Cholangiocyte primary cilia are chemosensory organelles that detect biliary nucleotides via P2Y\textsubscript{12} purinergic receptors

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Masyuk AI, Gradilone SA, Banales JM, Huang BQ, Masyuk TV, Lee S-O, Splinter PL, Stroope AJ, LaRusso NF. Cholangiocyte primary cilia are chemosensory organelles that detect biliary nucleotides via P2Y\textsubscript{12} purinergic receptors. Am J Physiol Gastrointest Liver Physiol 295: G725–G734, 2008. First published August 7, 2008; doi:10.1152/ajpgi.90265.2008.—Cholangiocytes, the epithelial cells lining intrahepatic bile ducts, contain primary cilia, which are mechano- and osmosensory organelles detecting changes in bile flow and osmolality and transducing them into intracellular signals. Here, we asked whether cholangiocyte cilia are chemosensory organelles by testing the expression of P2Y purinergic receptors and components of the cAMP signaling cascade in cilia and their involvement in nucleotide-induced cAMP signaling in the cells. We found that P2Y\textsubscript{12} purinergic receptor, adenylyl cyclases (i.e., AC4, AC6, and AC8), and protein kinase A (i.e., PKA RI-\textalpha and PKA RII-\textalpha regulatory subunits), exchange protein directly activated by cAMP (EPAC) isomorph 2, and A-kinase anchoring proteins (i.e., AKAP150) are expressed in cholangiocyte cilia. ADP, an endogenous agonist of P2Y\textsubscript{12} receptors, perfused through the lumen of isolated rat intrahepatic bile ducts or applied to the ciliated apical surface of normal rat cholangiocytes (NRCs) in culture induced a 1.9- and 1.5-fold decrease of forskolin-induced cAMP levels, respectively. In NRCs, the forskolin-induced cAMP increase was also lowered by 1.3-fold in response to ATP-\gamma-S, a nonhydrolyzed analog of ATP but was not affected by UTP. The ADP-induced changes in cAMP levels in cholangiocytes were abolished by chloral hydrate (a reagent that removes cilia) and by P2Y\textsubscript{12} siRNAs, suggesting that cilia and ciliary P2Y\textsubscript{12} are involved in nucleotide-induced cAMP signaling. In conclusion, cholangiocyte cilia are chemosensory organelles that detect biliary nucleotides through ciliary P2Y\textsubscript{12} receptors and transduce corresponding signals into a cAMP response.

CHOLANGIOCYTES, the epithelial cells lining intrahepatic bile ducts (IBDs), contain primary cilia, nonmotile, solitary organelles extending from the apical plasma membrane into the ductal lumen (21, 28, 29). In many cell types, including cholangiocytes, primary cilia function as sensory organelles detecting multiple (i.e., mechano-, osmo-, chemo-) stimuli and transducing them into intracellular signaling (12, 16, 27, 40, 46). However, although increasing evidence suggests the ability of primary cilia to act as mechano- and osmosensors (16, 24, 28, 34–37, 40–42, 49, 50, 55), less data support their chemosensory functions. To function as chemosensory organelles, primary cilia should possess receptors and associated signaling cascades through which signals induced by specific ligands are transmitted into the cell. Such mechanism exists in Caenorhabditis elegans neuronal primary cilia, which express specific G protein-coupled receptors linked to the cGMP signaling pathway (5). Several recent observations also suggest that, in vertebrates, primary cilia express receptors and components of signaling cascades that might provide chemosensory functions of these organelles. For example, neuronal primary cilia in the rat brain express somatostatin receptor 3 and 5-HT\textsubscript{6} serotonin receptors (9, 17, 57). Sternal chondrocyte primary cilia in chick embryo contain extracellular matrix receptors such as \alpha\textsubscript{2}, \alpha\textsubscript{3}, and \beta\textsubscript{1}-integrins and NG2 (22, 31); \alpha\textsubscript{3}, \alpha\textsubscript{5}-, and \beta\textsubscript{1}-integrins are also found in primary cilia in Madin-Darby canine kidney cells and in rat renal epithelial cells where they may function as chemoreceptors (39). In cultured fibroblasts, primary cilia express the platelet-derived growth factor receptor alpha and the PI3-kinase/Akt and Mek1/2-Erk1/2 signaling pathways, thus possessing necessary components for their chemosensory function (12, 46, 48).

To directly address a chemosensory function of cholangiocyte cilia, we extended our previous observation on ciliary adenylyl cyclase (AC) 6, a component of the cAMP signaling cascade (28), by hypothesizing that specific G protein-coupled receptors linked to the cAMP signaling pathway might be involved in a chemosensory function of primary cilia in biliary epithelia. Taking into account that bile contains ATP and ADP, which are considered extracellular signaling molecules affecting cholangiocyte functions via apically located P2Y purinergic receptors (11, 14, 15, 32, 33, 43, 45), we further hypothesized that some P2Y receptors are expressed in cholangiocyte cilia, providing a chemosensory function. A family of P2Y receptors consists of eight members (i.e., P2Y\textsubscript{1}, P2Y\textsubscript{2}, P2Y\textsubscript{4}, and P2Y\textsubscript{6}) associated with the intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) signaling pathway, whereas P2Y\textsubscript{12}, P2Y\textsubscript{13}, and P2Y\textsubscript{14} are associated with cAMP signaling. In contrast, P2Y\textsubscript{11} is associated with both [Ca\textsuperscript{2+}]\textsubscript{i} and cAMP signaling (1, 10, 56). Among four P2Y receptors (i.e., P2Y\textsubscript{11}-\textsubscript{14}) associated with the cAMP signaling pathway, P2Y\textsubscript{12} and P2Y\textsubscript{13} are most suitable for a chemosensory function of cholangiocyte cilia in rodents. This conclusion is based on three critical observations. First, rat and...

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mouse are P2Y11-deficient animals (1, 56); thus P2Y11 could not be considered as a potential ciliary-associated purinergic receptor in rats. Second, P2Y12 and P2Y13 are activated by ADP and ATP, i.e., two nucleotides that are extracellular signaling molecules in bile (1, 11, 56); thus these two P2Y purinergic receptors could be potentially involved in a chemosensory function of cholangiocyte cilia. Third, P2Y14 is activated by UDP-glucosamine but not by ATP or ADP (56). There are no reports on the presence of UDP-glucose in bile, and its potential role as an extracellular signaling molecule in biliary epithelia is unclear; thus a potential role of P2Y14 in cholangiocytes is not supported by existing studies. We therefore tested 1) the expression of P2Y12 and P2Y13 in rat cholangiocytes and their cilia, 2) the expression of components of the cAMP signaling cascade in cholangiocyte cilia, 3) the effects of nucleotides on cAMP levels in cholangiocytes, and 4) the involvement of cholangiocyte cilia in nucleotide-induced cAMP signaling.

MATERIAL AND METHODS

All chemicals were of highest purity commercially available and were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Animals and models. Male Sprague-Dawley rats (225–250 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN), housed in a temperature-controlled room (22°C) with 12-h light/dark cycles, and maintained on a standard diet with free access to water. All experimental procedures were approved by the Animal Use and Care Committee of the Mayo Foundation. Rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip) for in vivo procedures. Livers were harvested, fixed in 10% formaldehyde, and embedded in paraffin. IBDS ranging in luminal diameter from 100 to 125 μm and in length from 1.0 to 1.5 mm were isolated from rat liver and microperfused as previously described (26, 28). Normal rat cholangiocytes (NRCs) were isolated from rat liver and cultured as described (3). Cilia were isolated from cultured normal mouse cholangiocytes (NMCs) by the slide-pull technique (21).

Solutions. The composition of standard Ringer-HCO3 buffer (KRB) (in mM) was: 120.0 NaCl, 5.9 KCl, 1.2 Na2HPO4, 1.0 MgSO4, 25.0 NaHCO3, 1.25 CaCl2, and 5.0 D-glucose, pH 7.4.

RNA isolation and RT-PCR. Total RNA was obtained from freshly isolated rat IBDS, cholangiocytes, hepatocytes, brain, NRCs, and NMCs with the use of TRIzol reagent (Invitrogen, Carlsbad, CA). The expression of P2Y12 and P2Y13 were detected by RT-PCR using specific primers. The PCR products were sequenced, and the identities of the amplicons were verified by data base homology searches (BLAST; NCBI, National Institutes of Health).

Western blotting. Freshly isolated rat cholangiocytes, hepatocytes, brain and NMCs were resuspended in RIPA Sample Buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Samples (10 to 40 μg) of protein extracts were run in 7.5% SDS-PAGE and electrotransferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with 5% nonfat dried milk in PBS-0.5% Tween, the samples were incubated overnight at 4°C with antibodies against P2Y12 and P2Y13 (Alomone Laboratories, Jerusalem, Israel; dilution 1:100). After three washes with PBS-0.5% Tween, the samples were incubated with peroxidase-conjugated secondary antibodies (dilution 1:5,000) for 45 min at room temperature. Bands were visualized with ECL Plus Western Blotting Detection Kit (BD Biosciences, Franklin Lakes, NJ).

Immunogold scanning electron microscopy. Isolated IBDS were immersed in 4% phosphate-buffered glutaraldehyde (pH 7.4) for 1 h, treated with 0.1% Triton X-100 for 5 min, and then rinsed with phosphate buffer three times. The samples were incubated overnight at 4°C with anti-P2Y12 (Alomone, dilution 1:100) and then incubated for 2 h at room temperature with anti-rabbit IgG conjugated with 1.4 nm of nanogold. The samples were fixed in 1% glutaraldehyde for 10 min, and gold enhancement was performed with a gold enhancement kit (Nanoprobes, Yaphank, NY). The samples were dehydrated, critical-point dried, and carbon coated. Images were generated at 8 kV by a Hitachi S-4700 microscope (Hitachi, Pleasanton, CA).

Immunofluorescence confocal microscopy. Immunofluorescence microscopy was performed with a confocal microscope Zeiss LSM 510 (Carl Zeiss, Thornwood, NY) with a 100× Plan-Apochromat 1.4 NA oil objective. Isolated split-open IBDS, and rat liver samples were incubated with two antibodies, one against a ciliary marker, acetylated α-tubulin (dilution 1:200, Sigma), and the other against protein of interest, i.e., P2Y12, P2Y13 (Alomone, dilution 1:100), AC4, AC5, AC6, AC7 (FabGennix International, Frisco, TX; dilution 1:100), AC8 (Santa Cruz Biotechnology, dilution 1:100), PKA RI-α, PKA RII-α, and PKA RII-β (Santa Cruz Biotechnology, dilution 1:50), PKA RI-β (Calbiochem, San Diego, CA; dilution 1:50), exchange protein directly activated by cAMP (EPAC1) and EPAC2 (Santa Cruz Biotechnology, dilution 1:50) followed by incubation for 1 h at room temperature with fluorescent secondary antibodies (Molecular Probes, Eugene, OR; dilution 1:100). Nuclei were stained in blue with 4′,6-diamino-2-phenylindole (Sigma). Corresponding negative controls (i.e., no primary antibodies) were performed in all experiments. Images were taken with magnification ×100 and afterwards cropped using Adobe Photoshop to show the ciliary location of each protein.

Measurement of cAMP. A single IBD was equilibrated for 30 min in the perfusion chamber; then forskolin (FSK, 100 μM) was added into the bathing solution (KRB) containing 0.5 mM IBMX, an inhibitor of phosphodiesterases. Afterwards, an IBD was perfused through its lumen for 15 min with standard KRB (control, no ADP) or with KRB containing 100 μM ADP; during perfusion IBDS remained in the bathing KRB containing FSK and IBMX. After perfusion, an IBD was removed from the micro perfusion system, and cAMP levels were measured by using the Bridge-It cAMP designer cAMP assay (Mediomics, St. Louis, MO). Results were expressed in pmol of cAMP per μg DNA. The amount of DNA in a single IBD was determined by using the DNeasy Tissue Kit (Qiagen, Valencia, CA).

NRCs were stimulated for 15 min at 37°C with FSK (100 μM) in the presence of 0.5 mM IBMX and simultaneously treated with ADP (100 μM), ATP-γS (10 μM), or UTP (100 μM). cAMP levels were measured by the Bridge-It cAMP designer cAMP assay (Mediomics) and expressed in pmol of cAMP per well.

Statistical analysis. All values are expressed as mean ± SE. Statistical analysis was performed by the Student’s t-test, and results were considered statistically different at P < 0.05.

RESULTS

Expression of P2Y12 and P2Y13 in rat cholangiocytes and cilia. RT-PCR and Western blotting show that both P2Y12 and P2Y13 are expressed in rat cholangiocytes (Fig. 1, A and B). P2Y12 and P2Y13 are also expressed in rat hepatocytes and brain, which we used as a positive control (Fig. 1, A and B). To test the expression of P2Y12 and P2Y13 in cholangiocyte cilia, paraffin sections of rat liver were stained with antibodies to a ciliary marker, acetylated α-tubulin, and to either P2Y12 or P2Y13. Figure 1C shows the IBD with cilia (stained in red)
extending from the cholangiocyte apical plasma membrane into the ductal lumen. Images in green and overlay images in yellow suggest that P2Y12 is expressed in the cholangiocyte apical plasma membrane and cilia. In contrast, P2Y13 is not detected in cholangiocyte cilia (data not shown).

Expression of P2Y12 in cholangiocyte cilia was confirmed by immunogold scanning electron microscopy (Fig. 1D) (51). The scanning electron microscopy image of the lumen of isolated split-open IBD (Fig. 1D, a) shows the cholangiocyte apical surface with a single cilium; the image of the same field generated by backscattered electrons identified the position of the gold-labeled secondary antibodies, which are seen as bright white dots localized to the ciliary membrane.

Expression of components of the cAMP signaling cascade in cholangiocyte cilia. Given that P2Y12 is a G protein-coupled receptor associated with the cAMP signaling pathway, ACs should also be localized to the same cellular compartment, i.e., to the ciliary membrane. Figure 2 shows that at least three ACs (i.e., AC6, AC4, and AC8) are expressed in cilia. AC5 and AC7 are not present in cilia. AC9 was not tested in this study because high quality antibodies against this protein were not available.

Recent developments suggest that cAMP signaling requires tight regulation via a flexible downstream machinery to ensure an appropriate cellular response (30). The components of such machinery are PKA and EPAC, which are recruited to a given subcellular compartment by A-kinase anchoring proteins (AKAPs) (6, 52). According to our recent observation, rat cholangiocytes express all four regulatory subunits of PKA (i.e., PKA RI-α, PKA RI-β, PKA RII-α, and PKA RII-β) and both isoforms of EPAC (i.e., EPAC1 and EPAC2) (4). Dual labeling of rat liver sections with antibodies against acetylated α-tubulin and proteins of interest (i.e., four regulatory subunits of PKA and both EPAC isoforms) shows that PKA (i.e., two regulatory subunits, PKA RII-α and PKA RI-β) and EPAC (isoform 2) are expressed in cholangiocyte cilia (Figs. 3 and 4). AKAP150, which is known to form a functional complex with AC6, PKA, and EPAC (6, 52), is also expressed in cholangiocyte cilia (Fig. 4). All tested components of the cAMP signaling pathway are not exclusively localized to cholangiocyte cilia; they are also localized intracellularly and on the cholangiocyte plasma membrane. However, their expression in the ciliary membrane suggests that cilia possess key elements of the cAMP signaling cascade.

ADP decreases cAMP levels in cholangiocytes in a P2Y12-dependent manner. P2Y12 is a G protein-coupled receptor linked to ACs via Gπ, and its activation by the most potent endogenous agonist, ADP, leads to inhibition of ACs and to a decrease in cAMP levels (1, 56). To determine whether ADP affects cAMP signaling in cholangiocytes via P2Y12 receptors, experiments were performed on microperfused IBFs and on ciliated NRCs in which cAMP levels were increased by FSK (100 μM). In nontreated IBFs and NRCs, the basal levels of cAMP were 0.99 ± 0.26 pmol/μg DNA and 0.10 ± 0.06 pmol/well, respectively. ADP (100 μM) alone did not affect the basal cAMP levels in either microperfused IBFs or in NRCs, whereas FSK increased cAMP concentrations to 9.78 ± 1.35 pmol/μg DNA and to 14.26 ± 0.06 pmol/well, respec-
Fig. 2. Adenylyl cyclases (ACs) are expressed in cholangiocyte primary cilia. Isolated split-open rat IBDs were stained with antibodies to a ciliary marker, acetylated α-tubulin (shown in red), and antibodies to AC4, AC5, AC6, AC7, and AC8 (shown in green). Overlay images in yellow show that AC4, AC6, and AC8 but not AC5 and AC7 are expressed in cholangiocyte cilia.

Perfusion of IBDs and incubation of NRCs for 15 min at 37°C with ADP (100 μM) resulted in a decrease of FSK-induced cAMP levels from 9.78 ± 1.35 to 5.08 ± 1.06 pmol/μg DNA (i.e., by 1.9-fold; *P* < 0.05) and from 14.26 ± 0.06 to 9.30 ± 1.12 pmol/well (i.e., 1.5-fold; *P* < 0.05), respectively (Fig. 5, A and B). ATP-γS, a nonhydrolyzed analog of ATP, which is considered as another agonist for P2Y12, did not affect basal levels of cAMP but lowered the FSK-stimulated cAMP increase by 1.3-fold (*P* < 0.05). In contrast, UTP, a known agonist for P2Y2 and P2Y4,
but not for P2Y12 (1), did not affect either basal cAMP levels or FSK-induced cAMP increase (Fig. 5C), suggesting that P2Y12 is likely involved in effects of ADP and ATP-γS on FSK-induced cAMP levels in rat cholangiocytes.

To directly address the involvement of P2Y12 receptors in the ADP-induced changes in cAMP levels, experiments were performed on NRCs pretreated with a nonspecific inhibitor of P2Y receptors, suramin (50 μM), and on microperfused IBDs in which P2Y12 receptors were silenced by siRNAs. Data in Fig. 6 show that suramin completely abolished the ADP-induced cAMP decrease in NRCs. Likewise, P2Y12 siRNAs, which inhibit the expression of P2Y12 on both mRNA and protein levels by 86 ± 5% and 53 ± 8%, respectively (Fig. 7A and B), abolished the ADP-induced cAMP decrease in microperfused IBDs (Fig. 7C). Thus the data suggest that ADP affects cAMP signaling in rat cholangiocytes via P2Y12 purinergic receptors.

ADP decreases cAMP levels in cholangiocytes in a ciliary-dependent manner. To further address whether cholangiocyte cilia are involved in the ADP-induced cAMP signaling response, experiments were performed on IBDs and NRCs after pharmacological removal of cilia by chloral hydrate. Chloral hydrate deciliates different cell types, including cholangiocytes, via an unknown mechanism but do not cause other significant structural and/or functional alterations in the cells (16, 28, 42). Functional studies revealed that ADP does not affect cAMP levels in deciliated IBDs (i.e., 5.55 ± 1.69 compared with 6.88 ± 2.12 pmol/μg DNA in IBDs stimulated with FSK alone; Fig. 8A) and in deciliated NRCs (i.e., 13.29 ± 1.23 pmol/μg DNA in ADP-stimulated NRCs compared with 16.78 ± 1.58 pmol/μg DNA in NRCs stimulated with FSK alone; Fig. 8B).

Fig. 3. PKA is expressed in cholangiocyte primary cilia. Paraffin sections of rat liver were stained with an antibody to a ciliary marker acetylated α-tubulin (shown in red) and antibodies to PKA regulatory subunits (shown in green). Cholangiocyte nuclei were visualized by staining with DAPI (shown in blue). On overlay of the images, colocalization of acetylated α-tubulin and PKAI-β and PKAI-α (shown in yellow) was seen, indicating that 2 of 4 PKA subtypes are expressed in cholangiocyte cilia.
compared with 13.87 ± 0.66 pmol/well in NRCs stimulated with FSK alone; Fig. 8B). Thus cholangiocyte cilia play an important role in the ADP-induced intracellular cAMP signaling response.

**DISCUSSION**

The key findings of this study relate to a chemosensory function of cholangiocyte primary cilia. We demonstrated that 1) P2Y12 and P2Y13 purinergic receptors associated with the cAMP signaling pathway are expressed in rat cholangiocytes; 2) P2Y12 and components of the AKAP signaling complex (i.e., AKAP150, ACs, PKA, and EPAC) are expressed in cholangiocyte cilia; 3) ADP inhibits FSK-induced cAMP signaling in cholangiocytes; and 4) ADP-induced changes in cAMP levels are P2Y12 and ciliary dependent. Taken together, these observations suggest that cholangiocyte primary cilia function as chemosensory organelles.

On the basis of molecular structures and signal transduction mechanisms, P2Y receptors are divided into two subgroups; the first group consists of P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11, which are coupled to Gq and associated with activation of phospholipase C and Ca^{2+} release from the IP3-sensitive in...
tracellular stores (1, 10). In addition, P2Y11 is also coupled to Gs and associated with activation of ACs and an increase in intracellular cAMP (1, 56). The second group of P2Y receptors consists of recently cloned P2Y12, P2Y13, and P2Y14, which are coupled to Gi and associated with inhibition of ACs and decreased cAMP levels (1, 56).

This study suggests that one of the P2Y receptors, i.e., P2Y12, is involved in chemosensory functions of cholangiocyte cilia. P2Y12 purinergic receptor was originally identified on mRNA level in human platelets and was considered to have restricted expression, being abundantly present in platelets and to a smaller extent in brain (19). A similar selective expression of P2Y12 receptor was also described in rat tissues (19). Later, P2Y12 transcript was found in freshly isolated rat hepatocytes (13) but not in cultured rat hepatocytes and human liver (13, 54, 62). Thus our data are the first to demonstrate the expression of P2Y12 in rat cholangiocytes on both mRNA and protein levels, its localization to primary cilia, and involvement in a chemosensory function of these organelles.

In rat cholangiocytes, P2Y12 is activated by two nucleotides (i.e., ADP and ATP-γS). ADP is the most potent endogenous agonist for P2Y12. ATP-γS may activate P2Y12 but with less efficiency (1, 56). ATP is a partial agonist for P2Y12 and may also act as a relatively low-affinity antagonist for this receptor (8). ATP is released in bile by both hepatocytes and cholangiocytes via unknown mechanisms, whereas biliary ADP is likely a product of degradation of ATP (11, 32, 33). We previously demonstrated that ATP, ATP-γS, and ADP affect [Ca^{2+}]_i signaling in rat cholangiocytes via P2Y1, P2Y2, and P2Y4 purinergic receptors localized to the apical plasma membrane (14). This study shows that the cAMP signaling pathway in rat cholangiocytes is also affected by ADP and ATP-γS. Moreover, the effects of nucleotides on cAMP levels in cholangiocytes depend on cilia and ciliary P2Y12.
Our previous observations suggest that cholangiocyte primary cilia extending for several microns from the apical plasma membrane into the bile ductal lumen function as mechano- and osmosensory organelles through which bile flow and bile osmolality induce intracellular Ca\textsuperscript{2+} and cAMP signaling responses (16, 28). Several proteins [i.e., polycystin-1, polycystin-2, AC6, and transient receptor potential vanilloid (TRPV)4] localized to the ciliary membrane play a critical role in ciliary sensory functions (16, 28). In this work, we report additional ciliary protein (i.e., P2Y\textsubscript{12}) that is involved in sensory functions of primary cilia. P2Y\textsubscript{12} is not localized exclusively to cilia. This protein is also expressed on the cholangiocyte apical plasma membrane. However, its localization to cilia appears to be critically important for the nucleotide-induced changes in cAMP levels in cholangiocytes and for ciliary sensory functions.

The mechanisms of sensory functions of primary cilia in general, and cholangiocyte cilia in particular, are not fully understood. The present notion on a mechanoresponse function of primary cilia is based on observations that the flow-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} in renal epithelial cells and IBDs relates to influx of extracellular Ca\textsuperscript{2+} into the cell via a ciliary mechanoresponse complex formed by polycystin-1 and polycystin-2 (28, 34). However, recent findings suggest that the flow-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} is caused or augmented by a flow or stretch-induced ATP release from epithelial cells, which acts as a paracrine-signaling molecule activating epithelial P2 receptors (23, 38, 59). It is likely that the flow-induced release of ATP requires a mechanosensory function of primary cilia; however, the involvement of cilia in this process remains to be proven (38).

The osmosensory function of primary cilia in biliary epithelia relates to functional properties of a ciliary-associated Ca\textsuperscript{2+} channel, TRPV4, and is also linked to ATP release. We recently showed (16) that hypotonicity induces a release of ATP from ciliated cholangiocytes but not from cholangiocytes that are devoid of cilia after the treatment with chloral hydrate.

Thus, taken together, these observations and our new findings on ciliary-associated P2Y\textsubscript{12} suggest the complexity of the mechanisms providing sensory functions of primary cilia. These observations also allow us to hypothesize that ciliary-associated P2Y\textsubscript{12} purinergic receptor may have specific roles in ciliary sensory functions. Indeed, we previously showed that, although the flow-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} in cholangiocytes was abolished by removal of cilia, deciliated cholangiocytes still respond to ATP by an increase in [Ca\textsuperscript{2+}]\textsubscript{i} (28), whereas, the nucleotide-induced changes in cAMP levels in cholangiocytes were completely abolished by removal of cilia. Thus, biliary ATP and other nucleotides may induce a [Ca\textsuperscript{2+}]\textsubscript{i} increase in cholangiocytes via apically located P2Y\textsubscript{1}, P2Y\textsubscript{2}, and P2Y\textsubscript{4} receptors, i.e., through ciliary-independent mechanisms (14, 59). In contrast, biliary nucleotides affect cAMP signaling in cholangiocytes only if primary cilia remain structurally and functionally intact. Moreover, nucleotide-induced cAMP signaling in cholangiocytes requires P2Y\textsubscript{12} and its localization to cilia.

This study also shows that P2Y\textsubscript{12} may be linked to the ciliary AKAP signaling complex. The components of this complex include ACs, PKA, and EPAC. ACs are known to be expressed in different types of cilia. For example, olfactory sensory cilia possess AC2, AC3, and AC4 (2, 58). Motile cilia in airway epithelia have soluble AC (47). AC3 was recently reported in primary cilia of the adult mouse brain (7). PKA and EPAC, which are targeted in proximity to cAMP gradients by AKAPs, represent other components of the cAMP signaling cascade in cholangiocyte cilia. EPAC proteins (i.e., EPAC1 and EPAC2, also known as RapGEF3 and RapGEF4, respectively) belong to a family of the cAMP-regulated guanine nucleotide exchange factors regulating cellular processes via PKA-independent mechanisms (20). The localization of PKA and EPAC to cholangiocyte cilia may suggest the interconnectivity between EPAC- and PKA-mediated signaling in these organelles. PKA and AKAPs were previously found in Paramecium cilia (18), in mammalian motile cilia (25, 44), and in the Chlamydomonas flagella (60, 61). In this work, we demonstrate for the first time the expression of PKA, EPAC, and AKAP in mammalian primary cilia.

Our previous (28) and present studies show that fluid flow and biliary nucleotides do not affect the basal levels of cAMP in microperfused isolated rat IBDs but inhibit the FSK-stimulated cAMP increase via cilia and ciliary proteins, PC1, PC2, AC6, and P2Y\textsubscript{12}. This suggests that a chemosensory function of cholangiocyte cilia is critically important for downregulation of the cAMP signaling pathway initially activated by other signaling molecules, e.g., secretin. In a working model depicted in Fig. 9, we propose that mechanisms providing mechano-, osmo-, and chemosensory functions of cholangiocyte cilia are overlapping, and, in fact, one stimulus (e.g., bile flow or bile osmolarity) may generate a second stimulus (i.e., ATP release), affecting cilia in a coordinated fashion. Our

![Fig. 9. A working model of coordinated sensory functions of cholangiocyte cilia. (See DISCUSSION for details.)](http://ajpgi.physiology.org/)

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model shows that 1) mechanical and/or osmotic stimuli (i.e., bile flow and bile toxicity) affect [Ca\(^{2+}\)], in cholangiocytes via ciliary-dependent and ciliary-independent mechanisms (16, 28, 59), simultaneously inducing a release of ATP into the ductal lumen; 2) both ATP and ADP induce an increase in [Ca\(^{2+}\)], via P2Y\(_1\), P2Y\(_2\), and P2Y\(_4\) purinergic receptors but do not affect cAMP signaling; and 3) when the cAMP signaling pathway is initially activated by other stimuli (e.g., by secretin, a secretogog acting in cholangiocytes via secretin receptors expressed on the basolateral plasma membrane), biliary ATP and ADP inhibit cAMP signaling via cilia and ciliary P2Y\(_{12}\).

In conclusion, this study is the first to identify the expression of P2Y\(_{12}\) and several components of the cAMP signaling cascade in mammalian primary cilia that are critically important for ciliary sensory functions.

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