Characterization of regulatory volume decrease in freshly isolated mouse cholangiocytes

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Cho, Won Kyoo. Characterization of regulatory volume decrease in freshly isolated mouse cholangiocytes. Am J Physiol Gastrointest Liver Physiol 283: G1320–G1327, 2002; 10.1152/ajpgi.00256.2002.—Cell volume regulation plays a vital role in many cell functions. Recent study indicates that both K\(^+\) and Cl\(^-\) channels are important for the regulatory volume decrease (RVD) of cholangiocarcinoma cells, but its physiological significance is unclear due to the tumorous nature of the cells used. This present study reports the RVD of normal mouse cholangiocytes by using freshly isolated bile duct cell clusters (BDCC). A relatively simple and practical method of measuring the cross-sectional area of BDCCs by quantitative videomicroscopy was used to indirectly measure their volumes. Mouse cholangiocytes exhibited RVD, which was arrested by 5-nitro-2’-(3-phenylpropylamino)-benzoate, DIDS, and glibenclamide, suggesting its dependence on certain chloride channels, such as volume-activated chloride channels. It is also inhibited by barium chloride but not by tetraethylammonium chloride, indicating its dependence on certain potassium channels. However, cAMP agonists had no significant effect on the RVD of BDCCs. This indirect method described can be used to study the RVD of cholangiocytes from normal as well as genetically altered mouse livers.


Recently, we have developed a method for preparing novel intact polarized isolated bile duct units (IBDU) from mouse liver, which consists of clustered cholangiocytes lining a central lumen (8). This method eliminates the need for difficult and time-consuming microdissection and produces many functional and polarized IBDUs from 30- to 100-μm-sized murine bile ducts after several steps of enzymatic digestion and mechanical separation (8). In the present study, we used primary murine bile duct cell clusters (BDCC), which were prepared by the same isolation method used for IBDUs but which lack enclosed lumens. Because of the possibility that changes in the osmolarity of the perfusion medium may alter biliary secretion, and therefore luminal volume, thus possibly invalidating or complicating the interpretation of cell volume measurements, only BDCCs were included selectively in this study for the purpose of studying changes in cell volume.

In the present study, we present an easy method of measuring changes in cell volume using quantitative videomicroscopy and the validation of this method using other cell volume-measurement techniques. We also present some evidence that the primary BDCCs isolated from normal mouse liver demonstrate an intact RVD, which involves K\(^+\) and Cl\(^-\) conductances, thereby confirming the results obtained from a biliary tumor cell line (23). Furthermore, we present additional data on further characterization of these K\(^+\)- and Cl\(^-\)-conductance pathways using various inhibitors and agonists of ion transporters.

MATERIALS AND METHODS

Materials. Bovine serum albumin, penicillin/streptomycin, EDTA, HEPES, (x+)glucose, insulin, DMSO, hyaluronidase, deoxyribonuclease (DN-25), tetraethylammonium chloride (TEA), barium chloride, sucrose, DIDS, and 5-nitro-2’-(3-phenylpropylamino)-benzoate (NPPB) were purchased from Sigma (St. Louis, MO). Matrigel was from Collaborative Biomedical (Bedford, MA), collagenase D was from Roche Applied Sciences (Indianapolis, IN), and pronase was from Calbiochem (San Diego, CA). Liebowitz-15 (L-15), MEM,

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α-MEM, i-glutamine, gentamicin, and fetal calf serum were from Gibco (Grand Island, NY). Monoclonal anti-cytokeratin 19 antibody was from Amersham. All other chemicals were of highest purity commercially available.

**Solutions.** The compositions of the Krebs-Ringer bicarbonate (KRb) and HEPES buffer solutions (containing (in mM) 135 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.25 CaCl2, 10 HEPES, 1 MgSO4, and 5 glucose, pH 7.4, 37°C) have been described previously (1, 26). 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. Hypotonic solution was the same as the isotonic solution by removing 40% of the NaCl in the HEPES solution (pH 7.4 at 37°C) but without the sucrose. The actual osmolalities of the solutions used were determined by a Vapor Pressure Osmometer 5500 (Wescor, Logan, UT).

**Isolation of BDCC.** Normal male C57BL6 (Harlan Laboratory, Indianapolis, IN) mice at age 5–12 wk were housed and allowed free access to water and Purina rodent chow (St. Louis, MO). Animal care and studies were performed in compliance with institutional animal care and use committee guidelines. Mouse BDCCs lacking enclosed lumen were prepared using the same methods to isolate mouse IBDU’s as previously described by our lab (8). Briefly, mice were prepared and their livers were perfused via the portal vein with Hank’s buffers with collagenase D, and were then harvested. After the hepatic capsule was removed, the intrahepatic bile ducts were mechanically dissociated from hepatic parenchymal tissue by shaking in cold L-15 medium and incubated with scissors in solution A, containing various enzymes in-cluding pronase as previously described (8). The minced tissue was then transferred to a culture tissue culture wells (Corning) and incubated at 37°C in a humidified atmosphere of 5% CO2–5% O2–90% balance air-5% CO2-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.** Cytokeratin (CK) immunocytochemistry using CK-19 antibody (8, 16, 26) was performed in BDCCs 48–72 hr after plating. Immunofluorescent images were obtained using Olympus IX-70 inverted fluorescent microscope (Olympus America, Melville, NY) with a cooled charge-coupled device (CCD) video camera (Hamamatsu Photonics Systems, Bridgewater, NJ) con-nected to a Power Mac computer with image-analysis software (Improvision, Boston, MA). In addition, confocal micrograph of the mouse BDCCs, immunofluorescently stained with CK-19 antibody, were obtained using a Bio-Rad 1024 MRC laser-scanning confocal microscope (Hercules, CA) equipped with a Kr/Ar laser.

Quantitative of regulatory volume response with videomicroscopy. BDCCs cultured overnight on Matrigel-coated glass coverslips were preincubated in KRb 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. Video images of these BDCCs were obtained at 1- to 5-min intervals while maintaining the same focal plane at the maximum cross-sectional area (CSA). Osmoregulatory responses of BDCCs were determined by assessing the changes in CSA of BDCCs using an Olympus IX-70 (Olympus America) or Leica DMR (Leica Microsystems, Bannockburn, IL) inverted microscope with Nomarski optics equipped with CCD video camera (Hamamatsu Photonics Systems) connected to a computer with OpenLab image-analysis software (Improvision). After a 10- to 20-min prestimulation period with isotonic HEPES buffer alone, BDCCs were exposed to hypotonic HEPES buffer for 40 min with or without various inhibitors or agonists dissolved in the solution. Each BDCC served as its own internal control and changes in CSA were calculated as a percentage of baseline values at time 0. 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.

Previously, measurements of CSAs of hepatocytes and cholangiocytes were shown to reflect changes in volume (19). Relative CSA measurements of BDCCs obtained by quantitative videomicroscopy were validated by three independent methods: 1) sequential light, 2) fluorescence microscopy of BDCCs loaded with intracellular fluorescent dye BCECF and computer-assisted measurements of the corresponding CSA and volume calculations, and 3) laser-scanning confocal microscopy of BCECF-loaded BDCCs and computer-assisted three-dimensional reconstruction and volume calculation.

**Quantitative fluorescent microscopy.** Fluorescent micrographs were obtained using Olympus IX-70 inverted fluorescent microscope (Olympus America) with a cooled CCD video camera (Hamamatsu Photonics Systems) connected to a Power Mac computer with OpenLab image-analysis software (Improvision). Serial cross-sectional fluorescent images of BCECF-loaded BDCCs were obtained with fluorescent excitation at 490 nm, and the emission collected >505 nm as focal plane was advanced in 1-μm increments through the cell thickness by using a BioPoint Z-stepper (Ludl Electronic Products, Hawthorne, NY). The CSAs of the BDCCs from the serial fluorescent images were analyzed using the image-analysis program and were used to calculate their volumes.

**Laser-scanning confocal microscopy.** Cell images were collected with an inverted BioRad laser-scanning confocal microscope. Serial confocal fluorescence images of BCECF-loaded BDCCs were collected as the focal plane was advanced in 1-μm increments through the cell thickness. Images from each focal plane were collected with fluorescence excitation at 488 nm, and the emission was collected >520 nm. Confocal images were then analyzed with computer programs for three-dimensional reconstruction and volume calculation. Statistical analysis. All data from videomicroscopic measurements are presented as the arithmetic means ± SE. Statistical differences were assessed by the unpaired or paired Student’s t-tests using the INSTAT statistical computer program (GraphPad Software, San Diego, CA). In addition, the curve fits for linear and power-regression analyses
were done using CA-Cricket Graph III (Computer Associates International, Islandia, NY).

RESULTS

Characterization of BDCCs. As is the case with rat tissue, bile duct fragments from mouse liver, isolated immediately after serial enzymatic digestions, appeared as tubulelike structures that formed spherical clusters of cells with 24–48 h in culture (Fig. 1). Viability of the BDCCs studied was >95%, as assessed by trypan blue exclusion, after 24–72 h in culture. As with the normal mouse IBDUs characterized previously (8), the cells comprising these BDCCs were identified as bile duct epithelial cells by positive immunocytochemistry using an antibody to CK-19, whereas negative controls using secondary antibody alone were consistently negative for immunostaining (Fig. 2).

Validation of quantitative videomicroscopic measurements of cell volume. As previously used for other cell types (17) and for hepatocytes and cholangiocytes (19), measurements of CSA have been used as indirect indexes of cell volumes in BDCCs. As shown in Fig. 3, the CSAs of BDCCs in isotonic or hypotonic HEPES solutions, as measured by quantitative videomicroscopy,

Fig. 1. Videomicroscopy of normal mouse bile duct cell clusters (BDCC) with hypotonic maneuver. Normal mouse BDCCs (top and bottom) were preincubated in modified isotonic HEPES solution (40% of NaCl replaced with equimolar sucrose) (top, left), then were exposed to hypotonic solution (isotonic solution without sucrose) for 40 min. Normal mouse BDCCs swelled in hypotonic solution by +24.6% (top) and +24.2% (bottom) of their initial sizes (top, middle), then slowly recovered toward their initial sizes of +5.6% (top) and +4.9% (bottom), indicating an intact RVD.

Fig. 2. Cytokeratin immunocytochemistry of 48 h-cultured mouse BDCC. A: light micrograph of the BDCC in B. B: immunofluorescent micrograph of a BDCC from mouse liver stained for α-cytokeratin 19 shows brightly positive intracellular stainings, whereas negative, unstained isolated bile duct units (IBDUs; not shown) have a minimal background autofluorescence.
were linearly correlated (linear correlation efficient, $R^2 = 0.95, n = 20$) with the cell volumes of BDCCs, as measured by three-dimensional volume reconstruction and calculations performed on serial optical sections of BDCCs. Similar results were obtained by serial fluorescence microscopy of BCECF-loaded BDCCs followed by three-dimensional volume-measurement analysis (data not shown). The volumes of BCECF-loaded BDCCs were also analyzed by laser-scanning confocal microscopy followed by three-dimensional volume-measurement analysis. As shown in Fig. 4, the cell volumes of BDCCs, determined using this methodology, showed a significant linear correlation ($R^2 = 0.98, n = 11$), with the corresponding CSA values obtained using quantitative videomicroscopy. These results confirmed the validity of using the CSA measurements of BDCCs as indirect indexes of BDCC cell volume. Furthermore, as shown in Figs. 3 and 4, the CSAs of BDCCs in the usual size range of the BDCCs used are linearly correlated with their corresponding cell volumes.

Study of RVD in normal mouse cholangiocytes. As expected from their compositions, the osmolarity of the isotonic solutions, measured by a vapor pressure osmometer, was $300.9 \pm 4.5$ mosM ($n = 12$) and that of the hypotonic solutions was $181.9 \pm 3.6$ mosM ($n = 13$). As shown in Fig. 1, exposure of normal BDCCs to hypotonic HEPES solution after isotonic HEPES solution caused rapid swelling of their cell volumes. These changes in cell volume were assessed by changes in their corresponding CSAs, as measured by quantitative videomicroscopy. As shown in Fig. 5, the relative CSAs of BDCCs rapidly increased to $1.24 \pm 0.02$ ($n = 15$) in 10 min after exposure to hypotonic HEPES solution, then gradually returned to a relative CSA of $1.06 \pm 0.02$ over the next 30 min. Importantly, the viability of BDCCs, as assessed by trypan blue exclusion, was unchanged after exposure to hypotonic solution. These results are consistent with the previous studies of Mz-ChA-1 cells from a human cholangiocarcinoma cell line, in which cholangiocytes exhibited an intact RVD after exposure to hypotonic solution (23).

Role of Cl$^-$ channels in RVD. To examine the involvement of Cl$^-$ channels in the observed RVD in cholangiocytes, NPPB, a Cl$^-$-channel inhibitor, was administered during the perfusion of hypotonic HEPES solution (5, 9, 24). As shown in Fig. 6, coad-
ministration of 10 μM NPPB with the hypotonic HEPES solution completely inhibited the RVD in BDCCs, and the relative CSA after 40 min of hypotonic challenge was 1.16 ± 0.02 (n = 29), which was statistically significant (P < 0.01) compared with the RVD seen with control BDCCs (1.07 ± 0.01, n = 35). These results indicate that the RVD of mouse cholangiocytes is dependent on Cl⁻ conductance, as shown previously (23). To further characterize the chloride channels involved in the RVD of BDCCs, the effect of a glibenclamide (100 μM, n = 12) on RVD was studied, as shown in Fig. 7. With hypotonic challenge, the relative CSA of the glibenclamide-treated BDCCs increased to about the same amount as untreated controls but only decreased to 1.14 ± 0.02 of the initial CSA (n = 26) after 40 min, compared with 1.07 ± 0.01 in untreated controls (n = 32), indicating significant (P < 0.01) inhibition of the RVD by glibenclamide. In addition, as shown in Fig. 8, another chloride channel blocker, DIDS (250 μM), also had a significant (P < 0.05) inhibitory effect on the RVD of BDCCs and the relative CSA of DIDS-treated BDCCs (n = 7) after hypotonic challenge was 1.15 ± 0.03, compared with 1.08 ± 0.01 in untreated controls (n = 11). These results indicate that certain chloride channels, which are inhibitable by NPPB, glibenclamide, and DIDS, play an important role in the RVD of BDCCs. There was no significant difference between the viabilities of BDCCs treated with these inhibitors and control BDCCs, as assessed by trypan blue exclusion.

Role of K⁺ channels in RVD. To study the role of K⁺ channels in the RVD of cholangiocytes, the effect of a K⁺-channel inhibitor, TEA, on RVD was examined. As shown in Fig. 9, coadministration of 10–1,000 μM TEA had no significant effect on the RVD, and the relative CSA at 40 min after hypotonic challenge returned toward basal CSA (n = 13). However, as shown in Fig. 10, coadministration of barium chloride (5 mM), which is another K⁺-channel inhibitor, during the hypotonic challenge had a significant inhibitory effect on the RVD. The relative CSA at 40 min after hypotonic challenge only decreased to 1.16 ± 0.02 in barium chloride-treated BDCCs (n = 6), compared with that in untreated controls 1.09 ± 0.02 (n = 11), indicating a significant (P < 0.05) inhibitory effect of barium chloride on the RVD. These findings suggest important roles for certain TEA-resistant, barium chloride-sensitive K⁺ channel(s) mediating RVD.
Effect of cAMP agonists on RVD. To examine the effect of cAMP agonists on the RVD in cholangiocytes, IBMX was administered during the hypotonic challenge and the RVD responses were compared with normal controls. As shown in Fig. 11, there was no significant difference in the RVD of BDCCs treated with IBMX (1 mM) compared with untreated controls. In addition, the effect of a more potent cAMP agonist forskolin on the RVD of BDCCs was studied. As shown in Fig. 12, the RVD of BDCCs treated with forskolin (10 μM; n = 10) appeared to be slower in the initial phase of RVD compared with that of untreated controls (n = 18), but there was no significant difference between them. These findings indicate that stimulation of cholangiocytes with cAMP has no significant effect on the RVD.

DISCUSSION

In this manuscript, we report the successful isolation and use of isolated BDCCs from normal mouse liver using the mouse IBDU isolation method developed and reported recently (8). Although we have previously made extensive use of IBDUs for the study of biliary secretion, we have excluded these lumen-containing IBDUs and have used in this study only BDCCs, which lack enclosed lumens, to facilitate the measurement of cell cluster volume by eliminating the need to consider possible changes in luminal volume due to hypotonic challenge. These BDCCs were determined to be of biliary origin by positive immunocytochemistry using a cholangiocyte-specific CK-19 antibody. Similar to mouse IBDUs, these BDCCs have less connective tissue around them than rat IBDUs and exhibit a more...
refractory pattern than rat IBDUs by light microscopy using Nomarski optics. Thus measurement of CSA by tracing of the borders of individual cell clusters is easier with mouse BDCCs than with rat IBDUs.

To assess changes in cell cluster volume during hypotonic challenge, we have used CSA as an indirect measure of volume, as done previously in other cell types by other investigators (17, 19). The validity of CSA measurements of BDCCs by quantitative videomicroscopy as indirect measurements of cell cluster volume was confirmed by three independent methods: sequential light microscopy, fluorescence microscopy, and laser-scanning confocal microscopy of BCECF-loaded BDCCs, each of which was followed by computer-assisted measurements of the corresponding CSAs and volumes. As shown in Figs. 2 and 3, CSA measurements of BDCCs are very well correlated with their corresponding volume measurements, confirming the validity of CSA measurements of BDCCs as indirect measurements of their corresponding volumes. It is notable that the CSA measurements of BDCCs are linearly correlated with the corresponding volume measurements in the usual ranges of volumes of BDCCs. Thus this relatively simple method of quantitative videomicroscopy will allow accurate measurements of volume changes by measuring CSA to further study underlying mechanisms of cell volume regulation in primary bile duct cell preparations. Furthermore, given the inherent limitations of mouse cholangiocyte preparations due to their low cell number and yield in mouse liver, this method provides the only practical method to study cell volume regulation in primary mouse cholangiocytes from normal as well as from various knockout mouse livers. In fact, we tried to use a couler counter with cell sizer to measure cell volume changes of primary cholangiocyte preparations due to their low cell number and suboptimal purity of the cholangiocytes obtained from mouse livers and some methodological problems with cellular debris resulting from isolation procedure.

As previously reported in other cell types (3, 14, 17, 24) as well as in Mz-ChA-1 cells from human cholangiocarcinoma cell lines (23), normal mouse cholangiocytes exhibit an intact RVD, dependent on both Cl− and K+ conductances. As shown in Fig. 1, normal mouse BDCCs swell rapidly after exposure to hypotonic buffer, then gradually return toward their original cell volumes, as reflected by the changes in CSA shown in Fig. 5, indicating an intact RVD in these cell clusters. The RVD observed in normal BDCCs is significantly inhibited by general Cl−-channel blockers such as NPPB and DIDS, indicating the significant role of Cl− channels in the RVD of cholangiocytes (Figs. 5 and 7). In various cell types, DIDS has been shown to inhibit volume-activated chloride channels (24), calcium-activated chloride channels (2, 4), outwardly rectifying chloride channels (6), and ATP release (6) but does not inhibit CFTR (2, 11). Thus the inhibition of RVD by DIDS implies that chloride conductance via CFTR may not play a major role in RVD in cholangiocytes, and this conclusion is consistent with the finding that CAMP agonists had no significant effect on RVD. The RVD of BDCCs is also inhibited by glibenclamide (Fig. 7), which is a known inhibitor of CFTR, volume-activated chloride channels, as well as ATP-sensitive K+ (KATP) channels (25). However, RVD was not affected by coadministration of TEA at 10−1,000 μM (Fig. 9), suggesting that the KATP channel had no significant role in RVD because the KATP channel is inhibited by TEA (18). Therefore, these findings as a whole indicate an important role of the volume-sensitive chloride channel in RVD in cholangiocytes but cannot exclude a potential contributory role of other chloride channels such as calcium-activated chloride channels.

Next, the role of K+ channels in the RVD of BDCCs is examined. As shown in Fig. 10, the RVD of BDCCs is inhibited by BaCl2, a K+ channel blocker, whereas it is resistant to TEA, another K+ channel blocker, indicating the significant role of certain K+ channels in the RVD of cholangiocytes. A recent study of the RVD of Mz-ChA-1 cells from a human cholangiocarcinoma cell line (20) showed that a volume-sensitive SKCa channel is involved in the RVD of human cholangiocarcinoma cells. It is notable that these SKCa channels are known to be sensitive to BaCl2 but resistant to TEA, consistent with our results from normal mouse cholangiocytes (27). However, further characterization of these ion channels in normal cholangiocytes, using detailed patch-clamping studies, is needed to better define these pathways.

Our present study also examined the effects of CAMP agonists on the RVD of cholangiocytes. The CAMP agonists IBMX (Fig. 11) and forskolin (Fig. 12) had no significant effects on the RVD of BDCCs. These findings indicate that an activation of CAMP-dependent Cl− or K+ conductive pathways does not significantly stimulate the RVD of cholangiocytes and/or that CAMP-dependent pathways are not rate-limiting steps in the RVD of cholangiocytes. Thus CFTR may not play a major role in providing chloride conductive pathways for the RVD of cholangiocytes, but volume-activated chloride channels may have a more important role in the RVD of cholangiocytes. However, it does not exclude the possibility that CFTR may be important for regulation of various Cl− or K+ channels and conductive pathways mediating RVD of cholangiocytes. In fact, our preliminary data in Ctr−/− mouse BDCCs indicate that CFTR is important for regulating K+ conductive pathways involved in the RVD of cholangiocytes (unpublished observation).

In conclusion, our present study provides a simple method to quantify cell volume changes by measuring CSAs of the primary BDCCs isolated from mouse livers. It is the only practical method to study cell volume regulation in mouse cholangiocytes. In the present study, we have shown that cholangiocytes from normal mouse livers can regulate their cell volumes toward their basal cell volumes during hypotonic challenges, as shown previously in cholangiocarcinoma cells (23). As in cholangiocarcinoma cell lines, the RVDS of normal cholangiocytes are mediated by chloride channels,
which are inhibited by NPPB, DIDS, and glibenclamide but are not stimulated by cAMP agonists and by potassium channels, which are inhibited by barium chloride but not by TEA. With the use of this method and primary BDCC preparations, one can further study the underlying ion transports and mechanisms involved in the RVD of cholangiocytes from normal as well as genetically altered mouse livers.

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