Regulation of biliary secretion through apical purinergic receptors in cultured rat cholangiocytes

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Schlenker, Thorsten, Joelle M.-J. Romac, Ala I. Sharara, Richard M. Roman, Stephen J. Kim, Nicholas LaRusso, Rodger A. Liddle, and J. Gregory Fitz. Regulation of biliary secretion through apical purinergic receptors in cultured rat cholangiocytes. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1108–G1117, 1997.—To evaluate whether ATP in bile serves as a signaling factor regulating ductular secretion, voltage-clamp studies were performed using a novel normal rat cholangiocyte (NRC) model. In the presence of amiloride (100 µM) to block Na⁺ channels, exposure of the apical membrane to ATP significantly increased the short-circuit current (Isc) from 18.2 ± 5.9 to 52.8 ± 12.7 µA (n = 18). The response to ATP is mediated by basolateral-to-apical Cl⁻ transport because it is inhibited by 1) the Cl⁻ channel blockers 4,4'-disothiocyanostilbene-2,2'- disulfonyl acid (1 mM), diphencylaminanillic acid (1.5 mM), or 5-nitro-2-(3-phenylpropylamino)benzoic acid (50 or 100 µM) in the apical chamber, 2) the K⁺ channel blocker Ba²⁺ (5 mM), or 3) the Na⁺-K⁺-2Cl⁻ cotransport inhibitor bumetanide (200 µM) in the basolateral chamber. Other nucleotides stimulated an increase in Isc with a rank order potency of UTP > ATP = adenosine 5'-O-(3')-thiotriphosphate, consistent with P₄₅₀ purinergic receptors. ADP, AMP, 2-methylthioadenosine 5'- triphosphate, and adenosine had no effect. A cDNA encoding a rat P₂u receptor (rP₂uR) was isolated from a liver cDNA library, and functional expression of the corresponding mRNA in Xenopus laevis oocytes resulted in the appearance of ATP-stimulated currents with a similar pharmacological profile. Northern analysis identified hybridizing mRNA transcripts in NRC as well as other cell types in rat liver. These findings indicate that exposure of polarized cholangiocytes to ATP results in luminal Cl⁻ secretion through activation of P₂u receptors in the apical membrane. Release of ATP into bile may serve as an autocrine or paracrine signal regulating cholangiocyte secretory function.

The formation of bile by the liver depends on complementary interactions between two distinct cell types, including hepatic parenchymal cells, which account for ~80% of liver mass, and bile duct epithelial cells (cholangiocytes), which account for ~2–5% of liver mass (45). Secretion is initiated by hepatocytes that actively transport bile acids and other organic solutes into the canalicular space between cells (32). Subsequently, canalicular bile enters the lumen of the extensive network of intrahepatic ducts where it undergoes dilution and alkalization as a result of cholangiocyte Cl⁻ and HCO₃⁻ secretion (10, 31). Despite the comparatively small number of duct cells, cholangiocyte secretion is thought to account for up to 40% of human bile flow (32). Moreover, intrahepatic ducts represent an important target of injury in several disease states, including sclerosing cholangitis, primary biliary cirrhosis, and liver transplant rejection. However, the small size and intrahepatic location of cholangiocytes have limited investigation of the cellular mechanisms involved in ductular secretion.

Recent studies of isolated cholangiocytes and intrahepatic duct units from rat liver have focused on the role of secretin-stimulated bile flow (19, 31, 38). Secretin binds to receptors in the basolateral membrane (13) that are positively coupled to adenyl cyclase and stimulates cAMP (18, 21), Cl⁻/HCO₃⁻ exchange (31), and opening of Cl⁻ channels (29). These Cl⁻ channels are localized to the apical membrane and are associated with expression of cystic fibrosis transmembrane conductance regulator (CFTR) (2, 10, 48), the protein product of the cystic fibrosis (CF) gene (2). Defective regulation of Cl⁻ channels in the genetic disease CF is likely to contribute to the cholestasis and biliary obstruction observed in 5–30% of these patients (15). These and other findings provide indirect support for the assumption that transepithelial transport of Cl⁻ represents an important driving force for fluid and electrolyte secretion across intrahepatic bile ducts (10, 15).

Although the hepatic manifestations of CF may occasionally be severe, it is notable that the prevalence of biliary disease is much lower than pancreatic or pulmonary injury in CF (15). The reasons for these differences are not known. In the CF mouse model, the expression of alternative Cl⁻ channels unrelated to CFTR is an important determinant of organ-level disease (9). Thus one potential explanation for these clinical differences is that biliary secretion might also be regulated by secretin- and CFTR-independent pathways. Indeed, Cl⁻ channels that are distinct from CFTR have been identified in biliary cells and cell lines (14, 28, 39, 40). Definition of their physiological role(s) has been limited by the lack of information regarding their cellular localization to the apical (luminal) or basolateral membranes, by the inability to measure transepithelial potentials across polarized cell monolayers, and by the limited information regarding the other receptors and physiological factors involved in regulation of secretory function.

Extracellular ATP is a potent signaling molecule that regulates many transport and metabolic pathways in the liver (17). These effects are mediated by activation of one or more purinergic (P₂u) receptors, which are
expressed by hepatocytes (22), nonparenchymal liver cells (16), and intra- and extrahepatic biliary cells (11, 27, 33, 47). Based on signaling properties and agonist specificity, at least five subclasses of P2 receptors have been identified in different cell types (18), including P2Y1, P2Y2, and P2Y receptors, which signal through G proteins, P2X receptors, which are ligand-gated cation channels, and P2Z receptors expressed by mast cells.

In nonpolarized biliary cells, ATP and UTP are equally potent in their ability to increase intracellular Ca2+ concentration, consistent with expression of receptors of the P2Y subclass (27, 47). Studies in isolated duct cells, P2X receptors, which are ligand-gated cation channels, and P2Z receptors expressed by mast cells. In nonpolarized biliary cells, ATP and UTP are equally potent in their ability to increase intracellular Ca2+ concentration, consistent with expression of receptors of the P2Y subclass (27, 47). Studies in isolated duct cells, P2X receptors, which are ligand-gated cation channels, and P2Z receptors expressed by mast cells. Studies in isolated duct cells, P2X receptors, which are ligand-gated cation channels, and P2Z receptors expressed by mast cells.

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Northern blot analysis. Total RNA from freshly isolated rat hepatocytes, HTC rat hepatoma cells, and NRC cells was prepared using the guanidine thiocyanate and phenol method (Tel-Test, Friendswoods, TX). RNA preparations were separated on formaldehyde-containing agarose gels (1%) and then blotted on nylon membranes (Schleicher-Schuell, Keene, NH) and ultraviolet cross-linked for 2 min. A probe spanning nucleotides 933 to 1,690 from the rP2uR cDNA clone was obtained by polymerase chain reaction amplification and was radiolabeled with [32P]dCTP using Klenow (Life Science, Arlington Heights, IL). The blot was probed in QuickHyb (Stratagene) according to the manufacturer’s instructions. The membrane was then prehybridized for 15 min in QuickHyb at 68°C. Two washes in 2× saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature each were followed by a wash in 0.1× SSC, 0.1% SDS for 30 min at 60°C.

RESULTS

Basal properties of NRC monolayers. NRC cells plated on collagen-coated inserts formed polarized monolayers after 8 to 14 days (Fig. 1). As cells became confluent there was an increase in transepithelial resistance indicating formation of tight junctions. Inserts with values >1,000 Ω·cm² as measured with an EVOHM voltmeter produced reliable and reproducible results in voltage-clamp studies in which transepithelial ion transport could be evaluated more directly (20). When mounted in the recording chamber, inserts rapidly (<7 min) equilibrated and developed a basal Isc of 18.2 ± 5.9 µA (n = 18) with a lumen-negative potential of −3.2 ± 1.9 mV. The average transepithelial resistance measured in the Ussing chamber of 809 ± 436 Ω·cm² was consistently lower than values measured with the EVOHM voltmeter, as reported for other cell types (20).

Nucleotides stimulate transepithelial transport through activation of receptors in apical membrane. To assess whether exposure of the apical membrane to nucleotides regulates transepithelial transport, ATP (0.1–100 µM) was added selectively to the solution bathing the luminal surface. Exposure to ATP caused a rapid increase in Isc in all monolayers tested. The response occurred rapidly and peaked within ~5 min as shown in Fig. 2. The increase in Isc (ΔIsc) of 34.6 ± 12.2 µA (n = 18) was temporally associated with development of more negative luminal potentials of −12.0 ± 7.3 mV and a decrease in transepithelial resistance by 11 ± 16%, consistent with opening of conductance pathways. The Isc response tended to return toward basal values over 15–60 min despite the continued presence of ATP and ΔV recovered to −5.1 ± 5.9 mV over the same period. To minimize any effect of day-to-day variability in the magnitude or time course of the response, subsequent studies using test reagents were compared with control values measured on the same study day and are presented as relative values.

![Fig. 1. Vertical section of normal rat cholangiocyte (NRC) cells. Cells were cultured on collagen-coated filters as described and formed polarized monolayers with tight junctions between cells. In this vertical section, the apical membrane is oriented toward the top and the filter is evident at the bottom. Hematoxylin and eosin stain; original magnification, ×400.](http://ajpgi.physiology.org/)

![Fig. 2. Measurement of ATP-stimulated transepithelial transport. Short-circuit current (Isc) was measured under voltage-clamp conditions. In this representative recording, amiloride was added to the apical chamber to inhibit apical Na⁺ channels. Subsequent exposure of the apical membrane to ATP caused a rapid increase in Isc that was maximal within ~5 min and then decreased despite the continued presence of ATP.](http://ajpgi.physiology.org/)
Pharmacological properties of apical receptor. These findings are consistent with the presence of ATP receptors in the apical membrane that stimulate transepithelial ion transport. A similar response was observed with the nonhydrolyzable analog ATPγS, indicating that receptor activation does not depend on ATP hydrolysis and that the breakdown products of ATP are not responsible for the effect. To further evaluate the pharmacological properties of the receptor, other test nucleotides were added to the apical bath at a concentration of 50 µM. The rank order potency for stimulation of ∆Isc showed approximately equal responses to ATPγS, UTP, and ATP, and the concentration of UTP required to produce half-maximal increases in ∆Isc was ~2 µM (Fig. 3). ATP showed a similar concentration dependence (data not presented). Other nucleotides, including ADP, AMP, 2-MeS-ATP, and adenosine, failed to increase Isc above basal levels. These findings are consistent with the pharmacological properties of receptors of the P2u subclass (18).

Identification of conductances contributing to Isc. The ATP-induced increase in Isc was associated with generation of a lumen-negative potential, consistent with activation of apical-to-basolateral Na+ or basolateral-to-apical Cl- transport, or both. Therefore, in selected studies, the Na+ channel blocker amiloride (100 µM) was added to the apical chamber. Preincubation in amiloride did not block nucleotide-stimulated increases in ∆Isc (n = 81). Similarly, exposure to amiloride after stimulation of ∆Isc by ATP had no significant inhibitory effect (n = 10). These findings indicate the ATP-stimulated increase in Isc is not due to opening of amiloride-sensitive Na+ channels. It is notable, however, that amiloride decreased basal Isc values in ~30% of monolayers. The magnitude of the decrease tended to be larger in monolayers with higher initial Isc values, suggesting that apical Na+ uptake may contribute to Isc under basal conditions. Consequently, subsequent studies were performed in the presence of amiloride (100 µM) to minimize any potential contribution of apical Na+ conductances.

Many cellular models of transepithelial Cl- transport involve functional interactions among at least three transport mechanisms, including 1) opening of

![Graph A](image1)

![Graph B](image2)

Fig. 3. Characterization of the response to different nucleotides. A: peak response (∆Isc) to different nucleotides at a concentration of 50 µM is shown. Test reagents were added to the apical chamber only, and values represent the means ± SD of 3 to 10 monolayers. B: relative dose-response relationship for UTP (n = 3–5 monolayers per point). ∆Isc/∆Iscmax, maximal current response to 100 µM UTP.
purinergic stimulation of cholangiocyte secretion

**Fig. 4.** Effect of NPPB on ATP-stimulated transport. In the presence of amiloride, exposure to NPPB (100 µM) is increased analog of ATP, increased findings support an important contribution of Cl−

Cl− conductance(s) in the apical membrane by efflux of Cl− out of the cell and into the lumen, 2) bumetanide-sensitive Na+−K+−2Cl− cotransport across the basolateral membrane to maintain intracellular Cl− concentrations (12), and 3) opening of Ba2+-sensitive K+ channels in the basolateral membrane to recycle K+. The effects were rapidly reversed by exposure to NPPB, a putative Cl− channel blocker. All reagents were added to the apical chamber only. Note the temporal relationship between changes in I_sc (top) and lumen-negative potentials (bottom).

**Fig. 5.** Effect of transport inhibitors on ATP-stimulated Cl− secretion. NRC monolayers were preincubated with NPPB (n = 8) and DIDS (n = 10) caused significant inhibition of ATP-stimulated transport, and exposure of the basolateral membrane to the K+ channel blocker Ba2+ (n = 8) or the Na+−K+−2Cl− cotransporter bumetanide (n = 8) had similar effects. *P < 0.05.

Molecular cloning of rat liver P2u receptor. Complementary DNAs encoding P2u receptors have recently been identified in mouse brain (24), rat alveolar type II cells (37), rat pituitary gland (8), and human tissues (34). To further characterize the receptor involved in hepatobiliary signaling, a rat liver cDNA library was screened by plaque hybridization using a Bsr D1 restriction digest cDNA fragment of the rat receptor from mouse brain as a probe (24). A single clone of 2,164 bp was plaque purified (rP2uR), and sequence analysis revealed a 1,125-bp open reading frame, 444-bp 5′ untranslated region, and 592-bp 3′ untranslated region. The largest open reading frame encoded a putative protein of 374 amino acids with a predicted molecular mass of 42 kDa. Seven putative transmembrane domains were identified from the deduced sequence, typical for the G protein-coupled class of receptors. The liver amino acid sequence was identical to other rat P2u proteins obtained from pituitary gland or alveolar type II cell cDNA. Sequence comparison with the cloned murine and human P2u receptors showed >95% homology at the amino acid level (Fig. 6). However, the 5′ untranslated region of the rP2uR cDNA harbors a 200 bp fragment immediately upstream of the putative AUG start codon that is not present in the mouse or the human P2u receptor.
Identification of P2u mRNA by Northern analysis.

To assess whether mRNA corresponding to rP2uR is present in hepatobiliary cells, Northern analysis was performed under high stringency conditions using a probe encompassing nucleotides 933 to 1,690. P2u mRNA was abundant in NRC, and was also detected as a 33-kb band in RNA from hepatocytes and HTC hepatoma cells (Fig. 7). Analysis of RNA from other rat tissues revealed hybridizing transcripts in lung, skeletal muscle, and liver tissue but not in brain or kidney tissues (data not shown).

Functional characterization of cloned rP2uR. The functional properties of the protein encoded by rP2uR were assessed by heterologous expression. cRNA was prepared by in vitro transcription, and after injection of rP2uR cRNA (50 ng) into Xenopus oocytes, the response to extracellular nucleotides was assessed by two electrode voltage clamp studies. Exposure to ATP or UTP (100 µM) elicited peak inward currents of 200 nA (holding potential −50 mV) in oocytes injected with rP2uR cRNA but not in control oocytes or oocytes injected with water (Fig. 8A) or antisense cRNA.

Activation of inward currents by nucleotides presumably reflects opening of endogenous Ca2+-dependent Cl− channels. This was evaluated more directly by demonstration that ATP-stimulated currents 1) reverse near the Cl− equilibrium potential of −20 to −30 mV (not shown), 2) are inhibited by niflumic acid (300 µM), an inhibitor of Ca2+-dependent Cl− channels, and 3) are inhibited by intracellular injection of ethylene glycol-bis(β-aminoethyl ether)−N,N,N′,N′-tetraacetate acid (EGTA; estimated final concentration 10 mM) to block the receptor-stimulated rise in cytosolic Ca2+ concentration (Fig. 8B). The pharmacological properties were analogous to the properties of NRC monolayers, with equivalent responses to UTP and ATP and half-maximal current activation at −0.3 µM (Fig. 8C). The rank order potency (100 µM) was UTP = ATP > ADP >> AMP. There was no response in injected oocytes to adenosine, 2-MeS-ATP, or AMP-PCP.

DISCUSSION

These studies of NRC monolayers provide direct support for the concept that transepithelial transport of Cl− represents an important determinant of fluid and electrolyte secretion across intrahepatic biliary epithelium (10) and identify extracellular nucleotides as potent regulatory factors modulating biliary secretory function through activation of P2u receptors in the apical (luminal) membrane. Thus ATP released into bile may represent an autocrine and/or paracrine factor coupling the separate hepatic and ductular components of bile formation through stimulatory effects on duct cells.

Previous studies of biliary cells and cell lines have provided pharmacological support for expression of purinergic receptors. In isolated cells, exposure to nucleotides increases intracellular Ca2+ concentrations and membrane Cl− permeability (27, 30, 47). In isolated bile duct units, P2u receptors are present in the basolateral membrane (33). However, basolateral receptor stimulation has no apparent effect on secretion or cell volume as assessed by optical techniques. Further
definition of the physiological role(s) of purinergic signaling has been limited in part by the lack of access to the apical domain of polarized cells and by the lack of molecular insights regarding the properties of biliary purinergic receptors. A role in regulation of secretion has been postulated because 1) in gallbladder from the amphibian Necturus, ATP added to the apical (mucosal) surface of the epithelium stimulates ionic conductances (11), 2) ATP is present in mammalian bile in concentrations sufficient to activate receptors (7), and 3) ATP and UTP act as potent secretagogues in airway and certain other epithelial cell types that express apical receptors (26, 34). The mechanisms involved are complex, with evidence for expression of receptors in both the apical and basolateral membranes, and for coupling of receptors to both Ca$^{2+}$-dependent and Ca$^{2+}$-independent signaling pathways according to the model under investigation (6, 44). However, in airway epithelium, stimulation of Cl$^{-}$ secretion by extracellular nucleotides involves channels other than CFTR and has been...
The concentration of ATP required to stimulate $I_{sc}$ is similar to the average concentration of ATP in human bile of $\sim 1.5$ µM (7). Consequently, it is attractive to speculate that purinergic signaling mechanisms might contribute to regulation of secretion across intrahepatic ducts in vivo. If so, there are several important issues that remain to be addressed. First, the actual amount of ATP (or UTP) present within the lumen of intrahepatic ducts is not known, and the cellular origin and molecular mechanisms involved in ATP release into bile have not been established. Both hepatic and biliary cell lines in culture release nucleotides (7, 41), and express ATP-binding cassette (ABC) proteins, which have been implicated in transport of ATP in other cell types (1, 4, 36, 42). These ABC proteins include P-glycoproteins (mdr) in the apical (canalicular) membrane of hepatocytes (3) and CFTR in the apical membrane of intrahepatic duct cells (10). However, the role of ABC proteins in ATP transport remains controversial (35). In addition, ectonucleotidase activity and Na$^+$-adenosine cotransport across the canalicular membrane could also modulate local nucleotide concentrations (3). Thus there are multiple potential sites for regulation of luminal nucleotide concentrations through variations in the rate of ATP release, ATP degradation, or bile flow. Any ATP escaping degradation would have direct access to the apical surface of duct cells as bile flows out of the canaliculus and into the lumen of intrahepatic ducts.

Second, recent studies indicate that P$_2$ receptors are also present in the basolateral membrane of cholangiocytes (33). In isolated ducts, basolateral receptor stimulation increases cytosolic Ca$^{2+}$ concentration, but there is no apparent effect on secretion or cell volume (33). Consistent with these observations, we observed in preliminary studies that ATP added to the basolateral solution induced relatively small $I_{sc}$ responses ($4.5 \pm 1.4$ µA, $n = 10$), averaging only 10–15% of those induced by apical exposure. ATP$_S$ ($n = 3$) and UTP ($n = 4$) were without effect. Thus apical receptors appear to contribute more importantly to local regulation of secretion, and the additional type(s) and physiological roles of basolateral purinergic receptors remain to be determined.

Finally, the intracellular signals that couple receptor binding to channel opening have not been defined. In airway cells ATP stimulates Cl$^-$ secretion through activation of multiple signal transduction pathways, including both adenosine 3',5'-cyclic monophosphate (cAMP)-dependent and independent mechanisms (44). In preliminary studies of NRC monolayers, the cAMP inhibitor Rp-cAMPs failed to inhibit the response to ATP (data not presented), and in isolated biliary cells, both cytosolic Ca$^{2+}$ concentration and Ca$^{2+}$-calmodulin-dependent kinases increase membrane Cl$^-$ permeability (30, 40). If these findings are relevant to cholangiocytes in vivo, it seems likely that apical ATP modulates Cl$^-$ secretion through one or more cAMP-independent pathways not directly related to CFTR.
In summary, these studies of NRC cells indicate that extracellular ATP may serve as an autocrine and/or paracrine factor that contributes to regulation of ductular secretion through activation of P2u receptors in the apical membrane. Local control of biliary function by factors present in bile represents an important complement to the effects of secretin and other basolateral signals. Moreover, apical P2u receptors represent an attractive therapeutic target for pharmacological approaches aiming to modulate the volume and composition of bile and to bypass the Cl\(^{-}\) secretory defect associated with CF.

This work was supported in part by the National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-46082 and DK-43278, and by the Deutsche Forschungsgemeinschaft Grant Sfb 380/1–3.

References


2. Anderson, M. P., R. J. Gregory, S. Thompson, D. Souza, S. T. Schlenker, J. M.-J. Romac, and A. L. Sharara contributed to the effects of secretin and other basolateral paracrine factor that contributes to regulation of ductular function by activation of P2u receptors in the apical membrane. Local control of biliary function by factors present in bile represents an important complement to the effects of secretin and other basolateral signals. Moreover, apical P2u receptors represent an attractive therapeutic target for pharmacological approaches aiming to modulate the volume and composition of bile and to bypass the Cl\(^{-}\) secretory defect associated with CF.


