Published in final edited form as:

Gastroenterology. 2007 November ; 133(5): 1592–1602.

Cyclic AMP regulates bicarbonate secretion in cholangiocytes through release of ATP into bile

Noritaka Minagawa^{1,3}, Jun Nagata^{1,3}, Kazunori Shibao^{1,3}, Anatoliy I. Masyuk⁴, Dawidson A. Gomes¹, Michele A. Rodrigues¹, Gene LeSage⁵, Yasutada Akiba⁶, Jonathan D. Kaunitz⁶, Barbara E. Ehrlich², Nicholas F. LaRusso⁴, and Michael H. Nathanson^{1,7}

¹ Department of Medicine, Yale University School of Medicine, New Haven, CT

² Department of Pharmacology, Yale University School of Medicine, New Haven, CT

³ Department of Surgery 1, University of Occupational and Environmental Health, Kitakyushu, Japan

⁴ Department of Medicine, Mayo Clinic College of Medicine, Rochester, MN

⁵ Department of Medicine, University of Texas, Houston, TX

⁶ Department of Medicine, University of California, Los Angeles, CA

Abstract

Background & Aims—Bicarbonate secretion is a primary function of cholangiocytes. Either cAMP or cytosolic Ca²⁺ can mediate bicarbonate secretion, but these are thought to act through separate pathways. We examined the role of the inositol 1,4,5-trisphosphate receptor (InsP3R) in mediating bicarbonate secretion, because this is the only intracellular Ca²⁺ release channel in cholangiocytes.

Methods—Intrahepatic bile duct units (IBDUs) were microdissected from rat liver, then luminal pH was examined by confocal microscopy during IBDU microperfusion. Cyclic AMP was increased using forskolin or secretin, and Ca²⁺ was increased using acetylcholine (ACh) or ATP. Apyrase was used to hydrolyze extracellular ATP, and suramin was used to block apical P2Y ATP receptors. In selected experiments IBDU were pre-treated with siRNA to silence expression of specific InsP3R isoforms.

Results—Both cAMP and Ca²⁺ agonists increased luminal pH. The effect of ACh on luminal pH was reduced by siRNA for basolateral (types I and II) but not apical (type III) InsP3R isoforms. The effect of forskolin on luminal pH was reduced by a CFTR inhibitor and by siRNA for the type III InsP3R. Luminal apyrase or suramin blocked the effects of forskolin but not ACh on luminal pH.

Conclusions—Cyclic AMP-induced ductular bicarbonate secretion depends upon an autocrine signaling pathway that involves CFTR, apical release of ATP, stimulation of apical nucleotide receptors, and then activation of apical, type III InsP3Rs. The primary role of CFTR in bile duct secretion may be to regulate secretion of ATP rather than to secrete chloride and/or bicarbonate.

⁷ Address for correspondence: Michael H. Nathanson, Digestive Diseases, Room TAC S241D, Yale University School of Medicine, New Haven, CT 06520-8019, Phone 203-785-7312, FAX 203-785-4306, Email: michael.nathanson@yale.edu.

None of the authors have a conflict of interest to disclose.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

The biliary tree plays an important role in conditioning the canalicular bile that is secreted by hepatocytes. Although diseases of the biliary tree can be due to a range of etiologies, their common endpoint is cholestasis. Chronic cholestatic conditions in turn are a frequent indication for liver transplantation. According to the organ procurement transplantation network (<http://www.optn.org>), nearly one in seven liver transplants performed in the US in 2005 were in patients whose primary diagnosis was a cholestatic disorder such as primary biliary cirrhosis or primary sclerosing cholangitis. Cholangiocytes are the polarized, secretory epithelia that line the biliary tree and are the targets of disease in most chronic cholestatic disorders. Cholangiocytes are capable of increasing the rate of bile flow by as much as 50% (1), but under normal conditions the primary role of cholangiocytes is to modify the composition rather than the volume of bile flow (2). In particular, ductular secretion is responsible for regulating the concentration of biliary bicarbonate (2).

Biliary bicarbonate secretion occurs through two separate pathways. The first pathway is stimulated by hormones such as secretin and is mediated by cAMP. The downstream effect of cAMP is to activate the cystic fibrosis transmembrane conductance regulator (CFTR), the apical chloride channel that is defective in cystic fibrosis. Chloride released into the ductular lumen then is thought to be exchanged for bicarbonate via a chloride-bicarbonate exchanger (3–5). In some systems bicarbonate may be released directly via CFTR, and limited evidence suggests this may occur in cholangiocytes as well (2). The cAMP/CFTR pathway is thought to be of primary importance in maintaining ductular bile flow, and indeed cystic fibrosis can result in chronic cholestasis and secondary biliary cirrhosis (6). The second secretory pathway in cholangiocytes is stimulated by neurotransmitters such as acetylcholine (ACh) and autocrine agents such as ATP, and is mediated by cytosolic Ca^{2+} (7). The downstream effect of Ca^{2+} is to activate Ca^{2+} -dependent chloride channels on the apical plasma membrane, which in turn release chloride that is exchanged for bicarbonate via a chloride-bicarbonate exchanger (3–5). The relative importance of cAMP-dependent and Ca^{2+} -dependent ductular secretion is not understood. However, loss of expression of the inositol 1,4,5-trisphosphate (InsP3) receptor (InsP3R) appears to be a general feature of cholestatic conditions (8). Because the InsP3R is the only intracellular Ca^{2+} release channel in cholangiocytes (9), this suggests that secretion mediated by cAMP and CFTR must depend in part upon InsP3R expression. The purpose of this study was to determine whether and how each of the three isoforms of the InsP3R affects cAMP- and CFTR-mediated bicarbonate secretion in cholangiocytes.

MATERIAL AND METHODS

Animals and materials

Male Sprague-Dawley rats (200–250 g, Charles River Laboratories, Boston, MA) were used for all studies. Animals were maintained on a standard diet and housed under a 12-hour light-dark cycle. ACh, ATP, apyrase, suramin, and the adenylyl cyclase activator forskolin were purchased from Sigma Chemical Co. (St. Louis, MO). The membrane-impermeant, pH-sensitive dye 2',7'-bis(2-carboxyethyl-5-(and-6)-carboxyfluorescein (BCECF) dextran (70,000 MW), fluo-4/acetoxymethyl ester (AM), BAPTA/AM, the lipophilic membrane dye DiD and the nuclear stain TO-PRO-3 were obtained from Invitrogen (Eugene, OR). The selective, small-molecule CFTR inhibitor CFTR_{inh}-172 was chemically synthesized and purified by HPLC as described previously (10;11), and was microperfused into the lumen of bile ducts 30 min before the start of experiments. Type I InsP3R antibodies from affinity-purified specific rabbit polyclonal antiserum directed against the 19 C-terminal residues of the mouse type I InsP3R were commercially obtained from Affinity Bioreagents (Golden, CO). Type II InsP3R antibodies from affinity-purified specific rabbit polyclonal antiserum directed against the 18 C-terminal residues of the rat type II insP3R were kindly provided by Richard

Wojcikiewicz (State University of New York, Syracuse). Monoclonal antibodies directed against the N-terminus of the type III InsP3R were commercially obtained (Transduction Laboratories, Lexington, KY). All other chemicals were of the highest quality commercially available.

Isolation of intrahepatic bile duct segments

Intrahepatic bile duct units (IBDUs) were microdissected from normal rat liver as described previously (12). Briefly, rats were anesthetized with pentobarbital sodium (50mg/kg ip), then the portal vein was exposed and cannulated and the liver was perfused with ice-cold saline. Subsequently, 2–3 ml of liquid trypan blue agar was injected into the portal vein to facilitate identification of portal tracts. The liver was surgically removed and immersed in ice-cold, pre-oxygenated HEPES-buffered saline (HBS, pH 7.4). After mechanical removal of the hepatic capsule and surface hepatocytes, intrahepatic bile ducts were exposed and microdissected using a Zeiss Stemi SV11 dissection microscope (Thornwood, NY). The IBDUs were cut into 1.0–1.5 mm segments and transferred to a culture chamber. Viability was assessed by trypan blue exclusion; only IBDU without evidence of trypan blue uptake were used. A modified approach to this was taken to isolate bile duct segments for Ca^{2+} measurements (8;9). Briefly, rat livers were isolated and then perfused with buffer containing collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN). The portal tissue residue was separated mechanically, and then cut into strips. DiD and fluo-4/AM were first co-injected into the common bile duct to facilitate identification of bile duct epithelia within portal strips.

siRNA studies

Potential target sites within the rat InsP3R genes were selected and then searched with NCBI BlastN to confirm specificity for each InsP3R isoform as described previously (13). The siRNAs for the type I, II, and III InsP3R were prepared by a transcription-based method using the Silencer kit according to the manufacturer's instruction (Ambion, Austin, TX). The sense and antisense oligonucleotides of siRNAs were, respectively, as follows: type I, 5'-AAAGTTGTAGCTGCTGGTGCTCCTGTCTC- 3' and 5'-AAAGCACCAGCAGCTACAACCTCTGTCTC- 3'; type II, 5'-AACAGCCTAATCAAGATCTCCCCCTGTCTC- 3' and 5'-AAGGAGA TCTTGATTAGGCTGCCTGTCTC- 3'; type III, 5'-ATGGTGCTGGCAAACCTTGTTCCTGTCTC- 3' and 5'-AAACAAGTTTGCCA GCACCATCCTGTCTC- 3'; scrambled (siRNA negative control), 5'-AACACCTATAACAACGGTAGTC CTGTCTC -3' and 5'-AAACTACCGTTGTTATAGGTGCCTGTCTC -3'. siRNA constructs were tested for efficacy in CHO cells as described previously (13) and then used in IBDUs(14). IBDUs were maintained in culture with siRNAs or the corresponding scrambled siRNA for 24 hr at 37°C prior to use in microperfusion studies. siRNAs were delivered to both CHO cells and cholangiocytes using the TransMessenger™ Transfection Reagent lipid carrier (Qiagen, Valencia, CA).

Biliary pH measurements

The luminal pH of perfused IBDUs was measured using the cell-impermeant pH-sensitive dye BCECF dextran, as an index of ductular bicarbonate secretion (12;15;16). Luminal pH is an indirect measure of bicarbonate secretion, and other transport processes in cholangiocytes can increase luminal pH as well, but available evidence suggest that bicarbonate secretion is the primary process to increase luminal pH in cholangiocytes stimulated with the agonists used in this study (3–5). Individual isolated IBDUs were placed in a specially designed, temperature-controlled chamber mounted on the stage of a Zeiss LSM 510 Laser Scanning Confocal Microscope equipped with a krypton/argon mixed gas laser (Thornwood, NY). Concentric glass pipettes made from soft fine glass tubes (Drummond Scientific Co., Broomall, PA) were

attached to a microperfusion apparatus built to specification, and used to position and perfuse the IBDUs. One end of an individual IBDU was drawn into the tip of a glass holding pipette by gentle suction. The lumen was then cannulated with a concentric perfusion pipette that contained the perfusion solution. Solutions were delivered near the tip of the perfusion pipette at a rate of 1 μ l/min via a fluid-exchange pipette system connected with a variable-speed syringe pump (model 55-2222 Harvard microliter syringe pump, Harvard Apparatus, Inc., Holliston, MA). The opposite end of an IBDU was stabilized using a holding pipette. The external surface of the microperfused IBDU was simultaneously bathed in a buffer that was continuously oxygenated and exchanged. The temperature of the fluid was maintained at 37°C. Luminal BCECF dye was excited at a wavelength of 488 nm, and fluorescence emission was monitored at 505–550 nm. Confocal images were obtained at a rate of 5–20 images/min. In situ calibration curves were generated at the end of each experiment by perfusion with 10 μ M nigericin plus monensin in serial solutions of pH 6.8, 7.4, and then 8.0, as described (12). Non-ratio images of BCECF-dextran were collected to determine luminal pH, and potential errors from such measurements include photobleaching and changes in the volume of distribution of the dye. Luminal fluorescence decreased by only 0.2% after 5 min of continuous imaging ($p=0.3$; $n=6$ IBDU), so that photobleaching was negligible in this experimental system. Similarly, lumen width did not increase after 25 min of stimulation with either forskolin or ACh ($86 \pm 11 \mu$ m both before and after stimulation; $p>0.95$; $n=6$). Therefore, quantitative, calibrated measurements of luminal pH were made using this approach.

Immunoblots

Western blots were performed as previously described (13) using protein from CHO cells to test the efficacy of isoform-specific siRNAs for InsP3Rs. Protein was extracted by lysing CHO cells with M-PERTM mammalian protein extraction reagent (Pierce, Rockford, IL). The samples were subjected to sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% Tris-HCl gels. Gels were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and then membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS-T, 25mmol/L Tris-HCl, pH 7.5, 150mmol/L NaCl) plus 0.1% Tween 20 for 1 hr. Blots were incubated with InsP3R isoform-specific antibody (anti-type I InsP3R [1:1000], anti-type II InsP3R [1:100] or anti-type III InsP3R [1:1000]) in 5% nonfat dry milk in TBS-T at 4°C overnight, followed by incubation for 1 hr with peroxidase-conjugated immunoglobulin G secondary antibody (Bio-Rad, anti-rabbit [1:4000] or anti-mouse [1:5000]) in TBS-T. Blots were visualized by enhanced chemiluminescence using the ECL plus kit (Amersham Biosciences, Arlington Heights, IL). A Bio-Rad GS-700 imaging densitometer was used for quantitative analysis of the blots.

Immunofluorescence

Confocal immunofluorescence was performed as described previously (8). IBDUs were incubated in normal rat cholangiocyte medium with or without InsP3R-siRNA (5 μ M) for 24 hr at 37°C. IBDUs were then fixed with cold acetone for 10 min and then permeabilized in 0.1% Triton X-100 at room temperature. After blocking steps, IBDUs were labeled with primary antibodies (anti-type I InsP3R rabbit polyclonal [1:100], anti-type II InsP3R rabbit polyclonal [1:5] or anti-type III mouse monoclonal [1:100]). Following the primary antibody incubation, IBDUs were washed with PBS and incubated with secondary antibody. Secondary antibodies were Alexa 488 anti-mouse [1:500] or Alexa 555 anti-rabbit [1:500]. For negative control studies, IBDUs were incubated with secondary antibodies, but anti-InsP3R (primary) antibodies were omitted. IBDUs also were labeled with the nuclear stain TO-PRO-3 [1:200]. Some specimens were triple-labeled to detect type I and III InsP3R and the nucleus or type II and III InsP3R and the nucleus. Triple-labeled specimens were serially excited at 488 nm and observed at 505–550 nm to detect Alexa 488, at 543 nm and observed at >585 nm to detect Alexa 555, and excited at 633 nm and observed at >650 nm to detect TO-PRO-3. Double-

labeling was performed to detect type III InsP3R and the nucleus. Double-labeled specimens were excited at 488 nm and observed at 505–550 nm to detect Alexa 488, then excited at 633 nm and observed at >650 nm to detect TO-PRO-3. All images were collected using a Zeiss LSM 510 Meta Laser Scanning Confocal Microscope (Thornwood, NY).

Ca²⁺ measurements

DiD (25 μ mol/L) and fluo-4/AM (18 μ mol/L) were co-injected into the common bile duct, then bile duct segments were isolated as described above, transferred to a perfusion chamber, and observed using a Zeiss LSM 510 confocal imaging system. Tissue was excited at 647 nm and observed at greater than 680 nm to identify individual DiD-labeled bile duct cells, then cytosolic Ca²⁺ was monitored in these cells by exciting the specimen at 488 nm and detecting fluo-4 emission signals above 515 nm. Increases in Ca²⁺ were expressed as percent increases in fluorescence intensity of fluo-4 (8;9). In separate studies, cytosolic Ca²⁺ was measured in confluent monolayers of polarized rat cholangiocytes in culture (17). The cells were plated on a 96 well microtiter plate at 5–10 $\times$ 10³ cells/well and grown overnight. Cells were loaded with the Ca²⁺-sensitive ratio dye fura-2/AM and then fluorescence was monitored using a Pathway Imaging System (BD Biosciences, Rockville, MD). The cells were alternately exposed to excitation wavelengths of 340 and 380 nm and the fluorescence emission was collected at 510 nm. Measurements were obtained from 30–80 individual regions of interest (including both single cells and groups of cells) spanning 6–8 individual wells.

Statistics

Results are expressed as mean values $\pm$ S.E.M., except where otherwise noted. SigmaPlot version 9.01 (Systat Software, San Jose, CA) and Prism version 3.02 (GraphPad Software, San Diego, CA) were used for data analysis. Statistical significance was tested using student's *t* test or one-way ANOVA and a *p* value <0.05 was taken to indicate a significant difference.

RESULTS

Measurement of bicarbonate secretion in microdissected, microperfused bile ducts

Intrahepatic segments of bile ducts were microdissected from rat liver as described previously (12), then perfused with the cell-impermeant pH dye BCECF-dextran while examined by confocal microscopy in order to monitor bicarbonate secretion into the lumen (Figure 1a). In selected experiments, IBDU were maintained in culture overnight prior to microperfusion, which enabled IBDU to be treated with siRNA constructs prior to study. Responses to the cAMP agonist forskolin (100 μ M) were similar among freshly isolated IBDU and IBDU that had been in culture for 24 hr (Figure 1b).

Comparison of cyclic AMP-mediated and Ca²⁺-mediated bicarbonate secretion

Cyclic AMP-mediated secretion was induced by stimulation with forskolin (100 μ M), which activates adenylyl cyclase directly. Forskolin induced a sustained increase in luminal pH (*n*=3; Figure 1b), similar to what has been reported previously (12). This response was similar to the pattern observed during stimulation with secretin (100 nM; data not shown), which activates G_s and then adenylyl cyclase via the secretin receptor (3). Treatment of IBDU with the CFTR inhibitor CFTR_{inh}-172 blocked forskolin-induced increases in luminal pH (*p*<0.05, *n*=3; Figure 1b). Ca²⁺-mediated secretion was induced either by stimulation with ACh (100 μ M; *n*=3), which activates M3 muscarinic receptors, or with ATP (100 μ M; *n*=3), which activates P2Y nucleotide receptors.

Cholangiocytes express both of these receptors, each of which links to G_q, PLC activation, and then InsP3 formation in order to increase cytosolic Ca²⁺ (15;18). Stimulation with either

Ca^{2+} agonist induced a sustained increase in luminal pH (Figures 2a–b). Treatment of IBDU with the CFTR inhibitor CFTR_{inh}-172 did not block ACh-induced increases in luminal pH (n=3; Figure 2a). These findings show that confocal imaging of luminal pH in microperfused IBDU can be used to monitor agonist-induced ductular bicarbonate secretion, and demonstrate that cAMP-mediated secretion requires CFTR.

Effect of InsP3 receptor isoforms on ductular bicarbonate secretion

Ca^{2+} signaling in cholangiocytes is mediated entirely by the InsP3R, because this is the only type of intracellular Ca^{2+} release channel expressed in these cells (9). Cholangiocytes express all three isoforms of the InsP3R; the types I and II InsP3R are found throughout the cell, whereas the type III isoform is concentrated in the apical region (9). In order to understand the relative role of each isoform in the regulation of bicarbonate secretion, we used isoform-specific siRNA (13) to eliminate them from IBDU. The combination of siRNA for types I and II InsP3R was effective in simultaneously reducing expression of both isoforms in CHO cells (Figure 3a), whereas siRNA for type III InsP3R selectively reduced expression of that isoform (Figure 3b). Confocal immunofluorescence of IBDU in overnight culture demonstrated that the types I and II InsP3R are in the basolateral region of cholangiocytes (Figure 4a–b), whereas the type III InsP3R is concentrated in the apical region (Figure 4c), similar to what has been observed in cholangiocytes in the intact liver (8;9). Treatment of IBDU with siRNA for types I and II InsP3R selectively reduced expression of those isoforms in cholangiocytes (Figure 4a–b), whereas treatment with siRNA for type III InsP3R decreased expression of that isoform (Figure 4c). These findings demonstrate that this siRNA approach can be used to obtain microdissected IBDU with selective reduction of either type I and II (basolateral) or type III (apical) InsP3R.

IBDU with reduced expression of either type I and II or type III InsP3R were stimulated with ACh (100 μM) or forskolin (100 μM), to understand the relative roles of these isoforms in regulating each mode of bicarbonate secretion. The ACh-induced increase in luminal pH was reduced by 67% to 0.14 ± 0.03 pH units (n=3; $p < 0.05$) in IBDU lacking type I and II InsP3R, whereas the ACh-induced increase in luminal pH was not attenuated in IBDU lacking the type III InsP3R (n=3; Figure 5a–b). Similarly, the area under the curve (AUC) for ACh-induced changes in luminal pH over time was decreased in IBDU lacking type I and II InsP3R but not in IBDU lacking type III InsP3R ($p < 0.05$; Figure 5c). In contrast, the forskolin-induced increase in luminal pH was not significantly reduced in IBDU lacking type I and II InsP3R (n=3), but was reduced by 56% to 0.36 ± 0.03 pH units in IBDU lacking the type III InsP3R (n=3, $p < 0.05$; Figure 6a–b). Likewise, the AUC for forskolin-induced changes in luminal pH over time was decreased in IBDU lacking type III InsP3R but not in IBDU lacking type I and II InsP3R ($p < 0.05$; Figure 6c). These unexpected findings suggest that both Ca^{2+} - and cAMP-mediated secretory pathways depend upon InsP3Rs in cholangiocytes. This in turn suggests that not only ACh, but also forskolin would increase cytosolic Ca^{2+} in cholangiocytes. To test this, the effects of forskolin on Ca^{2+} were measured in intrahepatic bile duct segments isolated from rat livers, and then Ca^{2+} signaling in individual cells within the bile duct segments was examined using confocal microscopy (Figure 7) (8; 9). Bile ducts were stimulated with forskolin (100 μM), or with ACh (100 μM) as a positive control that is known to cause an InsP3-mediated increase in Ca^{2+} in this cell type (9). Both ACh (Figure 7a) and forskolin (Figure 7b) increased Ca^{2+} in cholangiocytes, although the forskolin-induced increase was more gradual. Suramin (50 μM) blocked the Ca^{2+} increase induced by forskolin ($p < 0.001$; Figure 7b–c). In separate studies, the effects of forskolin on Ca^{2+} were examined with fura-2 in polarized rat cholangiocytes in cell culture (17). Forskolin (100 μM) increased cytosolic Ca^{2+} in this cell system as well. This demonstrates that forskolin, like ACh, increases cytosolic Ca^{2+} in cholangiocytes, but the forskolin-induced Ca^{2+} signal is mediated by activation of P2Y receptors. Together, these findings suggest that the classical Ca^{2+} -mediated pathway for bicarbonate secretion depends

upon basolateral InsP3Rs, whereas cAMP-mediated bicarbonate secretion depends upon apical InsP3Rs.

Luminal ATP is necessary for cyclic AMP-mediated bicarbonate secretion

Cholangiocytes express apical P2Y nucleotide receptors (15), which link to InsP3R-mediated Ca^{2+} signaling (7). Because forskolin-mediated secretion involves CFTR (Figure 1b) (3–5), and activation of CFTR in other systems may result in ATP release (19–21), we investigated whether forskolin-mediated secretion involves autocrine signaling via luminal ATP. IBDU lumens were perfused with either apyrase to hydrolyze luminal ATP or suramin to block apical P2Y receptors (22). Apyrase reduced the forskolin-induced increase in luminal pH by 86%, and suramin reduced the forskolin-induced increase by 90% ($n=6$, $p<0.01$; Figure 8a–b). Apyrase and suramin both reduced the AUC for forskolin-induced alkalization of the lumen as well ($p<0.05$; Figure 8c). In contrast, neither apyrase nor suramin attenuated the increase in luminal pH or the AUC that was induced by ACh ($n=6$; Figure 8d–f). Finally, treatment of IBDU with suramin prevented the forskolin-induced increase in Ca^{2+} in cholangiocytes (Figure 7b–c), and treatment of IBDU with the cytosolic Ca^{2+} chelator BAPTA prevented the forskolin-induced increase in luminal pH ($p<0.001$; Figure 8a–c). These findings provide evidence that the ductular bicarbonate secretion that is induced by cAMP results from release of ATP into the lumen followed by stimulation of apical P2Y receptors, followed by an increase in cytoplasmic Ca^{2+} , and then Ca^{2+} -mediated bicarbonate secretion.

DISCUSSION

Bicarbonate secretion is a primary function of the cholangiocyte (2). The prototypical and most extensively characterized pathway for bicarbonate secretion is initiated by stimulation of the secretin receptor, which leads to formation of cAMP, followed by activation of CFTR, and then CFTR-dependent chloride transport, and finally chloride-bicarbonate exchange. Stimulation of receptors that link to G_q rather than G_s , and then to InsP3-mediated Ca^{2+} signals and activation of Ca^{2+} -dependent chloride channels and chloride-bicarbonate exchange are thought to represent an alternative or secondary secretory pathway (3–5). Evidence from bivascularly perfused rat liver studies suggests a slightly different model, in which Ca^{2+} -induced bicarbonate excretion depends on chloride channels as well as bicarbonate exchange, but secretin-induced bicarbonate excretion occurs independent of chloride-bicarbonate exchange (2). Evidence in other secretory epithelia similarly suggests that CFTR may directly secrete bicarbonate (23;24). Here we report the unexpected result that cAMP-mediated secretion also depends upon InsP3R expression and Ca^{2+} signaling. Specifically, our findings suggested that increases in cAMP result in apical secretion of ATP, leading to stimulation of apical P2Y nucleotide receptors and then activation of apical, type III InsP3Rs (Figure 9). We used the P2Y receptor antagonist suramin to provide evidence that forskolin induces ATP release. Although one report suggests that suramin can also block CFTR (25), in that study suramin was found to be cell-impermeant and had to be applied to the cytoplasmic side of CFTR to be inhibitory. A more recent study suggests suramin does not inhibit CFTR (26), and in our study we applied suramin extracellularly (to the luminal side of cholangiocytes) and we found apyrase and BAPTA to similarly block forskolin-induced bicarbonate secretion. Together, these results suggest that cAMP acts through an autocrine loop that involves ATP release, activation of P2Y receptors, and then an increase in cytosolic Ca^{2+} . This in turn suggests a convergence of secretory pathways in the cholangiocyte at the level of InsP3R-mediated Ca^{2+} release. Both ACh and forskolin were found to increase cytosolic Ca^{2+} in cholangiocytes, providing further evidence that InsP3R-mediated Ca^{2+} signaling occurs even in cAMP-mediated ductular secretion.

Forskolin-induced bicarbonate secretion was blocked by inhibition of CFTR, as well as by knockdown of type III InsP3R and by inhibition of autocrine ATP signaling, suggesting that these steps are linked in cAMP-induced secretion (Figure 9). The current findings also clarify the significance of the previous observation that loss of InsP3R expression is uniformly observed in patients with cholestatic disorders including primary biliary cirrhosis, primary sclerosing cholangitis, extrahepatic biliary obstruction, and biliary atresia (8). It had not been clear whether loss of InsP3Rs was the cause or effect of cholestasis. However, the current work shows that loss of InsP3Rs specifically reduces ductular bicarbonate secretion. Because both cAMP- and Ca^{2+} -mediated secretion depend upon expression of InsP3Rs, loss of InsP3Rs thus indeed may serve as a unifying event that is responsible for cholestasis.

The presence of ATP in bile is well established (16;27). However, the endogenous pathways that lead to the appearance of ATP in bile under normal conditions have been less clear. Pathological conditions such as cell swelling result in release of ATP from both hepatocytes (28) and cholangiocytes (29). In addition, the therapeutic bile acids ursodeoxycholate and tauroursodeoxycholate induce hepatocytes to secrete ATP into bile (16). The current work establishes cAMP-induced, autocrine release of ATP from cholangiocytes as a physiological route by which ATP appears in bile. Similarly, the presence of apical P2Y receptors on cholangiocytes is well established (15;30) but their physiological role has been less clear. Stimulation of these receptors causes a more potent increase in cytosolic Ca^{2+} and bicarbonate secretion than occurs after stimulation of basolateral P2Y receptors or M3 muscarinic receptors (15), even though all of these receptors links to G_q -mediated InsP3 formation. Previous studies of polarized nasal epithelia suggest that stimulation of either apical or basolateral P2Y receptors selectively mobilizes only ipsilateral Ca^{2+} stores (31), and the current work suggests that cholangiocytes behave similarly. Furthermore, our findings provide a molecular basis for this observation by providing evidence that basolateral membrane receptors link to activation of nearby type I and II InsP3Rs, whereas apical membrane receptors link to activation of nearby type III InsP3Rs. Because InsP3Rs are most heavily expressed in the apical region (8;9), this may explain why stimulation of apical P2Y receptors is more effective than stimulation of basolateral P2Y receptors for increasing Ca^{2+} and inducing bicarbonate secretion. A number of other epithelia similarly express apical P2Y receptors plus apical Ca^{2+} -dependent chloride channels, including pancreatic (32) and submandibular (33;34) duct cells and airway epithelia (35;36), so the autocrine system for regulation of secretion described here for cholangiocytes may be of more general significance.

What is the mechanism by which ATP is released from cholangiocytes? Available evidence suggests two possibilities. First, ATP may be released from apical vesicles by exocytosis. This mechanism is well-described in neurons, where ATP serves as a neurotransmitter and is released from presynaptic vesicles (37). This mode of ATP secretion may also occur in epithelia, because vesicular transport depends upon microtubules (3), and microtubule inhibitors block ATP release in Schwann cells (38). Moreover, cAMP stimulates vesicular exocytosis in hepatocytes (39;40) as well as in cholangiocytes (41). Alternatively, ATP secretion may be indirectly or directly due to CFTR. Several studies have provided evidence that CFTR causes (21) or regulates (20) cellular ATP release. For example, atomic force microscopic measurements have detected ATP release from individual CFTR channels (19), while electrophysiologic studies suggest that CFTR regulates an associated but distinct ATP-conducting channel (42). On the other hand, cell systems that co-express CFTR and P2Y receptors (43) have failed to produce the autocrine signaling pattern that is reported here, so the role of CFTR in mediating ATP release is controversial, and may be cell type-specific. However, our observation that a specific CFTR inhibitor blocks forskolin-induced bicarbonate secretion is consistent with the idea that CFTR is related to ATP release in cholangiocytes. Moreover, there is recent evidence that ursodeoxycholate induces ATP release from cholangiocytes, but that this does not occur in CFTR-defective mice (Mario Strazzabosco,

unpublished observation). That observation provides a more direct link between CFTR and ATP secretion in cholangiocytes. Therefore, CFTR is likely to be important for cAMP-induced ATP secretion in cholangiocytes, although the mechanism by which CFTR mediates this remains a question.

The current work provides new insight into current and future treatment strategies for cholestatic disorders. Because cAMP-mediated secretion depends upon stimulation of apical P2Y receptors, agents that increase biliary ATP would be expected to stimulate ductular secretion. Ursodeoxycholate induces hepatocytes to secrete ATP and increases the concentration of ATP in bile (16), so it could be predicted on this basis that this bile acid would stimulate bile flow and bicarbonate secretion and would be of potential benefit in cholestasis. Indeed, these are precisely the properties this bile acid displays (44), although the mechanism by which ursodeoxycholate exhibits its therapeutic effect is controversial and may be multifactorial (45). Direct luminal administration of ATP or other nucleotides would be expected to have a similar beneficial effect on bile flow, and aerosolized UTP has been tried to treat pulmonary manifestations of cystic fibrosis (46;47), which has a similar pathophysiology. However, this therapeutic approach has not been effective (46;47), presumably because ecto-ATPases in the bronchial tree rapidly degrade exogenous nucleotides (48). Canalicular ATPases (49) would be expected to similarly degrade nucleotides that are exogenously administered to the biliary tree, and delivery of drugs to the biliary tree represents an additional challenge. Aerosolized administration of long-lasting nucleotide analogs is under investigation to treat pulmonary manifestations of cystic fibrosis (50;51), so targeting of such agents to the liver could provide a novel approach to treat cholestasis.

Acknowledgements

This work was supported by NIH grants DK45710, DK61747, and DK34989.

References

1. Nathanson MH, Burgstahler AD, Mennone A, Dranoff JA, Rios-Velez L. Stimulation of bile duct epithelial secretion by glybenclamide in normal and cholestatic rat liver. *J Clin Invest* 1998;101:2665–2676. [PubMed: 9637700]
2. Hirata K, Nathanson MH. Bile duct epithelia regulate biliary bicarbonate excretion in normal rat liver. *Gastroenterology* 2001;121:396–406. [PubMed: 11487549]
3. Boyer JL; Nathanson, MH. Bile formation. In: Schiff, ER.; Sorrell, MF.; Maddrey, WC., editors. *Diseases of the liver*. 1999. p. 119–146.
4. Lazaridis KN, Strazzabosco M, Larusso NF. The cholangiopathies: disorders of biliary epithelia. *Gastroenterology* 2004;127:1565–1577. [PubMed: 15521023]
5. Kanno N, LeSage G, Glaser S, Alpini G. Regulation of cholangiocyte bicarbonate secretion. *Am J Physiol Gastrointest Liver Physiol* 2001;281:G612–G625. [PubMed: 11518673]
6. Colombo C, Battezzati PM, Crosignani A, Morabito A, Costantini D, Padoan R, Giunta A. Liver disease in cystic fibrosis: A prospective study on incidence, risk factors, and outcome. *Hepatology* 2002;36:1374–1382. [PubMed: 12447862]
7. Nathanson MH, Burgstahler AD, Mennone A, Boyer JL. Characterization of cytosolic Ca²⁺ signaling in rat bile duct epithelia. *Am J Physiol* 1996;271:G86–G96. [PubMed: 8760111]
8. Shibao K, Hirata K, Robert ME, Nathanson MH. Loss of inositol 1,4,5-trisphosphate receptors from bile duct epithelia is a common event in cholestasis. *Gastroenterology* 2003;125:1175–1187. [PubMed: 14517800]
9. Hirata K, Dufour JF, Shibao K, Knickelbein R, O'Neill AF, Bode HP, Cassio D, St Pierre MV, Larusso NF, Leite MF, Nathanson MH. Regulation of Ca(2+) signaling in rat bile duct epithelia by inositol 1,4,5-trisphosphate receptor isoforms. *Hepatology* 2002;36:284–296. [PubMed: 12143036]
10. Akiba Y, Jung M, Ouk S, Kaunitz JD. A novel small molecule CFTR inhibitor attenuates. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G753–G759. [PubMed: 15905414]

11. Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galletta LJ, Verkman AS. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J Clin Invest* 2002;110:1651–1658. [PubMed: 12464670]
12. Masyuk AI, Gong AY, Kip S, Burke MJ, LaRusso NF. Perfused rat intrahepatic bile ducts secrete and absorb water, solute, and ions. *Gastroenterology* 2000;119:1672–1680. [PubMed: 11113089]
13. Mendes CC, Gomes DA, Thompson M, Souto NC, Goes TS, Goes AM, Rodrigues MA, Gomez MV, Nathanson MH, Leite MF. The type III inositol 1,4,5-trisphosphate receptor preferentially transmits apoptotic Ca²⁺ signals into mitochondria. *J Biol Chem* 2005;280:40892–40900. [PubMed: 16192275]
14. Splinter PL, Masyuk AI, LaRusso NF. Specific inhibition of AQP1 water channels in isolated rat intrahepatic bile duct units by small interfering RNAs. *J Biol Chem* 2003;278:6268–6274. [PubMed: 12468529]
15. Dranoff JA, Masyuk AI, Kruglov EA, LaRusso NF, Nathanson MH. Polarized expression and function of P2Y ATP receptors in rat bile duct epithelia. *Am J Physiol Gastrointest Liver Physiol* 2001;281:G1059–G1067. [PubMed: 11557527]
16. Nathanson MH, Burgstahler AD, Masyuk A, Larusso NF. Stimulation of ATP secretion in the liver by therapeutic bile acids. *Biochem J* 2001;358:1–5. [PubMed: 11485545]
17. Alpini G, Phinzy JL, Glaser S, Francis H, Benedetti A, Marucci L, LeSage G. Development and characterization of secretin-stimulated secretion of cultured rat cholangiocytes. *Am J Physiol Gastrointest Liver Physiol* 2003;284:G1066–G1073. [PubMed: 12540366]
18. Alvaro D, Alpini G, Jezequel AM, Bassotti C, Francia C, Fraioli F, Romeo R, Marucci L, Le Sage G, Glaser SS, Benedetti A. Role and mechanisms of action of acetylcholine in the regulation of rat cholangiocyte secretory functions. *J Clin Invest* 1997;100:1349–1362. [PubMed: 9294100]
19. Schneider SW, Egan ME, Jena BP, Guggino WB, Oberleithner H, Geibel JP. Continuous detection of extracellular ATP on living cells by using atomic force microscopy. *Proc Natl Acad Sci U S A* 1999;96:12180–12185. [PubMed: 10518596]
20. Schwiebert EM, Egan ME, Hwang TH, Fulmer SB, Allen SS, Cutting GR, Guggino WB. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* 1995;81:1063–1073. [PubMed: 7541313]
21. Reisin IL, Prat AG, Abraham EH, Amara JF, Gregory RJ, Ausiello DA, Cantiello HF. The cystic fibrosis transmembrane conductance regulator is a dual ATP and chloride channel. *J Biol Chem* 1994;269:20584–20591. [PubMed: 7519611]
22. Schlosser SF, Burgstahler AD, Nathanson MH. Isolated rat hepatocytes can signal to other hepatocytes and bile duct cells by release of nucleotides. *Proc Natl Acad Sci U S A* 1996;93:9948–9953. [PubMed: 8790437]
23. Poulsen JH, Fischer H, Illek B, Machen TE. Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator. *Proc Natl Acad Sci U S A* 1994;91:5340–5344. [PubMed: 7515498]
24. Clarke LL, Harline MC. Dual role of CFTR in cAMP-stimulated. *Am J Physiol* 1998;274:G718–G726. [PubMed: 9575854]
25. Bachmann A, Russ U, Quast U. Potent inhibition of the CFTR chloride channel by suramin. *Naunyn Schmiedeberg's Arch Pharmacol* 1999;360:473–476. [PubMed: 10551285]
26. Yamamoto-Mizuma S, Wang GX, Hume JR. P2Y purinergic receptor regulation of CFTR chloride channels in mouse cardiac myocytes. *J Physiol* 2004;556:727–737. [PubMed: 14978203]
27. Chari RS, Schutz SM, Haebig JE, Shimokura GH, Cotton PB, Fitz JG, Meyers WC. Adenosine nucleotides in bile. *Am J Physiol* 1996;270:G246–G252. [PubMed: 8779965]
28. Wang Y, Roman R, Lidofsky SD, Fitz JG. Autocrine signaling through ATP release represents a novel mechanism for cell volume regulation. *Proc Natl Acad Sci U S A* 1996;93:12020–12025. [PubMed: 8876255]
29. Roman RM, Feranchak AP, Salter KD, Wang Y, Fitz JG. Endogenous ATP release regulates Cl[−] secretion in cultured human and rat biliary epithelial cells. *Am J Physiol* 1999;276:G1391–G1400. [PubMed: 10362642]
30. Salter KD, Fitz JG, Roman RM. Domain-specific purinergic signaling in polarized rat cholangiocytes. *Am J Physiol Gastrointest Liver Physiol* 2000;278:G492–G500. [PubMed: 10712270]

31. Paradiso AM, Mason SJ, Lazarowski ER, Boucher RC. Membrane-restricted regulation of Ca²⁺ release and influx in polarized epithelia. *Nature* 1995;377:643–646. [PubMed: 7566178]
32. Zsembery A, Strazzabosco M, Graf J. Ca²⁺-activated Cl⁻ channels can substitute for CFTR in stimulation of pancreatic duct bicarbonate secretion. *FASEB J* 2000;14:2345–2356. [PubMed: 11053257]
33. Lee MG, Zeng W, Muallem S. Characterization and localization of P2 receptors in rat submandibular gland acinar and duct cells. *J Biol Chem* 1997;272:32951–32955. [PubMed: 9407074]
34. Zeng W, Lee MG, Muallem S. Membrane-specific regulation of Cl⁻ channels by purinergic receptors in rat submandibular gland acinar and duct cells. *J Biol Chem* 1997;272:32956–32965. [PubMed: 9407075]
35. Cressman VL, Lazarowski E, Homolya L, Boucher RC, Koller BH, Grubb BR. Effect of loss of P2Y₂ (2) receptor gene expression on nucleotide regulation of murine epithelial Cl⁻ transport. *J Biol Chem* 1999;274:26461–26468. [PubMed: 10473606]
36. Homolya L, Watt WC, Lazarowski ER, Koller BH, Boucher RC. Nucleotide-regulated calcium signaling in lung fibroblasts and epithelial cells from normal and P2Y₂ (2) receptor (–/–) mice. *J Biol Chem* 1999;274:26454–26460. [PubMed: 10473605]
37. Sneddon P, McLaren GJ, Kennedy C. Purinergic cotransmission: Sympathetic nerves. *Seminars in the Neurosciences* 1996;8:201–205.
38. Liu GJ, Werry EL, Bennett MR. Secretion of ATP from Schwann cells in response to uridine triphosphate. *Eur J Neurosci* 2005;21:151–160. [PubMed: 15654852]
39. Boyer JL, Soroka CJ. Vesicle targeting to the apical domain regulates bile excretory function in isolated rat hepatocyte couplets. *Gastroenterology* 1995;109:1600–1611. [PubMed: 7557144]
40. Hayakawa T, Bruck R, Ng OC, Boyer JL. DBcAMP stimulates vesicle transport and HRP excretion in isolated perfused rat liver. *Am J Physiol* 1990;259:G727–G735. [PubMed: 2173414]
41. Kato A, Gores GJ, LaRusso NF. Secretin stimulates exocytosis in isolated bile duct epithelial cells by a cyclic AMP-mediated mechanism. *J Biol Chem* 1992;267:15523–15529. [PubMed: 1322400]
42. Sugita M, Yue Y, Foskett JK. CFTR Cl⁻ channel and CFTR-associated ATP channel: distinct pores regulated by common gates. *EMBO J* 1998;17:898–908. [PubMed: 9463368]
43. Watt WC, Lazarowski ER, Boucher RC. Cystic fibrosis transmembrane regulator-independent release of ATP. Its implications for the regulation of P2Y₂ receptors in airway epithelia. *J Biol Chem* 1998;273:14053–14058. [PubMed: 9593757]
44. Dumont M, Erlinger S, Uchman S. Hypercholeresis induced by ursodeoxycholic acid and 7-ketolithocholic acid in the rat: possible role of bicarbonate transport. *Gastroenterology* 1980;79:82–89. [PubMed: 7380227]
45. Beuers U, Boyer JL, Paumgartner G. Ursodeoxycholic acid in cholestasis: potential mechanisms of action and therapeutic applications. *Hepatology* 1998;28:1449–1453. [PubMed: 9828205]
46. Knowles MR, Olivier KN, Hohneker KW, Robinson J, Bennett WD, Boucher RC. Pharmacologic treatment of abnormal ion transport in the airway epithelium in cystic fibrosis. *Chest* 1995;107:71S–76S. [PubMed: 7842817]
47. Bennett WD, Olivier KN, Zeman KL, Hohneker KW, Boucher RC, Knowles MR. Effect of uridine 5'-triphosphate plus amiloride on mucociliary clearance in adult cystic fibrosis. *Am J Respir Crit Care Med* 1996;153:1796–1801. [PubMed: 8665037]
48. Picher M, Burch LH, Boucher RC. Metabolism of P2 receptor agonists in human airways: implications for mucociliary clearance and cystic fibrosis. *J Biol Chem* 2004;279:20234–20241. [PubMed: 14993227]
49. Lin SH. Localization of the ecto-ATPase (ecto-nucleotidase) in the rat hepatocyte plasma membrane. Implications for the functions of the ecto-ATPase. *J Biol Chem* 1989;264:14403–14407. [PubMed: 2527234]
50. Noone PG, Hamblett N, Accurso F, Aitken ML, Boyle M, Dovey M, Gibson R, Johnson C, Kellerman D, Konstan MW, Milgram L, Mundahl J, Retsch-Bogort G, Rodman D, Williams-Warren J, Wilmott RW, Zeitlin P, Ramsey B. Safety of aerosolized INS 365 in patients with mild to moderate cystic fibrosis: results of a phase I multi-center study. *Pediatr Pulmonol* 2001;32:122–128. [PubMed: 11477729]

51. Deterding R, Retsch-Bogart G, Milgram L, Gibson R, Daines C, Zeitlin PL, Milla C, Marshall B, Lavange L, Engels J, Mathews D, Gorden J, Schaberg A, Williams J, Ramsey B. Safety and tolerability of denufosal tetrasodium inhalation solution, a novel P2Y2 receptor agonist: results of a phase 1/phase 2 multicenter study in mild to moderate cystic fibrosis. *Pediatr Pulmonol* 2005;39:339–348. [PubMed: 15704203]

Figure 1a

Figure 1b

Figure 1.

Measurement of pH in microdissected, microperfused bile ducts stimulated with forskolin (100 μ M). **(a)** Confocal image of a microperfused bile ductule. **(b)** Forskolin-induced bicarbonate secretion is similar in freshly isolated bile ducts and ducts in culture for 24 hr, while the CFTR inhibitor CFTR_{inh}-172 blocks forskolin-induced increases in ductular pH. Values here and in subsequent tracings are mean $\pm$ SEM (n=3 experiments under each condition).

Figure 2a

Figure 2b

Figure 2.

Effects of ACh and ATP on ductular pH. Each agent was added to the bathing medium for basolateral stimulation. **(a)** ACh (100 μM)-induced increase in ductular pH (n=3 ductules). The CFTR inhibitor CFTR_{inh}-172 does not block the effects of ACh. **(b)** ATP (100 μM)-induced increase in ductular pH (n=3 ductules).

Figure 3a

Figure 3b

Figure 3.

Validation of siRNA for each isoform of the InsP3R. **(a)** co-treatment of CHO cells with siRNA for type I and II InsP3R reduces expression of both isoforms. Densitometric values are mean $\pm$ SEM of 2 replicates ($p < 0.01$). **(b)** Treatment of CHO cells with siRNA for type III InsP3R reduces expression of that isoform. Densitometric values are mean $\pm$ SEM of 3 replicates ($p < 0.01$). The isoform specificity of each siRNA construct has been demonstrated previously (13).

a **Figure 4****b** **Figure 4**

C

Figure 4

Figure 4.

Use of siRNA to decrease expression of specific InsP3R isoforms in microdissected bile ducts. **(a)** Type I InsP3R (InsP3R-1) is distributed throughout cholangiocytes (left) and expression is undetectable after treatment with isoform-specific siRNA (right). Ducts are triple-labeled for type I (red) and type III (green) InsP3R and nuclei (blue). **(b)** Type II InsP3R (InsP3R-2) is distributed throughout cholangiocytes (left) and expression is undetectable after treatment with isoform-specific siRNA (right). Ducts are triple-labeled for type II (red) and type III (green) InsP3R and nuclei (blue). **(c)** Type III InsP3R (InsP3R-3) is concentrated in the apical region of cholangiocytes (left) and expression is undetectable after treatment with isoform-specific siRNA (right). Ducts are double-labeled for type III InsP3R (green) and nuclei (blue). Cross-sections of each duct are based on three-dimensional reconstructions of confocal images collected from serial focal planes.

Figure 5a **Figure 5b**

Figure 5c

Figure 5.

Loss of the types I and II InsP3R selectively impairs ACh-induced bicarbonate secretion. **(a)** Tracings show that ACh (100 μ M) increases ductular pH under control conditions and after treatment with siRNA for type III InsP3R, but the effect of ACh is blocked after treatment with siRNA for types I and II InsP3R. Values are mean $\pm$ SEM of $n=3$ experiments under each condition. **(b)** Bar graph summary of the peak increase in luminal pH under each experimental condition (* $p<0.05$). **(c)** Bar graph summary of the area under the curve (AUC) for luminal pH during 25 min of stimulation with ACh under each experimental condition (* $p<0.05$).

Figure 6a **Figure 6b**

Figure 6c

Figure 6.

Loss of the type III InsP3R selectively impairs forskolin-induced bicarbonate secretion. **(a)** Tracings show that forskolin (100 μ M) increases ductular pH in bile ducts in culture for 24 hr that have been treated with scrambled siRNA (control group) and after treatment with siRNA for types I and II InsP3R, but the effect of forskolin is significantly reduced after treatment with siRNA for type III InsP3R. Values are mean $\pm$ SEM of n=3 experiments under each condition. **(b)** Bar graph summary of the peak increase in luminal pH under each experimental condition (*p<0.05). **(c)** Bar graph summary of the AUC for luminal pH under each experimental condition (*p<0.05).

Figure 7.

Forskolin increases cytosolic Ca^{2+} in cholangiocytes. **(a)** ACh (100 μM) induces a rapid, transient increase in Ca^{2+} , as has been described previously (9). **(b)** Forskolin (100 μM) induces a gradual, progressive increase in Ca^{2+} , which is blocked by suramin (50 μM). Tracings depict Ca^{2+} signals measured by confocal microscopy in individual cholangiocytes within isolated intrahepatic bile duct segments. **(c)** Bar graph summary of results. Each result is mean $\pm$ SEM of the peak response elicited in >20 cells from at least three separate bile duct preparations under each experimental condition.

Figure 8a **Figure 8b**

Figure 8c **Figure 8d**

Figure 8e

Figure 8f

Figure 8.

Forskolin- but not ACh-induced bicarbonate secretion is mediated by luminal ATP. (a) pH tracings, (b) bar graph summary of the peak increase in luminal pH, and (c) bar graph summary of the AUC for luminal pH after treatment with forskolin under control conditions and after luminal administration of the ATP hydrolyzing agent apyrase (3 U/mL), the P2Y receptor antagonist suramin (50 μ M), or the cytosolic Ca^{2+} chelator BAPTA/AM (30 μ M). (d) pH tracings, (e) bar graph summary of the peak increase in luminal pH, and (f) bar graph summary

of the AUC for luminal pH after treatment with ACh under control conditions and after luminal administration of either apyrase or suramin. Values are mean $\pm$ SEM of 3 separate measurements (*p<0.05).