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Bicarbonate Secretion of Mouse Cholangiocytes Involves $\text{Na}^+\text{-HCO}_3^-$ Cotransport in Addition to Na^+ -Independent $\text{Cl}^-/\text{HCO}_3^-$ Exchange

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Bicarbonate secretion from cholangiocytes is required for appropriate adjustment of primary canalicular bile along the biliary tract. In human and rat cholangiocytes, bicarbonate secretion is mediated by anion exchanger (AE) 2, an electroneutral Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ AE also involved in intracellular pH (pH_i) regulation. In *Ae2_{a,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 *Ae2_{a,b}^{+/+}* and *Ae2_{a,b}^{-/-}* mouse cholangiocytes by microfluorimetric measurement of pH_i changes upon established perfusion maneuvers. Cl^- withdrawal by isethionate-based perfusions showed that *Ae2_{a,b}^{+/+}* but not *Ae2_{a,b}^{-/-}* mouse cholangiocytes can display $\text{Cl}^-/\text{HCO}_3^-$ 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 $\text{Na}^+\text{-HCO}_3^-$ cotransport (NBC). NBC activity is greater in *Ae2_{a,b}^{-/-}* than *Ae2_{a,b}^{+/+}* mouse cholangiocytes, and membrane-depolarization experiments showed that it is electrogenic. Consistent with the potential role of *Slc4a4/Nbc1* as the involved transporter, *Ae2_{a,b}^{-/-}* mouse cholangiocytes exhibit up-regulated expression of this electrogenic NBC carrier. Whereas Ae2-mediated $\text{Cl}^-/\text{HCO}_3^-$ exchange in *Ae2_{a,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 *Ae2_{a,b}^{-/-}* mouse cholangiocytes. Polarized *Ae2_{a,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 $\text{Cl}^-/\text{HCO}_3^-$ exchange and $\text{Na}^+\text{-HCO}_3^-$ cotransport. (HEPATOLOGY 2010;51:891-902.)

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; I_{sc} , short-circuit current; KRB, Krebs-Ringer bicarbonate; mRNA, messenger RNA; NBC, $\text{Na}^+\text{-HCO}_3^-$ cotransport; NRC, normal rat cholangiocyte; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; pH_i , intracellular pH.

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Bile flow is produced through the coordinated secretory function of hepatocytes and cholangiocytes.¹ 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 HCO_3^- , which is partially achieved through catalyzed hydration of CO_2 and subsequent H^+ extrusion through Na^+/H^+ exchange. Moreover, 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 HCO_3^- influx mediated by $\text{Na}^+-\text{HCO}_3^-$ cotransport (NBC).² In human cholangiocytes, however, basolateral influx of HCO_3^- occurs mainly through Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchange.³

In both human⁴ and rat⁵ cholangiocytes, the secretion of HCO_3^- to bile occurs through a Na^+ -independent electroneutral $\text{Cl}^-/\text{HCO}_3^-$ 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 (pH_i) regulation.⁶ Secretin-stimulated bicarbonate-rich hydrocholerisis has been postulated to involve the synchronized action of AE2 and two additional flux proteins, the cyclic adenosine monophosphate (cAMP)-responsive Cl^- channel cystic fibrosis transmembrane conductance regulator (CFTR) and the water channel aquaporin-1.⁷ Upon secretin/secretin-receptor interaction and subsequent increase in cAMP levels and protein kinase A activation,⁸⁻¹⁰ intracellular vesicles with all three flux proteins core-distribute to the luminal membrane of cholangiocytes.⁷ Accompanying phosphorylation and activation of CFTR¹¹ lead to apical efflux of Cl^- , which is ultimately exchanged with HCO_3^- through AE2.^{1,5,9,10} Resultant secretion of HCO_3^- is followed by osmotically driven efflux of water through aquaporin-1, ending up in the typical bicarbonate-rich hydrocholerisis induced by secretin.^{7,12} The effect of this mechanism may be reinforced through the recently described CFTR-associated release of adenosine triphosphate (ATP) from cholangiocytes to bile.¹³ 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 HCO_3^- through AE2. Bicarbonate-enriching processes elicited by secretin might also involve CFTR-independent effects of cAMP on the AE2-mediated $\text{Cl}^-/\text{HCO}_3^-$ exchange, as observed *in vitro* in human cholangiocytes in the presence of CFTR inhibitors³ and in hepatocyte-derived cells⁴ that lack CFTR. Acetylcholine (ACh) may further assist biliary HCO_3^- secretion through

Ca^{2+} -dependent Cl^- efflux from cholangiocytes and by potentiating the effect of secretin on both intracellular cAMP levels and $\text{Cl}^-/\text{HCO}_3^-$ exchange in a calcineurin-dependent manner.¹³⁻¹⁵

We previously hypothesized that abnormalities of AE2 in primary biliary cirrhosis (PBC) could play a pathogenic role in the disease,¹⁶⁻¹⁸ and generated a mouse model targeted for the three major Ae2a, Ae2b1, and Ae2b2 isoforms (*Ae2_{a,b}^{-/-}* mice).¹⁹ These mice showed biochemical, serologic, histopathologic, and immunologic features compatible with PBC.²⁰ pH_i assessment in lymphocytes²⁰ and fibroblasts²¹ of *Ae2_{a,b}^{-/-}* mice confirmed the expected intracellular alkalinization, while cholangiocytes isolated from these mice were found to have normal pH_i values.²⁰ We therefore decided to analyze the mechanisms involved in HCO_3^- secretion in cultured *Ae2_{a,b}^{+/+}* and *Ae2_{a,b}^{-/-}* mouse cholangiocytes. Our present data show that wild-type but not *Ae2_{a,b}^{-/-}* mouse cholangiocytes exhibit $\text{Cl}^-/\text{HCO}_3^-$ 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.^{4,5} Characteristically, however, mouse cholangiocytes can display an additional transport activity for HCO_3^- secretion that is more pronounced in *Ae2_{a,b}*-deficient cholangiocytes. This additional activity is Cl^- -independent and Na^+ -dependent (consistent with NBC activity), electrogenic, and responds differentially to agonists such as cAMP, ATP, and ACh.

Material and Methods

Cultured Cholangiocytes from Normal and *Ae2_{a,b}^{-/-}* Mice. Cultured cholangiocytes were obtained from male *Ae2_{a,b}^{+/+}* and *Ae2_{a,b}^{-/-}* mice, starting with isolation of intrahepatic bile duct units, followed by their seeding and culture on collagen monolayer²⁰ (see Supporting Information). Normal rat cholangiocytes (NRCs) were similarly obtained from male Wistar rats. Animal interventions were approved by the Institutional Animal Care and Use Committee.

Determination of HCO_3^- Transport Activities. Cultured cholangiocytes were assessed for HCO_3^- transport activities by microfluorimetry (see Supporting Information). Cells initially perfused with Krebs-Ringer bicarbonate (KRB) medium were monitored for pH_i variations upon two types of perfusion maneuvers^{2,3,18} (see detailed buffers in Supplementary Table 1). First, to test $\text{Cl}^-/\text{HCO}_3^-$ 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).^{3,18} These Cl^- -free based maneuvers are designed to elicit efflux of intracellular Cl^- in exchange with extracellular HCO_3^- , increasing pH_i . In the case of AE2,

which physiologically functions as a HCO_3^- -extruder (i.e., acid-loader), $\text{Cl}^-/\text{HCO}_3^-$ exchange activity is measured in a reverse mode. The exchange activity (expressed as transmembrane base fluxes J_{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_i . In this case, HCO_3^- secretion rates (J_{OH^-}) are calculated from the tangent of the experimental plot of the recovery toward resting pH_i . The stimulatory effect of either a cAMP/stimulation mixture (cAMP/SM) (100 μM N⁶2-O-dibutyryl-cAMP, 3 μM forskolin, and 100 μM 3-isobutyl-1-methyl-xanthine),^{5,18,22} 100 μM ACh¹³ 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 NRCs²⁴ (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} ($\mu\text{A}/\text{cm}^2$) 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 $\pm$ 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 human⁴ and rat⁵ 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 $\text{Ae2}_{a,b}^{+/+}$ and $\text{Ae2}_{a,b}^{-/-}$ 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 $\text{Cl}^-/\text{HCO}_3^-$ exchange ($J_{\text{OH}^-} = 20.97 \pm 0.72 \text{ mmol/L/minute}$). Likewise, Cl^- withdrawal induced intracellular alkalinization in normal ($\text{Ae2}_{a,b}^{+/+}$) mouse cholangiocytes, which reveals that these cells also display $\text{Cl}^-/\text{HCO}_3^-$ exchange activity ($J_{\text{OH}^-} = 10.63 \pm 0.95 \text{ mmol/L/minute}$) (Fig. 1B). In $\text{Ae2}_{a,b}^{-/-}$ mouse cholangiocytes, however, no intracellular alkalinization was observed after Cl^- withdrawal (Fig. 1C), demonstrating that the $\text{Cl}^-/\text{HCO}_3^-$ 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 $\text{Cl}^-/\text{HCO}_3^-$ 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 $\text{Ae2}_{a,b}^{+/+}$ and $\text{Ae2}_{a,b}^{-/-}$ 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 $\text{Ae2}_{a,b}^{-/-}$ than normal mouse cholangiocytes ($J_{\text{OH}^-} = 10.92 \pm 2.02$ versus $1.82 \pm 0.95 \text{ mmol/L/minute}$; $P < 0.001$), which might be partially due to the counterbalancing Ae2-mediated alkalinization that takes place in normal but not $\text{Ae2}_{a,b}^{-/-}$ 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

Fig. 1. Mouse cholangiocytes display Ae2-mediated Cl⁻/HCO₃⁻ AE activity. (A) Representative trace from control experiments with NRCs showing that removal of Cl⁻ (both in the presence of Na⁺ through perfusion with isethionate-based medium, and in the absence of Na⁺ through perfusion with *N*-methyl-D-glucamine-based medium), results in intracellular alkalinization (left). This observation is as expected, because NRCs were described to possess AE activity,²² which upon Cl⁻ removal is forced to operate in the reverse mode with Cl⁻ efflux in exchange to HCO₃⁻ influx. When Cl⁻/HCO₃⁻ exchange activities in the presence and in the absence of Na⁺ were calculated, values were found to be rather similar (right), also consistent with the Na⁺ independency of the Ae2-mediated Cl⁻/HCO₃⁻ exchange in these rat cells.⁵ To prevent acidification of the pH_i through Na⁺/H⁺ exchange activity after Na⁺ removal, amiloride (1 mM) was added along these experiments (as well as along following experiments with mouse cholangiocytes). (B,C) Maneuvers of Cl⁻ removal from Ae2_{a,b}^{+/+} and Ae2_{a,b}^{-/-} mouse cholangiocytes, respectively. Removal of Cl⁻ alone induces intracellular alkalinization in Ae2_{a,b}^{+/+} but not Ae2_{a,b}^{-/-} mouse cholangiocytes, indicating that the increase in pH_i in the former is mediated by a reverse-mode operating Ae2. Unlike NRCs, simultaneous removal of Na⁺ and Cl⁻ revealed that murine cells of both genotypes possess an additional Na⁺-dependent HCO₃⁻-secreting activity that acidifies the pH_i. This Na⁺-dependent acidifying activity becomes more apparent in Ae2_{a,b}^{-/-} than Ae2_{a,b}^{+/+} mouse cholangiocytes. For each type of cell, n values were obtained from three different cell preparations.

recovery—indicative of HCO₃⁻ extrusion—in the presence of Cl⁻ (J_{OH⁻} = 5.39 ± 0.24 mmol/L/minute) but not in its absence (Fig. 2A), confirming again that HCO₃⁻ secretion only occurs through Cl⁻/HCO₃⁻ ex-

change in these cells. By contrast, Ae2_{a,b}^{+/+} mouse cholangiocytes showed no inhibition of the HCO₃⁻ extrusion rate despite Cl⁻ withdrawal (J_{OH⁻} = 2.87 ± 0.33 mmol/L/minute in the presence of Cl⁻ versus 2.83 ± 0.29 mmol/L/minute in the absence of this anion) (Fig. 2B), thus unmasking a Cl⁻-independent HCO₃⁻ extrusion activity. This observation was even clearer in Ae2_{a,b}^{-/-} mouse cholangiocytes. In spite of their Ae2 deficiency, these cells were able to display HCO₃⁻ extrusion activity regardless of the presence or absence of extracellular Cl⁻ (J_{OH⁻} = 4.91 ± 1.12 versus 5.89 ± 0.80 mmol/L/minute, respectively; Fig. 2C). In fact, HCO₃⁻ extrusion rate values were significantly higher in Ae2_{a,b}^{-/-} than Ae2_{a,b}^{+/+} mouse cholangiocytes (P < 0.05 in the presence of Cl⁻ and P < 0.01 in its absence). This Cl⁻-independent and Na⁺-driven activity to secrete HCO₃⁻ observed in mouse cholangiocytes is by definition a Na⁺-HCO₃⁻ 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 HCO₃⁻ transporters)²⁵ in both Ae2_{a,b}^{+/+} (J_{OH⁻} = 2.81 ± 0.23 versus 1.03 ± 0.44 mmol/L/minute in the presence of DIDS; P < 0.001) and Ae2_{a,b}^{-/-} (J_{OH⁻} = 6.15 ± 0.86 versus 3.05 ± 0.63 mmol/L/minute; P < 0.05) mouse cholangiocytes.

Na⁺-Driven Cl⁻-Independent HCO₃⁻ 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 K⁺.²⁷ Upon membrane depolarization, an electrogenic-driven HCO₃⁻ extrusion would be diminished, whereas an electroneutral transport would remain unchanged.²⁷ Whereas HCO₃⁻ secretion activity in NRCs was maintained (consistent with its complete mediation by electroneutral Ae2 activity) (Fig. 3A), the secretion rate was diminished in Ae2_{a,b}^{+/+} mouse cholangiocytes (J_{OH⁻} = 3.09 ± 0.19 versus 1.54 ± 0.16 mmol/L/minute; P < 0.001) (Fig. 3B) and totally abolished in Ae2_{a,b}^{-/-} mouse cholangiocytes (J_{OH⁻} = 4.83 ± 0.46 versus 0 mmol/L/minute; P < 0.001) (Fig. 3C), indicating that the Na⁺-HCO₃⁻ cotransport displayed by mouse cholangiocytes is electrogenic.

Slc4a4 Expression Is Up-Regulated in Ae2_{a,b}^{-/-} Mouse Cholangiocytes. Among NBC carriers, Slc4a4 and Slc4a5 have been reported to be electrogenic,^{26,28} whereas Slc4a7 is electroneutral.^{27,29} We therefore analyzed the messenger RNA (mRNA) expression of these cotransporters as well as the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger Slc4a8 in mouse cholangiocytes. Both Ae2_{a,b}^{+/+} and Ae2_{a,b}^{-/-} mouse cholangiocytes showed expression of Slc4a4 mRNA, which was 1.8-fold up-reg-

ulated in $Ae2_{a,b}^{-/-}$ versus $Ae2_{a,b}^{+/+}$ 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 $Ae2_{a,b}^{-/-}$ mouse cholangiocytes (Fig. 3E).

HCO₃⁻ Secretion Is Stimulated by cAMP in $Ae2_{a,b}^{+/+}$ Cholangiocytes but Inhibited in $Ae2_{a,b}^{-/-}$ Cholangiocytes. Rise of intracellular cAMP levels is known to stimulate Ae2 activity in human and rat cholan-

giocytes, resulting in an increase of HCO_3^- secretion to bile.¹ As shown in Fig. 4A, application of a cAMP/SM to $Ae2_{a,b}^{+/+}$ mouse cholangiocytes increased the HCO_3^- extrusion rate ($J_{OH^-} = 2.81 \pm 0.23$ versus 4.10 ± 0.48 mmol/L/minute; $P < 0.05$), whereas the same application to $Ae2_{a,b}^{-/-}$ mouse cholangiocytes diminished HCO_3^- extrusion ($J_{OH^-} = 6.15 \pm 0.86$ versus 3.64 ± 0.45 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 $Ae2_{a,b}^{-/-}$ mouse cholangiocytes have raised levels compared with $Ae2_{a,b}^{+/+}$ mouse cholangiocytes (Fig. 4C).

Acetylcholine Stimulates HCO₃⁻ Secretion in $Ae2_{a,b}^{+/+}$ but not in $Ae2_{a,b}^{-/-}$ 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 $Ae2_{a,b}^{+/+}$ mouse cholangiocytes ($J_{OH^-} = 3.63 \pm 0.48$ versus 6.79 ± 0.56 mmol/L/minute; $P < 0.001$). However, the presence of ACh did not affect HCO_3^- secretion in $Ae2_{a,b}^{-/-}$ mouse cholangiocytes ($J_{OH^-} = 4.15 \pm 0.66$ versus 4.02 ± 0.65 mmol/L/minute) (Fig. 5B).

ATP Inhibits HCO₃⁻ Secretion in $Ae2_{a,b}^{-/-}$ 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 μ M ATP did not modify the HCO_3^- secretion rate in $Ae2_{a,b}^{+/+}$ mouse cholangiocytes ($J_{OH^-} = 5.53 \pm 0.66$ versus 4.68 ± 0.52 mmol/L/

Fig. 2. Na⁺-dependent HCO₃⁻-secreting activity displayed by mouse cholangiocytes does not depend on Cl⁻, consistent with NBC. (A) Control experiments with NRCs to test HCO₃⁻ secretion by using propionate-based maneuvers. The weak intracellular acidification initially induced by perfusion with propionate is sufficient to overstimulate acid extrusion mechanisms, which upon propionate withdrawal (through perfusion with KRB medium) results in pronounced intracellular alkalization. Increased pH_i may then activate acid-loading mechanisms such as Ae2, which physiologically functions extruding HCO₃⁻ in exchange with Cl⁻ entrance. Indeed, withdrawal of Cl⁻ from perfusion medium totally blocks intracellular acidification, consistent with the notion that recovery of resting pH_i in rat cholangiocytes involves HCO₃⁻ secretion through Cl⁻/HCO₃⁻ exchange. HCO₃⁻ secretion rate is calculated from the acidification curves after propionate removal and administration of KRB with and without Cl⁻. (B,C) Propionate-based maneuvers on $Ae2_{a,b}^{+/+}$ and $Ae2_{a,b}^{-/-}$ mouse cholangiocytes, respectively. Unlike NRCs, murine cells of both genotypes are able to secrete HCO₃⁻ in a Cl⁻-independent manner, because Cl⁻ withdrawal does not affect their HCO₃⁻ secretion rate. The Cl⁻-independent HCO₃⁻ secretion rate is higher in $Ae2_{a,b}^{-/-}$ than $Ae2_{a,b}^{+/+}$ mouse cholangiocytes and should correspond to the NBC activity uncovered with the *N*-methyl-D-glucamine-based perfusion maneuvers (Fig. 1). (D) Cl⁻-independent HCO₃⁻ secretion in both $Ae2_{a,b}^{+/+}$ and $Ae2_{a,b}^{-/-}$ mouse cholangiocytes is sensitive to the nonspecific inhibitor of HCO₃⁻ transporters DIDS (0.5 mM), as expected for an NBC-mediated activity. For each type of cell, n values were obtained from three different cell preparations.

Fig. 3. NBC activity of mouse cholangiocytes is electrogenic. To assess the electrogenicity of the Na^+ - HCO_3^- cotransport in mouse cholangiocytes observed with the propionate-based maneuvers, cells were perfused with KRB or KRB with K^+ at a depolarizing concentration (50 mM) after removal of propionate (always in the presence of Cl^-). (A) Control experiments with NRCs showing that cell membrane depolarization does not significantly affect HCO_3^- secretion, as expected for an $Ae2$ -mediated electroneutral Cl^-/HCO_3^- exchange activity.⁵ (B,C) Propionate-based maneuver testing the effect of cell membrane depolarization in $Ae2_{a,b}^{+/+}$ and $Ae2_{a,b}^{-/-}$ mouse cholangiocytes, respectively. Depolarizing conditions result in partial inhibition of HCO_3^- secretion in $Ae2_{a,b}^{+/+}$ mouse cholangiocytes while completely blocking this secretion in $Ae2_{a,b}^{-/-}$ mouse cholangiocytes. For each type of cell, n values were obtained from four different cell preparations. (D) Relative mRNA expression of the NBC cotransporter genes $Slc4a4$ and $Slc4a7$ in mouse cholangiocytes. Glyceraldehyde 3-phosphate dehydrogenase-normalized values in $Ae2_{a,b}^{-/-}$ mouse cholangiocytes are given as percentages of those in $Ae2_{a,b}^{+/+}$ mouse cholangiocytes (100%). (E) Western blotting showing the expression of $Slc4a4$ protein in extracts of mouse cholangiocytes (left); β -actin was employed as a normalizing loading control. Band intensity values (normalized for β -actin) in four different lanes with $Ae2_{a,b}^{-/-}$ mouse-cholangiocyte protein extracts are represented (right) as fold change relative to values in $Ae2_{a,b}^{+/+}$ cholangiocytes.

minute) (Fig. 6A), and interestingly resulted in diminished rate in $Ae2_{a,b}^{-/-}$ mouse cholangiocytes ($J_{OH^-} = 9.00 \pm 0.83$ versus 5.99 ± 0.34 mmol/L/minute; $P < 0.001$) (Fig. 6B).

$Ae2_{a,b}^{-/-}$ 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.^{24,30,31} 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 $Ae2_{a,b}^{+/+}$ mouse cholangiocytes resulted in increased values of short-circuit current intensity due to Cl^- secretion (ΔI_{sc} : 3.45 ± 0.38 $\mu A/cm^2$), which was similar to that reported for polarized NRCs.²⁴ However, cAMP stimulation of $Ae2_{a,b}^{-/-}$ mouse cholangiocytes resulted in less intense Cl^- secretion (ΔI_{sc} : 0.95 ± 0.13 $\mu A/cm^2$) (Fig.

7A). When we measured the area under the curve (AUC) of I_{sc} , the cAMP response in $Ae2_{a,b}^{-/-}$ mouse cholangiocytes was found to be significantly lower than that in $Ae2_{a,b}^{+/+}$ mouse cholangiocytes ($AUC\ 10.63 \pm 1.071$ 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 $Ae2_{a,b}^{-/-}$ than $Ae2_{a,b}^{+/+}$ mouse cholangiocytes (13.7% versus 100%; $P < 0.001$) (Fig. 7B).

On the other hand, ATP stimulation led to significantly higher Cl^- secretion in $Ae2_{a,b}^{-/-}$ compared with $Ae2_{a,b}^{+/+}$ mouse cholangiocytes ($AUC\ 49.68 \pm 6.02$ ve-

Fig. 4. HCO_3^- secretion is increased by cAMP stimulation in $Ae2_{a,b}^{+/+}$ mouse cholangiocytes but inhibited in $Ae2_{a,b}^{-/-}$ cholangiocytes. (A,B) Representative traces (left) showing that administration of cAMP/SM ($100\ \mu M$ N⁶,2'-O-dibutyryl-cAMP, $3\ \mu M$ forskolin, and $100\ \mu M$ 3-isobutyl-1-methyl-xanthine)^{5,18} accelerated the recovering acidification after the alkalinization elicited by propionate removal in perfused $Ae2_{a,b}^{+/+}$ mouse cholangiocytes, but decreased this acidification in $Ae2_{a,b}^{-/-}$ mouse cholangiocytes, respectively. Calculated $J_{O_H^-}$ (right) indicate that these opposite changes are both statistically significant. For each type of cell, n values were obtained from three different cell preparations. (C) Baseline intracellular cAMP levels are elevated in $Ae2_{a,b}^{-/-}$ compared with $Ae2_{a,b}^{+/+}$ mouse cholangiocytes; moreover, the cAMP/SM increased the intracellular cAMP levels in both $Ae2_{a,b}^{+/+}$ and $Ae2_{a,b}^{-/-}$ mouse cholangiocytes.

Fig. 5. HCO_3^- secretion is increased by ACh in $Ae2_{a,b}^{+/+}$ mouse cholangiocytes but remains unchanged in $Ae2_{a,b}^{-/-}$ cholangiocytes. (A) Representative traces showing that propionate removal and subsequent alkalinization in $Ae2_{a,b}^{+/+}$ mouse cholangiocytes is followed by accelerated recovering acidification in the presence of $100\ \mu M$ ACh (left). Indeed, calculated $J_{O_H^-}$ (right) are significantly higher in the presence of ACh. (B) In contrast to normal mouse cholangiocytes, ACh shows no effect on the recovering acidification (HCO_3^- secretion rates) in $Ae2_{a,b}^{-/-}$ mouse cholangiocytes. For each type of cell, n values were obtained from three different cell preparations.

sus 193.3 ± 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 $Ae2_{a,b}^{-/-}$ compared with $Ae2_{a,b}^{+/+}$ 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.^{4,5} Previously, we hypothesized that abnormalities in the liver expression^{16,17} and function¹⁸ of AE2 and concomitant alterations of HCO_3^- secretion in the biliary epithelium,³² may be involved in the pathogenesis of PBC. More recently, this hypothesis has been supported by our findings in $Ae2_{a,b}^{-/-}$ 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.

Fig. 6. ATP inhibits HCO₃⁻ secretion in *Ae2_{a,b}^{-/-}* mouse cholangiocytes but shows no effect in *Ae2_{a,b}^{+/+}* mouse cholangiocytes. (A) Representative traces (left) showing that the presence of 10 μ M ATP in perfused *Ae2_{a,b}^{+/+}* mouse cholangiocytes has no effect on the recovering acidification after propionate removal and subsequent alkalization. Calculated J_{OH⁻} (right) were similar in the presence or absence of ATP. (B) Representative traces from *Ae2_{a,b}^{-/-}* mouse cholangiocytes (left) show that the presence of ATP decreases the recovering acidification in these cells. Calculated J_{OH⁻} (right) were diminished in the presence of ATP. Amiloride (1 mM) was employed during the entire experiment to prevent an activation of the Na⁺/H⁺ exchange activity by ATP, which could interfere in pH_i variations. For each type of cell, n values were obtained from three different cell preparations.

Studies involved monitoring pH_i 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 alkalization upon Cl⁻ withdrawal was completely absent in *Ae2_{a,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 pH_i 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 *Ae2_{a,b}^{-/-}* than *Ae2_{a,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 pH_i from intracellular alkalization)—also confirmed that the Na⁺-driven HCO₃⁻ secretory activity observed in both *Ae2_{a,b}^{+/+}* and *Ae2_{a,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.

Our findings of up-regulated expression of *Slc4a4* mRNA in *Ae2_{a,b}^{-/-}* (versus normal mouse cholangiocytes) and absent *Slc4a5* expression in both *Ae2_{a,b}^{-/-}* and *Ae2_{a,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 *Slc4a4* protein in the *Ae2_{a,b}^{-/-}* cells. *Slc4a4* up-regulation and subsequently increased NBC activity in *Ae2_{a,b}^{-/-}* mouse cholangiocytes may account for an elevated HCO₃⁻ secreting activity in these cells, which might be viewed as an attempt to compensate Ae2 deficiency for pH_i regulation. In this sense, cholangiocytes from *Ae2_{a,b}*-deficient mice appear to be more capable of maintaining their resting pH_i²⁰ than *Ae2_{a,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 *Ae2_{a,b}^{-/-}* mouse cholangiocytes (Fig. 4). Baseline NBC activity of these *Ae2_{a,b}^{-/-}* cells is surprisingly high in spite of elevated cAMP production (also observed in *Ae2_{a,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_3^- secretion (similarly to what had been observed in NRCs with cAMP stimulation),^{5,22} probably because stimulation of Ae2-mediated $\text{Cl}^-/\text{HCO}_3^-$ 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_3^- secretion is initiated by Cfr activation and Cl^- efflux into bile; $\text{Cl}^-/\text{HCO}_3^-$ exchange would follow as a consequence of outside-to-inside transmembrane gradient of Cl^- at a relatively high intracellular HCO_3^- concentration.^{9,15,33,34} Moreover, cAMP activation of Cfr was recently associated with apical release of ATP.¹³ Autocrine/paracrine purinergic stimulation

through the potent apically located ATP-responsive P2Y receptors and subsequent activation of Ca^{2+} -dependent Cl^- channels with additional efflux of Cl^- to bile could therefore result in further increased $\text{Cl}^-/\text{HCO}_3^-$ exchange at the apical membrane.^{13,23,30,31,35} In our model of perfused cholangiocytes, however, released Cl^- becomes diluted in the vast volume of perfusion media, with no chance for the alleged indirect stimulatory effects of the cAMP/SM on $\text{Cl}^-/\text{HCO}_3^-$ exchange after outside-to-inside transmembrane gradient of Cl^- . Thus, cAMP appears to exert alternative stimulatory effects on the Ae2-mediated $\text{Cl}^-/\text{HCO}_3^-$ exchange in our $\text{Ae2}_{a,b}^{+/+}$ mouse cholangiocytes.

ACh was also reported to indirectly stimulate HCO_3^- secretion in rat isolated bile ducts.^{13,15} ACh binds to M3 muscarinic receptors at the basolateral membrane of cholangiocytes, leading to increased intracellular Ca^{2+} levels, activation of Ca^{2+} -dependent Cl^- channels and further Cl^- efflux into the bile duct lumen, and subsequent $\text{Cl}^-/\text{HCO}_3^-$ exchange for HCO_3^- secretion to bile.¹⁵ In our perfused $\text{Ae2}_{a,b}^{+/+}$ mouse cholangiocytes, we found that ACh increased HCO_3^- secretion similarly to cAMP. However, in contrast to cAMP, ACh showed no effect in our perfused $\text{Ae2}_{a,b}^{-/-}$ mouse cholangiocytes. Our data therefore suggest that ACh may stimulate HCO_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_3^- secretion in our perfused $\text{Ae2}_{a,b}$ -deficient mouse cholangiocytes while

Fig. 7. Altered Cl^- secretion in polarized $\text{Ae2}_{a,b}^{-/-}$ mouse cholangiocytes. Short-circuit current (I_{sc} , indicative of Cl^- conductance) was analyzed in polarized $\text{Ae2}_{a,b}^{+/+}$ and $\text{Ae2}_{a,b}^{-/-}$ mouse cholangiocytes mounted in Ussing chambers. (A) Representative I_{sc} tracings in $\text{Ae2}_{a,b}^{+/+}$ and $\text{Ae2}_{a,b}^{-/-}$ 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_{sc} values (right) are lower in $\text{Ae2}_{a,b}^{-/-}$ than $\text{Ae2}_{a,b}^{+/+}$ mouse cholangiocytes. (B) Relative Cfr mRNA levels are lower in $\text{Ae2}_{a,b}^{-/-}$ than $\text{Ae2}_{a,b}^{+/+}$ mouse cholangiocytes. Glyceraldehyde 3-phosphate dehydrogenase-normalized values in $\text{Ae2}_{a,b}^{-/-}$ mouse cholangiocytes are given as percentages of those in $\text{Ae2}_{a,b}^{+/+}$ mouse cholangiocytes (100%) ($n = 6$ each). (C) Representative I_{sc} trace in $\text{Ae2}_{a,b}^{+/+}$ and $\text{Ae2}_{a,b}^{-/-}$ mouse cholangiocytes (left) in response to 100 μM ATP. Upon ATP administration, calculated I_{sc} values (right) are greater in $\text{Ae2}_{a,b}^{-/-}$ than $\text{Ae2}_{a,b}^{+/+}$ mouse cholangiocytes. (D) Representative tracings of Fluo-4 fluorescence emission at 535 nm captured every 2 seconds, indicative of intracellular Ca^{2+} variations in $\text{Ae2}_{a,b}^{+/+}$ and $\text{Ae2}_{a,b}^{-/-}$ 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_0). Upon ATP stimulation, calculated fluorescence values (right) are greater in $\text{Ae2}_{a,b}^{-/-}$ than $\text{Ae2}_{a,b}^{+/+}$ mouse cholangiocytes. For each type of cell, n values were obtained from three different cell preparations. Once monitored, values for I_{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 Cfr 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 Cfr 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 $\text{Cl}^-/\text{HCO}_3^-$ 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 *Cfr*^{-/-} mice, respectively),^{37,38} in which cAMP stimulation elicited no HCO_3^- secretion. These findings seemingly argued for CFTR/Cfr 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—while they exhibited cAMP-stimulated Cl^- current, even though this was certainly diminished. Reduced cAMP-stimulated Cl^- current in *Ae2_{a,b}*-deficient mouse cholangiocytes might be due to both Cfr–Ae2 interaction failures and low *Cfr* gene expression. ATP stimulation, however, elicited higher values of Cl^- currents in *Ae2_{a,b}*^{-/-} 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}*^{-/-} than *Ae2_{a,b}*^{+/+} mouse cholangiocytes.

In conclusion (see working model on Fig. 8), our data indicate that Ae2, in close interaction with Cfr, mediates

Fig. 8. Working model of HCO_3^- secretion in mouse cholangiocytes. Similar to both normal rat and human cholangiocytes, the $\text{Cl}^-/\text{HCO}_3^-$ AE activity in mouse cholangiocytes is entirely mediated by Ae2. Physiologically, this activity results in HCO_3^- secretion. But in contrast to rat and human cholangiocytes, mouse cholangiocytes possess an additional carrier for HCO_3^- secretion that functions as an electrogenic NBC. Because *Slc4a4* is the only electrogenic NBC found to be expressed in mouse cholangiocytes, this carrier is presumably involved in the NBC-mediated HCO_3^- secretion described here. Moreover, *Ae2_{a,b}*^{-/-} mouse cholangiocytes exhibit up-regulated expression of *Slc4a4* compared with *Ae2_{a,b}*^{+/+} mouse cholangiocytes, which is consistent with the increased HCO_3^- -secreting NBC activity encountered in the former. This NBC activity in *Ae2_{a,b}*^{-/-} mouse cholangiocytes is decreased by both cAMP and ATP, but not by ACh. Finally, the mechanisms for Cl^- secretion (which *in vivo* is crucially required by Ae2 for $\text{Cl}^-/\text{HCO}_3^-$ exchange and subsequent HCO_3^- secretion at the biliary epithelium) are also altered in *Ae2_{a,b}*^{-/-} mouse cholangiocytes, with diminished (but not abolished) activity of Cfr and up-regulated activity of Ca^{2+} -dependent Cl^- channels.

biliary HCO_3^- secretion in mouse cholangiocytes through $\text{Cl}^-/\text{HCO}_3^-$ exchange, which is directly activated by both cAMP and ACh. Additionally, mouse cholangiocytes possess a $\text{Na}^+-\text{HCO}_3^-$ cotransport activity, which under circumstances of diminished Ae2 activity allows the biliary cells to maintain basal pH_i and HCO_3^- secretion. Unlike Ae2, NBC activity in mice appears to be negatively regulated by cAMP and ATP. Finally, Ae2 deficiency in $\text{Ae2}_{a,b}^{-/-}$ mouse cholangiocytes is associated with impaired Cftr activity and overactivation of Ca^{2+} -dependent Cl^- channels.

$\text{Ae2}_{a,b}^{-/-}$ mouse cholangiocytes resemble PBC cholangiocytes concerning baseline pH_i homeostasis. $\text{Ae2}_{a,b}$ -deficient mouse cholangiocytes can maintain their resting pH_i through the supplementary NBC-mediated HCO_3^- secreting activity, which is absent in human cholangiocytes.^{4,18} However, PBC cholangiocytes may in turn maintain resting pH_i because they generally display residual AE2 activity,¹⁸ due to diminished (rather than absent) AE2 expression in most PBC samples.¹⁷ Such a similar outcome might contribute to the fact that our $\text{Ae2}_{a,b}^{-/-}$ mice indeed reproduce several features of PBC²⁰ and may therefore be employed as an animal model of the human disease.

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