Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 9080–9084, October 1993
Physiology

Regulation of bicarbonate-dependent ductular bile secretion assessed by lumenal micropuncture of isolated rodent intrahepatic bile ducts

STUART K. ROBERTS*, SUSAN M. KUNTZ*, GREGORY J. GORES*, AND NICHOLAS F. LARUSSO*†‡

Departments of *Internal Medicine and †Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905

Communicated by Ralph T. Holman, May 27, 1993 (received for review March 23, 1993)

ABSTRACT **While intrahepatic bile duct epithelial cells secrete bile through transport of ions and water, the physiological mechanisms regulating ductular bile secretion are obscure, in part because of the lack of suitable experimental models. We report here the successful micropuncture of the lumen of isolated intrahepatic bile ducts and direct measurements of ductular ion secretion. Intact, polarized bile duct units (BDUs) were isolated from livers of normal rats by enzymatic digestion and microdissection. BDUs were cultured and mounted on a microscope in bicarbonate-containing buffer, and the lumens were microinjected with 2′,7′-bis(2-carboxyethyl)-5-(and -6)carboxyfluorescein (BCECF)-dextran. Lumenal pH was measured by ratio imaging of BCECF fluorescence using digitized video fluorescent microscopy. After 36 hr in culture, the ends of BDUs sealed, forming closed compartments. After lumenal microinjection of BCECF-dextran, fluorescence was stable at the pH-insensitive wavelength, indicating no dye leakage. Serial changes in pH of extralumenal buffers containing pH-gradient collapsing ionophores allowed us to establish reliable standard curves relating fluorescence ratio to lumenal pH ($r = 0.99$; $P < 0.001$). By this approach, the basal pH inside the lumen of BDUs was 7.87 ± 0.08 units ($n = 9$), 0.47 unit higher ($P < 0.001$) than the bathing buffer pH. Addition of 100 μM forskolin increased ($P = 0.02$) the lumenal pH from 7.78 ± 0.06 to 7.97 ± 0.06 units ($n = 5$); the forskolin effect was completely abolished by incubation of BDUs in HCO_3^-/CO_2-free buffer. Moreover, forskolin caused a 50-fold increase in cAMP levels in BDUs. The observations are consistent with cAMP-dependent, active lumenal HCO_3^- secretion by BDUs. Furthermore, they demonstrate the suitability of the BDU model for studying regulatory and mechanistic aspects of ductular bile secretion.**

Ductular bile secretion by intrahepatic bile duct epithelial cells, or cholangiocytes, contributes up to 40% to total bile flow (1). It involves the secretion of ions and water both spontaneously and in response to agonists, principally hormones (2). For example, both secretin and somatostatin, two gastrointestinal hormones, affect ductular bile secretion by interacting with cell-surface receptors on cholangiocytes (3, 4). While the effects of the hormones are different (i.e., secretin stimulates while somatostatin inhibits ductular bile secretion), both appear to accomplish their effect by altering the levels of cAMP in cholangiocytes (5, 6).

At the cellular level, little else is known about the transport mechanism(s) involved in ductular ion and water secretion. The paucity of information is due in part to the lack of suitable experimental models to study ductular bile. While physiologic studies in whole animals (7, 8) suggest HCO_3^- transport is likely involved in the vectorial transport of water by bile ducts, these indirect experimental approaches are limited and cannot conclusively identify mechanism(s). Although the recent development of methods to isolate relatively pure cholangiocytes has allowed direct transport studies (6, 9, 10), the loss of polarity in these isolated cell preparations makes the relevance of these findings to lumenal membrane secretion problematic. Clearly, the ability to perform direct studies on intact, contiguous, polarized biliary epithelia would represent a major advance and permit elucidation of regulatory and mechanistic aspects of ductular bile secretion. Thus, our goal was to develop and apply an experimental model to directly study regulatory and mechanistic aspects of ductular bile secretion.

METHODS AND MATERIALS

Preparation and Characterization of Bile Duct Units (BDUs). Male rats (250–320 g) were anesthetized with pentobarbital (50 mg/kg; i.p.). The hepatic artery was cannulated with PE-10 intramedic polyethylene tubing (Becton Dickinson). The portal vein was cannulated and perfused with 250 ml of preoxygenated buffer A (115 mM NaCl/5 mM KCl/0.8 mM KH_2PO_4/25 mM Hepes/2.5 mM glucose) containing 0.5 mM EGTA (pH 7.4; 37°C). The liver was removed and perfused with 150 ml of buffer A containing 1 mM $CaCl_2$ and 0.02% type II collagenase (Worthington) for 10 min at 37°C using a perfusion apparatus previously described by us (11). During this time, 4 ml of 1% agar in saline was remelted and 6 drops of 0.4% trypan blue solution in saline was added to it. After liver perfusion, 2–3 ml of liquid trypan blue agar was injected into the portal vein and 1 ml was injected into the hepatic artery. The liver was then placed in ice-cold, preoxygenated buffer B (115 mM NaCl/5 mM KCl/0.8 mM KH_2PO_4/25 mM Hepes/2 mM $CaCl_2$/0.8 mM $MgSO_4$/2.5 mM glucose, pH 7.4).

Interlobular and primary branches of the portal vein, hepatic artery, and bile ducts were exposed by removing surface hepatocytes by mechanical dissociation. Under a dissecting microscope, bile ducts were microdissected by using the blue agar-filled portal vein and hepatic artery for reference and placed in cold buffer B on ice. Further microdissection was performed under higher magnification to remove residual hepatocytes, components of the portal vein and hepatic artery, and excess connective tissue.

For morphologic studies, isolated BDUs were cultured overnight at 37°C on rat tail collagen in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (Biocell Laboratories) containing antibiotic–antimycotic (GIBCO/BRL). For microinjection studies, isolated bile ducts were cut into 2- to 3-mm segments and cultured for 36–48 hr in a suspension in cholangiocyte growth medium.

γ-Glutamyl transpeptidase histochemical staining of unfixed BDUs was performed as described (12). Immunohisto-

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Abbreviations: BDU, bile duct unit; BCECF, 2′,7′-bis(2-carboxyethyl)-5-(and -6)carboxyfluorescein.

‡To whom reprint requests should be addressed.

chemical detection of cytokeratin 19 and vimentin was performed as described (13).

For scanning and transmission electron microscopy, BDUs were fixed, processed, and viewed as described (13).

The length of freshly isolated BDUs was determined from measurements of 21 ducts from three normal rats. The mean duct and lumen diameters of isolated BDUs were determined from 165 sections collected at 2-mm intervals from 12 ducts isolated from three normal rats.

cAMP concentrations in cultured BDUs were measured by radioimmunoassay and expressed relative to DNA content as described (6).

Microinjection Studies. Lumenal pH was measured in BDUs by multiparameter digitized video microscopy as described (14).

Isolated BDUs were cultured for 36–48 hr to ensure a proper seal at both ends (see Fig. 4*A*). BDUs were attached to glass coverslips with Cell-Tak tissue adhesive (Collaborative Biomedical Products, Bedford, MA) and placed in culture dishes containing Krebs/Ringer/Hepes bicarbonate (KRHB) solution preequilibrated with 95% O_2/5% CO_2, pH 7.4. Coverslips were mounted on the microscope stage for microinjection and fluorescent measurements in KRHB buffer (pH 7.4; 37°C). 2′,7′-Bis(2-carboxyethyl-5-(and -6)carboxyfluorescein (BCECF)-dextran, M_r 70,000 (Molecular Probes), a cell-impermeant, fluorescent pH indicator, was dissolved (100 μM) in an injection buffer containing 140 mM NaCl and 5 mM Na_2HPO_4 (pH 7.2). Micropipettes were pulled from borosilicate glass capillary tubes as described (15) and loaded with BCECF-dextran. Micropuncture of the lumen of BDUs and injection of the fluorescent dye were performed as described (15).

The pH in the lumen of BDUs was measured by ratio imaging of BCECF-dextran fluorescence at excitation wavelengths of 490 and 440 nm (14). *In situ* calibration curves were generated in BCECF-dextran-loaded BDUs by methods similar to those described (16). The extralumenal buffer contained the two pH gradient collapsing ionophores nigericin (10 μM) and monensin (10 μM) (Sigma), and the pH was adjusted over the range 6.7–8.3 with either 1 M KOH or 1 M HCl.

Statistical Analysis. All values are expressed as means ± SEM. Statistical differences between means were determined by Student's *t* test; results were considered significant at the $P < 0.05$ level. One-way ANOVA was used to compare differences in cAMP levels in BDUs after forskolin stimulation.

RESULTS

Morphologic Study of Isolated Intrahepatic BDUs. The isolation method described yielded a pure preparation of intact, whole intrahepatic bile ducts; hepatocytes and components of the portal vein and hepatic artery were not present (Fig. 1*A*). BDUs appeared as intact ducts with a lumen surrounded by a single layer of columnar or cuboidal epithelium (Fig. 1). A thin layer of connective tissue surrounded the ducts (Fig. 1 *A* and *B*). Histochemical staining with γ-glutamyl transpeptidase, a marker for cholangiocytes, was positive in BDU epithelium (Fig. 1*C*). BDUs also stained positively for cytokeratin 19, another specific marker for cholangiocytes (Fig. 1*D*), but negatively for vimentin (Fig. 1*E*), a marker for mesenchymal cells.

Electron microscopy showed that isolated BDUs retained their *in situ* morphologic polarity. By scanning electron microscopy, the lumenal surface of BDUs had a cobblestone appearance with numerous microvilli covering the apical cell surface (Fig. 2*A*). The basal domain of the BDU epithelium contained multiple folds and interdigitations that lay adjacent to extracellular components. These components were essentially devoid of mesenchymal cells (Fig. 2*B*). By transmission electron microscopy, BDUs had an intact epithelium with a basally located, irregularly shaped nucleus and a high nucleus/cytoplasm ratio, characteristic of cholangiocytes *in situ*. Abundant microvilli distinguished the apical surface (Fig. 2*C*). Small, relatively sparse mitochondria were located in the perinuclear cytoplasm. Clear vesicles of variable sizes were present mainly in the apical cytoplasm (Fig. 2*D*). Similar transmission electron microscopy findings were also seen in BDUs cultured for 48 hr (data not shown), indicating that cultured BDUs remain polarized.

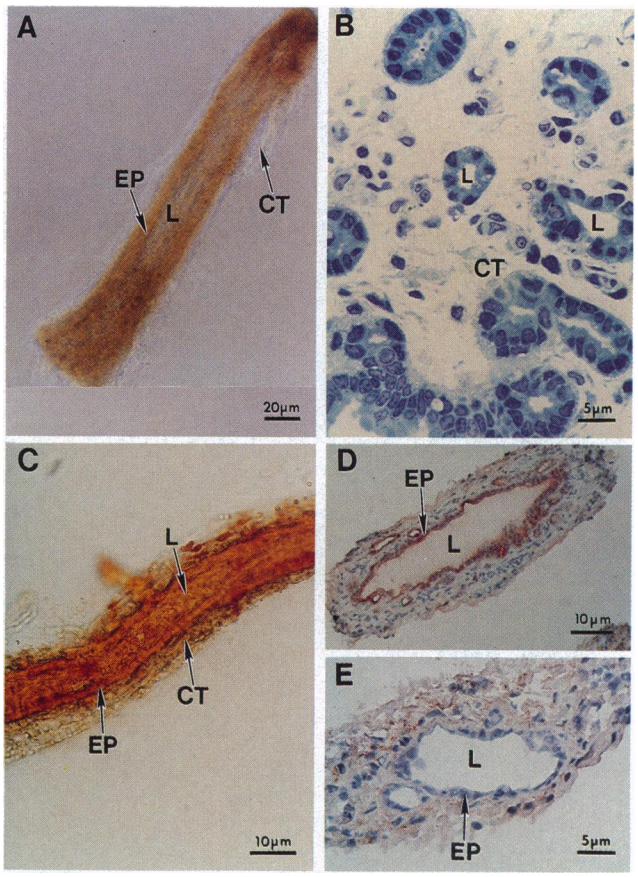

FIG. 1. Light micrographs of freshly isolated intrahepatic BDUs. (*A* and *B*) Structural morphology of BDUs in an unstained whole-mount preparation (*A*) and in 1-μm cross-sections stained with methylene blue (*B*). The lumen (L) is surrounded by a single layer of epithelial cells (EP) with a thin outer layer of connective tissue (CT). (*C–E*) Staining for cholangiocyte-specific proteins in BDUs. (*C*) γ-Glutamyl transpeptidase histochemical staining showing the epithelium stains strongly positive. (*D* and *E*) Immunohistochemical staining was performed for cytokeratin 19 (*D*), a specific marker for cholangiocytes, and for vimentin (*E*), a marker for mesenchymal cells. Note the positive staining of the epithelium in *D* and the negative staining in *E*.

Cultured BDUs were viable based on the observation that the epithelial cells completely excluded trypan blue (data not shown). Morphometric data for BDUs isolated from normal rats are summarized in Table 1.

Measurements of cAMP. Isolated BDUs cultured for 48 hr were biologically responsive based on forskolin-stimulated measurements of cAMP (Fig. 3). Exposure to forskolin, a potent activator of adenylate cyclase, caused a dose-dependent increase in cAMP, reaching a maximum of 1855 ± 307 fmol of cAMP per μg of DNA at 100 μM forskolin. This represented a 50-fold increase above the basal levels of 37 ± 8.3 fmol of cAMP per μg of DNA ($P < 0.0001$).

Microinjection of the BDU Lumen. After overnight culture, the ends of BDUs sealed, accompanied by bulbous swelling

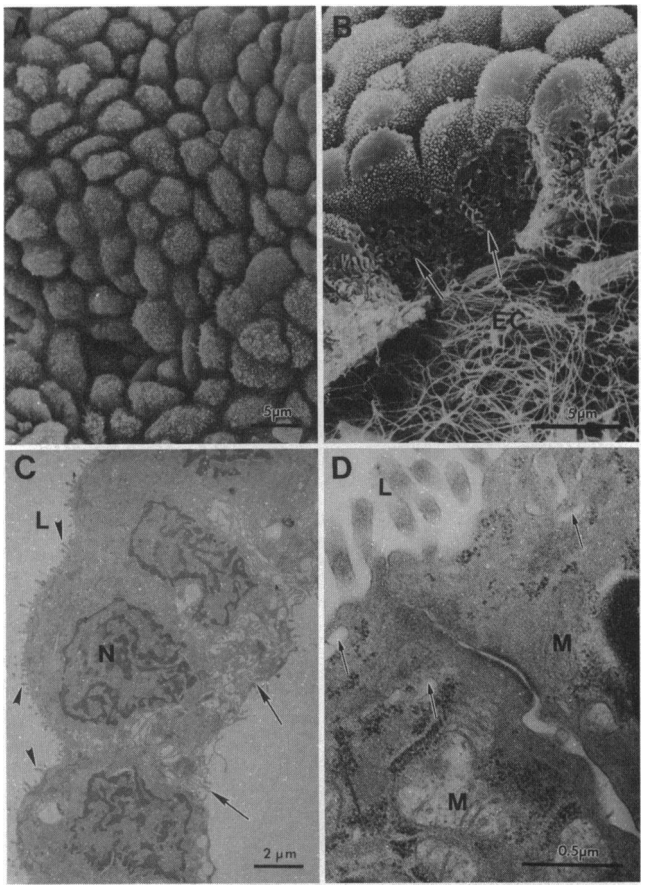

FIG. 2. Electron micrographs of freshly isolated BDUs. (*A* and *B*) Scanning electron microscopy. (*A*) The lumenal surface had a cobblestone appearance with numerous microvilli. (*B*) Cross-sectional scanning electron microscopy with the basal domain of BDUs containing folds and interdigitations (arrows) that lay adjacent to extracellular components (EC) devoid of cells. (*C* and *D*) Transmission electron microscopy. (*C*) Note the apical microvilli (arrowheads) projecting into the lumen (L), basally located nucleus (N), and numerous folds and interdigitations at the basal surface (arrows). (*D*) Cells had few mitochondria (M) with clear vesicles of variable size (arrows), mainly in the apical region. L, BDU lumen.

formation (Fig. 4*A*). These observations indicated that BDUs formed enclosed units and were suitable for microinjection of the lumen and direct studies of ductular bile secretion. The technique used for microinjection studies is illustrated in Fig. 4*B*. It shows the appearance of a BDU after the lumen was microinjected with a droplet of oil from a micropipette. Note the small size of the micropipette tip in comparison with the lumenal diameter and the containment of oil within the lumen. Based on the findings described above, we microinjected the lumen of BDUs with the cell-impermeant BCECF-dextran, achieving lumenal access with the dye in 76% ± 6% of attempts. After microinjection of the dye, BCECF-dextran is seen to be confined to the lumen (Fig. 4*C*). After successful lumenal microinjection, we observed stable BCECF fluorescence for at least 30 min at the pH-insensitive (440 nm) excitation wavelength (data not shown), an observation in-

Table 1. Morphometric data on isolated BDUs

Parameter	Mean (±SEM)	Range
Duct length, mm	6.8 (±1.48)	0.4–21
Duct diameter, μm	98 (±0.01)	20–454
Duct lumen diameter, μm	80 (±0.01)	10–433

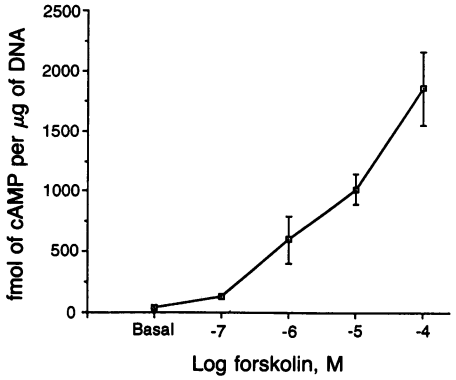

FIG. 3. Dose–response effect of forskolin on cAMP levels in BDUs (n = 5).

dicating no dye leakage or photobleaching. The lumenal pH could be reliably calibrated by using a previously described (16) *in situ* technique in which serial changes were made in the pH of the bathing buffer in the presence of ionophores that collapse the pH gradient. Fig. 4*D* shows the relationship between the measured fluorescent ratio in the lumen of BDUs and the pH value of the extralumenal buffer, over the pH range 6.7–8.3 (r = 0.99; $P < 0.001$). Using this approach, we measured the lumenal pH under both basal and forskolin-stimulated conditions. The basal pH of BDUs in HCO_3^-/CO_2-containing buffer was 7.87 ± 0.08 units (range, 7.61–8.30; n

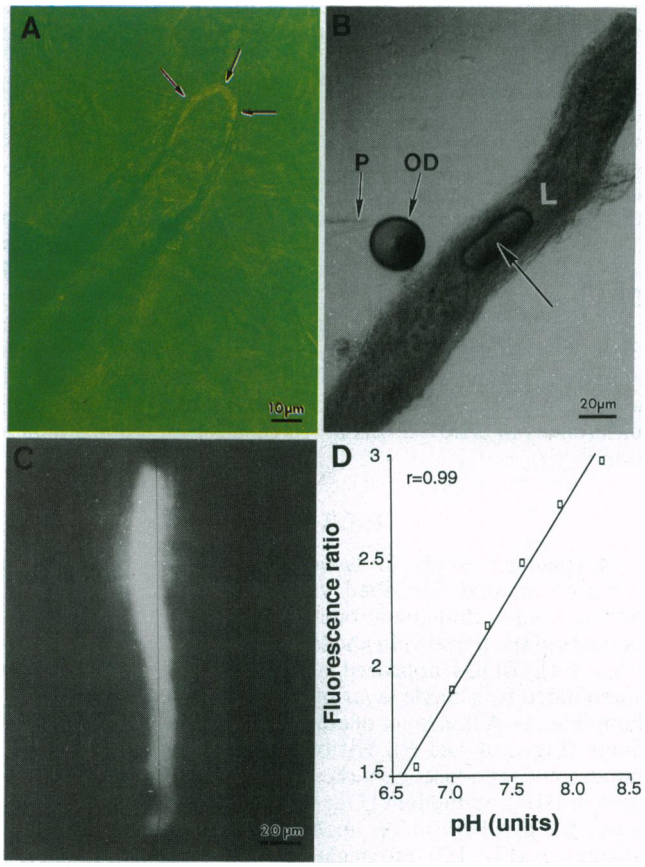

FIG. 4. Validation of the microinjection technique for BDUs. (*A*) Phase-contrast micrograph showing the sealed end of a BDU (arrows) after overnight culture. (*B*) Illustration of the technique used showing the BDU lumen (L) containing oil (arrow) after microinjection of an oil droplet (OD) with a micropipette (P). (*C*) BDU after microinjection of BCECF-dextran showing confinement of the dye to the lumen. (*D*) Relationship between lumenal BCECF fluorescence and the pH of the extralumenal buffer.

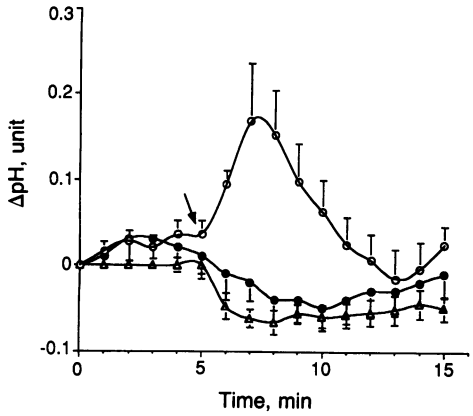

FIG. 5. Time course of the change in lumenal pH in BDUs after forskolin. After lumenal microinjection, measurements of BCECF fluorescence were taken at 1-min intervals for 15 min. At time $t = 5$ min (arrow), forskolin prepared in dimethyl sulfoxide was added to HCO_3^-/CO_2-containing buffer (open circles). Control experiments are shown in which an equivalent amount of dimethyl sulfoxide (solid circles) (final concentration, 1%) was added at the same time point ($n = 4$). Experiments with forskolin were also performed in HCO_3^--free buffer (open triangles), prepared by isosmotic substitution with NaCl and gassing with 100% O_2 ($n = 6$). The buffer was changed from HCO_3^--containing to HCO_3^--free buffer at time $t = 0$.

= 9). This pH value was significantly higher by 0.47 unit than the pH in the bathing buffer ($P < 0.001$), a finding that strongly suggested that BDUs were actively secreting HCO_3^-. Addition of forskolin (final concentration, 100 μM) to HCO_3^--containing buffer resulted in a significant increase in lumenal pH of 0.19 unit above basal values, from 7.78 ± 0.06 to 7.97 ± 0.06 units ($P = 0.02$; $n = 5$). The time course of the pH increase after forskolin is depicted in Fig. 5. During the 5-min control period, the pH in the lumen was stable. Within 1 min of exposure to forskolin, the lumenal pH had begun to increase, reaching a peak at 2 min after exposure. Since the fact that the forskolin-stimulated increase in lumenal pH could have represented either a stimulation of lumenal HCO_3^- secretion or an inhibition of lumenal H^+ secretion, we examined the importance of HCO_3^-/CO_2 in the bathing buffer on the forskolin effect on lumenal pH. As Fig. 5 shows, the effect of forskolin on lumenal pH was HCO_3^-/CO_2 dependent. When forskolin was added to HCO_3^-/CO_2-free buffer, no increase in lumenal pH was observed above the basal values ($n = 6$). Indeed, the change in lumenal pH after addition of forskolin to HCO_3^-/CO_2-free buffer was similar over time to that observed after the addition of the vehicle, dimethyl sulfoxide, during control experiments.

DISCUSSION

This paper reports an experimental model to directly study ductular bile secretion. Our major findings relate (*i*) to methodology for isolating BDUs; (*ii*) to morphologic, morphometric, and biochemical observations in BDUs; and (*iii*) to microinjection studies on BDUs using digitized video fluorescent microscopy. We describe a technique for isolating intact rodent BDUs that combines enzymatic digestion with microdissection. After isolation, BDUs are viable and retain their *in situ* morphologic polarity, surface organization, and ultrastructural features. As important is the observation that cultured BDUs formed enclosed functional units enabling microinjection of fluorescent dye indicators to directly study transport processes.

There have been very few reports on the technique for isolating intrahepatic bile ducts from rat liver (17, 18). Gall and Bhathal (17) combined enzymatic liver digestion with mechanical agitation and gauze filtration, while Oda *et al.* (18) applied differential centrifugation to liver homogenates to isolate intrahepatic bile ducts. Both techniques produced preparations that were less than ideal since isolated intrahepatic ducts were contaminated with hepatocytes, blood vessels, cellular debris, and connective tissue. Also, intrahepatic bile duct fragments have been isolated from pig liver by microdissection of liver biopsy specimens, although formal characterization of these bile ducts has not been reported (19, 20). Our isolation technique was adapted from the method used to successfully isolate interlobular ducts from the rat pancreas (21). This method produced a pure preparation of principally medium-sized bile ducts, free of overt cellular contamination and excess connective tissue as assessed by histology, histochemistry, and immunohistochemistry.

Bile ducts isolated by our method retained their *in situ* morphology. They appear as intact ducts with a lumen enclosed by a single layer of epithelial cells. BDUs retain morphologic polarity with apical and basolateral domains easily distinguished by ultrastructural features; these include the presence of apical microvilli and apically oriented junctional complexes as well as a basally located, irregularly shaped nucleus, characteristics previously described in bile duct epithelia *in situ* (22) and in *freshly* isolated cells (13). The presence of apical microvilli and apically oriented vesicles strongly suggests that BDUs are capable of absorptive and secretory activities. Additional evidence supporting this was apparent from several changes that occurred in the appearance of BDUs after culture for 36–48 hr. During this time, the ends of BDUs sealed and bulbous swellings formed, alterations suggesting spontaneous secretion of fluid into the lumen of BDUs.

Two distinct populations of BDUs (i.e., medium and large ducts) were isolated; small ducts were not isolated because of the technical difficulties involved. Medium-sized ducts, which likely correspond to interlobular and septal bile ducts, were the predominant form of bile ducts isolated and used for transport studies.

BDUs remained viable and biologically responsive after isolation based on their ability to (*i*) exclude trypan blue, (*ii*) seal at the ends, and (*iii*) increase cAMP levels in response to forskolin. The dose-dependent increase in cAMP levels in BDUs was quantitatively similar to the response observed in isolated cholangiocytes (6, 9) following adenylate cyclase activation.

In these studies, a micropipette has been introduced into the lumen of intact intrahepatic bile ducts to directly study ductular secretion. We micropunctured the lumen of isolated BDUs, injected a cell-impermeant, fluorescent pH indicator, and used digitized video fluorescent microscopy to study HCO_3^- transport across the lumenal membrane. No appreciable mixing of lumenal dye with the bathing buffer occurred after successful microinjection. The approach using microinjection and fluorescence microscopy allowed us to measure lumenal pH in the basal state and after perturbations. Our major findings include the observations that (*i*) the basal lumenal pH of BDUs was 0.47 unit higher than the pH in the bathing buffer; (*ii*) forskolin increases the lumenal pH of BDUs 0.19 unit above the basal level; and (*iii*) the forskolin-induced alkalinization of the lumenal contents is abolished by the removal of HCO_3^-/CO_2 from the bathing buffer. Taken together, these observations strongly suggest active ductular HCO_3^- secretion into the lumen of BDUs. The importance of these findings is 3-fold. First, the data provide definitive evidence that intrahepatic bile ducts are involved in secretory activities. Second, the data demonstrate directly that biliary epithelia can secrete HCO_3^-. Third, the data show the suitability of the BDU model for studying regulatory and mechanistic aspects of ductular bile secretion.

Although little is known about the subcellular mechanisms regulating hormone-induced ductular bile secretion, recent

evidence suggests that it is mediated by the adenylate cyclase/cAMP second messenger system. Lenzen *et al.* (23) showed that the secretin-stimulated choleresis *in vivo* is associated with activation of the cAMP system in isolated cholangiocytes. We have shown that secretin and forskolin both stimulate exocytosis in isolated cholangiocytes by a cAMP-dependent mechanism (6). Furthermore, secretin has recently been reported to stimulate Cl^- secretion in isolated cholangiocytes by this pathway (9). These findings are paralleled by observations in the pancreas, an organ that, like the liver, secretes a HCO_3^-- and Cl^--rich fluid in association with changes in cAMP in response to secretin (21, 24, 25). Our findings that forskolin causes an increase in cAMP levels and stimulates lumenal HCO_3^- secretion in BDUs add further credence to the regulatory role of the cAMP pathway in ductular bile secretion.

The physiologic mechanisms underlying biliary HCO_3^- secretion are poorly understood. Recent studies in pig liver indicate that secretin-induced ductular HCO_3^- secretion is dependent on both the activity of carbonic anhydrase in bile duct cells (19) and on the arterial P_{CO_2} level (20), findings similar to those previously reported in the epithelial cells that line the ducts in the pancreas (26). Our finding that forskolin-stimulated HCO_3^- secretion is dependent on the presence of HCO_3^-/CO_2 in the bathing buffer is consistent with the concept that secreted HCO_3^- is derived from CO_2 within the cell, originating from plasma. Also, the cystic fibrosis transmembrane conductance regulator, a cAMP-regulated Cl^- channel, as well as a Cl^-/HCO_3^- exchanger, has recently been demonstrated in isolated rat cholangiocytes (9, 10). However, the unpolarized nature of the cells used in these studies has left the precise location of these channels and hence their relevance to lumenal secretion obscure. While our results are compatible with the hypothesis that HCO_3^- is secreted into the lumen via a Cl^-/HCO_3^- exchanger in the apical plasma membrane, further studies are required to formally characterize the mechanisms governing ductular HCO_3^- transport.

While the principal reason for applying lumenal micropuncture techniques to the liver is to determine the physiological mechanisms regulating ductular bile secretion, these techniques could also be used to answer other key questions pertaining to ductular bile formation. Such questions would include but not be limited to (*i*) whether intrahepatic bile ducts are functionally heterogeneous with respect to secretion and absorption; and (*ii*) how hormones regulate ductular bile secretion. Moreover, lumenal micropuncture studies of intrahepatic bile ducts could directly evaluate the effects of drugs implicated in modulating ductular bile secretion. The availability of the model described here now makes feasible experimental approaches directed in all these areas.

We would like to thank Mr. S. F. Bronk for his help in the microinjection experiments and Ms. Maureen Craft for excellent secretarial assistance. This work was supported by Grants DK 24031 and DK 45876 from the National Institutes of Health and the Mayo Foundation.

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