Impaired Regulatory Volume Decrease in Freshly Isolated Cholangiocytes from Cystic Fibrosis Mice

IMPLICATIONS FOR CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR EFFECT ON POTASSIUM CONDUCTANCE*

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Various K^+ and Cl^- channels are important in cell volume regulation and biliary secretion, but the specific role of cystic fibrosis transmembrane conductance regulator in cholangiocyte cell volume regulation is not known. The goal of this research was to study regulatory volume decrease (RVD) in bile duct cell clusters (BD-CCs) from normal and cystic fibrosis (CF) mouse livers. Mouse BDCCs without an enclosed lumen were prepared as described (Cho, W. K. (2002) *Am. J. Physiol.* 283, G1320–G1327). The isotonic solution consisted of HEPES buffer with 40% of the NaCl replaced with isomolar amounts of sucrose, whereas hypotonic solution was the same as isotonic solution without sucrose. The cell volume changes were indirectly assessed by measuring cross-sectional area (CSA) changes of the BDCCs using quantitative videomicroscopy. Exposure to hypotonic solutions increased relative CSAs of normal BDCCs to 1.20 ± 0.01 (mean $\pm$ S.E., $n = 50$) in 10 min, followed by RVD to 1.07 ± 0.01 by 40 min. Hypotonic challenge in CF mouse BDCCs also increased relative CSA to 1.20 ± 0.01 ($n = 53$) in 10 min but without significant recovery. Coadministration of the K^+-selective ionophore valinomycin restored RVD in CF mouse BDCCs, suggesting that the impaired RVD was likely from a defect in K^+ conductance. Moreover, this valinomycin-induced RVD in CF mice was inhibited by 5-nitro-2′-(3-phenylpropylamino)-benzoate, indicating that it is not from nonspecific effects. Neither cAMP nor calcium agonists could reverse the impaired RVD seen in CF cholangiocytes. Our conclusion is that CF mouse cholangiocytes have defective RVD from an impaired K^+ efflux pathway, which could not be reversed by cAMP nor calcium agonists.

Under physiological conditions, osmoregulation plays a crucial role in cholangiocytes, which are exposed to various osmotic stresses from the uptake of solutes and electrolytes and bile secretion (1, 2). A recent study on a human cholangiocarcinoma cell line (3), as well as our results in a study on primary bile duct cell clusters (BDCCs)[1] from normal mouse livers (4), indicates that cholangiocytes can regulate their cell volumes back to base line from hypotonicity-induced swelling. As in other cell types, these adaptive mechanisms of regulatory volume decrease (RVD) in cholangiocytes are mediated by certain K^+ and Cl^- conductances (3, 4).

Cystic fibrosis (CF) is the most common inherited multisystem disease in the Caucasian population and is caused by a defect in the cystic fibrosis transmembrane conductance regulator (CFTR). Many CF patients develop a spectrum of hepatobiliary diseases that are thought to be due to secretory dysfunctions from the CFTR Cl^- channel defects. In recent years, CF liver diseases have become the second most common cause of mortality in CF patients as they live longer, but the underlying pathophysiological mechanisms are not well understood. Recent immunocytochemical studies of the liver have shown that CFTR is expressed only on the bile duct epithelium but not on hepatocytes (5). The CFTR is one of the major Cl^- channels mediating ion transport in the bile duct epithelium, but the direct role of CFTR in osmoregulation in cholangiocytes is not well known, although such defects in CFTR are likely to cause defects in osmoregulation. Thus, the purpose of the present study is to examine RVD in the cholangiocytes isolated from Ctfr$-/-$ or CF mouse livers and compare it with RVD in normal cholangiocytes. To accomplish this purpose, we have used BDCCs, which are prepared by the same isolation method as mouse isolated bile duct units (IBDUs) reported recently by our laboratory (6) but lack the enclosed lumen of IBDUs. These mouse cell preparations are primary cholangiocytes, and thus are considered to be closer to the physiologic state than other cholangiocyte cell lines and have proven to be quite powerful tools for studying cholangiocyte biology and physiology. The use of BDCCs can avoid likely problems with changes in luminal volumes with changes in osmotic solutions, and we have successfully used these BDCCs to study RVD in normal cholangiocytes (4).

In the present study, we present compelling evidence that, unlike normal mouse BDCCs, those from Cftr$-/-$ mouse livers

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[1] The abbreviations used are: BDCCs, bile duct cell clusters; CF, cystic fibrosis; RVD, regulatory volume decrease; CFTR, cystic fibrosis transmembrane conductance regulator; IBDUs, intrahepatic bile duct units; NPPB, 5-nitro-2′-(3-phenylpropylamino)-benzoate; CSA, cross-sectional area; IBMX, isobutylmethylxanthine; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein; SKCa, small conductance Ca^{2+}-sensitive K^+ channel; VACC, volume-activated chloride channel.

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have an impaired RVD as suspected from the defect in CFTR. Surprisingly, however, the impaired RVD in these CF BDCCs is mainly caused by defects in K^+ conductances, rather than Cl^- conductances, and is thus restored when K^+ conductances are provided by valinomycin, a K^+ ionophore. In addition, the observed RVD of CF BDCCs during administration of valinomycin is inhibited by NPPB, a general chloride channel blocker, indicating that the effects of valinomycin are not from nonspecific effects of valinomycin. In addition, these findings also indicate that there is a functioning non-CFTR chloride conductance pathway(s) present in CF cholangiocytes, which can mediate RVD in these cells.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, penicillin/streptomycin, EDTA, heparin, HEPES, D(+)-glucose, insulin, dimethyl sulfoxide (Me$_2$SO), hyaluronidase, and deoxyribonuclease (DN-25) were purchased from Sigma. Matrigel was from Collaborative Biomedical (Bedford, MA), collagenase D was from Roche Applied Science, and Pronase was from Calbiochem. Liebowitz-15 (L-15), minimum essential medium, α-minimum essential medium, L-glutamine, gentamicin, and fetal calf serum were from Invitrogen. Monoclonal anti-cytokeratin 19 antibodies were from Amersham Biosciences. The CFTR antibody was a generous gift from Dr. Christopher Marino. All other chemicals were of the highest purity commercially available.

Solutions—The compositions of the Krebs-Ringer bicarbonate and HEPES buffer solutions (containing (in mM) 135 NaCl, 4.7 KCl, 1.2 KH$_2$PO$_4$, 1.25 CaCl$_2$, 10 HEPES, 1 MgSO$_4$, 5 glucose, pH 7.4, 37 °C) have been described previously (7, 8). Isotonic and hypotonic solution compositions are as described previously (4). The isotonic solution was made by replacing 40% of the NaCl in the HEPES solution (pH 7.4 at 37 °C) with an equimolar amount of sucrose. The hypotonic solution was made the same as the isotonic solution but without sucrose. The actual osmolarities of the solutions used were determined by a vapor pressure osmometer 5500 (Wescor, Inc., Logan, UT).

Isolation of Bile Duct Cell Cluster—The male Cftr−/− mice at age 4–14 weeks were obtained from Dr. Bev Koller at the University of North Carolina. Control normal male C57BL6 mice at the same ages were obtained from the Harlan Laboratory (Indianapolis, IN). These mice were housed and allowed free access to Colyte-supplemented water and Purina rodent chow (St. Louis, MO) to minimize complications of intestinal obstruction described previously (9, 10). Animal care and studies were performed in compliance with institutional animal care and use committee guidelines. Mouse BDCCs were prepared as previously described (4, 6). Briefly, mice were prepped and anesthetized, and their portal veins were perfused with Hanks' buffers with collagenase B; then the liver was harvested as described (4, 6). The non-parenchymal tissue was obtained by removing the hepatic capsule and then mechanically dissociating hepatocytes. The non-parenchymal tissue was finely minced with scissors and then further digested serially by various enzymes including collagenase, DNase, and Pronase or hyaluronidase in modified minimum essential medium solution as described previously (4, 6). After each serial enzymatic digestion step, fragments were filtered through 100- and 30-μm meshes (Tetko, Lancaster, NY), and those remaining on the 30-μm mesh were collected in 3–6 ml of modified α-minimum essential medium plating medium as described (4, 6). Fragments were then plated on small coverslips (2–4 mm), coated with Matrigel (Collaborative Research) in 12-mm-diameter tissue culture wells (Corning), and incubated at 37 °C in an air/5% CO$_2$-equilibrated incubator. Experiments were carried out 36–56 h after plating. Cell viability was assessed by trypan blue exclusion in plated BDCCs at the end of the functional studies.

Characterization of Mouse BDCC—Immunocytochemistry using cytokeratin-19 or CFTR antibody (6, 8, 11) was performed in BDCCs 48–72 h after plating. Immunofluorescent images of the mouse BDCCs, immunostained with cytokeratin-19 or CFTR antibody, were obtained using an Olympus IX-70 inverted fluorescent microscope (Olympus America, Inc., Melville, NY) with a cooled charge-coupled device video camera (Hamamatsu Photonics Systems, Bridgewater, NJ) connected to a Power Mac computer with image analysis software (Improvision, Inc., Boston, MA).

Quantitation of Regulatory Volume Response with Videomicroscopy— Applying the methods used for measuring a cross-sectional area (CSA) of hepatocytes and cholangiocytes (12) as well as other cell types (13), we recently have shown and validated that the CSA measurements of
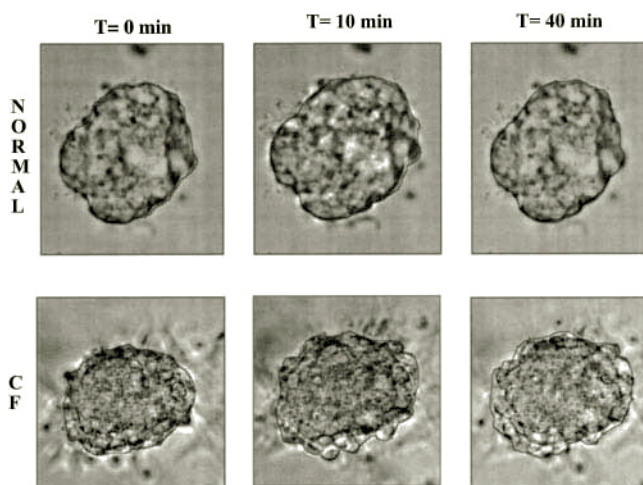
FIG. 1. **Videomicroscopy of cystic fibrosis mouse bile duct cell clusters with hypotonic maneuver.** Normal (*top panels*) and CF mouse BDCCs (*bottom panels*) were preincubated in modified isotonic HEPES solution (40% of NaCl replaced with equimolar sucrose) and then were exposed to hypotonic solution (isotonic solution without sucrose) for 40 min. Both normal and CF mouse BDCCs swelled in hypotonic solution by 20% within 10 min but, unlike normal BDCCs, CF mouse BDCCs did not return toward their initial sizes with time, indicating an impaired regulatory volume decrease.

mouse BDCCs obtained by quantitative videomicroscopy were highly correlated (linear correlation coefficient, $r^2 > 0.94$) with the cell volume measurements by 3 independent methods: 1) sequential Nomarski light, 2) fluorescence microscopy of BDCCs loaded with BCECF and computer-assisted measurements of the corresponding CSA and volumes, and 3) laser-scanning confocal microscopy of intracellular fluorescent dye, BCECF-loaded BDCCs, and computer-assisted three-dimensional reconstruction and volume calculation (4). Thus, in the present study, the CSA measurements of BDCCs by quantitative videomicroscopy were used as indirect indices of cell volume measurements.

BDCCs cultured overnight on Matrigel-coated glass coverslips were preincubated in isotonic solution for 10–20 min after being placed in a thermostated specimen chamber on a microscope stage. Coverslips were scanned for 5–10 min to select relatively spheroid BDCCs with sharp borders and without connections to other contiguous BDCCs and without any enclosed lumen. Videoimages of these BDCCs were obtained at 1–5-min intervals while maintaining the same focal plane at the maximum cross-sectional area. Osmoregulatory responses of BDCCs were determined by assessing the changes in CSA of BDCCs using an Olympus IX-70 (Olympus America) or a Leica DMIR (Leica Microsystems, Inc., Bannockburn, IL) inverted microscope with Nomarski optics equipped with a CCD video camera (Hamamatsu Photonics Systems) connected to a computer with OpenLab image analysis software (Improvision). Following a 10–20-min prestimulation period with isotonic HEPES buffer alone, BDCCs were exposed to a hypotonic HEPES buffer for 40 min with or without various inhibitors or chemicals dissolved in the solution. Each BDCC served as its own internal control, and changes in the cross-sectional area were expressed as a percentage of base-line values at time 0. The viability of each BDCC was assessed by the addition of trypan blue to the specimen chamber after each experiment. The BDCCs with positive trypan blue staining were excluded from data analysis. However, there was no significant change in viability, assessed by trypan blue staining, in experimental groups exposed to various inhibitors or chemicals compared with controls.

Statistical Analysis—All data from videomicroscopic measurements are presented as the arithmetic mean ± S.E., and cell purity and viability are presented as the arithmetic mean ± S.D. Statistical differences were assessed by the unpaired or paired Student's *t* tests using the INSTAT statistical computer program (GraphPad Software, San Diego, CA).

RESULTS

Characterizations of BDCCs—As with the BDCCs from normal mouse livers, BDCCs from Cftr−/− mouse livers formed spheroid clusters of cells with 24–48 h in culture (Fig. 1). Viability of the BDCCs was over 95% as assessed by trypan
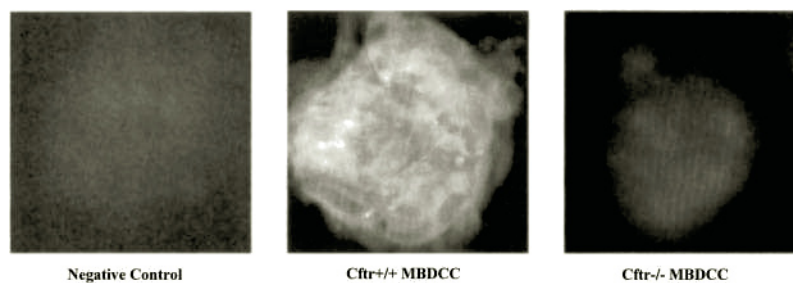
FIG. 2. **Immunofluorescent micrograph of normal and CF mouse BDCCs.** Normal and CF mouse BDCCs were immunofluorescently stained for CFTR. Whereas the normal mouse BDCC had bright immunofluorescent staining for CFTR (*middle*), the CF mouse BDCC (*right*) had no significant immunofluorescent staining compared with negative control (*left*).

FIG. 3. **Quantitative videomicroscopic measurements of normal and CF mouse BDCC cross-sectional area changes with hypotonic maneuver.** The CSAs of both normal and CF mouse BDCCs, as measured by quantitative videomicroscopy, increased by 20% with hypotonic maneuver. Whereas the CSAs of normal BDCCs returned toward their initial sizes (+6% in 40 min), those of CF BDCCs did not return to their initial sizes, indicating an impaired RVD in CF mouse BDCCs.
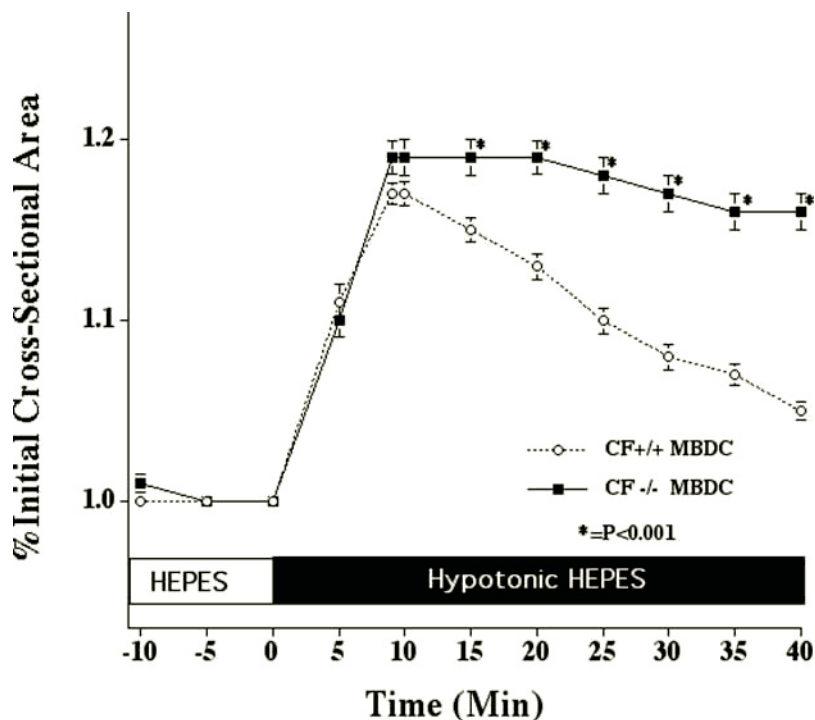
blue exclusion 24–72 h after culture. As with normal mouse BDCCs characterized previously (6), these BDCCs from normal and CF mouse livers were identified as bile duct epithelial cells by positive immunocytochemistry using a cytokeratin-19 antibody, whereas negative controls with a secondary antibody alone were consistently negative for immunostaining. As expected, the BDCCs isolated from Cftr−/− mouse livers had no significant immunostaining with the CFTR antibody, compared with the negative controls, whereas BDCCs from normal mouse livers had a bright CFTR immunostaining (Fig. 2).

Study of Regulatory Volume Decrease in Normal Mouse Cholangiocytes—The osmolarity of the isotonic solutions, measured by an osmometer, was 300.9 ± 4.5 mosM ($n = 12$, mean $\pm$ S.D.), and that of the hyposomolar solutions was 181.9 ± 3.6 mosM ($n = 13$). As shown in Fig. 1, BDCCs rapidly and significantly increased in size within the first 10 min of exposure to hypotonic solution. Exposing normal BDCCs to hypotonic HEPES solution from isotonic HEPES solution caused rapid increases in CSA as shown in Fig. 1, indicating swelling of the cholangiocytes. Measurements of cross-sectional areas using quantitative videomicroscopy showed that the relative CSA of normal BDCCs rapidly increased to 1.20 ± 0.01 (mean $\pm$ S.E.; $n = 50$) in 10 min after exposure to hypotonic HEPES solution (Fig. 3) and then gradually returned toward 1.07 ± 0.01 of initial CSA over the next 30 min. Previous work from our laboratory has confirmed that CSA measurements of

BDCCs accurately (correlation coefficient $r^2 > 0.94$) reflect their corresponding cell volume measurements using sequential phase-contrast and fluorescence microscopy of BCECF-loaded BDCCs as well as by laser-scanning confocal microscopy followed by three-dimensional volume measurement analysis (4). These results are consistent with the previous studies on Mz-ChA-1 cells from human cholangiocarcinoma cell lines (3) and normal BDCCs (4) that showed cholangiocytes exhibit intact regulatory volume decrease after exposure to a hypotonic solution.

Study of Regulatory Volume Decrease in Cystic Fibrosis Mouse Cholangiocytes—To study the RVD of cholangiocytes in the absence of Cftr, BDCCs isolated from Cftr−/− mouse livers were subjected to the same hypotonic challenges. As shown in Fig. 1, exposure of the BDCCs from Cftr−/− mouse livers caused rapid swelling of cholangiocytes, indicated by a rapid increase in the relative CSA to 1.20 ± 0.01 of initial CSA ($n = 53$) in 10 min (Fig. 3). Compared with normal BDCCs, this swelling of cholangiocytes from Cftr−/− livers had a similar time course and magnitude of increase in CSA in the swelling phase. However, unlike in normal BDCCs, the relative CSA did not return to the initial CSA and remained at 1.16 ± 0.01 of initial CSA after 40 min in the hypotonic solution, indicating an impairment of RVD in CF mouse cholangiocytes (Fig. 3).

Effect of Potassium Ionophore on RVD—Given the fact that RVD in normal cholangiocytes is dependent on both Cl− and

FIG. 4. **Effect of valinomycin on RVD in CF mouse BDCCs.** Coadministration of valinomycin (1 μM), a potassium ionophore, during hypotonic maneuver normalized the impaired RVD in CF mouse BDCCs, suggesting that the impaired RVD in CF cholangiocytes is probably due to a defect in K$^+$ conductance to provide a K$^+$ efflux pathway.
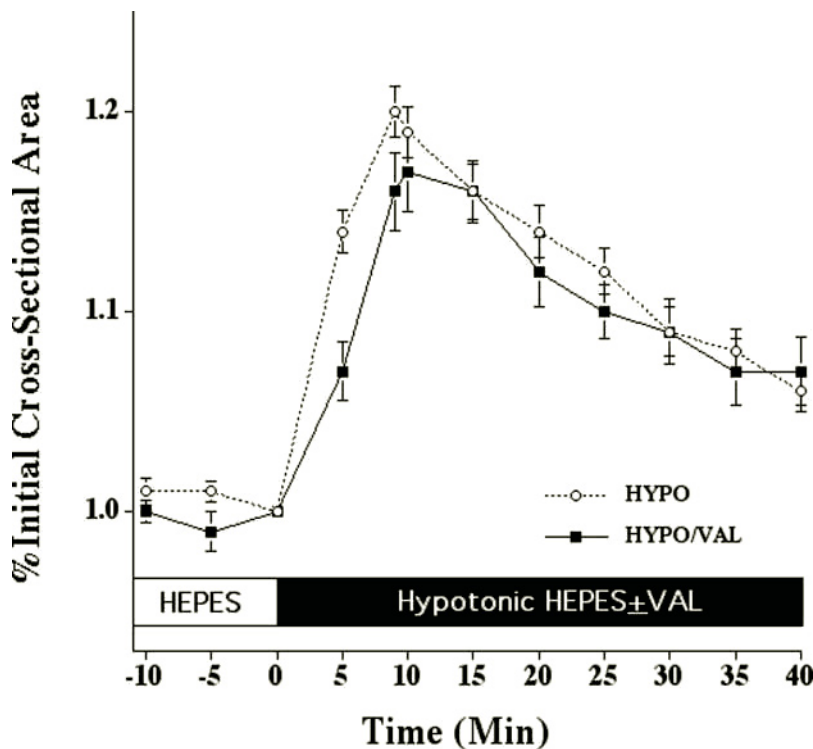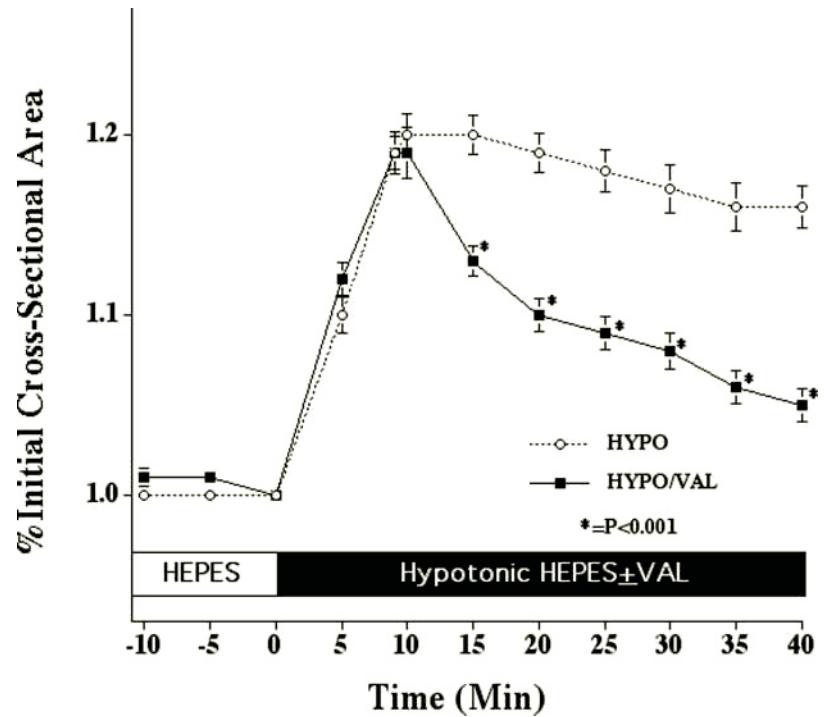

FIG. 5. **Effect of valinomycin on RVD in normal mouse BDCCs.** Coadministration of valinomycin (1 μM) during hypotonic maneuver had no significant effect on RVD in normal mouse BDCCs compared with the untreated controls.

K$^+$ conductances, as shown by our study using normal freshly isolated mouse cholangiocytes (4) and others in the cholangiocarcinoma cell line (3), this impaired RVD seen in CF mouse cholangiocytes can be from defects either in Cl$^-$ or K$^+$ conductances. Although Cftr Cl$^-$ channels provide major Cl$^-$ conductances in cholangiocytes, previous electrophysiological studies have characterized a number of other non-Cftr Cl$^-$ channels present and functioning in cholangiocytes (14, 15). Therefore, we then studied the effect of valinomycin, a K$^+$ ionophore, on RVD in Cftr$-/-$ mouse cholangiocytes to examine whether providing K$^+$ conductance can rectify the impaired RVD in these cells. Surprisingly, as shown in Fig. 4, an administration

of 1 μM valinomycin completely restored the RVD in Cftr$-/-$ mouse BDCCs, and relative CSA returned to 1.05 $\pm$ 0.01 (n = 35) ($p < 0.01$ compared with negative control) of initial CSA in 40 min, whereas the CSA of untreated BDCCs did not show a significant decrease. There was no significant change in cell viability with valinomycin administration. Furthermore, coadministration of valinomycin in normal mouse BDCCs during hypotonic challenge had no significant effect on RVD compared with controls (Fig. 5).

Characterization of Valinomycin-induced RVD in CF Cholangiocytes—To further characterize the RVD seen in Cftr$-/-$ BDCCs with valinomycin, the effect of a chloride chan-

FIG. 6. **Effect of NPPB on valinomycin-induced RVD in CF mouse BD-CCs.** Coadministration of NPPB (10 μM), a general chloride channel blocker, with valinomycin completely inhibited the valinomycin-induced RVD seen in CF BD-CCs, indicating that it is not a nonspecific effect of valinomycin but is dependent on the functioning of the chloride channel(s).
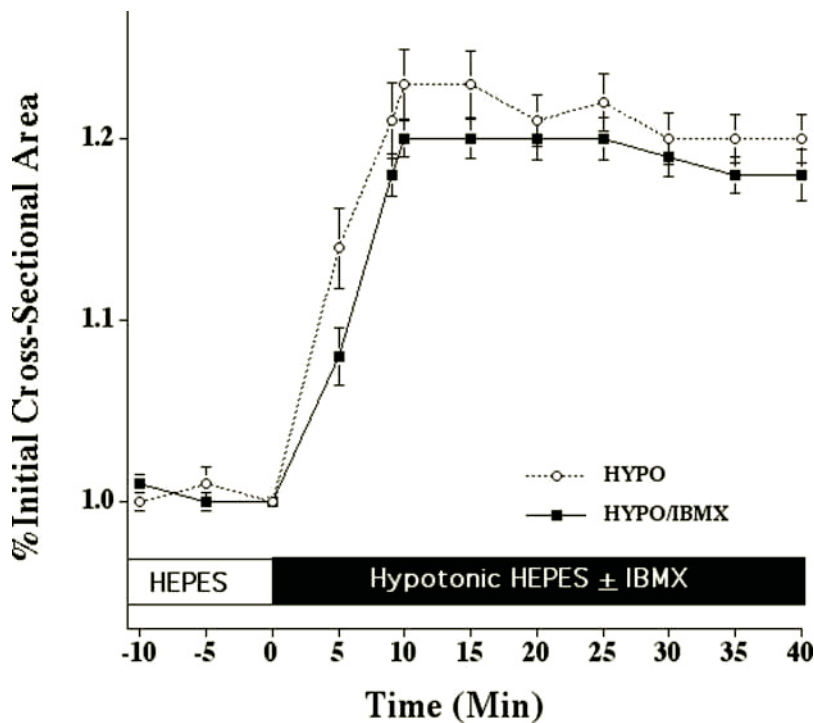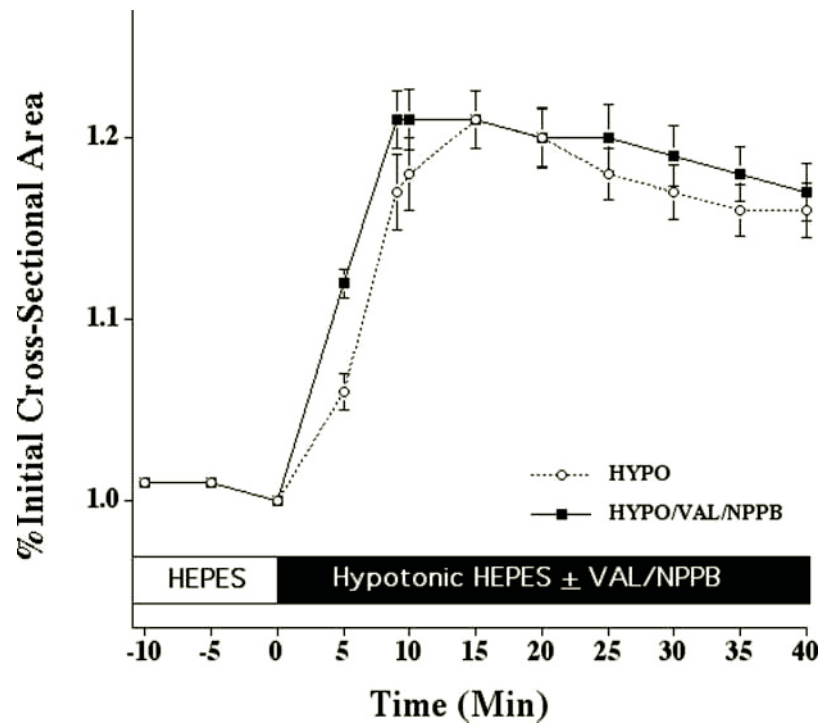

FIG. 7. **Effect of IBMX on RVD of CF mouse BDCCs.** Coadministration of IBMX (1 mM) during hypotonic maneuver had no significant effect on the RVD of normal CF BDCCs.

nel blocker, NPPB, on the valinomycin-induced RVD was studied to examine the specificity and dependence of Cl$^-$ conductances during the observed RVD. As shown in Fig. 6, coadministration of 10 μM NPPB, in addition to 1 μM valinomycin, during hypotonic challenge completely inhibited RVD. These results indicate that the RVD seen with valinomycin is dependent on non-CFTR Cl$^-$ conductance(s), which is most likely a volume-activated chloride channel as shown by our recent study in normal cholangiocytes (4). Again, there was no significant change in viabilities of BDCCs, assessed by trypan blue staining, in any of these groups. Taken together, these results indicate that the limiting factor in the impaired

RVD seen in Cftr$-$/$-$ cholangiocytes is a defect not in Cl$^-$ conductances but in K$^+$ conductances. Moreover, there are other Cl$^-$ conductances, which are functioning in Cftr$-$/$-$ cholangiocytes even with known CFTR defect and can mediate RVD when K$^+$ conductances are provided.

Effect of cAMP Agonists on RVD in CF Cholangiocytes— Because stimulation of cAMP in certain cell types, such as non-pigmented ciliary epithelial cells (16), has been shown to enhance RVD, the effect of cAMP agonists on the impaired RVD in CF cholangiocytes was examined. As shown in Fig. 7, the administration of a cAMP agonist, isobutylmethylxanthine (IBMX) (1 mM), during the hypotonic challenge had no signifi-

FIG. 8. **Effect of forskolin on RVD of CF mouse BDCCs.** Coadministration of forskolin (10 μM) during hypotonic maneuver had no significant effect on the RVD of normal CF BDCCs.
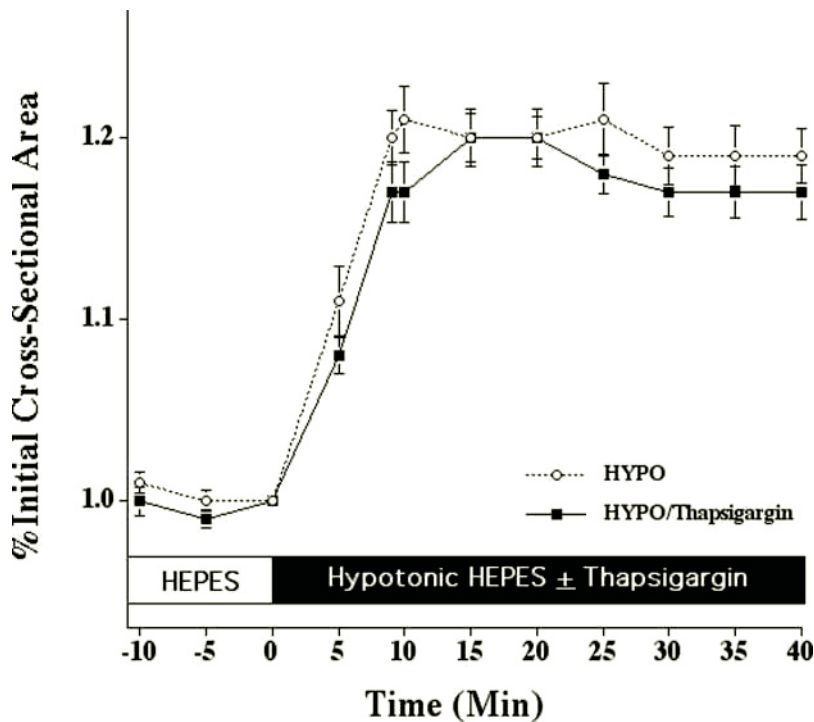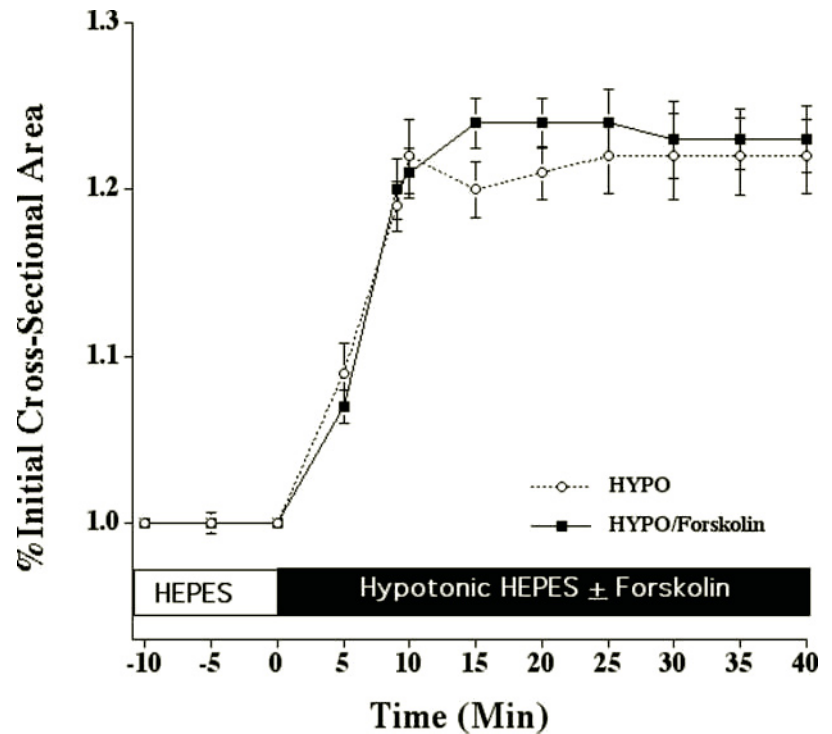

FIG. 9. **Effect of thapsigargin on RVD of CF mouse BDCCs.** Coadministration of thapsigargin (2 μM) during hypotonic maneuver had no significant effect on the RVD of CF mouse BDCCs.

cant effect on the impaired RVD of BDCCs compared with that of the untreated controls. In addition, the effect of a more potent cAMP agonist, forskolin, on RVD of BDCCs was also studied. As shown in Fig. 8, the RVD of BDCCs treated with forskolin (10 μM) ($n = 10$) appeared to be slower in the initial phase of RVD when compared with that of untreated controls ($n = 18$), but there was no statistically significant difference between them. These findings indicate that, as previously shown in normal cholangiocytes (4), the stimulation of CF cholangiocytes with cAMP has no significant effect on the impaired RVD in CF cholangiocytes.

Effect of Calcium Agonists on RVD in CF Cholangiocytes—The calcium pathway is known to be important for RVD in many cell types, and stimulation of the calcium pathway in certain cells has been shown to enhance RVD (16–22), although RVD in certain cell types is not dependent on calcium (23–26). To examine whether an increase in intracellular calcium can rectify the impaired RVD in CF cholangiocytes, the effect of calcium agonists on the RVD in CF BDCCs was studied. As shown in Figs. 9 and 10, the administration of calcium agonists, thapsigargin (2 μM) or ionomycin (1 μM), respectively, during the hypotonic challenge had no significant effect on

FIG. 10. **Effect of ionomycin on RVD of CF mouse BDCCs.** Coadministration of ionomycin (1 μM) during hypotonic maneuver had no significant effect on the RVD of CF mouse BDCCs.
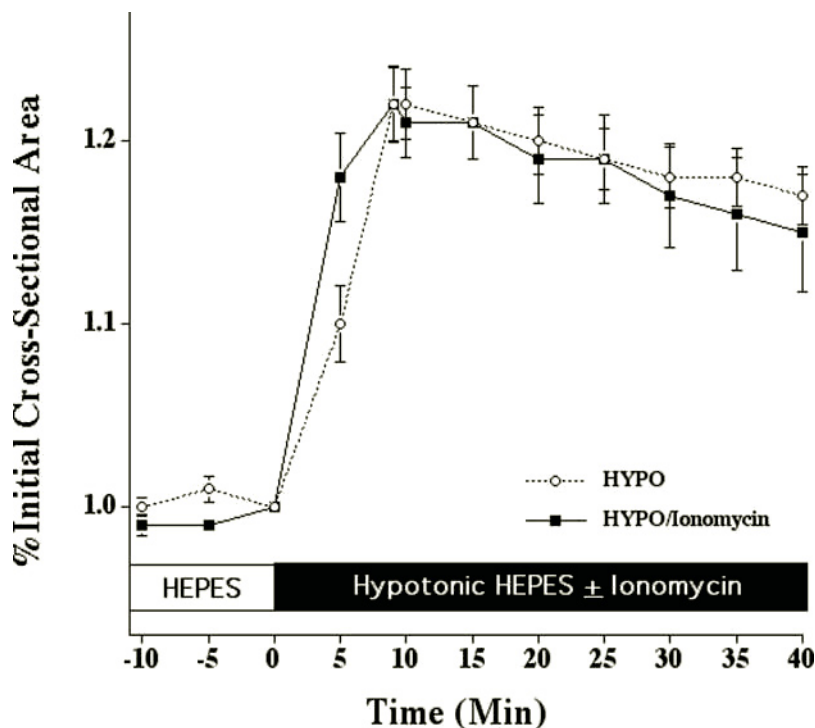
RVD in CF BDCCs. These findings indicate that unlike some other cell types, the stimulation of CF cholangiocytes with calcium agonists had no significant effect on the impaired RVD observed in CF cholangiocytes.

DISCUSSION

In this manuscript, we report the first successful isolation and use of intact intrahepatic bile duct fragments from CF mouse liver using the mouse intrahepatic bile duct unit isolation method developed and reported recently (4, 6). Although previously we have extensively used IBDUs with enclosed lumen for biliary secretion studies, in this study we have excluded those IBDUs and used only those bile duct cell clusters without lumen to simplify cell volume measurements without interference from changes in lumen volume with hypotonic challenge. These BDCCs are determined to be of biliary origin by positive immunocytochemistry using a cholangiocyte specific cytokeratin-19 antibody. In addition, the mouse BDCCs from normal mouse livers had a bright CFTR immunostaining, whereas the CF BDCCs had no significant immunostaining compared with negative controls (Fig. 2). Previous electron microscopic studies demonstrated that these cell clusters consisted of typical cholangiocytes, resembling previous morphologic descriptions of isolated rat cholangiocytes (27, 28) or rat and mouse IBDUs (6, 11, 29) with large, lobulated, basally situated nuclei and sparse mitochondria. Unlike rat IBDUs but like mouse IBDUs, these BDCCs have less connective tissue around them, and the mouse cholangiocytes exhibit a more refractory pattern by light microscopy using Nomarski optics. Thus, it is easier to outline the borders of these mouse BDCCs to measure CSAs than those of rat IBDUs.

To assess cell volume changes with hypotonic challenges, we have used CSA as an indirect measure of cell volume as have other investigators for cholangiocytes (12) or for other cell types (13). In addition, we recently have shown and validated that the CSA measurements of mouse BDCCs obtained by quantitative videomicroscopy were highly correlated (linear correlation coefficient, $r^2 > 0.94$) with the cell volume measurements by 3 independent methods: 1) sequential Nomarski light, 2)

fluorescence microscopy of BDCCs loaded with BCECF and computer-assisted measurements of the corresponding CSA and volumes, and 3) laser-scanning confocal microscopy of intracellular fluorescent dye, BCECF-loaded BDCCs, and computer-assisted three-dimensional reconstruction and volume calculation (4).

As previously reported, in Mz-ChA-1 cells from human cholangiocarcinoma cell lines (3) and in freshly isolated BDCCs (4), cholangiocytes have intact RVD, which is important for this active epithelium to cope with changes in osmolarity from absorption and secretion of ions and substances. However, we now show that BDCCs from CF mouse livers have an impaired RVD, which has important implications for various vital cellular functions such as hepatobiliary metabolism, ion transport, bile secretion, and gene expression (30). Furthermore, RVD is shown to stimulate bile flow and bile salt secretion in isolated perfused rat livers (31), and cell volume regulation is thought to play a critical role in bile secretion, thus this impaired RVD in CF cholangiocytes may directly contribute to the pathophysiologic mechanisms underlying the biliary cholestatic liver diseases seen in CF patients. In fact, there is some literature indicating that coupled regulatory volume increase and RVD may provide underlying ion transport mechanisms in secretory epithelia (32–34).

Because the RVD seen in cholangiocytes is mediated by chloride and potassium conductances, as demonstrated in human cholangiocarcinoma cell lines (3) and in freshly isolated normal BDCCs (4), the observed impaired RVD in CF cholangiocytes can be from either defective chloride or potassium conductances. Contrary to our initial reasoning that the deficient chloride conductance from the absence of CFTR chloride channel accounts for the observed impaired RVD, the results that valinomycin, a potassium ionophore, rectified the impaired RVD in CF cholangiocytes indicate that the rate-limiting defect is not chloride conductance but potassium conductance. These results provide the first compelling evidence that CFTR has regulatory interactions on potassium conductance(s) responsible for the RVD in cholangiocytes as shown in other cell types (35). In fact, CFTR is known to function as both a chloride channel and an epithelial transport regulator, inter-

acting with various sodium (36) and chloride (37–39) channels, as well as potassium channels (40, 41). Although the identity of the potassium conductance pathway(s) responsible for the RVD in CF cholangiocytes is not clear, our previous study in normal cholangiocytes (4) provides some helpful clue that it is most likely a tetraethylammonium chloride-resistant but barium chloride-inhibitable potassium conductance pathway(s) assuming that the same ion transport pathways are functional in CF cholangiocytes. A recent study using human cholangiocarcinoma Mz-ChA-1 cells identified apamin-sensitive SKCa channels as a potential potassium conductive pathway mediating the RVD in cholangiocytes (42). However, the present study indicates that calcium agonists could not overcome the impaired RVD in CF cholangiocytes even though such an increase in intracellular calcium was expected to stimulate the SKCa channels, unless the activation of SKCa channels requires the presence of functioning CFTR in the cholangiocytes. In addition, calcium agonists had no significant effect on RVD in normal cholangiocytes.[2] Therefore, the role of the calcium-activated ion transport pathway(s) in RVD in cholangiocytes is not entirely clear, and further study is needed.

The precise underlying mechanism(s) for the defect in RVD in CF mouse cholangiocytes is not clearly characterized, but some recent studies in various cell types propose an important role of ATP in cell volume regulation (43–46). Although it is somewhat controversial whether the CFTR can conduct ATP or regulate ATP-conducting pathways, ATP transported out of the cell during RVD is thought to interact with the purinergic receptor pathway to bring about cell volume regulation (43–46). Therefore, an administration of extracellular ATP to the CF cells should restore RVD as shown in CF mouse renal cells (47, 48). However, no clear cause-and-effect relationship was observed with the ATP release and the VACC activation in the human intestinal epithelial cell line lacking CFTR (49) or in the murine mammary carcinoma cell line (50). Thus, further studies are needed to determine the precise underlying mechanisms of RVD in CF cholangiocytes and the role of extracellular ATP in RVD and its effect on VACC in CF cholangiocytes.

It is also notable that the valinomycin-induced RVD in CF cholangiocytes is faster than RVD in normal cholangiocytes, which may suggest that the rate-limiting step of the RVD in normal cholangiocytes also may be potassium conductance. However, an alternative explanation is that the expression of CFTR in normal cholangiocytes may have some inhibitory regulatory influence on VACCs, as it has been shown recently that an ATP-hydrolyzable conformation of NBD2 is essential for the regulation of the VACC by the CFTR (39, 51). This explanation is also supported by the fact that the administration of valinomycin during the RVD in normal cholangiocytes does not increase the rate of RVD in normal cholangiocytes (Fig. 5), arguing against the explanation that the rate-limiting step in RVD in normal cholangiocytes is potassium conductance. Thus, the absence of functional CFTR in CF cholangiocytes may lead to loss of its inhibitory influence on VACCs, which is reflected by the faster RVD in CF cholangiocytes induced by valinomycin than the RVD in normal cholangiocytes.

Moreover, the correction of the impaired RVD in CF cholangiocytes with the administration of valinomycin was not due to nonspecific effects of the potassium ionophore but was completely inhibited by NPPB, a general chloride channel blocker, demonstrating an involvement of the NPPB-inhibitable chloride conductive pathway(s) in this RVD. Although we cannot definitively rule out the nonspecific effect of NPPB, we have used NPPB at a relatively low (10 μM) concentration in an attempt to minimize nonspecific effects of NPPB, and we have not observed any changes in cell morphologies or viabilities by trypan blue exclusion at the end of the experiments. Considering the fact that both chloride and potassium efflux are required for RVD in cholangiocytes (3, 4), the observed inhibition of NPPB on the valinomycin-induced RVD in CF cholangiocytes may be from its well known inhibitory effect on the chloride channel or from its possible but less likely nonspecific inhibitory effect on the potassium channel. However, any possible nonspecific inhibitory effect of NPPB on the potassium channel has no significant bearing here because valinomycin, a potassium ionophore, should have provided an alternative potassium conductive pathway to bypass such inhibition. Thus, even if NPPB inhibited the potassium channel, it cannot account for the observed inhibitory effect of NPPB on the valinomycin-induced RVD in CF cholangiocytes. Therefore, the most logical conclusion is that NPPB-inhibited chloride efflux is required for the valinomycin-induced RVD in CF cholangiocytes. These findings together, in turn, support our conclusion that the loss of CFTR function in CF cholangiocytes resulted in the defective potassium conductive pathway responsible for RVD.

Furthermore, these results provide evidence for the presence of a non-CFTR chloride conductive pathway(s) functioning in CF cholangiocytes. Previously, a number of non-CFTR chloride channels have been characterized in cholangiocytes, such as the calcium-activated chloride channel, volume-activated chloride channel, and high conductance anion channel (3, 14, 52). However, it was not known whether any of these non-CFTR chloride channels were functioning in CF cholangiocytes. Thus, the present finding provides the first evidence that an NPPB-inhibitable non-CFTR chloride channel(s) is functioning to mediate RVD in CF cholangiocytes. Such a chloride conductive pathway(s) can provide an alternative chloride conductive pathway to compensate for the absence of a CFTR chloride channel in the CF biliary epithelium, knowledge that is potentially useful for developing therapies for CF cholestatic liver diseases.

In the present study, cAMP agonists were employed in an attempt to correct for the impaired RVD in CF cholangiocytes because cAMP is shown to stimulate RVD in some cell types (16, 53). However, neither IBMX nor forskolin had any significant stimulatory effect on RVD in CF cholangiocytes, and they could not reverse the impaired RVD in CF cholangiocytes. This result is consistent with our findings in normal cholangiocytes that cAMP agonists had no stimulatory effect on RVD (4). Instead, forskolin had a mild inhibitory effect on RVD in normal cholangiocytes (4) as shown in myocytes (54), whereas IBMX had no significant effect on RVD in normal cholangiocytes (4). Although the mechanism underlying this inhibitory effect of forskolin on RVD seen in normal cholangiocytes is not clear, it is possibly from its inhibitory effect on VACCs as shown in myocytes (54). Thus, the measures to stimulate the cAMP pathway(s) by potent cAMP agonists to overcome the defective CFTR chloride channels in CF cholangiocytes may be counterproductive because of their possible inhibitory effect on cell volume regulation in cholangiocytes, critical for biliary secretion.

The calcium pathway plays an important role in cell volume regulation in many cell types (17, 18, 20, 22) whereas in some cell types, RVD is not dependent on calcium (23–26). In addition, in some cells such as cerebellar astrocytes (55), RVD and changes in intracellular calcium are not related. Thus intracellular calcium is thought to be irrelevant as a transduction signal for RVD, whereas an increase in intracellular calcium in certain cells such as human non-pigmented ciliary epithelial cells (16) has been shown to enhance RVD. A recent patch-

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[2] W. K. Cho, V. J. Siegrist, and W. Zinzow, manuscript in preparation.

clamping study demonstrated the important role of SKCa channel-mediating RVD in cholangiocytes (42), thus an increase in intracellular calcium was expected to help reverse the impaired RVD in CF cholangiocytes. However, neither ionomycin nor thapsigargin had any significant effect on the impaired RVD in CF cholangiocytes, suggesting that the stimulation of a calcium-dependent pathway(s) cannot compensate for the impaired RVD in CF cholangiocytes. These results are consistent with the findings in normal cholangiocytes that calcium agonists had no significant effect on the RVD in normal BDCCs.[2] Therefore, the role of calcium in RVD in cholangiocytes is not clear, but these results suggest that calcium-dependent pathways do not appear to play a major role in RVD in cholangiocytes. Alternatively, the defect from CFTR loss may be a downstream event from calcium signal. Further studies are needed to clarify the precise role of calcium-dependent pathways in RVD in cholangiocytes.

As a summary, the present study demonstrates for the first time that cholangiocytes from CF mouse liver have an impaired cell volume regulation and that this impaired RVD is most likely from the defect in potassium conductance in the absence of CFTR, which may serve an important pathophysiological mechanism for CF cholestatic liver disease. Moreover, our indirect evidence indicates that the volume-activated chloride conductance pathway(s) is functional in CF cholangiocytes, and that such a pathway(s) can potentially provide an alternative chloride conductance pathway necessary to compensate for the absence of functional CFTR chloride conductance in CF cholangiocytes to overcome biliary secretory defect in CF liver diseases.

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