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DIFERENTIALLY EXPRESSED ADENYLYL CYCLASE ISOFORMS MEDIATE SECRETORY FUNCTIONS IN CHOLANGIOCYTE SUBPOPULATION

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Abstract

cAMP is generated by adenylyl cyclases (ACs) a group of enzymes with different tissue specificity and regulation. We hypothesized that AC isoforms are heterogeneously expressed along the biliary tree, are associated with specific secretory stimuli and are differentially modulated in cholestasis.

Methods: Small (SDC) and large (LDC) cholangiocytes were isolated from controls and from lipopolysaccharide-treated (LPS) or α-naphthylisothiocyanate-treated (ANIT) rats. ACs isoforms expression was assessed by real-time PCR. Secretion and cAMP levels were measured in intrahepatic bile duct units after stimulation with secretin, forskolin, HCO_3^-/CO_2, cholinergic and β-adrenergic agonists, with or without selected inhibitors or after silencing of AC8 or sAC with siRNA.

Results: Gene expression of the Ca^{2+}-insensitive isoforms (AC4, AC7) was higher in SDC, while that of the Ca^{2+}-inhibitable (AC5, AC6, AC9), the Ca^{2+}/calmodulin stimulated AC8, and the soluble sAC, was higher in LDC. Ca^{2+}/calmodulin-inhibitors and AC8 gene silencing inhibited choleresis and cAMP production stimulated by secretin and acetylcholine, but not by forskolin. Secretion stimulated by isoproterenol and calcineurin-inibitors was cAMP-dependent and GABA-inhibitable, consistent with activation of AC9. Cholangiocyte secretion stimulated by isohydric changes in $[HCO_3^-]_i$, was cAMP-dependent and inhibited by sAC-inhibitior and by sAC gene silencing. Treatment with LPS or ANIT increased expression of AC7 and sAC, while decreasing that of the others ACs.

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Conclusion: These studies demonstrate a previously unrecognized role of AC in biliary pathophysiology. In fact: 1) ACs isoforms are differentially expressed in cholangiocyte subpopulations, 2) AC8, AC9, and sAC mediate cholangiocyte secretion in response to secretin, β-adrenergic agonists, or changes in $[HCO_3^-]_i$, respectively, 3) ACs gene expression is modulated in experimental cholestasis.

Keywords

Large and small cholangiocytes; cAMP; Secretin; sAC; LPS; ANIT

INTRODUCTION

The intrahepatic biliary tree is lined by cholangiocytes, specialized epithelial cells that regulate the alkalinity(1) and volume of bile(2). Cholangiocyte secretion is modulated by autocrine/paracrine signals and gastrointestinal hormones including secretin(3). Cholangiocytes display morphological and functional heterogeneity(4,5). Cholangiocytes lining the smaller cholangioles (SDC), do not contribute to secretin-regulated ductal secretion(4,5) and are involved in the reparative/regenerative reponse to liver damage(2). Cholangiocytes lining the larger bile ducts (LDC) contribute to secretin-regulated bile secretion(4,5) and express the secretin receptor, the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and the Cl^-/HCO_3^- exchanger (AE2)(4-7). In addition to secretin, cholinergic and β-adrenergic agonists, as well as the intracellular HCO_3^- content, influence bile secretion trough the cAMP/PKA pathway(2,3).

Stimulation of secretin receptor promotes cAMP gradient by adenylyl cyclase (AC) and thereby a protein kinase A (PKA)-dependent phosphorylation of CFTR(2). This is a complex Cl^- channel that facilitates Cl^- and HCO_3^- efflux(8), but also performs important cell regulatory functions, such as modulation of apical Cl^-/HCO_3^- exchange(9), vesicular transport(10) and apical ATP release(11). The cAMP/PKA pathway also regulates vesicular trafficking, cell proliferation and signal transduction from ciliary mechanosensors(12). Cholangiocyte AC activity is altered in inflammatory conditions leading to cholestasis(13). The ability of proinflammatory cytokines and nitric oxide to block secretin-stimulated cholangiocyte secretion, by inhibiting AC function, suggested that AC represents an important player in cholangiocyte pathophysiology(13,14).

Nine membrane-bound isoforms of AC (AC1 to AC9)(15) and one cytosolic isoform, named soluble AC (sAC), are known(16). AC activity is relevant for many important functions in liver, but little information is available concerning the distribution and function of ACs isoforms in liver cells and their specific role in the regulation of ductal secretion(17). Recently, AC6, AC4 and AC8 were shown to be expressed in cholangiocyte cilia(12,18), indicating a preferential coupling between ciliary function and specific AC isoforms. AC isoforms have different expression patterns, a unique regulation and respond to specific stimuli. This allows the integration of multiple and contrasting extracellular signals into a net cellular cAMP content. Therefore, understanding the expression profile of ACs in a given cell type is a relevant piece of information.

We have hypothesized that AC isoforms are differentially expressed by cholangiocyte subpopulations, that each AC isoform is functionally coupled to specific mediators able to stimulate bile secretion and that the pattern of expression of ACs is affected by cholestatic injuries. Our results uncover an hiterto unrecognized complexity in the integration of secretory stimuli acting through the cAMP/PKA pathway in the biliary tree both in physiological and pathophysiological conditions.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma Chemical Co., unless otherwise indicated. Further details on materials can be found in the Supplementary section. The sAC inhibitor KH7 was a kind gift from Drs. Buck and Levin (Weill Cornell Medical College, New York)(19).

Animals and Cholestatic Models

Male Sprague Dawley rats (weighing 150-200 g) were used. Animals received care according to protocols approved by the University of Padua Institutional Veterinary Medicine Service, and by the Institutional Animal Care and Use Committee of Texas A&M Health Science Center, Texas. Rats were fed a diet containing 0.1 % ANIT (70 to 80 mg/kg) for up to 28 days(20). The endotoxin (LPS from *Salmonella typhimurium*) was given by a single i.p. injection (1 mg/Kg·bwt in 0.9% sodium chloride)(21); control rats received an IP saline injection. Rats were anesthetized with pentobarbital sodium (50 mg/Kg·bwt).

Isolation of Hepatocytes and Small and Large Cholangiocytes and Large Intrahepatic Bile Duct Units

Hepatocytes were obtained from normal and cholestatic rats as described(22). Cholangiocytes (SDC, ~8 μm diameter, and LDC, ~14 μm)(5) were purified by counterflow elutriation followed by immunoaffinity separation utilizing a monoclonal antibody(5). Large IBDU (mean diameter 20.8 μm), i.e. sealed fragments of isolated bile ducts, which retain polarity and secrete into a closed lumen, were prepared and purified as described, and were plated for 48 hours over a thin layer of Matrigel™ (Collaborative Research Products, Bedford MA)(13). Cultured normal rat intrahepatic cholangiocytes (NRC), a polarized rat cholangiocyte cell line, was generated and cultured as described(23,24).

PCR and Quantitative Real Time PCR Analysis

Total RNA was isolated from freshly isolated cholangiocytes and NRC. The following tissues served as control, kidney for AC1, AC2, AC3, AC4, AC5, heart for AC6, and brain for AC7, AC8, AC9 and sAC for qualitative PCR. For details on primers and real-time PCR conditions see suppl. Table 1 and Methods, respectively.

Assessment of Ductular Secretion By Video-Optical Planimetry in IBDU

Expansion of IBDU lumen over time was quantified by video-optical planimetry, as a measure of ductal secretion rate(13,25). After a 10 minute baseline period, IBDU were exposed to 50 nM secretin, forskolin (10 μM), acethylcholine (10 μM) or isoproterenol (10 μM), in the presence or the absence of specific inhibitors at the concentrations indicated in the results and figures. In experiments studying the effect of isohydric increases in intracellular [HCO_3^-], cells were equilibrated in HCO_3^--free HEPES-buffered media or in KRB-buffer-25 (25 mM HCO_3/5% CO_2) and exposed for 30 minutes respectively to KRB-buffer-25 or to KRB-buffer-50 (50 mM HCO_3/10% CO_2)(7,26,27). Serial images of IBDU were acquired by a JVC TKC 1380 video-camera (Galileo Siscam, 50132 Firenze, Italy) every 5 minutes for 30 minutes. Luminal areas were determined from the recorded images, using an image processor ARKON from Nikon (Galileo Siscam). Data are expressed as % variation over the 30 minutes recording, as respect to baseline(13,25).

RNAi Silencing

Silencer Cy3-Labeled custom siRNA for AC8 was purchased from Ambion (Austin, TX) according to a previous published sequence 5′-GGUUUGUCGUCCUAGAAAUTT-3′(26). Scramble negative control was purchased from Ambion (Austin, TX). NRC were plated on a

10cm plate at approximately 60% confluence and allowed grow for 24 hr. NRC were transfected according the manufacturer's protocol with the TransIT-TKO tranfection reagent was obtained from Mirus (Madison, WI). The final concentration of siRNA utilized was 30 nM. Twenty-four hours after transfection cells were harvested and stimulated with secretin (100 nM) for the analysis of intracellular cAMP levels or processed for the isolation of total RNA. In IBDU, AC8 SiRNA and Scramble RNA were added immediately after isolation, for 24 hour at concentration of 30 nM. To silence sAC in IBDU we used siRNA against four different sequences of sAC and Alexa fluor 488 negative control siRNA (Qiagen, Valencia, CA) (19) using the same procedure described above. Fourty-eight hours after transfection IBDU were used to measure fluid secretion by videoptical planimetry.

Intracellular Cyclic Adenosine Monophosphate Assay

cAMP was measured in cell extracts by RIA (see Supplementary Methods for details).

Statistical Analysis

Results are shown as mean±SD. Statistical comparisons were made using Student's t-tests, or using ANOVA, with Tukey's post test where appropriate. The Graph-Pad software (Biosoft, Cambridge UK) was used; p values <0.05 were considered significant.

RESULTS

Gene expression pattern of AC Isoforms in Normal Cholangiocytes and Hepatocytes

Expression of mRNA for AC isoforms was evaluated in freshly isolated hepatocytes, SDC, LDC and NRC by qualitative RT-PCR using β-actin as internal control (see supplementary table 1 for the list of primers). In hepatocytes, expression of all AC isoforms was observed except for AC2 and AC8. AC isoforms 1-3 were not expressed in cholangiocytes that, on the contrary, expressed AC8 (Supplementary figure 1). Real-time PCR revealed quantitative differences in AC gene expression between SDC and LDC. Interestingly, transcripts of the so-called "Ca^{2+} insensitive" AC4 and AC7, were on the average 2.4 and 6.3-fold, more abundant ($p<0.05$) in SDC than LDC. On the other hand, transcripts of the Ca^{2+}-inhibitable isoforms (AC5, AC6, AC9), of the Ca^{2+}-calmodulin activated AC8, and of the soluble AC (sAC) were more abundant (respectively, 2.4, 4.5, 2.3, 6.8 and 5.9-fold) in LDC (see supplementary table 2). AC gene expression in NRC cells was quantitatively similar to that of large cholangiocytes.

Secretin-stimulated and acethylcholine-stimulated ductal secretion and cAMP production in normal IBDU are Ca^{2+}/calmodulin dependent

Secretin and acethylcholine stimulate cAMP production and fluid secretion in large cholangiocytes(2-4). The effects of cholinergic agonists on cAMP production in cholangiocytes is $[Ca^{2+}]_i$-dependent. AC8 is the only Ca^{2+}-activated AC expressed by cholangiocytes, thus, to study if secretin-stimulated fluid secretion was functionally linked to AC8 in LDC, we evaluated the effects of inhibition of calmodulin, a Ca^{2+}-binding protein that is required to stimulate AC8(27). As shown in Figure 1A, and supplementary table 3 exposure of IBDU to secretin (50 nM), forskolin (10 μM) or acetylcholine (10 μM) stimulated fluid secretion and the effect was inhibited by the PKA inhibitor H89 (10 μM). In IBDUs stimulated with secretin or acetylcholine, inhibition of calmodulin with ophiobolin-A (1 μM)(28) or W7 (1 μM)(29), resulted in a significant decrease in secretion. Conversely, inhibition of calmodulin in IBDU treated with 10 μM forskolin (stimulating of all ACs except AC9 and sAC)(30,31) did not affect cholangiocyte secretion (Figure 1A). Calmodulin may interfere with secretory events other than AC stimulation; thus we measured the effects of ophiobolin A and W7 on basal and secretin-stimulated cAMP

formation in IBDU. Ophiobolin A and W7 had no effect on basal cAMP levels, but significantly decreased secretin-stimulated cAMP production (Figure 1B). These data indicate that a calmodulin-dependent AC modulates secretin signaling.

Effect of Secretin on Intracellular cAMP levels and on fluid secretion in AC8-silenced NRC and IBDU

We utilized a siRNA approach to test the involvement of AC8 in secretin-stimulated cAMP production and fluid secretion, using NRC and IBDU, respectively. NRC is a polarized normal cholangiocyte cell line whose AC expression resembles that of large cholangiocytes. By real-time PCR we established that transfection with AC8 siRNA results in a greater than 90% reduction in the fold-expression of the messager RNA for AC8. Scramble siRNA did not alter basal levels of AC8 gene expression (Figure 2A). Secretin did not increase cAMP levels in silenced NRC, whereas secretin increased cAMP levels in NRC exposed to scramble siRNA (Figure2B). Forskolin instead increased cAMP levels in both silenced and not-silenced NRC (Figure 2B). We measured fluid secretion by video-optic planimetry in IBDU following silencing of AC8. Secretin-stimulated fluid secretion was reduced in silenced IBDU, (68±29 % vs 24±16 %), whereas the forskolin-stimulated secretion (110±56 %) was not (97±46 % in forskolin-treated silenced IBDU) (Figure 2C). These data provide strong evidence for the role of AC8 in the regulation of secretin-stimulated ductal secretion.

β adrenergic-induced cholangiocyte secretion is mediated by AC9

IBDU, exposed to the calcineurin inhibitors FK506 or cyclosporin-A, in the absence of secretin, showed a significant secretory response that was inhibited by treatment with H89 (Figure 3 and Supplementary table 3). Calcineurin inhibits the activity of AC9, an AC isoform expressed in large cholangiocytes. Consistent with the activation of AC9 by β2-adrenergic receptor agonist isoproterenol, reported in airways epithelial cells (32), we tested the effects of the isoproterenol (10 μM) stimulated secretion in IBDU. This effect was inhibited by GABA (10 μM) (Figure 3 and Supplementary table 3). GABA is known to promote calcineurin-dependent dephoshorylation of AC9(33), indicating that AC9 in LDC is tonically inhibited by calcineurin-dependent processes, but can be activated by β2-agonists and mediates substantial bile secretion.

Soluble AC is involved in regulation of cholangiocyte secretion

Soluble AC is not regulated by G-proteins, but rather responds to increases in intracellular HCO_3^- concentrations(34). The dependence of liver cell secretory properties on the presence of HCO_3^- in the media is a well known phenomenon, thought to be a consequence of HCO_3^- transport by Cl^-/HCO_3^- exchange or CFTR(8,9). Earlier studies showed that in the presence of HCO_3^- or cAMP in the culture media, vesicular trafficking to the apical membrane was dramatically increased by cAMP(35). Thus, we studied if sAC played a role in the regulation of cholangiocyte fluid secretion. By inducing isohydric increases in intracellular HCO_3^- concentrations (i.e changing HCO_3^- without changing intracellular pHi) we stimulated fluid secretion in IBDU, as measured by video-optical planimetry from the changes in ductular area over time(25). As shown in figure 4, changing from HEPES buffered media to a KRB 25mM/5% CO_2 increased fluid secretion by 76±34 %, whereas changing from 25 mM/5 % CO_2 to 50 mM /10% CO_2 stimulated fluid secretion by 101±34 %. The stimulation of secretion achieved by increasing [HCO_3^-] was almost equal to that stimulated by administration of secretin or forskolin (Figure 1A). As expected for sAC, these changes were inhibited by H89 (10 μM), by preventing the generation of HCO_3^- from CO_2 with acetazolamide (100 μM), and by the specific sAC inhibitor KH7 (30 μM)(19). Moreover, silencing sAC gene expression with siRNA, significantly inhibited HCO_3-stimulated fluid secretion (increase over 30 minutes = 22±15% and 10±6% respectively), as

respect to IBDU treated with scramble siRNA (Figure 4). These findings are consistent with the involvement of sAC in the regulation of cholangiocyte fluid secretion.

Gene expression of ACs in Cholangiocytes from Cholestatic Rats

By real-time PCR, we studied AC isoform gene expression in SDC and LDC isolated from rats treated with ANIT or with LPS. LPS causes a cholestasis associated with reduced secretin-stimulated cholangiocyte secretion(13,14,36), whereas, in ANIT-treated rats, cholestasis results from toxic injury to cholangiocytes and is associated with proliferation of both SDC and LDC(20). As shown in figure 5, expression of the Ca^{2+}-insensitive, PKC-stimulated AC4 and AC7 was upregulated by ANIT in both SDC and LDC; expression of the Ca^{2+}-inhibitable isoforms AC5, AC6, and AC9 was down-regulated in SDC and LDC after both LPS and ANIT, while the Ca^{2+}-calmodulin stimulated AC8 was decreased by LPS in both SDC and LDC. Finally, the gene expression of sAC was upregulated after ANIT and LPS treatments, but only in SDC.

DISCUSSION

This study demostrates that the intrahepatic biliary epithelium expresses multiple AC isoforms with an heterogenous pattern of expression among different cholangiocyte subpopulation. The Ca^{2+}-insensitive ACs are more expressed in small cholangiocytes, whereas the Ca^{2+}-activated ACs are more expressed in large cholangiocytes. The study also demonstrates that specific AC isoforms are functionally linked to certain secretory stimuli. In fact, AC8 and AC9 mediate cholangiocyte secretion induced by secretin and β2-adrenergic receptor agonists, respectively, whereas sAC couples secretion to the metabolic status of the cell.

Evaluation of the gene expression profile of AC isoforms in hepatocytes and cholangiocytes from different segments of the intrahepatic biliary tree of normal and cholestatic rat livers indicated that liver cells express all AC, except for isoform 1 and 2. In comparison to hepatocytes, cholangiocytes do not express AC3, but expresses AC8. Quantitative differences in AC gene expression between LDC and SDC were found. The Ca^{2+}-activated AC isoforms (AC5, AC6, AC9) and the HCO_3^--activated isoform, sAC(16), were more expressed in large cholangiocytes. The expression of AC isoforms positively (AC8)(37) or negatively (AC5, AC6 and AC9)(37) regulated by Ca^{2+} and of isoforms that are regulated by intracellular HCO_3^- (sAC)(16) is consistent with the secretory function of large cholangiocytes and its regulation by intracellular Ca^{2+} and HCO_3^-(12,38). The "Ca^{2+}-insensitive" AC4 and AC7(39,40), were instead preferentially expressed by small cholangiocytes. Expression of these PKC-regulated ACs in small cholangiocytes is consistent with their ability to proliferate and participate to the repair of liver damage.

Intrahepatic rat cholangiocytes expressed AC8. Previous studies have identified AC8 in nervous tissue(41) and pancreatic islets(42). Thus, AC8 expression by cholangiocytes further supports the concept that similarities exist between neuroendocrine tissue and the intrahepatic biliary tree(43). Higher expression of AC8 in LCD compared to SDC suggests that AC8 is involved in secretin-stimulated bile secretion. Consistent with this hypothesis, we showed that in LCD models, secretin-stimulated secretion and cAMP generation were inhibited by two different calmodulin inhibitors and by silencing AC8 expression. When secretion was induced by forskolin, a general stimulator of AC isoforms except for AC9 and sAC(30,31), administration of calmodulin inhibitors and of AC8 siRNA did not cause a significant inhibitory effect. AC8 is the only known calmodulin-activated AC in cholangiocytes; in fact, AC9 activity is inhibited by calmodulin. Consistent with the data on secretion, secretin-stimulated cAMP production was inhibited by calmodulin inhibitors(Figure 1b). Furthermore, silencing of AC8 gene expression in NRC cells(23,24)

blocked secretin-, but not forskolin-stimulated cAMP production and fluid secretion, consistent with a functional relationship between secretin and AC8.

Previous studies in isolated perfused liver lead to the speculation that a Ca^{2+}-activated adenylyl cyclase isoform was activated by secretin(44). Alvaro et al.(38) showed that cholinergic stimulation was synergistic with secretin in promoting Cl^{-}/HCO_3^{-} exchange, and that this synergism occurred at the level of a Ca^{2+}-dependent stimulation of cAMP production. The Ca^{2+}/calmodulin system is activated by capacitative Ca^{2+} entry into the cell(44). In our study, we documented that acetylcholine stimulated cholangiocyte secretion in a calmodulin-dependent way, suggesting that the secretin and cholinergic agonists converge at the level of AC8. This mechanism is important because, during digestion, secretin targets the biliary epithelium during parasympathetic predominance(38).

Cholangiocytes also expressed AC9, a Ca^{2+}-inhibitable AC, typically unresponsive to forskolin. AC9 activity is stimulated by β2-adrenergic receptor agonists and inhibited by calcineurin, a protein-phosphatase activated by $Ca^{2+/}$Calmodulin. AC9 is more expressed in LDC, suggesting that it may also regulate bile secretion. In fact, administration of the β2-agonist isoproterenol(32), stimulated secretion in IBDU. This effect was inhibited by GABA, an inhibitor of AC9(33). Administration of FK506 and cyclosporin stimulated an H89-inhibitable secretion in IBDU. This is consistent with the concept that AC9 in cholangiocytes is tonically inhibited by calcineurin and may be derepressed by beta-adrenergic stimulation.

In addition to the transmembrane AC isoforms that generate cAMP in response to G-protein coupled receptors, an important source of cAMP is provided by the sAC(30,34). Soluble AC is not stimulated by forskolin and G proteins, but it is uniquely dependent on $[HCO_3^{-}]$ and therefore can act as a cellular metabolic sensor. Here we show that sAC is expressed in cholangiocytes and that cholangiocyte secretion stimulation is sensitive to increases in HCO_3^{-}. This effect was inhibited by H89, acetazolamide, KH7 and sAC gene silencing with siRNA. These data demonstrate the role of sAC(30,34) in the regulation of cholangiocyte secretion. Likely, cholangiocyte sAC maintains a tonic level of cAMP and fluid secretion during the interdigestive phase.

As shown in Figure 6, based on the expression of AC isoforms, their different regulatory mechanisms and functional interactions with specific agonists, we propose that the level of cAMP stimulating CFTR-dependent secretion is the results of the integration of different stimuli acting on Ca^{2+}dependent, Ca^{2+}-independent and HCO_3-dependent ACs. Thus, the intracellular Ca^{2+} concentration resulting from the flow-dependent bending of apical cilia, and the intracellular concentration of $[HCO_3]$i, resulting from the cellular metabolic status determine a “resting” level of cAMP that eventually will be further modulated by gastrointestinal hormones, cholinergic agonists and β-adrenergic agonists, acting via AC8 or AC9. Our studies therefore highlight an important cross-talk between the Ca^{2+} and cAMP-dependent pathways at the level of ACs.

Other studies indicate that the InsP3 receptor-mediated Ca^{2+} pathway is inhibited in cholestatic conditions(45), leaving cAMP/PKA as the main regulatory pathway. Our study uncovered the existence of a previously unrecognized modulation of AC gene expression in cholangiocytes during experimental cholestasis. While calcium-activated ACs were down-regulated in both cholangiocyte subpopulations, calcium-insensitive ACs and sAC become upregulated. The PKC-stimulated AC7, was strongly up-regulated, particularly in conditions in which cholangiocyte proliferation prevails (ANIT). Expression of the $[HCO_3]$i-activated sAC increased in SDC, likely compensating for the metabolic disturbance induced by cholestasis.

In conclusion, our findings indicate that AC regulation is a fundamental regulatory node in the complex array of stimuli targeting cholangiocytes. Differential expression of AC isoforms with different regulatory properties is an important strategy to achieve specificity. Together with our previous studies(13,14), the present study further indicates that modulation of different ACs plays an important pathophysiological role in biliary tree diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AC	adenylyl cyclase
ANIT	α-naphthylisothiocyanate
ATP	adenosine 5′-triphosphate
cAMP	3′-5′-cyclic adenosine monophosphate
CFTR	cystic fibrosis transmembrane regulator
γ-GT	γ-glutamyltranspeptidase
IBDU	intrahepatic bile duct units
LDC	Large Duct Cholangiocytes
LPS	lipopolysaccharide
NRC	normal rat cholangiocytes
PKA	protein kinase A
PKC	protein kinase C
sAC	soluble adenylyl cyclase
SDC	Small Duct Cholangiocytes.

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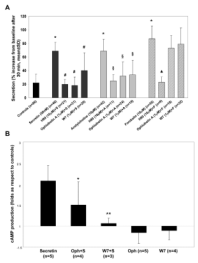

Figure 1. Calmodulin inhibitors diminish secretin and acetylcholine but not forskolin stimulated fluid secretion and intracellular cAMP levels in normal rat IBDU

(A) Secretion was measured as luminal expansion over time. S=secretin; A=acetylcholine; F=forskolin; H89 is an inhibitor of PKA. (*P<0.001 vs controls; #P<0.001 vs secretin; §P<0.001 vs acetylcholine; ▲ P<0.001 vs forskolin). **(B)** Controls or ophiobolin-A(1μM)-pretreated cells or W7(1μM)-pretreated cells were stimulated with secretin (50 nM) for 30 minutes. Ophiobolin-A and W7 inhibited secretin-stimulated increase in cAMP, but not basal levels of cAMP. Columns represent the mean±SD of indicated replicas normalized to controls (*P<0.05 vs. Secretin 50 nM; **P<0.01vs. Secretin 50 nM).

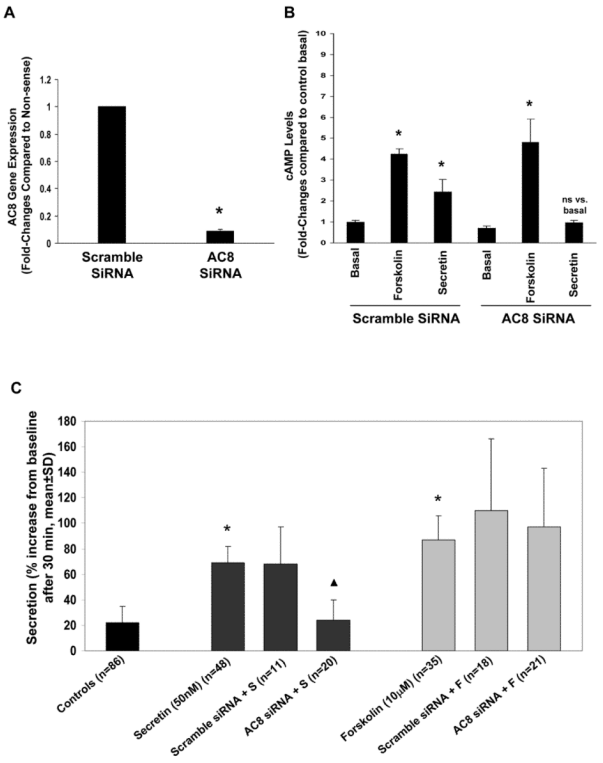

Figure 2. (A) Evaluation of AC8 message expression in NRC following AC8 silencing

AC8 siRNA results in a greater than 90% reduction in the fold-expression in AC8 mRNA. (mean±SEM of 3 experiments). **P*<0.05 vs. scramble SiRNA. (**B**) Effect of forskolin and secretin on cAMP levels in AC8-silenced. Contrary to NRC exposed to scramble siRNA, secretin did not increase cAMP levels in AC8 silenced cells. Forskolin increased cAMP levels of both silenced and non-silenced NRC (mean±SEM of 7 experiments; **P*<0.05 vs. the corresponding basal value). (C) Effects of AC8 silencing on secretin- stimulated and forskolin-stimulated secretion in IBDU. A significant reduction in secretin but not forskolin luminal secretion in AC8 silenced IBDU was found (*P<0.001 vs controls; ▲P< 0.001 vs secretin+scramble siRNA).

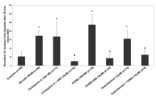

Figure 3. Evidence for beta-adrenergic activated calcineurin-inhibitable AC9

Secretion was measured as luminal expansion in IBDU as in Figure 1 and 2A. H89-dependent stimulation of secretion by calcineurin inhibitors cyclosporin-A or FK508 ($p<0.001$) is consistent with the presence of AC9. Secretion was also stimulated by administration of isoproterenol, a β-adrenergic agonists known to stimulate AC9 in airways(32)($p<0.001$). Co-administration of GABA inhibited the isoproterenol-stimulated secretion (*$P<0.001$ vs controls; ▲$P<0.001$ vs cyclosporinA; #$P<0.001$ vs FK506; § $P<0.05$ vs isoproterenol).

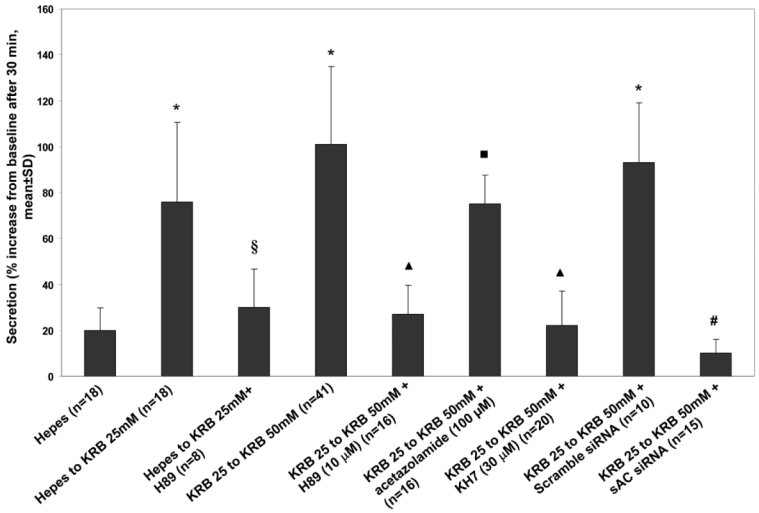

Figure 4. Increases in intracellular bicarbonate concentrations stimulated fluid secretion in normal rat IBDU

Secretion was measured as in Figure 1 and 3. To avoid changes in pHi, these manoeuvres were conducted in isohydric conditions buffering with 5% CO_2 (in KRB 25 mM) or 10 % CO_2 (in KRB 50 mM). HCO_3^--stimulated fluid secretion was inhibited by H89 (10μM), by the carbonic anhydrase inhibitor acetazolamide (100 μM), by the specific sAC inhibitor KH7 (30 μM) and by gene silencing of sAC with siRNA (50nM). (**P* < 0.001vs Hepes; § P<0.001 vs. Hepes to KRB 25 mM; ▲ *P*<0.001 vs KRB 25 mM to KRB 50 mM; ■*P* < 0.01 vs KRB 25 mM to KRB 50 mM; # P<0.001 vs KRB 25 mM to KRB 50 mM+scramble siRNA).

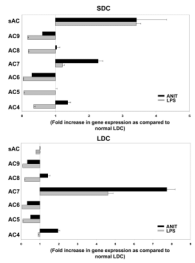

Figure 5. Expression levels of different ACs isoforms in small and large cholangiocytes isolated from LPS-treated and ANIT-fed rats

Plot **A** shows the results for SDC, while the results for LDC are shown in plot **B**. Colums represent averages ± standard deviations of the ratio between AC copy number/β-actin copy number in ANIT or LPS-treated animals vs AC copy number/β-actin copy number in untreated controls (cholangiocyte subpopulations isolated from normal rats) on two different isolation. Each isolation represent the pools of n=16 rats for normal cholangiocytes and n=8 rats for ANIT or LPS treatments.

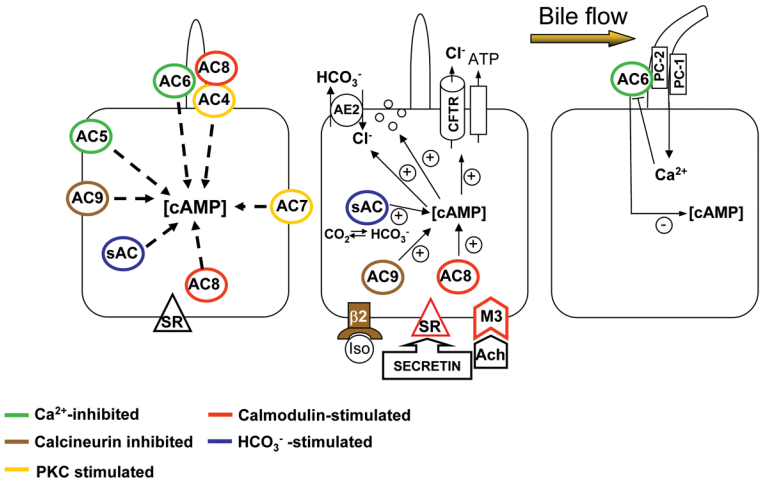

Figure 6. Cartoon showing ACs isoforms expression in large duct cholangiocytes subpopulations, with the proposed integration of cAMP signaling

(see discussion for details). ACs are color-coded to indicate their mechanisms of activation/inhibition. Membrane topography of AC is hypothetical except for the ciliary location of AC6, AC4 and AC8(12,18). AC8 is also functionally linked to stimulation of the basolateral secretin receptor (SR) and cholinergic stimuli. Stimulation of secretin receptor and activation of AC8 increases [cAMP] and apical CFTR-dependent secretion, including vesicular transport and AE2 activity. Increased bile flow will bend cholangiocyte primary cilia, triggering Ca^{2+} influx via Policystin 1 and 2 (PC1, PC2)(12). Ca^{2+} influx will in turn inhibit AC6, counteracting the effect of secretin(12). β-adrenergic agonist (isoproterenol) stimulates the calcineurin-inhibitable AC9, while modulated by calcineurin. sAC transduces the intracellular HCO3 concentration into cAMP levels. All these mechanisms concur to determine the level of cellular cAMP responsible for CFTR-dependent secretion.