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

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The response to ATP is mediated by basolateral-to-apical Cl− transport because it is inhibited by 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′-diisothiocyanostilbene-2,2′-disulfonic acid (1 mM), diphenylanthranilic 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 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 results 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 (P2) 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 P2Z, P2U, 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 P2U, and P2Y receptors, which signal through G proteins, P2X receptors, which are ligand-gated cation channels, and P2Z receptors expressed by mast cells.

METHODS

Materials. Purinergic receptor agonists included adenosine, adenosine 5′-monophosphosphate (AMP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate disodium salt (ATP), 2-methylthioadenosine 5′-triphosphate (2-MeS-ATP), adenosine 5′-O-(3-thiotriphosphate) tetralithium salt (ATPγS), and uridine 5′-triphosphate sodium salt (UTP). These were made fresh as stock solutions and added to the perfusion chamber to achieve the final concentrations indicated. Transport inhibitors included amiloride hydrochloride, 3-aminosulfonyl)-5-(butylamino)-4-phenoxybenzoic acid (bumetanide), niflumic acid, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), diphenylanilinacetic acid (DPC; Fluka, Switzerland), and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, Calbiochem, La Jolla, CA). The last three were dissolved in dimethyl sulfoxide (DMSO) and added to the concentrations indicated with a final DMSO concentration <0.1%. Other reagents were of high grade and were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Cell culture. NRC were cultured as recently described (46). These cells possess phenotypic features of biliary origin, including expression of the differentiated markers GT, CK-7, and CK-19, and form polarized monolayers with tight junctions between cells and microvilli in the apical membrane (46).

Voltage clamp measurements of short-circuit current. NRC were seeded at a density of 1 × 10⁵ cells/cm² on collagen-treated polycarbonate filters with a pore size of 0.4 µm (Costar, Cambridge, MA). Before study transmembrane resistances were measured with an epithelial tissue voltmeteter [EVOHM; World Precision Instruments (WPI), Sarasota, FL], and only inserts with a resistance >1,000 Ω·cm² were used for experiments. Transepithelial transport was measured under voltage-clamp conditions using a DVC 1,000 voltage-clamp amplifier (WPI). Cells were mounted in a Trans-24 mini-perfusion system for tissue culture cups (WPI). All experiments were carried out at 37°C, and basolateral and apical luminal sides were perfused continuously and independently in a closed system with a standard NaCl-rich buffer (as will be described), by bubbling O₂ through air-lift circulators. Test substrates were added to the apical or basolateral buffer solution as indicated. Transepithelial voltage (Vₑ) was clamped to 0 mV and short-circuit current (Iₛₑ) was recorded through agar bridges (3% agar in 3 M KCl) connected to Ag-AgCl electrodes (cartridge electrodes, WPI). Transepithelial resistance was calculated by measuring the current response to a 10 mV change in Vₑ. Data were collected in 0.5-min intervals or were digitized for storage on a computer. To minimize potential effects of day-to-day variability in the preparations, results are compared with same-day controls and presented as means ± SD. Student's t-test analysis for unpaired data was performed with SigmaPlot (Jandel Scientific), and P < 0.05 was considered significant.

Solutions. The standard NaCl-rich extracellular solution (pH 7.35) contained (in mM) 140 NaCl, 4 KCl, 1 KH₂PO₄, 2 MgCl₂, 1 CaCl₂, 5 glucose, and 10 N, 2-hydroxyethylpipеразине-N′-2-этиленсульфоnic acid (HEPES)NaOH. Solutions were pre-equilibrated with O₂ (100%) and maintained at 37°C. Electrogentic HCO₃⁻ secretion and Cl⁻/HCO₃⁻ exchange would not be anticipated to contribute substantially to the Iₛₑ response because the solutions were nominally HCO₃⁻ free and because exchange activity does not result in net transfer of charge.

cDNA cloning and expression. An oligo(dT) rat liver cDNA library in Zap II (Stratagene, La Jolla, CA) was screened by plaque hybridization according to the manufacturer's recommendation with a 700 bp Bsr DI restriction enzyme fragment encompassing the open reading frame from the cloned mouse P2U receptor cDNA (kindly provided by Dr. David julius, Univ. of California, San Francisco). A single clone (rP₂U) was isolated, and both strands were sequenced by the dideoxy chain termination method using Sequenase 2.0 (Life Science, Arlington Heights, IL).

In vitro transcription. The rP₂U cDNA cloned into pBlue-script SK (Stratagene) was linearized by restriction digest using Not I or Acc restriction enzymes and transcribed in vitro for expression in oocytes with T7 (sense cRNA) or with T3 (antisense cRNA) polymerase (Ambion, Austin, TX). The cRNA preparations were precipitated and resuspended in deionized water at a final concentration of 1 µg/µl. The integrity of the transcripts was verified by electrophoresis on agarose gel.

cDNA expression in oocytes. Oocytes were isolated from Xenopus laevis (Nasco, Racine, WI) using standard procedures (43), and the follicular cell layer was removed after treatment with collagenase (GLBCO-BRL, Gaithersburg, MD). Oocytes were incubated in ND-96 (in mM, 96 NaCl, 2 KCl, 18 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.5) (43) and maintained at 18°C. Twenty-four hours after isolation, healthy stage V and VI oocytes were microinjected with 50 nl of water or an equal volume containing rP₂U receptor sense or antisense cRNA (1 µg/µl). Two electrode voltage clamp studies were performed after 48–72 h using an Axoclamp 2 amplifier (Axon Instruments, Foster City, CA) at room temperature under continuous superfusion. Data were digitized for storage on a personal computer. Digitized recordings were analyzed using Axotape software (Axon Instruments).
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 (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 I_sc 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 I_sc in all monolayers tested. The response occurred rapidly and peaked within ~5 min as shown in Fig. 2. The increase in I_sc (ΔI_sc) 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 I_sc 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.

Fig. 2. Measurement of ATP-stimulated transepithelial transport. Short-circuit current (I_sc) 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 I_sc that was maximal within ~5 min and then decreased despite the continued presence of ATP.
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 ΔIsc showed approximately equal responses to ATPγS, UTP, and ATP, and the concentration of UTP required to produce half-maximal increases in ΔIsc 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 P2y 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 ΔIsc (n = 81). Similarly, exposure to amiloride after stimulation of ΔIsc 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...
Cl\(^-\) conductance(s) in the apical membrane with 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 Ba\(^{2+}\)-sensitive K\(^+\) channels in the basolateral membrane to recycle K\(^+\) and maintain the driving force for Cl\(^-\) efflux (25). Because amiloride had no effect on the amplitude or duration of ATP-stimulated increases in \(I_{sc}\), the effects of inhibitors of Cl\(^-\) transport were assessed. Taken together, the findings support an important contribution of Cl\(^-\) transport to ATP-stimulated currents. First, when added to the apical solution, putative anion channel blockers consistently inhibited the current response. In one experimental approach, monolayers were first exposed to ATP to stimulate \(\Delta I_{sc}\), and then NPPB (50 or 100 \(\mu M\), \(n = 6\)) or DPC (1.5 \(mM\), \(n = 4\)) was added to the apical solution. In each case, there was significant inhibition of \(\Delta I_{sc}\). A representative study for NPPB (100 \(\mu M\)) is shown in Fig. 4. In another experimental approach, monolayers were preincubated with NPPB or DIDS for 15 min, which also caused a significant decrease in \(\Delta I_{sc}\) compared with same-day controls (Fig. 5). Second, preincubation with bumetanide in the basolateral solution in concentrations sufficient to inhibit Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport (200 \(\mu M\)) inhibited the response to ATP (Fig. 5). Finally, preincubation with Ba\(^{2+}\) in the basolateral solution to inhibit \(K^+\) conductances (5 \(mM\)) also decreased \(\Delta I_{sc}\) (Fig. 5). Thus \(P_{2u}\) receptor stimulation increases \(I_{sc}\) through effects on transepithelial transport of Cl\(^-\).

Molecular cloning of rat liver \(P_{2u}\) receptor. Complementary DNAs encoding \(P_{2u}\) 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 \(P_{2u}\) receptor from mouse brain as a probe (24). A single clone of 2,164 bp was plaque purified (r\(P_{2u}\)R), 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 \(P_{2u}\) proteins obtained from pituitary gland or alveolar type II cell cDNA. Sequence comparison with the cloned murine and human \(P_{2u}\) receptors showed >95% homology at the amino acid level (Fig. 6). However, the 5' untranslated region of the r\(P_{2u}\)R cDNA harbors a 200 bp fragment immediately upstream of the putative AUG start codon that is not present in the mouse or the human \(P_{2u}\) 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 250 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′-tetraacetic 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 > 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\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent signaling pathways according to the model under investigation (6, 44). However, in airway epithelium, stimulation of Cl\textsuperscript{−} secretion by extracellular nucleotides involves channels other than CFTR and has been

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**Fig. 8.** Functional characterization of rP\textsubscript{2u} in *Xenopus laevis* oocytes. cRNA was prepared from the donor rP\textsubscript{2u}R as described in METHODS and injected into *Xenopus laevis* oocytes (50 ng). After 48–72 h, oocytes were voltage clamped at −50 mV, and representative current traces are shown. A: in water-injected oocytes, exposure to UTP (100 µM) had no effect (top). In contrast, UTP (0.35 µM) elicited large inward currents in oocytes injected with rP\textsubscript{2u} cRNA (bottom). B: in Ca\textsuperscript{2+}-free extracellular buffer, exposure to UTP elicited inward currents. Subsequent intracellular injection with EGTA to chelate intracellular Ca\textsuperscript{2+} completely inhibited the response to UTP, consistent with a role for Ca\textsuperscript{2+} mobilization in current activation. C: dose-response relationship for AMP, ADP, ATP, and UTP. Nucleotides were added to the bath, and values represent the means ± SE of inward currents in oocytes injected with rP\textsubscript{2u}R (n = 4–6). \(I_{\text{max}}\), maximal current response to 100 µM UTP.
proposed as an approach to treatment of the secretory defect associated with CF (23, 34).

NRC monolayers spontaneously developed high transepithelial resistances and, under voltage-damp conditions, exhibited basal \( I_{sc} \) values of 10–30 µA and lumen-negative transepithelial potentials. In the nominal absence of HCO\(_3\)-, basal transport was attributable for the most part to parallel function in the apical membrane of an amiloride-sensitive Na\(^+\) conductance and NPPB-sensitive Cl\(^-\) conductance(s). The relative contribution of the Na\(^+\) conductance varied among monolayers, with increases in the amiloride-sensitive component at higher basal values of \( I_{sc} \).

Exposure to nucleotides elicited a rapid increase in \( I_{sc} \) that was mediated by an increase in apical Cl\(^-\) permeability and stimulation of transepithelial Cl\(^-\) transport. In selected studies, the increase in \( I_{sc} \) was associated with lumen-negative potentials of \(-10\) mV or more and was unaffected by amiloride, consistent with net movement of Cl\(^-\) into the lumen. Moreover, inhibitors of Cl\(^-\) transport, including putative Cl\(^-\) channel blockers in the apical solution, and inhibitors of K\(^+\) channels and Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport in the basolateral solution significantly inhibited the response to ATP. These findings indicate that purinergic stimulation is positively coupled to transport mechanisms in both the apical and basolateral membranes of biliary cells. It is interesting that NPPB and DPC returned \( I_{sc} \) to basal values when added after ATP stimulation, but the inhibitory effects of Cl\(^-\) channel blockers were incomplete in preincubation studies. The reasons for the partial inhibition are not known and may reflect impaired access of blockers to channels caused by the dense microvilli in the apical membrane (46) or intrinsic differences in the pharmacological properties of the channels themselves. In other epithelia the response to purinergic stimulation involves several channel types, including both CFTR and outwardly rectifying Cl\(^-\) channels, which differ in their sensitivity to blockers (6, 42). Alternatively, a contribution of other electrogenic transport mechanisms cannot be excluded.

Both molecular and functional criteria indicate that purinergic receptors of the P\(_2\) subclass are involved in the secretory response. When applied to the apical membrane, ATP, UTP, and ATP\(_2\)S were equally potent in their effects on \( I_{sc} \). Moreover, rP\(_{2\alpha}\)R mRNAs are abundant in NRC cells, rat hepatocytes, and liver cell lines, and expression of rP\(_{2\alpha}\)R in oocytes gave similar results. The presence in liver differs from results obtained by Rice et al. (37) but confirms the Northern blot analysis performed by Lustig et al. (24) on mouse tissue. In addition, there is abundant physiological evidence for the presence of purinergic receptors in hepatobiliary cells (5, 17, 22, 27, 47). The rP\(_{2\alpha}\)R cDNA identified here shows \( \geq 95\% \) homology at the amino acid level with the mouse brain receptor and exhibits similar functional properties when expressed in oocytes (24). Moreover, current activation in oocytes is mediated through a Ca\(^{2+}\)-dependent pathway that appears analogous to the Ca\(^{2+}\)-mobilizing effects of UTP in biliary cells (47).

The concentration of ATP required to stimulate \( I_{sc} \) is similar to the average concentration of ATP in human bile of \(-1.5\) 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 \( I_{sc} \) response (4.5 ± 1.4 µA, \( n = 10 \)), averaging only 10–15% of those induced by apical exposure. ATP\(_2\)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 inhibitory 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 independent 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.

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