text{ versus } 4.68 \pm 0.52 \text{ mmol/L/minute})\) (Fig. 5B).
minute) (Fig. 6A), and interestingly resulted in diminished rate in $\text{Ae2}^{a,b}$ mouse cholangiocytes (0.83 versus 0.34 mmol/L/minute; $P < 0.001$) (Fig. 6B).

$\text{Ae2}^{a,b}$ Mouse Cholangiocytes Show Altered Cl$^-$ Secretion. Previous studies in Ussing chambers to analyze the Cl$^-$ conductance in NRCs with recovered cell polarity indicated that cAMP stimulation leads to apical secretion of Cl$^-$ through Cftr activation, while purinergic stimulation by ATP leads to apical Cl$^-$ efflux through activation of Ca$^{2+}$-dependent Cl$^-$ channels. We therefore decided to directly assess the Cl$^-$ conductance in polarized mouse cholangiocytes upon cAMP and ATP stimulations. As shown in Fig. 7A, cAMP stimulation of $\text{Ae2}^{a,b}$ mouse cholangiocytes resulted in increased values of short-circuit current intensity due to Cl$^-$ secretion ($\Delta I_{sc}: 3.45 \pm 0.38 \mu A/cm^2$), which was similar to that reported for polarized NRCs. However, cAMP stimulation of $\text{Ae2}^{a,b}$ mouse cholangiocytes resulted in less intense Cl$^-$ secretion ($\Delta I_{sc}: 0.95 \pm 0.13 \mu A/cm^2$) (Fig.
When we measured the area under the curve (AUC) of \( J_{\text{cAMP}} \), the cAMP response in \( Ae2\alpha,\beta^{-/-} \) mouse cholangiocytes was found to be significantly lower than that in \( Ae2\alpha,\beta^{+/+} \) mouse cholangiocytes (AUC 10.63 ± 1.071 versus 2.85 ± 0.22; \( P < 0.05 \) (Fig. 7A). Interestingly, these data are consistent with our observation that the levels of Cftr mRNA are much lower in \( Ae2\alpha,\beta^{-/-} \) than \( Ae2\alpha,\beta^{+/+} \) mouse cholangiocytes (13.7% versus 100%; \( P < 0.001 \) (Fig. 7B).

On the other hand, ATP stimulation led to significantly higher \( Cl^{-} \) secretion in \( Ae2\alpha,\beta^{-/-} \) compared with \( Ae2\alpha,\beta^{+/+} \) mouse cholangiocytes (AUC 49.68 ± 6.02 versus 19.3 ± 5.58, respectively; \( P < 0.05 \) (Fig. 7C). It is noteworthy that this effect was correlated with higher relative values for increased intracellular Ca\(^{2+} \) in response to ATP in \( Ae2\alpha,\beta^{-/-} \) compared with \( Ae2\alpha,\beta^{+/+} \) mouse cholangiocytes (AUC of fluorescence values: 68.05 ± 6.62 versus 32.54 ± 3.25, respectively; \( P < 0.001 \) (Fig. 7D).

Discussion

Biliary \( HCO_{3}^{-} \) secretion is important for the alkalinization and fluidization of bile as well as for pH\(_{i}\) homeostasis in cholangiocytes.\(^1\) In human and rat cholangiocytes, \( HCO_{3}^{-} \) secretion occurs essentially through AE2/Ae2-mediated Cl\(^{-}\)/\( HCO_{3}^{-} \) anion exchange.\(^3,4,5\) Previously, we hypothesized that abnormalities in the liver expression of AE2 and concomitant alterations of \( HCO_{3}^{-} \) secretion in the biliary epithelium,\(^3,2\) may be involved in the pathogenesis of PBC. More recently, this hypothesis has been supported by our findings in \( Ae2\alpha,\beta^{-/-} \) mice, in which important PBC features are reproduced.\(^20\) We therefore decided to explore the transport mechanisms for \( HCO_{3}^{-} \) secretion in cultured mouse cholangiocytes from these mice.
Studies involved monitoring pH2 variations after two different types of perfusion maneuvers, i.e. with Cl-–free media (mainly an isethionate-based medium), and propionate-based perfusions. Although Cl-–withdrawal is designed to provoke Cl-/HCO3- exchange running in the opposite direction to the physiological one for Ae2 (with HCO3- entry into the cell instead of exit), this maneuver provided relevant information. It showed that, as with NRCs, normal mouse cholangiocytes display Cl-/HCO3- exchange activity that is entirely mediated by Ae2; the reverse-mode alkalinization upon Cl- withdrawal was completely absent in Ae2a,b-deficient mouse cholangiocytes (Fig. 1). Thus, in agreement with our previous data from AE2 gene silencing in normal human and rat cholangiocytes,4,5 no additional Na+-independent Cl-/HCO3- exchangers (e.g., Ae1, Ae3, Slc26a3, Slc26a4, Slc26a6, or Slc26a7) or Na+-dependent exchange carriers (e.g., Slc4a8) mediate the AE activity observed in normal mouse cholangiocytes. But unlike the case with NRCs, in which alkalization remained after simultaneous removal of Cl- and Na+, mouse cholangiocytes of both genotypes exhibited an opposite effect resulting in pH2 acidification. This unexpected effect in murine cholangiocytes indicated the presence of an additional HCO3- secretory activity that is driven by Na+. Interestingly, this activity appeared more pronounced in Ae2a,b+/− than Ae2a,b+/+ mouse cholangiocytes (Fig. 1). This observation was confirmed with propionate-based maneuvers (Fig. 2). These maneuvers—designed for directly measuring the acid-loading activity (e.g., HCO3- secretion, when cells try to recover their resting pH2 from intracellular alkalinization)—also confirmed that the Na+-driven HCO3- secretory activity observed in both Ae2a,b+/+ and Ae2a,b−/− mouse cholangiocytes does not depend on extracellular Cl-, which is consistent with a Na+-HCO3- cotransport or NBC activity.25,26 This NBC activity is electrogenic, as it is sensitive to membrane depolarization induced by high extracellular concentration of K+ during the propionate maneuvers. Such electrogenic Cl-–independent and Na+-driven HCO3- secretory activity was not detected in NRCs and therefore appears mouse-specific.

![Ae2a,b+/− mouse cholangiocytes](B)

Our findings of up-regulated expression of Slc4a4 mRNA in Ae2a,b−/− (versus normal mouse cholangiocytes) and absent Slc4a4 expression in both Ae2a,b−/− and Ae2a,b+/+ mouse cholangiocytes (Fig. 3) suggest Slc4a4 might be the responsible electrogenic NBC carrier. This concept is supported by our finding of increased expression of the Sl4a4 protein in the Ae2a,b−/− cells. Slc4a4 up-regulation and subsequently increased NBC activity in Ae2a,b−/− mouse cholangiocytes may account for an elevated HCO3- secretion activity in these cells, which might be viewed as an attempt to compensate Ae2 deficiency for pH2 regulation. In this sense, cholangiocytes from Ae2a,b-deficient mice appear to be more capable of maintaining their resting pH220 than Ae2a,b−/− mouse fibroblasts, in which Slc4a4 is not expressed.21

Stoichiometry of the NBC activity displayed by Slc4a4 is cell type–dependent; 1:3 stoichiometry runs for Na+ and HCO3- efflux (acid load), whereas 1:2 works for influx of these ions into the cell (acid extrusion).26 Moreover, Slc4a4 stoichiometry may vary in the same cells, as reported for mouse kidney cells, in which protein kinase A–mediated phosphorylation in Ser982 shifts stoichiometry from 1:3 to 1:2, leading to decreased HCO3- efflux.28 This mechanism might also mediate the decrease in HCO3- secretory activity observed in cAMP/SM-stimulated Ae2a,b−/− mouse cholangiocytes (Fig. 4). Baseline NBC activity of these Ae2a,b-deficient cells is surprisingly high in spite of elevated cAMP production (also observed in Ae2a,b-deficient mouse fibroblasts),21 but further increased CAMP levels after cAMP/SM stimulation may trigger the decreasing effect. In the case of wild-type mouse cholangiocytes, cAMP/SM administration leads to...
overall increase in HCO\textsubscript{3} secretion (similarly to what had been observed in NRCs with cAMP stimulation),
probably because stimulation of Ae2-mediated Cl\textsuperscript{-}/HCO\textsubscript{3} exchange overcomes the concurrent decrease in the NBC activity.

Experiments performed in isolated bile duct units with preserved bile duct lumen (thus resembling the in vivo situation) suggested that the stimulatory effect of cAMP on biliary HCO\textsubscript{3} secretion is initiated by Cftr activation and Cl\textsuperscript{-} efflux into bile; Cl\textsuperscript{-}/HCO\textsubscript{3} exchange would follow as a consequence of outside-to-inside transmembrane gradient of Cl\textsuperscript{-} at a relatively high intracellular HCO\textsubscript{3} concentration. Moreover, cAMP activation of Cftr was recently associated with apical release of ATP. Autocrine/paracrine purinergic stimulation through the potent apically located ATP-responsive P2Y receptors and subsequent activation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels with additional efflux of Cl\textsuperscript{-} to bile could therefore result in further increased Cl\textsuperscript{-}/HCO\textsubscript{3} exchange at the apical membrane. In our model of perfused cholangiocytes, however, released Cl\textsuperscript{-} becomes diluted in the vast volume of perfusion media, with no chance for the alleged indirect stimulatory effects of the cAMP/SM on Cl\textsuperscript{-}/HCO\textsubscript{3} exchange after outside-to-inside transmembrane gradient of Cl\textsuperscript{-}. Thus, cAMP appears to exert alternative stimulatory effects on the Ae2-mediated Cl\textsuperscript{-}/HCO\textsubscript{3} exchange in our Ae2\textsubscript{a,b}+/+ mouse cholangiocytes.

ACh was also reported to indirectly stimulate HCO\textsubscript{3} secretion in rat isolated bile ducts. ACh binds to M3 muscarinic receptors at the basolateral membrane of cholangiocytes, leading to increased intracellular Ca\textsuperscript{2+} levels, activation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels and further Cl\textsuperscript{-} efflux into the bile duct lumen, and subsequent Cl\textsuperscript{-}/HCO\textsubscript{3} exchange for HCO\textsubscript{3} secretion to bile. In our perfused Ae2\textsubscript{a,b}+/+ mouse cholangiocytes, we found that ACh increased HCO\textsubscript{3} secretion similarly to cAMP. However, in contrast to cAMP, ACh showed no effect in our perfused Ae2\textsubscript{a,b}−/− mouse cholangiocytes. Our data therefore suggest that ACh may stimulate HCO\textsubscript{3} secretion through Ae2 without affecting the NBC activity. Interestingly, these effects were the opposite of those observed with ATP, which caused net inhibition of the NBC-mediated HCO\textsubscript{3} secretion in our perfused Ae2\textsubscript{a,b}−/− deficient mouse cholangiocytes while

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Fig. 7. Altered Cl\textsuperscript{-} secretion in polarized Ae2\textsubscript{a,b}−/− mouse cholangiocytes. Short-circuit current (I\textsubscript{sc}, indicative of Cl\textsuperscript{-} conductance) was analyzed in polarized Ae2\textsubscript{a,b}+/+ and Ae2\textsubscript{a,b}−/− mouse cholangiocytes mounted in Ussing chambers. (A) Representative I\textsubscript{sc} tracings in Ae2\textsubscript{a,b}+/+ and Ae2\textsubscript{a,b}−/− mouse cholangiocytes (left) in response to cAMP stimulation (500 µM 8-cpt-cAMP plus 450 µM 3-isobutyl-1-methyl-xanthine). Upon cAMP stimulation, calculated I\textsubscript{sc} values (right) are lower in Ae2\textsubscript{a,b}−/− than Ae2\textsubscript{a,b}+/+ mouse cholangiocytes. (B) Relative Cftr mRNA levels are lower in Ae2\textsubscript{a,b}−/− than Ae2\textsubscript{a,b}+/+ mouse cholangiocytes. Glycereraldehyde 3-phosphate dehydrogenase-normalized values in Ae2\textsubscript{a,b}−/− mouse cholangiocytes are given as percentages of those in Ae2\textsubscript{a,b}+/+ mouse cholangiocytes (100%) (n = 6 each). (C) Representative I\textsubscript{sc} trace in Ae2\textsubscript{a,b}+/+ and Ae2\textsubscript{a,b}−/− mouse cholangiocytes (left) in response to 100 µM ATP. Upon ATP administration, calculated I\textsubscript{sc} values (right) are greater in Ae2\textsubscript{a,b}−/− than Ae2\textsubscript{a,b}+/+ mouse cholangiocytes. (D) Representative tracings of Fluo-4 fluorescence emission at 535 nm captured every 2 seconds, indicative of intracellular Ca\textsuperscript{2+} variations in Ae2\textsubscript{a,b}+/+ and Ae2\textsubscript{a,b}−/− mouse cholangiocytes in response to administration of 100 µM of nonhydrolyzable ATP-γ-S (left); fluorescence values are relative to the baseline measurement (F/F\textsubscript{0}). Upon ATP stimulation, calculated fluorescence values (right) are greater in Ae2\textsubscript{a,b}−/− than Ae2\textsubscript{a,b}+/+ mouse cholangiocytes. For each type of cell, n values were obtained from three different cell preparations. Once monitored, values for I\textsubscript{sc} (A,C) and the relative Fluo-4 fluorescence (D) are given as the calculated AUC (arbitrary units) in the experimental plots.
having no overall effect on normal mouse cholangiocytes. Although different Ca\(^{2+}\) signaling pathways and/or different wave patterns of Ca\(^{2+}\) change might be involved in the differentiated effect of ATP on the NBC-mediated HCO\(_3^-\) transport versus that of ACh (for instance, calcium waves elicited by ATP can differ from those caused by ACh), additional Ca\(^{2+}\)-independent mechanism may also be implicated.

We finally assessed Cl\(^{-}\) efflux in polarized Ae2\(_{a,b}^{+/+}\) and Ae2\(_{a,b}^{−/−}\) mouse cholangiocytes in response to cAMP and ATP stimulation (through Cftr and Ca\(^{2+}\)-activated Cl\(^{-}\) channels, respectively). Interestingly, cAMP stimulation elicited lower (but did not abolish) Cl\(^{-}\) secretory activity in Ae2\(_{a,b}^{−/−}\) than Ae2\(_{a,b}^{+/+}\) mouse cholangiocytes. The diminished Cftr activity in the absence of Ae2 suggests a close functional interaction of these flux proteins in biliary cells, which is not surprising in view of their colocalization (together with aquaporin-1) in intracellular vesicles that migrate toward the apical membrane in response to cAMP.\(^7\) Thus far, no evidence for such functional interaction has been available, although previous studies in Xenopus oocytes and HEK293 cells have shown regulatory interactions of CFTR with electrogenic Cl\(^{-}/\)HCO\(_3^-\) exchangers.\(^{36}\) Our data may shed some light on former findings in CFTR-deficient human and mouse cholangiocytes (isolated from patients with cystic fibrosis and Cftr\(^{−/−}\) mice, respectively),\(^{37,38}\) in which cAMP stimulation elicited no HCO\(_3^-\) secretion. These findings seemingly argued for CFTR/Cftr as the carrier mediating the cAMP-stimulated HCO\(_3^-\) secretion in cholangiocytes, and thus questioned AE2/Ae2 involvement. However, our present data in Ae2\(_{a,b}^{−}\)-deficient mouse cholangiocytes, together with the aforementioned AE2-silencing results in human and rat cholangiocytes\(^4,5\) confirm the crucial role of AE2/Ae2. In fact, our Ae2\(_{a,b}^{−}\)-deficient cholangiocytes were completely unable to respond transporting HCO\(_3^-\) upon Cl\(^{-}\) removal—with and without cAMP stimulation—even though this was certainly diminished. Reduced cAMP-stimulated Cl\(^{-}\) current in Ae2\(_{a,b}^{−}\)-deficient mice might be due to both Cftr–Ae2 interaction failures and low Cftr gene expression. ATP stimulation, however, elicited higher values of Cl\(^{-}\) currents in Ae2\(_{a,b}^{−}\)-deficient mouse cholangiocytes, which suggests overactivation of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels attempting to maintain Cl\(^{-}\) homeostasis in these cells as reported for cystic-fibrosis cholangiocytes.\(^{37}\) This might be related with the fact that the ATP-stimulated increase in intracellular Ca\(^{2+}\) levels was more pronounced in Ae2\(_{a,b}^{−}\)-deficient mouse cholangiocytes.

In conclusion (see working model on Fig. 8), our data indicate that Ae2, in close interaction with Cftr, mediates cAMP-stimulated Cl\(^{-}\) channels, respectively). Interestingly, cAMP stimulation elicited lower (but did not abolish) Cl\(^{-}\) secretory activity in Ae2\(_{a,b}^{−/−}\) than Ae2\(_{a,b}^{+/+}\) mouse cholangiocytes. The diminished Cftr activity in the absence of Ae2 suggests a close functional interaction of these flux proteins in biliary cells, which is not surprising in view of their colocalization (together with aquaporin-1) in intracellular vesicles that migrate toward the apical membrane in response to cAMP.\(^7\) Thus far, no evidence for such functional interaction has been available, although previous studies in Xenopus oocytes and HEK293 cells have shown regulatory interactions of CFTR with electrogenic Cl\(^{-}/\)HCO\(_3^-\) exchangers.\(^{36}\) Our data may shed some light on former findings in CFTR-deficient human and mouse cholangiocytes (isolated from patients with cystic fibrosis and Cftr\(^{−/−}\) mice, respectively),\(^{37,38}\) in which cAMP stimulation elicited no HCO\(_3^-\) secretion. These findings seemingly argued for CFTR/Cftr as the carrier mediating the cAMP-stimulated HCO\(_3^-\) secretion in cholangiocytes, and thus questioned AE2/Ae2 involvement. However, our present data in Ae2\(_{a,b}^{−}\)-deficient mouse cholangiocytes, together with the aforementioned AE2-silencing results in human and rat cholangiocytes\(^4,5\) confirm the crucial role of AE2/Ae2. In fact, our Ae2\(_{a,b}^{−}\)-deficient cholangiocytes were completely unable to respond transporting HCO\(_3^-\) upon Cl\(^{-}\) removal—with and without cAMP stimulation—even though this was certainly diminished. Reduced cAMP-stimulated Cl\(^{-}\) current in Ae2\(_{a,b}^{−}\)-deficient mouse cholangiocytes might be due to both Cftr–Ae2 interaction failures and low Cftr gene expression. ATP stimulation, however, elicited higher values of Cl\(^{-}\) currents in Ae2\(_{a,b}^{−}\)-deficient mouse cholangiocytes, which suggests overactivation of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels attempting to maintain Cl\(^{-}\) homeostasis in these cells as reported for cystic-fibrosis cholangiocytes.\(^{37}\) This might be related with the fact that the ATP-stimulated increase in intracellular Ca\(^{2+}\) levels was more pronounced in Ae2\(_{a,b}^{−}\)-deficient mouse cholangiocytes.
biliary HCO\textsubscript{3}\textsuperscript{-} secretion in mouse cholangiocytes through Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange, which is directly activated by both cAMP and ACh. Additionally, mouse cholangiocytes possess a Na\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{-} cotransport activity, which under circumstances of diminished Ae2 activity allows the biliary cells to maintain basal pH\textsubscript{i} and HCO\textsubscript{3}\textsuperscript{-} secretion. Unlike Ae2, NBC activity in mice appears to be negatively regulated by cAMP and ATP. Finally, Ae2 deficiency in Ae2\textsubscript{a,b}\textsuperscript{-/-} mouse cholangiocytes is associated with impaired Cl\textsuperscript{-} activity and overactivation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels.

Ae2\textsubscript{a,b}\textsuperscript{-/-} mouse cholangiocytes resemble PBC cholangiocytes concerning baseline pH\textsubscript{i} homeostasis. Ae2\textsubscript{a,b}-deficient mouse cholangiocytes can maintain their resting pH\textsubscript{i} through the supplementary NBC-mediated HCO\textsubscript{3}\textsuperscript{-} secreting activity, which is absent in human cholangiocytes.\textsuperscript{4,18} However, PBC cholangiocytes may in turn maintain resting pH\textsubscript{i} because they generally display residual Ae2 activity,\textsuperscript{18} due to diminished (rather than absent) AE2 expression in most PBC samples.\textsuperscript{17} Such a similar outcome might contribute to the fact that our Ae2\textsubscript{a,b}\textsuperscript{-/-} mice indeed reproduce several features of PBC\textsuperscript{20} and may therefore be employed as an animal model of the human disease.

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