Endogenous ATP release regulates Cl⁻ secretion in cultured human and rat biliary epithelial cells

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Roman, Richard M., Andrew P. Feranchak, Kelli D. Salter, Yu Wang, and J. Gregory Fitz. Endogenous ATP release regulates Cl⁻ secretion in cultured human and rat biliary epithelial cells. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1391–G1400, 1999.—P2Y receptor stimulation increases membrane Cl⁻ permeability in biliary epithelial cells, but the source of extracellular nucleotides and physiological relevance of purinergic signaling to biliary secretion are unknown. Our objectives were to determine whether biliary cells release ATP under physiological conditions and whether extracellular ATP contributes to cell volume regulation and transepithelial secretion. With the use of a sensitive bioluminescence assay, constitutive ATP release was detected from human Mz-ChA-1 cholangiocarcinoma cells and polarized normal rat cholangiocyte monolayers. ATP release increased rapidly during cell swelling induced by hypotonic exposure. In Mz-ChA-1 cells, removal of extracellular ATP (apyrase) and P2 receptor blockade (suramin) reversibly inhibited whole cell Cl⁻ current activation and prevented cell volume recovery during hypotonic stress. Moreover, exposure to apyrase induced cell swelling under isotonic conditions. In intact normal rat cholangiocyte monolayers, hypotonic perfusion activated apical Cl⁻ currents, which were inhibited by addition of apyrase and suramin to bathing media. These findings indicate that modulation of ATP release by the cellular hydration state represents a potential signal coordinating cell volume with membrane Cl⁻ permeability and transepithelial Cl⁻ secretion.

PURINERGIC SIGNALING pathways contribute to the regulation of physiological functions in many, if not all, tissues. Within the extracellular environment, ATP and UTP act as versatile autocrine and paracrine effectors that bind selectively to membrane P2 receptors (4). The response to these agonists may be quite complex inasmuch as de- or transphosphorylation to other nucleotides and nucleosides produces agonists that recognize different P2 and P1 receptors, and cells often express many receptor subtypes coupled to distinct biological pathways (14, 15). In neurons and circulating blood cells, ATP stored in intracellular vesicles is released during exocytosis (37, 38). However, epithelial cells are also capable of releasing ATP and/or UTP in response to physiological stimuli, including changes in cell volume, membrane stress, hypoxia, and receptor stimulation (2, 19, 21, 24). One mechanism for cellular ATP efflux may be electrodiffusional, with charged ATP⁻ molecules permeating selective membrane channels down an ~10,000-fold concentration gradient (30, 34). Although not well defined, epithelial release of ATP would be expected to specifically modify tissue responses by regulating the local extracellular availability of purinergic agonists.

In liver, purinergic stimulation modulates many fundamental processes, including fatty acid and protein metabolism, glucose availability, and hepatic perfusion (13, 16, 23). Some of these effects appear to require sophisticated paracrine communication among different cell types. In the perfused liver model, for example, ATP released by hepatocytes during adenosine stimulation induces eicosanoid efflux from nonparenchymal cells, which then activate glycogenolysis (26). Recently, ATP in physiological concentrations has been documented in human bile (5). This is intriguing because ATP in bile would be expected to bind to P2Y2 receptors in the apical membrane of cholangiocytes. P2Y2 stimulation enhances secretion across polarized cholangiocyte monolayers, a response analogous in many ways to that observed in other epithelial models (31). Although such a mechanism could contribute directly to bile salt-independent modulation of bile flow, neither the cellular origin of luminal ATP nor the physiological stimuli that regulate ATP release have been identified.

In view of the pivotal role of cholangiocyte secretion in bile formation, the objectives of these studies were threefold: first, to characterize ATP release from nonpolarized and polarized biliary epithelia under basal conditions and to determine whether cell volume increases stimulate ATP efflux; second, to establish the role of extracellular ATP as an autocrine regulator of Cl⁻ permeability and cell volume; finally, to determine whether volume-sensitive ATP release modulates epithelial Cl⁻ secretion as an endogenous autocrine and paracrine signaling molecule.

MATERIALS AND METHODS

Cells. Studies in isolated cells were performed using Mz-ChA-1 cells from human cholangiocarcinoma and in polarized monolayers using normal rat cholangiocytes (NRCs). Mz-ChA-1 are model biliary cells that express cytoskeletal proteins, purinergic receptors, and regulated Cl⁻ channels analogous to primary cholangiocytes (20, 22). After increases in cell volume, Mz-ChA-1 cells exhibit a regulatory volume decrease (RVD) toward basal values mediated in part by opening of membrane Cl⁻ channels (29). NRCs are derived from intrahepatic cholangiocytes and also maintain expression of differentiated biliary markers in culture. Unlike Mz-ChA-1 cells, NRCs form polarized monolayers with intercellular tight junctions and apical microvilli (39); when they are inserted into Ussing chambers, exposure to secretory agonists leads to
an increase in short-circuit current \((I_{sc})\) (31). Cells were maintained and passaged as previously described (29, 31).

Solutions. Unless indicated, the standard extracellular NaCl solution used for all studies contained (in mM) 140 NaCl, 4 KCl, 1 KH\(_2\)PO\(_4\), 2 MgCl\(_2\), 1 CaCl\(_2\), 10 glucose, and 10 HEPES-NaOH (pH 7.40) with a total Cl\(^-\) of 150 mM. Solution osmolarity (vapor-pressure osmometer; model 5500, Wescon, Logan, UT) was \(-295\) mosM. Increases in cell volume were induced by exposure to hypotonic extracellular buffer, which was prepared by 1) lowering the NaCl concentration [Coulter multisizer, patch clamp (Cl\(^-\) currents), Ussing chamber], 2) adding water [luciferase-luciferin assay, patch clamp (ATP currents)], or 3) exchanging mannitol-containing with mannitol-free extracellular NaCl-rich buffer (Ussing chamber). Exposure to hypotonic stress (15–30% reduction in osmolarity) did not affect cell viability over 1 h (propidium iodide staining, data not shown).

Reagents. 5-Nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Amiloride, adenosine 5’-O-(3-thiotriphosphate) (ATP\(_S\)), apyrase, and suramin were obtained from Sigma (St. Louis, MO).

Bioluminescence ATP detection assay. The methods used are similar to those recently published (8, 35). Mz-CHA-1 cells grown to confluence in 35-mm dishes (surface area 9.62 cm\(^2\)) were used to determine “bulk” cellular ATP release. To measure “vectorial” ATP release from cholangiocyte monolayers, NRCs were plated on collagen-coated semipermeable supports (Millicell HA, diameter 12 mm, surface area 1.13 cm\(^2\), Millipore-Fisher, Bedford, MA) and were studied when transepithelial resistance exceeded 1,000 Ω·cm\(^2\) (EVOHM, World Precision Instruments, Sarasota FL). Before study cells were washed twice with PBS, and serum-free Optimem-1 medium containing 2 mg/ml luciferase-luciferin (lyophilized reagent, Calbiochem, La Jolla, CA) was added directly to cells. ATP released from cells into media catalyzes the oxidation of luciferin, generating bioluminescence. For Mz-CHA-1 cells, bulk ATP release into media was measured (initial volume 600 µl). Semipermeable supports containing NRC cells were placed on 35-mm culture dishes in a 200-µl volume of media to bath the apical side, and 200 µl of media were then added to the apical chamber. Openings in the support allow direct access to basolateral buffer. To selectively measure ATP efflux from either apical or basolateral NRC membranes, the luciferase-luciferin reagent was added to medium on one side of the monolayer only (the side in which ATP is detected), and medium without reagent was added to the other side (Fig. 1). Dishes containing Mz-CHA-1 cells and NRC-containing inserts were placed on a platform and lowered directly into a chamber in complete darkness within a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA), and cumulative bioluminescence over 15-s intervals was quantified in real time as arbitrary light units (ALU). Cell volume increases were induced by adding water to dilute media 20 and 40%; identical volumes of isotonic media were added in control studies to dissociate the effects of volume changes from membrane perturbation on ATP release. Luciferase-luciferin concentrations more than fivefold less did not change detected luminescence. All solutions (water, media, reagents) that were added to cells contained identical amounts of luciferase-luciferin so that the reagent would not be diluted. Addition of apyrase (2 U/ml) to extracellular media to hydrolyze ATP eliminated bioluminescence. All studies were performed at room temperature.

Bioluminescence assay standardization. Background luminescence (cells plus media without luciferase-luciferin) was checked before each experiment and was always <0.05 ALU. To approximate ATP released from cells, standard curves of ATP (ATP free acid, Calbiochem) at known concentrations were performed by adding serial dilutions of ATP stock (freshly made at the time of measurements) into 200 µl Optimem-1 medium containing 2 mg/ml luciferase-luciferin. Dilution of media 20–40% with water and addition of isotonic media (all solutions containing luciferase-luciferin) did not significantly affect luminescence detected for given concentrations of ATP. Multiple measurements for each dose of ATP were performed, and luminescence values were stable among all measurements (no evidence of bleaching or signal instability).

Cell volume. Mean Mz-CHA-1 cell volume was measured in cell suspensions by electronic cell sizing (Coulter multisizer, Hialeah, FL) using an aperture of 100 µm as previously described (30). Measurements of ~20,000 cells in suspension at intervals to 30 min after exposure to hypotonic buffer (30% less mosmol) were compared with basal values in isotonic buffer; values are expressed as relative volume normalized to the basal period.

Measurement of Cl\(^-\) and ATP currents. Membrane Cl\(^-\) and ATP currents were measured and analyzed using whole cell patch-clamp techniques as previously described (22, 30, 40). Isolated Mz-CHA-1 cells plated on coverslips were studied after ~24 h in a chamber perfused with extracellular buffer (chamber volume ~400 µl, flow 4–5 ml/min). For measurement of Cl\(^-\) currents, the intracellular (pipette) solution contained (in mM) 130 KCl, 10 NaCl, 2 MgCl\(_2\), 10 HEPES-KOH, 1 ATP, 0.5 CaCl\(_2\), and 1 EGTA (pH 7.3), corresponding to a free Ca\(^{2+}\) concentration of ~100 nM. To enhance detec-
tion of currents carried by charged ATP molecules, high concentrations of ATP were included in bath and pipette solutions as the predominant charge carrier (100 mM MgATP, 5 mM MgCl₂, 10 mM HEPES, pH ~7.4 TrisOH) (40).

Voltage-clamp measurements of Iₛₜc. NRCs were grown on collagen-treated polytetrafluoroethylene membrane filters (Corning Costar, Acton, MA) and used for experiments when the transmembrane resistance exceeded 1,000 Ω·cm² (31). After inserts were mounted in an Ussing chamber (Ijm's Instrument Manufacturing, Iowa City, IA), apical and basolateral membranes were perfused with standard NaCl-rich buffer that was continuously bubbled with 100% O₂ (~20 ml/reservoir). Buffer temperature was maintained at 37°C using a recirculating heated water bath (model 800; Fisher Scientific, Pittsburgh, PA). Transepithelial Iₛₜc was measured under voltage-clamp conditions using an epithelial voltage clamp amplifier (model EC-825; Warner Instruments, MRA International, Naples, FL). Cell volume increases were induced by simultaneous reduction of apical and basolateral buffer osmolarity by 30%.

Statistics. Results are presented as means ± SE, with n representing the number of cells for patch-clamp studies and the number of repetitions for other experiments. All experiments were repeated on at least two study days. For Ussing protocols, each experimental study was paired directly with a control study on the same day. Paired or unpaired Student's t-tests were used to assess statistical significance, and P < 0.05 was considered to be significant.

RESULTS

Increases in Mz-ChA-1 cell volume enhance membrane ATP permeability. To assess the possibility that ATP in bile is derived from cholangiocytes, initial studies examined whether human biliary cells exhibit basal membrane ATP permeability and whether increases in cell volume enhance cellular ATP efflux. As shown in Fig. 2A, Mz-ChA-1 cells released ATP into extracellular buffer as detected by the luciferase-luciferin assay (range ~11-43 ALU). In most experiments, addition of isometric media led to a small but significant increase in ATP release; this effect was most apparent during the initial compared with subsequent media additions. As shown in Fig. 2A, top, the addition of isometric media (200 µl added to 600 µl) increased bioluminescence from 31.43 ± 3.12 to 41.71 ± 3.38 ALU (P < 0.05), indicating that mechanical membrane perturbation represents a stimulus for ATP efflux (n = 8). However, induction of cell swelling by adding water to decrease media osmolarity by 20 and 40% led to rapid, sustained increases in extracellular ATP that were much greater than values in control studies in which similar volumes of isometric media were added. Depending on the study day, bioluminescence increased approximately two- to fourfold after a 20% media dilution; ATP levels peaked within 5 min and decreased gradually toward basal values over 30 min. These findings indicate that human biliary epithelial cells release ATP into extracellular media under basal conditions and that both membrane stress (isometric media addition) and increases in cell volume (hypotonic media addition) enhance ATP efflux independently.

Mz-ChA-1 cells express volume-sensitive ATP channels. In detecting extracellular ATP molecules, the bioluminescence assay does not discriminate between ATP release pathways. Evidence in other epithelial cells suggests that one mechanism for cellular ATP release is electrodiffusional, with charged ATP molecules (ATP⁻) permeating the cell membrane via selective channel pores (30, 34). Consequently, modified whole cell patch-clamp techniques (100 mM MgATP on
both sides of the membrane as the principal anionic charge carrier) were utilized to measure whole cell ATP currents in Mz-ChA-1 cells. As shown in Fig. 2B, in isotonic buffer a basal ATP conductance was detected, which was nearly linear and reversed near 0 mV, compatible with ATP currents previously described (n = 7) (27, 30). Similar to observations using the luciferase-luciferin assay, exposure to hypotonic buffer (−15% reduction in osmolarity) led to a >10-fold increase in ATP current density, increasing from −1.34 ± 1.61 to −16.82 ± 4.49 pA/pF at −80 mV (n = 7, P < 0.001). Thus human biliary cells possess membrane channels permeable to ATP, and conductive movement of charged ATP molecules represents one potential pathway for basal and volume-sensitive ATP efflux.

ATP release contributes to cell volume homeostasis. In previous studies, both P2 receptor binding and increases in cell volume have been identified as potent stimuli that increase membrane Cl⁻ permeability (22, 29). Therefore, additional studies were performed to assess the roles of volume-sensitive ATP efflux and P2 receptor stimulation in Mz-ChA-1 cell volume recovery (Fig. 3A). Under control conditions, exposure of cells to hypotonic buffer (−30% less mosmol) led to a rapid increase in mean cell volume (n = 6). Peak swelling at 2.5 min (relative volume 1.20 ± 0.01) was followed by RVD toward basal values despite continued incubation in hypotonic buffer (relative volume 1.08 ± 0.01 at 30 min). When the ATP scavenger apyrase (2 U/ml) or the P2 receptor blocker suramin (100 µM) were added to hypotonic buffer, RVD was significantly inhibited (n = 6 for each). However, addition of the nonhydrolyzable P2 receptor agonist ATPγS (20 µM) after peak swelling (5.5 min) nearly restored volume recovery in the presence of apyrase (n = 6) but not suramin (n = 5).

These observations indicate that volume-sensitive ATP release contributes importantly to Mz-ChA-1 cell volume recovery from swelling. Additional studies were performed to determine whether constitutive ATP release contributes to maintenance of cell volume in the absence volume changes. For these studies, cells were incubated in isotonic buffer at all times. As shown in Fig. 3B, simply adding apyrase (2 U/ml) to isotonic buffer led to a rapid increase in relative cell volume (1.07 ± 0.01 at 15 min, n = 6). This response appears to reflect a specific effect of ATP scavenging since coincubation with ATPγS (20 µM), which is not an apyrase substrate, prevented the increase in volume (1.01 ± 0.01 at 15 min, n = 6). Therefore, constitutive cellular ATP release appears to be important for regulation of basal cell volume homeostasis as well.

Autocrine stimulation of P2 receptors regulates volume-sensitive Cl⁻ channels. Because Mz-ChA-1 cell volume recovery depends on channel-mediated Cl⁻ efflux, we then explored the regulatory role of extracellular ATP as a modulator of the volume-sensitive Cl⁻ conductance using whole cell patch-clamp techniques. These results are summarized in Fig. 4. In control cells, exposure to hypotonic buffer (15% decrease in osmolarity) was followed within 2 min by a large increase in Cl⁻ current density from −1.41 ± 0.26 to −37.47 ± 5.48 pA/pF (−80 mV, n = 6, P < 0.001). The characteristics of the volume-activated Cl⁻ conductance included outward rectification and time-dependent inactivation at positive potentials as previously described. Addition of apyrase (3 U/ml, n = 4) to extracellular ATP or suramin (100 µM, n = 6) to block P2 receptors nearly abolished volume-dependent current activation (−2.19 ± 0.71 and −3.42 ± 0.78 pA/pF, respectively). Notably, current inhibition by apyrase and suramin was reversible; after washout of reagent-containing buffer, reexposure of individual cells to hypotonic buffer restored volume-dependent current activation.

Taken together these findings suggest the presence of an autocrine-paracrine signaling pathway for Mz-ChA-1 cells that includes 1) release of ATP into extracellular media during increases in cell volume, 2) stimulation of membrane P2 receptors by the localized increase in ATP, 3) activation of membrane Cl⁻ chan-
Cholangiocytes in vivo are highly polarized, and layers of the cell and volume recovery. Transepithelial secretion depends on opening of Cl\(_{\text{apical}}\) channels in the apical membrane. In contrast to Mz-ChA-1 cells, addition of isotonic buffer did not significantly affect ATP permeability. However, graded buffer dilutions led to a rapid, sustained increase in the rate of ATP release from both membranes; bioluminescence peaked at 19.20 ± 1.36 and 26.86 ± 1.47 ALU (apical) and at 11.39 ± 0.89 and 17.83 ± 1.41 ALU (basolateral) after 20 and 40% decreases in osmolarity, respectively. Thus polarized cholangiocytes exhibit basolateral and volume-sensitive ATP release.

Volume-activated transepithelial Cl\(^-\) currents require extracellular ATP. If volume-sensitive Cl\(^-\) channels are present in the apical membrane, then increases in cell volume would be anticipated to increase \(I_{\text{sc}}\), the electrophysiological equivalent of transepithelial secretion. Therefore, additional experiments were performed to assess the effect of cell volume on \(I_{\text{sc}}\) and the results are summarized in Fig. 6 and Table 1. Depending on the study day, exposure of monolayers to hypotonicity (30% less mosmol) led to a 41–69% increase in \(I_{\text{sc}}\) that peaked at \(~\sim\)1 min; a representative recording is shown in Fig. 6. Current magnitude and time course were similar after induction of swelling by exchange of mannitol-containing with mannitol-free buffer (same electrolyte composition) or standard buffer with buffer containing less NaCl. In the first group of control studies, simultaneous reduction of apical and basolateral buffer osmolarity increased \(I_{\text{sc}}\) from basal values (8.68 ± 1.21 \(\mu\text{A/cm}^2\)) by 4.52 ± 0.43 \(\mu\text{A/cm}^2\) within 1 min (\(n = 7, P < 0.01\)). Volume-activated transepithelial currents appear to be mediated by an increase in apical Cl\(^-\) permeability because 1) amiloride (100 \(\mu\text{M}\)) in the apical buffer to inhibit Na\(^+\) absorption via apical membrane Na\(^+\) channels had no effect (\(\Delta I_{\text{sc}}\) 0.51 \(\mu\text{A/cm}^2, n = 5\)) and 2) addition of the anion channel blocker NPPB (20 \(\mu\text{M}\)) to the apical bathing solution.

![Fig. 4](http://ajpgi.physiology.org/)

**Fig. 4.** Extracellular ATP modulates volume-dependent activation of Cl\(^-\) channels in Mz-ChA-1 cells. A: representative whole cell current tracings are shown. As shown in top tracing, exposure to hypotonic buffer (box) caused reversible increase in anion currents (downward deflection) at test potentials of 0 mV and \(~\sim\)80 mV at 10-s intervals. In the same cell, after equilibration in isotonic buffer, reexposure to hypotonic buffer induced similar currents. Volume-dependent current activation was inhibited by 1) removal of extracellular ATP with apyrase (3 U/ml) or suramin (100 \(\mu\text{M}\)) and 2) P2 receptor blockade by suramin (100 \(\mu\text{M}\), third tracing). Inhibitory effects of apyrase and suramin were reversible; after washout of each reagent, hypotonic exposure still activated anion currents. B: current-voltage (I-V) relations of whole cell Cl\(^-\) currents are depicted (means ± SE). Exposure to hypotonic buffer increased currents from basal (•) that were characterized by outward rectification and reversal near 0 mV (\(\triangledown\), \(n = 6\)). In contrast, exposure to apyrase (3 U/ml, ■, \(n = 4\)) and suramin (100 \(\mu\text{M}\), ○, \(n = 6\)) prevented volume-dependent current activation. In all studies hypotonic buffer contained 30% less milliosmol.

![Fig. 5](http://ajpgi.physiology.org/)

**Fig. 5.** Vectorial ATP release from rat cholangiocyte monolayers. Luciferase-luciferin was added selectively to either side of NRC monolayers to detect apical or basolateral ATP release (see MATERIALS AND METHODS). Basal luminescence was \(~\sim\)3-fold greater in apical (●, \(n = 7\)) compared with basolateral membranes (2.41 ± 0.05 ALU, \(n = 8, P < 0.001\)). In contrast to Mz-ChA-1 cells, addition of isotonic buffer did not significantly affect ATP permeability. However, graded buffer dilutions led to a rapid, sustained increase in the rate of ATP release from both membranes; bioluminescence peaked at 19.20 ± 1.36 and 26.86 ± 1.47 ALU (apical) and at 11.39 ± 0.89 and 17.83 ± 1.41 ALU (basolateral) after 20 and 40% decreases in osmolarity, respectively. Thus polarized cholangiocytes exhibit basolateral and volume-sensitive ATP release.
nearly abolished volume-dependent current activation $\Delta I_{sc} 0.54 \pm 0.27 \mu A/cm^2$, $n = 7$).

In previous studies, stimulation of apical membrane P2Y$_2$ (agonist preference of ATP$_\gamma$S \( \geq \) UTP \( \geq \) ATP, IC$_{50}$ \( \sim \) 1–2 µM) induced transepithelial Cl$^-$ currents in NRC monolayers. Therefore, the effect of cell volume on $I_{sc}$ was evaluated in the presence of apyrase to ascertain whether endogenously released ATP modulates volume-sensitive Cl$^-$ channel activation (Fig. 6, Table 1). In contrast to control monolayers, addition of apyrase (3 U/ml) to apical and basolateral perfusate 5 min before hypotonic challenge inhibited current activation by \( \sim 74\% \) ($\Delta I_{sc}$ 4.81 $\pm$ 0.32 and 1.27 $\pm$ 0.42 µA/cm$^2$ for control and apyrase, respectively, $P < 0.01$, $n = 5$).

Selective addition of apyrase to the apical perfusate decreased hypotonic-induced $\Delta I_{sc}$ by \( \sim 45\% \) ($n = 3$), suggesting that ATP released from both apical and basolateral membranes contributes to volume-sensitive currents. Removal of extracellular ATP did not alter cellular responsiveness because apical delivery of ATP$_\gamma$S (300 nM) still increased $I_{sc}$ 6.04 $\pm$ 0.58 µA/cm$^2$ in the presence of apyrase (3 U/ml in both chambers, $n = 3$).

Additional studies examined whether the effects of extracellular ATP require stimulation of P2 receptors. When suramin (100 µM) was added to apical and basolateral buffer 10 min before hypotonic exposure, volume-activated currents were inhibited by \( \sim 84\% \) ($\Delta I_{sc}$ 3.32 $\pm$ 0.13 and 0.53 $\pm$ 0.07 µA/cm$^2$ for control and suramin, respectively, $P < 0.01$, $n = 3$). These findings, in conjunction with observations that neither apical nor basolateral exposure to the P1 agonist adenosine alters basal $I_{sc}$ (basolateral effects unpublished), imply P2 receptor binding as a critical stimulus for volume-dependent current activation. In summary, vectorial release of ATP from cholangiocytes appears to contribute both to cell volume homeostasis and to a volume-sensitive increase in ductular secretion.

**DISCUSSION**

Intrahepatic bile ducts play a critical role in controlling the volume and composition of bile, but their small size and intrahepatic location have limited efforts to ascertain the cellular mechanisms involved (9). The identification of cystic fibrosis transmembrane conductance regulator (CFTR), the protein product of the cystic fibrosis gene, in cholangiocytes implies that transepithelial transport of Cl$^-$ represents one mechanism of secretion; hormonal stimulation is thought to stimulate

Fig. 6. Endogenous extracellular ATP regulates volume-dependent transepithelial Cl$^-$ secretion. Representative short-circuit current ($I_{sc}$, µA) tracings of NRC monolayers mounted in an Ussing chamber and perfused with NaCl-rich buffer are shown. A: in control study (left), exchanging isotonic buffer in both chambers with hypotonic buffer (boxes) increased $I_{sc}$ that peaked at \( \sim 1 \) min (positive deflection of current tracing). In contrast, addition of ATP scavenger apyrase (3 U/ml) to apical and basolateral perfusate (5 min preincubation) inhibited volume-dependent current activation (right). B: compared with control study (left), addition of P2 receptor blocker suramin (100 µM, 10 min preincubation) to both chambers also decreased $I_{sc}$ during hypotonic exposure (right). C: in this example, $I_{sc}$ was minimal during hypotonic challenge in presence of apical and basolateral apyrase (3 U/ml); however, subsequent addition of ATP$_\gamma$S (300 nM) to the apical membrane still increased transepithelial currents. Monolayers were pulsed every 90 s with 1 µA of current (spikes on tracings). Relative current values on y-axis (µA) vary among A, B, and C. Studies in A and B were performed in tandem on same day. In all studies, hypotonic buffer contained 30% less milliosmole.
Table 1. Extracellular ATP regulates volume-dependent apical Cl⁻ secretion across polarized cholangiocyte monolayers

<table>
<thead>
<tr>
<th>Condition</th>
<th>Basal</th>
<th>Peak</th>
<th>∆I_v</th>
<th>P</th>
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<td>Control</td>
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<td>NPPB (apical)</td>
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<td>5.86±0.18</td>
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<td>8.22±0.35</td>
<td>11.47±0.44</td>
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<tr>
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<tr>
<td>Suramin (apical, basolateral)</td>
<td>9.94±0.25</td>
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<td>0.53±0.07</td>
<td>&lt;0.01</td>
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<tr>
<td>Apynase (apical, basolateral)+ATPγS (apical)</td>
<td>7.00±0.26</td>
<td>13.04±0.34</td>
<td>6.04±0.58</td>
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</table>

Values are means ± SE. Transepithelial currents were measured in normal rat cholangiocyte monolayers mounted in an Ussing chamber. Cell swelling was induced by exchanging apical and basolateral isotonic buffer with buffer containing 30% less milliosmole. In all studies, hypotonic exposure led to increase in basal currents (basal short-circuit current (I_sc), which peaked within 1 min (peak I_sc; difference shown as ∆I_sc). Addition of the anion channel blocker 5-nitro-2-(3-phenylpropylamine)-benzoic acid (NPPB, 20 µM) to apical hypotonic buffer markedly inhibited current activation (n = 7). Volume-activated Cl⁻ secretion is largely dependent on P2 receptor stimulation by extracellular ATP because I_sc increases were inhibited by 1) addition of apynase (3 U/ml) to both apical and basolateral buffers (5 min preincubation, n = 5), 2) selective addition of apynase to apical perfusate (5 min preincubation, n = 3), and 3) addition of suramin (100 µM) to apical and basolateral buffers (10 min preincubation, n = 3). In the presence of apynase (3 U/ml in both chambers), addition of adenosine 5'-O-(3-thiotriphosphate) (ATPγS, 300 nM) to apical buffer significantly increased transepithelial currents (n = 3). Controls were paired with experimental studies on same study day.

Although purinergic receptors have been identified in epithelia, there is considerable controversy regarding the cellular origins of ATP and the mechanisms involved in its release. Membrane stress represents one stimulus for ATP release by hepatocytes (32) and other cell types (2, 11, 12), but the physiological relevance of mechanical stimulation to cholangiocytes is not readily apparent. For example, ATP in concentrations sufficient to bind to P2 receptors is present in human bile and is released into sinusoidal blood in the isolated perfused organ in the absence of obvious mechanical changes (5, 26). The present observations, using a sensitive luminometric assay, indicate that ATP release by biliary cells occurs independently of membrane perturbation or other stimulation and that extracellular ATP plays a physiological role in cell volume homeostasis. Exposure to apynase results in an increase in cell volume. This effect appears specific for extracellular ATP removal because intracellular ATP concentrations are unaffected (unpublished observations) and cell swelling is reversed by simultaneous exposure to ATPγS, a nonhydrolyzable P2 receptor agonist.
In contrast to the modest effects of media addition on ATP release, increases in cell volume resulted in rapid and sustained increases in extracellular ATP. Moreover, changes in membrane ATP permeability are reversible, recovering within 2 min of restoration of extracellular osmolarity. This finding is of great interest because all cells are subject to substantial osmotic challenges as a result of normal transport and metabolism (1) and because volume-sensitive ATP release has been demonstrated recently in several cell types with quite different biological functions including primary hepatocytes, hepatoma cells, fibroblasts, and ciliary epithelia (24, 28, 36, 40). Taken together, these findings suggest that volume-sensitive ATP release pathways may be highly conserved.

Exposure to ATP has been shown previously to increase Cl− permeability of cholangiocytes and other secretory epithelia. The present studies suggest two potential physiological roles for volume-sensitive ATP release. First, at the cellular level, release of ATP into the extracellular space and P2 receptor stimulation appear to be necessary for cell volume recovery during hypotonic exposure. Because a similar role for ATP release in cell volume regulation has been identified in both model and primary hepatocytes (28, 40), this might represent a mechanism shared by multiple liver cell types.

Complementary studies in NRC monolayers support a second potential physiological role for ATP release in the regulation of transepithelial Cl− secretion. In intact rat liver, hypotonic perfusion to induce cell swelling leads to an increase in bile acid secretion and bile formation (3). The present studies suggest that endogenous volume-sensitive release of ATP from cholangiocytes into bile may stimulate ductular secretion through binding of P2 receptors and opening of Cl− channels in the apical membrane (Fig. 7). It is notable that the inhibitory effects of ATP removal and P2 receptor blockade on volume-sensitive increases in $I_{\text{cl}}$ are incomplete, implying the presence of additional P2 receptor-independent anion conductance pathways as well. Hepatocytes, the principal liver parenchymal cell, also invest considerable cellular resources in the release and metabolism of ATP at the apical (canalicular) membrane (6). In a similar fashion, any ATP derived from hepatocytes that escapes luminal degradation would have direct access to the apical membrane of cholangiocytes located downstream as bile flows away from its canalicular origins within the network of intrahepatic ducts. Because volume-sensitive ATP release has recently been demonstrated from primary human hepatocytes (28), ATP may play a role in coordinating the separate hepatocyte and ductular components of bile formation, a process referred to as hepatobiliary coupling (Fig. 7). Such a mechanism is analogous to that involving the peptide hormone endothelin, which also exerts local control over ductular secretion by binding to specific receptors in the apical membrane of cholangiocytes (10).

Assuming that biliary ATP functions in this manner and bile formation, several critical questions remain to be answered. First, the molecular basis of membrane ATP permeability and the cellular signals that control the permeability pathway(s) have not been defined. Cytoplasmic ATP molecules are present inside cells in concentrations much greater than those observed extracellularly, and cytoplasmic ATP molecules exist largely in anionic forms. Thus opening of an ATP pore or channel represents an attractive option and is consistent with the presence of volume-sensitive currents carried by ATP detected by whole cell recordings (Fig. 1). Permeation of ATP molecules through selective membrane channels could account for the rapid rise in bioluminescence observed after hypotonic challenge. However, because of the high concentrations of ATP required to detect these currents (100 nM), the relevance of these findings to physiological cellular pathways is still conjectural. The specific advantages of the luciferase-luciferin assay are an increased sensitivity, an ability to measure ATP release from intact cells, and the detection of both charged and electroneutral ATP molecules released by any transport mechanism.

Second, it is not clear from these studies how much ATP is required to elicit cellular responses. Generation of ATP standard curves indicates that the amount of ATP present in extracellular media during cholangiocyte swelling is ~150–240 nM (Mz-ChA-1 cells) and ~90–115 nM (apical NRC membranes), values below the IC50 of ~300 nM observed for activation of P2Y2 receptors. These luminometric measurements, however, are likely to underestimate the local availability of ATP molecules at the membrane surface, where there is a dynamic interplay among release, degradation, and receptor binding. Third, changes in extracellular ATP levels detected by bioluminescence are dependent on the rates of ATP transport out of the cell and subsequent dephosphorylation by ecto-ATPases. At present, the relative contributions of these pathways are not established. Finally, whether P2 receptors in addition to the P2Y2 subtype contribute to volume-dependent current activation is unknown. Notably, the luciferase-luciferin assay does not detect ADP nor UTP, both of which may be enzymatically generated from ATP and contribute to cellular responses by stimulating distinct P2 receptor subtypes.

These and other studies suggest that regulated release of ATP may be a general property of epithelial cells. Evidence herein using biliary cell models supports the presence of dynamic functional interactions among cell volume, ATP release, and membrane Cl− permeability that contribute importantly to local regulation of Cl− secretion and bile formation. Thus characterization of the mechanisms involved in ATP release and metabolism may offer new strategies for the pharmacological manipulation of biliary secretion in genetic and acquired cholestatic liver diseases, where ductular secretion is deficient and therapeutic options are few. However, the universal presence and functional diversity of purinergic receptors in many different epithelial cell types suggest that alternative modes of ATP release are likely to exist in other cells, with different cellular...
strategies tuned to match local environmental cues and specific cellular functions.

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