Gastrointestinal, Hepatobiliary and Pancreatic Pathology

After Damage of Large Bile Ducts by Gamma-Aminobutyric Acid, Small Ducts Replenish the Biliary Tree by Amplification of Calcium-Dependent Signaling and *de Novo* Acquisition of Large Cholangiocyte Phenotypes

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Large cholangiocytes secrete bicarbonate in response to secretin and proliferate after bile duct ligation by activation of cyclic adenosine 3′, 5′-monophosphate signaling. The Ca²⁺-dependent adenylyl cyclase 8 (AC8, expressed by large cholangiocytes) regulates secretin-induced choleresis. Ca²⁺-dependent protein kinase C (PKC) regulates small cholangiocyte function. Because γ-aminobutyric acid (GABA) affects cell functions by activation of both Ca²⁺ signaling and inhibition of AC, we sought to develop an *in vivo* model characterized by large cholangiocyte damage and proliferation of small ducts. Bile duct ligation rats were treated with GABA for one week, and we evaluated: GABAₐ, GABA_B, and GABA_C receptor expression; intrahepatic bile duct mass (IBDM) and the percentage of apoptotic cholangiocytes; secretin-stimulated choleresis; and extracellular signal-regulated kinase1/2 (ERK1/2) phosphorylation and activation of Ca²⁺-dependent PKC isoforms and AC8 expression. We found that both small and large cholangiocytes expressed GABA receptors. GABA: (i) induced apoptosis of large cholangiocytes and reduced large IBDM; (ii) decreased secretin-stimulated choleresis; and (iii) reduced ERK1/2 phosphorylation and AC8 expression in large cholangiocytes. Small cholangiocytes: (i) proliferated leading to increased IBDM; (ii) displayed activation of PKCβII; and (iii) *de novo* expressed secretin receptor, cystic fibrosis transmembrane regulator, Cl⁻/HCO₃⁻ anion exchanger 2 and AC8, and responded to secretin. Therefore, in pathologies of large ducts, small ducts replenish the biliary epithelium by amplification of Ca²⁺-dependent signaling and acquisition of large cholangiocyte phenotypes. *(Am J Pathol 2010, 176:1790–1800; DOI: 10.2353/ajpath.2010.090677)*

Cholangiocytes line the intrahepatic biliary tree,[1,2] a network of interconnecting ducts of different sizes and functions.[1,2] A number of gastrointestinal hormones including secretin modify bile of canalicular origin before reaching the duodenum.[3,4] In humans, cholangiocytes are the target cells in a number of chronic cholestatic liver diseases characterized by biliary proliferation/loss.[5] Cholangio-

cytes proliferate or are damaged in animal models of cholestasis including bile duct ligation (BDL) or acute administration of carbon tetrachloride (CCl_4).[4,6–8] Secretin receptor (SR, expressed only by large cholangiocytes in rodent liver)[1,2,8,9] is a unique pathophysiological tool for evaluating at the functional level the degree of biliary growth/loss.[6–9] Whereas enhanced cholangiocyte growth is associated with increased SR expression and secretin-stimulated choleresis, biliary damage leads to decreased functional expression of SR.[7,8]

The human and rodent biliary epithelium is morphologically and functionally heterogeneous.[1,2,10–12] In rat liver, purified small cholangiocytes (≈ 8 μm in size) derive from small ducts (<15 μm in diameter), whereas large cholangiocytes (≈ 15 μm in size) originate from large ducts (>15 μm in diameter).[1,2] Whereas the secretory, apoptotic, and proliferative activities of large cholangiocytes are regulated by changes in cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent signaling,[1,6,8,9,11,13] the function of small cholangiocytes (normally mitotically dormant)[6,8] is regulated by the D-*myo*-inositol 1,4,5-trisphosphate (IP_3)/Ca^{2+}/calmodulin-dependent protein kinase I signaling pathway.[14,15] For example, large (but not small) rodent cholangiocytes express SR,[1,2,10] cystic fibrosis transmembrane regulator (CFTR),[1,2,10] and Cl^-/HCO_3^- exchanger[1,2,10] (recently identified as the Cl^-/HCO_3^- anion exchanger 2 [AE2]),[16] and secrete bile in response to secretin by activation of cAMP$\Rightarrow$protein kinase A (PKA)$\Rightarrow$CFTR$\Rightarrow$$Cl^-$/$HCO_3^-$ anion AE2.[1,2,9] The Ca^{2+}-dependent adenylyl cyclase 8 (AC8, expressed mainly by large cholangiocytes)[17] regulates secretin-stimulated choleresis of large bile ducts.[17] After BDL, large but not small cholangiocytes undergo mitosis (leading to enhanced large duct mass)[6,8,18] by activation of cAMP signaling.[6,8,18] A single dose of CCl_4 to rats induces a functional loss of large cAMP-responsive cholangiocytes, whereas small cholangiocytes (resistant to CCl_4-induced apoptosis) *de novo* proliferate to compensate for the loss of large biliary mass.[8] Although some studies suggest that activation of Ca^{2+}-dependent signaling may be important in the regulation of small cholangiocyte function,[14,15] the mechanisms by which small cholangiocytes replenish the biliary tree in response to the damage of large bile ducts is unknown.

Gamma-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in the vertebrate central nervous system.[19] In addition to the central nervous system, the liver represents the major site of synthesis and metabolism of GABA.[20] GABA actions are mediated by three GABA receptor subtypes ($GABA_A$, $GABA_B$, and $GABA_C$).[21] Studies have shown that GABAergic activity inhibits hepatic regeneration after partial hepatectomy in rats.[22] We have shown that GABA decreases both *in vivo* and *in vitro* cholangiocarcinoma growth.[21] However, no data exist regarding the role of GABA in the regulation of cholangiocyte hyperplasia in cholestasis. Because GABA can affect cell functions by both activation of Ca^{2+} signaling and inhibition of AC activity,[23] we tested the hypothesis that GABA regulates the proliferative, apoptotic, and secretory activities of small and large cholangiocytes by the differential activation/deactivation of Ca^{2+}- and cAMP-dependent signaling pathways.

Materials and Methods

Materials

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. The RIA kits for the measurement of intracellular cAMP ([^{125}I] Biotrak Assay System, RPA509) and IP_3 (D-*myo*-inositol 1,4,5-trisphosphate (IP_3) [^{3}H] Biotrak Assay System, TRK1000) levels were purchased from GE Health care (Piscataway, NJ). The antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) unless otherwise indicated. The CFTR monoclonal (IgG1) antibody (M3A7, previously used by us in rodent cholangiocytes)[10] was purchased from Thermo Fisher Scientific (Fremont, CA). The antibody (an affinity-purified rabbit anti-rat AE2 IgG) against Cl^-/HCO_3^- AE2[16] was purchased from α Diagnostic International (San Antonio, TX). The RNeasy Mini Kit to purify total RNA from cholangiocytes was purchased from Qiagen Inc, Valencia, CA.

Animal Models

Male 344 Fischer rats (150 to 175 g) were purchased from Charles River (Wilmington, MA) and kept in a temperature-controlled environment (22°C) with a 12:12 hours light/dark cycle. Animals were fed *ad libitum* and had free access to drinking water. The studies were performed in normal rats, and in rats that immediately after BDL or bile duct incannulation (BDI, for bile collection)[4] were treated with daily IP injections of NaCl or GABA (50 mg/kg body weight)[24] for 1 week. Before each procedure, animals were anesthetized with sodium pentobarbital (50 mg/kg IP) according to the regulations of the panel on euthanasia of the American Veterinarian Medical Association and local authorities. In all animals, we measured wet liver weight, body weight, and wet liver weight to body weight ratio, an index of liver cell growth including cholangiocytes.[4]

Purification of Small and Large Cholangiocytes

Virtually pure (by γ-glutamyl transpeptidase histochemistry)[25] and distinct subpopulations of small (mean diameter 8 μm) and large (mean diameter 14 μm) cholangiocytes[2,9] were isolated by counterflow elutriation.[2,6,9] Cell viability was approximately 98%.

Expression of $GABA_A$, $GABA_B$, and $GABA_C$ Receptors

The expression of GABA receptors was evaluated by: (i) immunohistochemistry in paraffin-embedded liver sections (4 to 5 μm thick) from the aforementioned groups of animals; and (ii) immunofluorescence in cell smears of purified small and large BDL cholangiocytes. For immu-

nohistochemistry,[26] endogenous peroxidase activity was blocked by a 30-minute incubation in methanolic hydrogen peroxide (2.5%). The endogenous biotin was blocked by Biotin Blocking System (Dako, Copenhagen, Denmark) according to the instructions supplied by the vendor. Sections were hydrated in graded alcohol and rinsed in PBS (pH 7.4), then the primary antibodies $GABA_A$ (Santa Cruz, #21336; 1: 50 dilution), $GABA_B$ (Santa Cruz, #14006; 1: 50 dilution), $GABA_C$ (Santa Cruz, #23362; 1: 50 dilution) were applied and incubated overnight at 4°C. Samples were rinsed with PBS for 5 minutes, incubated for 10 minutes at room temperature with secondary biotinylated antibody (Dako LSAB Plus System, Milan, Italy), then with Dako ABC (Dako LSAB Plus System, Milan, Italy) and finally developed with 3-3′ diaminobenzidine. To demonstrate the specificity of the immunoreactions, negative controls (the primary antibody was replaced with the same dilution—with normal serum from the same species) were performed for all immunoreactions. Sections were analyzed in a coded manner using a BX-51 light microscope (Olympus, Tokyo, Japan) with a video cam (Spot Insight; Diagnostic Instrument, Inc., Sterling Heights, MI) and processed with an Image Analysis System (IAS: Delta Sistemi, Rome, Italy). Immunofluorescence for GABA receptors was performed as described.[27] After staining, images were visualized using an Olympus IX-71 confocal microscope. For all immunoreactions, negative controls were included.

To evaluate the message expression of $GABA_A$, $GABA_B$, and $GABA_C$ in purified small and large cholangiocytes, we used the RT^2 Real-Time assay from SABiosciences (Frederick, MD).[14] A $\Delta\Delta C_T$ (delta delta of the threshold cycle) analysis was performed using brain tissue as the control sample.[14] The primers (purchased from SABiosciences) for $GABA_A$, $GABA_B$, and $GABA_C$ were designed according to the NCBI GenBank Accession number, NM 017289 for $GABA_A$[28]; NM 031028 for $GABA_B$[29]; and NM 017291 for $GABA_C$.[30] Data were expressed as relative mRNA levels ± SEM of GABA receptors to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ratio.

In Vivo *Effect of GABA on Liver Morphology, Cholangiocyte Apoptosis, and Proliferation*

We evaluated lobular morphology, necrosis, and portal inflammation by hematoxylin & eosin staining of paraffin-embedded liver sections (4 to 5 μm thick). Liver sections were examined in a coded fashion by BX-51 light microscopy (Olympus, Tokyo, Japan) equipped with a camera. Six slides were analyzed for each group and six nonoverlapping fields (magnification ×20) for each slide were evaluated for each parameter.

Apoptosis of small and large cholangiocytes was measured by quantitative terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) kit (Apoptag; Chemicon International, Inc) in liver sections (4 to 5 μm thick) from BDL rats treated with NaCl or GABA. Six slides were analyzed for each group using a BX-51 light microscopy (Olympus, Tokyo, Japan). Positive cholangiocytes were counted in six nonoverlapping fields (magnification ×20) for each slide, and the data are expressed as percentage of positive cells.

Immunoblots for BCL2-associated X protein (Bax, a pro-apoptotic protein)[31] expression was performed in protein (10 μg) from whole cell lysates from small and large cholangiocytes. Blots were normalized by β-actin.[14] The intensity of the bands was determined by scanning video densitometry using the phospho-imager, Storm 860, (GE Health care, Piscataway, NJ) and the ImageQuant TL software version 2003.02 (GE Health care, Little Chalfont, Buckinghamshire, England).

Proliferation of small (<15 μm diameter)[1] and large (>15 μm diameter)[1] bile ducts was measured by evaluating intrahepatic bile duct mass (IBDM) in liver sections. IBDM was measured as area occupied by cytokeratin-19 positive-bile duct/total area × 100. Proliferation was evaluated by immunoblots for proliferating cellular nuclear antigen (PCNA) in protein (10 μg) from whole cell lysate from purified small and large cholangiocytes. Blots were normalized to β-actin[14] and visualized as described above.

Membrane Translocation and Phosphorylation of Ca^{2+}-Dependent PKC Isoforms (Expression of cAMP-Dependent Signaling)

We next determined whether small BDL cholangiocytes proliferate and secrete (to compensate for GABA-induced damage of large cAMP-dependent BDL cholangiocytes) by both: (i) the activation of Ca^{2+}-dependent signaling evaluated by the enhanced translocation and phosphorylation of Ca^{2+}-dependent protein kinase C (PKC) isoforms (ie, αI, βI, βII and γ), which are important in the regulation of cholangiocyte function[7,32,33]; and (ii) the *de novo* acquisition of cAMP-dependent phenotypes (ie, expression of SR, CFTR, Cl^-/HCO_3^- AE2 and AC8, and cAMP and Cl^- efflux in response to secretin), which are key in the modulation of large cholangiocyte functions.[1,2,6,8,9,13,34]

The activation of Ca^{2+}-dependent PKCα, βI, βII, or γ was evaluated by: (i) immunofluorescence (membrane translocation) in cell smears; and (ii) immunoblots (phosphorylation)[7,14] in protein (10 μg) from whole cell lysate from small and large cholangiocytes. In purified small and large cholangiocytes, we also evaluated the phosphorylation of ERK1/2 (expressed by a ratio to the corresponding total protein),[18] a protein linked to cAMP-dependent signaling pathway.[18]

Evaluation of Secretory Activity of Small and Large Cholangiocytes

We performed experiments to demonstrate that following chronic GABA administration to BDL rats: (i) small cholangiocytes acquire functional phenotypes of large cholangiocytes; and (ii) there is down-regulation of cAMP-dependent secretory activity[1,2,8,9] in large cholangiocytes. To achieve this, we measured the protein expression for SR, CFTR, and Cl^-/HCO_3^- AE2 by immunohistochemistry in liver sections (4 to 5 μm thick), and
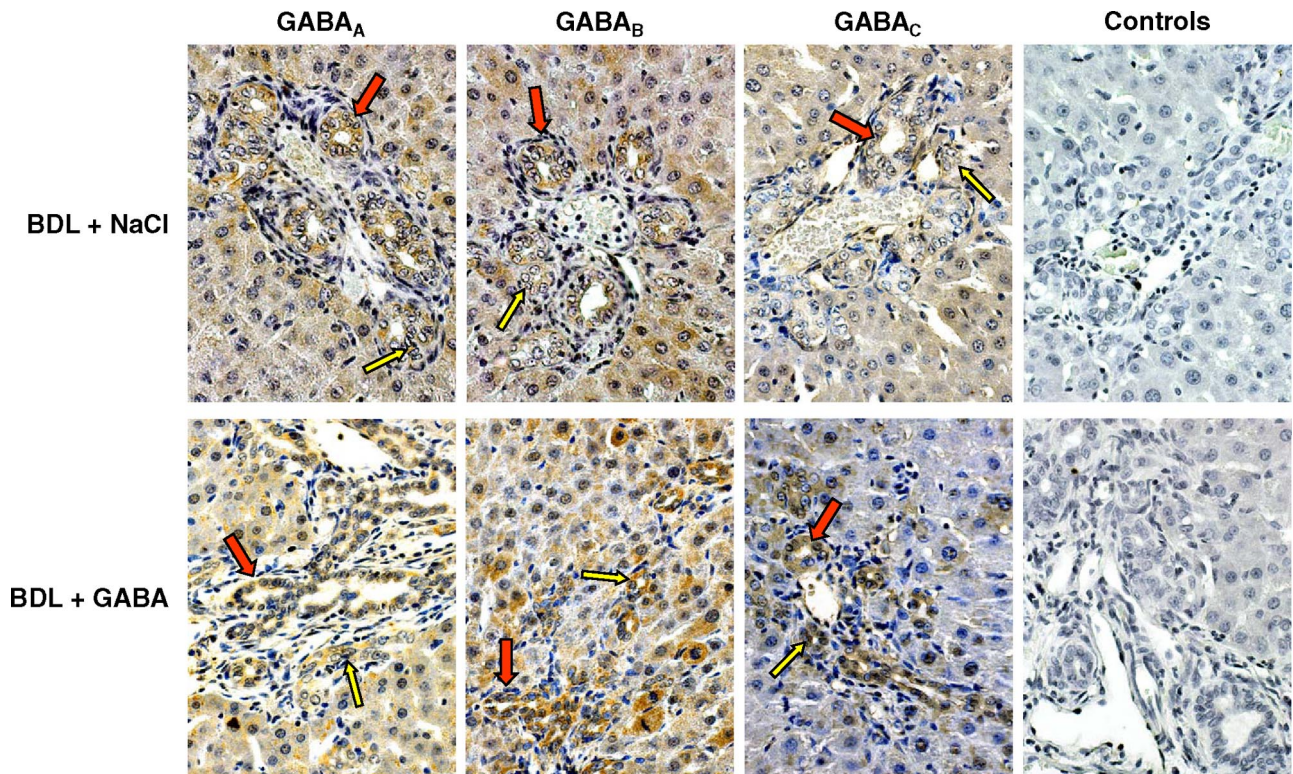
Figure 1. Representative immunohistochemistry for GABA$_A$, GABA$_B$, and GABA$_C$ in liver sections from BDL rats treated with NaCl or GABA for 1 week. Both small (**yellow arrows**) and large (**red arrows**) bile ducts express the three subtypes of GABA receptors. No staining was visible when primary antibodies were replaced with nonimmune serum from the same species. Original magnification, ×40.

immunofluorescence[14,27] in small and large cholangiocytes; (ii) AC8 protein expression in liver sections (4 to 5 μm) by immunohistochemistry,[14] and AC8 gene expression by real-time PCR in total RNA (1 μg) from small and large cholangiocytes; and (iii) basal and secretin-stimulated cAMP levels by RIA[6,7,13,34] and $^{36}Cl^-$ efflux (a functional index of CFTR activity).[9,35,36] The primers (purchased from SABiosciences, Frederick, MD) for AC8 were designed according to the NCBI GenBank Accession number NM 017142.[37]

The secretory activity of small and large cholangiocytes was also assessed by measuring basal and secretin-stimulated bile and bicarbonate secretion in bile fistula BDI rats.[4] After anesthesia, rats were surgically prepared for bile collection as described.[4] When steady-state bile flow was reached (60 to 70 minutes from the intravenous infusion of Krebs-Ringer-Henseleit solution, KRH), rats were infused with secretin (100 nmol/L) for 30 minutes followed by IV infusion of KRH for 30 minutes. Bicarbonate concentration in bile was determined by an ABL 520 Blood Gas System (Radiometer Medical A/S, Copenhagen, Denmark).

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Differences between groups were analyzed by Student unpaired *t* test when two groups were analyzed and analysis of variance when more than two groups were analyzed, followed by an appropriate *post hoc* test.

Results

Cholangiocytes Express GABA Receptors

Immunohistochemistry in liver sections from normal (not shown) and BDL (Figure 1) rats treated with NaCl or GABA shows that both small (yellow arrows) and large (red arrows) bile ducts express GABA$_A$, GABA$_B$, and GABA$_C$ receptors. By immunofluorescence, there was positive immunoreactivity for the three GABA receptors in small and large BDL cholangiocytes (Figure 2A). Negative controls are shown in Figure 2A. By real-time PCR, both small and large cholangiocytes from BDL rats treated with NaCl or GABA express the message for the three GABA receptors (Figure 2B).

Effect of GABA on Liver Histology, Liver and Body Weight, Apoptosis, and Proliferation of Small and Large Bile Ducts

No significant differences in wet liver weight, body weight, liver to body weight ratio, degree of portal inflammation, necrosis, and lobular damage were observed in BDL rats treated with NaCl or GABA for 1 week (not shown). Chronic *in vivo* administration of GABA to normal rats did not alter cholangiocyte apoptosis or proliferation (not shown). Administration of GABA to BDL rats induced apoptosis of large bile ducts (red arrows, by TUNEL in liver sections) (Figure 3A and Table 1) and purified large

A **Small BDL cholangiocytes**   **Large BDL cholangiocytes**

B
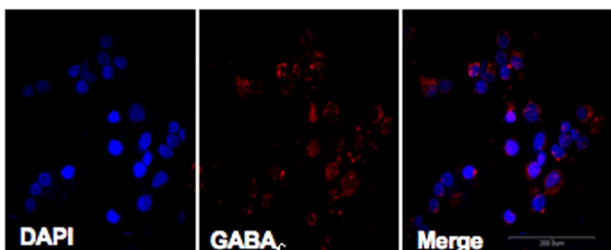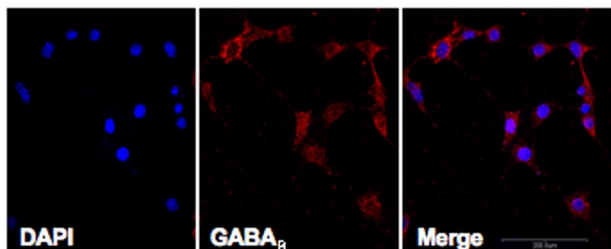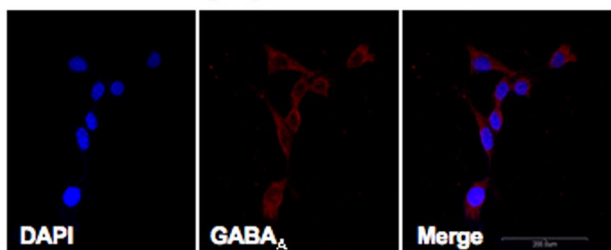
Figure 2. A: By immunofluorescence, both small and large cholangiocytes from BDL rats express the three GABA receptor subtypes. Specific immunoreactivity of representative fields is shown in red; cell nuclei were stained with DAPI (blue). Scale bar = 200 μm. **B:** Real-time PCR shows that purified small and large cholangiocytes from BDL rats treated with NaCl or GABA express the message for all three GABA receptors. Data are mean ± SEM of 3 experiments.
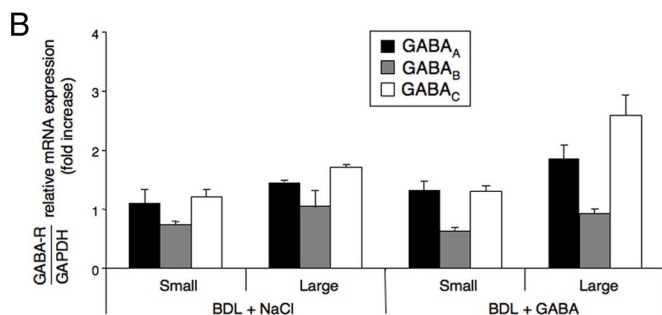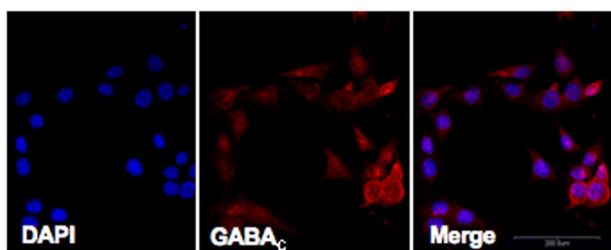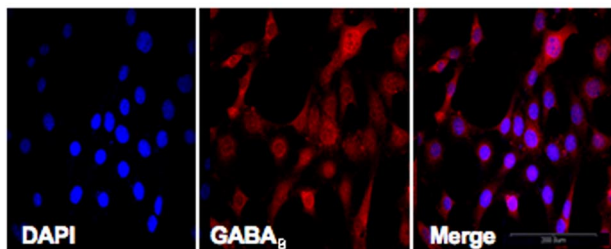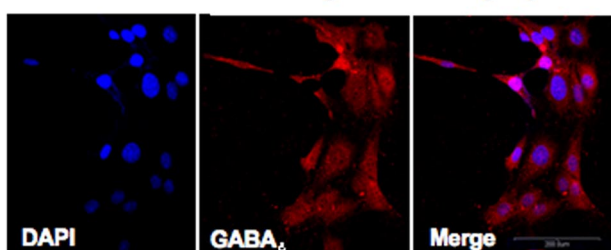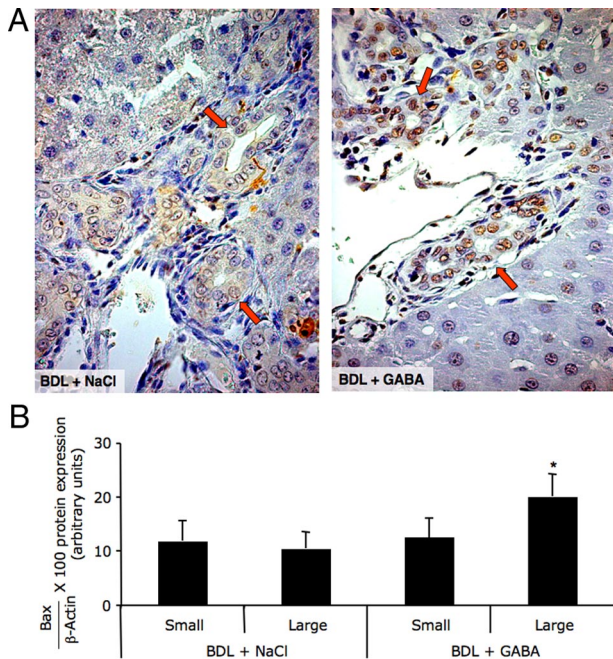
cholangiocytes (by Bax immunoblots; Figure 3B). No changes in apoptosis were seen in small ducts (Figure 3A) and purified small cholangiocytes (Figure 3B) from BDL rats treated with NaCl or GABA.

In agreement with previous studies,[6] after BDL only large cholangiocytes proliferated leading to increased large IBDM (Figure 4A and Table 1). Concomitant with GABA-induced apoptosis of large cholangiocytes (Figure 3, A and B), there was a significant decrease in large (red arrows) IBDM (Figure 4A and Table 1) and the *de novo* proliferation of small ducts (yellow arrows) leading to an increase in small IBDM (Figure 4A and Table 1). The overall IBDM was similar between the BDL rats treated with NaCl or GABA (Table 1). After BDL, large cholangiocytes displayed higher proliferative activity compared with small BDL cholangiocytes (Figure 4B).[6,8] Furthermore, there was decreased proliferation in large cholangiocytes and increased PCNA protein expression in purified small cholangiocytes from BDL rats treated with

GABA compared with small and large cholangiocytes from BDL rats treated with NaCl (Figure 4B).

Membrane Translocation and Phosphorylation of Ca^{2+}-Dependent PKC Isoforms and Phosphorylation of ERK1/2

We observed membrane translocation (by immunofluorescence, Figure 5A) and enhanced phosphorylation of PKCβII (by immunoblots, Figure 5B) in small cholangiocytes from BDL GABA-treated rats compared with small cholangiocytes from BDL rats treated with NaCl. No membrane translocation of PKCα, PKCβI, and PKCγ was observed in small cholangiocytes from BDL rats treated with GABA compared with small cholangiocytes from BDL rats treated with NaCl (not shown). After GABA administration to BDL rats, there was: (i) decreased phosphorylation of ERK1/2 in large cholangiocytes;

Figure 3. Evaluation of cholangiocyte apoptosis by TUNEL analysis in liver sections (**A**), and Bax immunoblots in small and large cholangiocytes (**B**) from BDL rats treated with NaCl or GABA for 1 week. GABA induced apoptosis of large ducts (**A, red arrows**, for quantitative data see Table 1) and large cholangiocytes (**B**) from BDL rats treated with GABA compared with BDL rats treated with NaCl. **A** and **B**: No changes in apoptosis were seen in small ducts and small cholangiocytes from BDL rats treated with NaCl or GABA. **A**: Original magnification, ×40. **B**: Data are mean ± SEM of 8 blots. *$P < 0.05$ versus large cholangiocytes from BDL rats treated with NaCl for 1 week.

and (ii) increased ERK2 (but not ERK1) phosphorylation in small cholangiocytes compared with small and large cholangiocytes from BDL rats treated with NaCl (Figure 5C).

Table 1. Evaluation of % of Tunel-Positive Cholangiocytes in Small and Large Bile Ducts and Measurement of Bile Duct Mass by Cytokeratin 19 Staining

Groups	Bile ducts	Apoptosis	IBDM
Normal + NaCl	Small	ND	0.05 ± 0.01
	Large	ND	0.21 ± 0.03
Normal + GABA	Small	ND	0.07 ± 0.01
	Large	ND	0.19 ± 0.02
BDL + NaCl	Small	12.04 ± 2.77	0.90 ± 0.08
	Large	15.14 ± 2.46	4.65 ± 0.34
BDL + GABA	Small	10.62 ± 1.85	1.70 ± 0.15†
	Large	40.77 ± 2.68*	2.61 ± 0.31‡

Small bile ducts = <15 μm diameter; large bile ducts = >15 μm diameter. Apoptosis of small and large bile ducts was measured by TUNEL analysis in liver sections. Proliferation of small and large bile ducts was measured by evaluating intrahepatic bile duct mass (IBDM) in liver sections. IBDM was measured as area occupied by CK19-positive bile duct/total area × 100. ND indicates not detected. Data are expressed as mean ± SEM.
*$P < 0.05$ versus the number of large bile ducts (positive by TUNEL) from BDL rats treated with NaCl.
†$P < 0.05$ versus the number of small bile ducts from BDL NaCl-treated rats.
‡$P < 0.05$ versus the number of large bile ducts from BDL NaCl-treated rats.
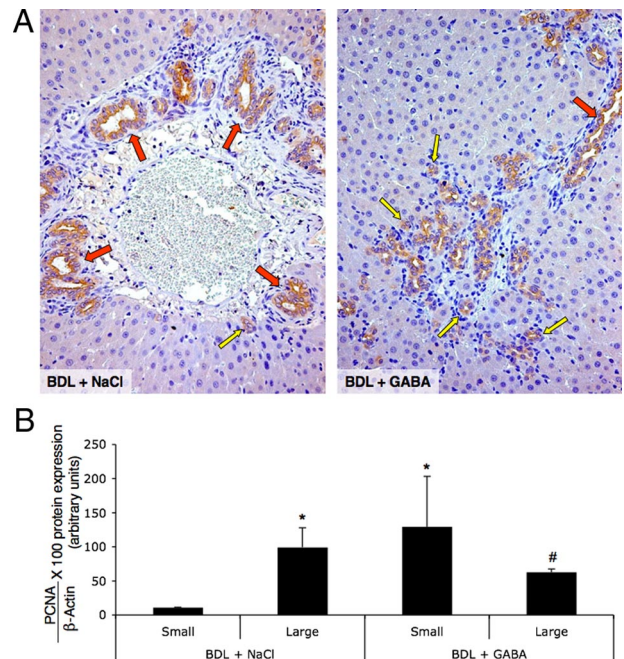
Figure 4. Measurement of IBDM of small and large bile ducts by immunohistochemistry for cytokeratin-19 in liver sections (**A**), and PCNA protein expression in protein (10 μg) from whole cell lysate from small and large cholangiocytes (**B**) from BDL rats treated with NaCl or GABA for 1 week. **A**: After BDL, large (**red arrows**) bile ducts proliferate leading to an increase in large IBDM; there were no changes in the number of small (**yellow arrow**) bile ducts. After GABA administration, there were decreased large IBDM and the *de novo* proliferation of small bile ducts (**yellow arrows**) leading to an increase in small IBDM (for quantitative data see Table 1). Original magnification, ×20. Data are mean ± SEM of 36 cumulative values obtained from the six slides evaluated per each group of animals. **B**: After BDL, only large cholangiocytes displayed higher proliferative activity. There was decreased proliferation in large cholangiocytes and increased PCNA protein expression in purified small cholangiocytes from BDL rats treated with GABA compared with BDL rats treated with NaCl. Data are mean ± SEM of 8 blots. *$P < 0.05$ versus small cholangiocytes from BDL rats treated with NaCl for 1 week. #$P < 0.05$ versus large cholangiocytes from BDL rats treated with NaCl for 1 week.

Evaluation of Biliary Markers and Secretory Activity of Small and Large Cholangiocytes

The protein for SR, CFTR, and Cl^-/HCO_3^- AE2 was expressed only by large bile ducts (red arrows, Figure 6A) and large cholangiocytes (by immunofluorescence, not shown)[1,2] from BDL rats. By immunofluorescence, small BDL cholangiocytes do not express SR, CFTR, and Cl^-/HCO_3^- AE2 (Figure 6A). In BDL rats treated with GABA, small ducts (yellow arrows, Figure 6A) and small cholangiocytes (Figure 6A) express *de novo* SR, CFTR, and Cl^-/HCO_3^- AE2. Parallel to our previous studies,[17] immunohistochemistry in liver sections shows that AC8 was mostly expressed by large cholangiocytes from BDL rats treated with NaCl (Figure 6B). In liver sections from BDL rats treated with GABA, the expression of AC8 in liver sections seemed lower in large ducts and present *de novo* in small bile ducts (Figure 6B). Similarly, by real-time PCR we demonstrated that: (i) AC8 was present in large and at lower levels in small BDL cholangiocytes[17]; and (ii) AC8 mRNA expression decreased in large cholangiocytes and significantly increased in small cholangiocytes from BDL rats treated with GABA (Figure 6B).
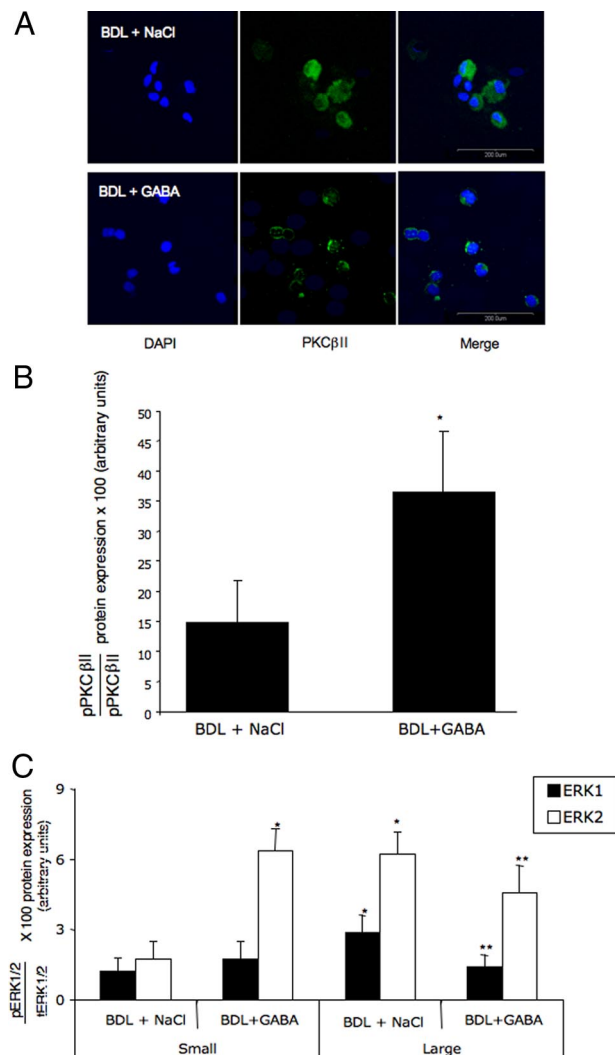
Figure 5. Evaluation of the membrane translocation (**A**) and phosphorylation (**B**) of PKCβII in small cholangiocytes from BDL rats treated with NaCl or GABA for 1 week. We observed enhanced membrane translocation (by immunofluorescence [**A**]) and phosphorylation (by immunoblots [**B**]) of PKCβII in small cholangiocytes from BDL GABA-treated rats compared with small cholangiocytes from BDL rats treated with NaCl. **A:** Specific immunoreactivity of representative fields is shown in green; cell nuclei were stained with DAPI (blue). Bar = 200 μm. **C:** Evaluation of ERK1/2 phosphorylation in small and large cholangiocytes from BDL rats treated with NaCl or GABA for 1 week. After GABA administration to BDL rats, there was: (i) decreased phosphorylation of ERK1/2 in large cholangiocytes; and (ii) increased ERK2 (but not ERK1) phosphorylation in small cholangiocytes compared with small and large cholangiocytes from BDL rats treated with NaCl. Data are mean ± SEM of 8 blots. *$P < 0.05$ versus small cholangiocytes from BDL rats treated with NaCl for 1 week. **$P < 0.05$ versus large cholangiocytes from BDL rats treated with NaCl for 1 week.

Parallel to other studies,[6,9,32,38] secretin increased cAMP levels (Figure 7A) and Cl$^-$ efflux (Figure 7B) of large (but not small) BDL cholangiocytes compared with their corresponding basal values. In large cholangiocytes from BDL GABA-treated rats, there was a decrease in secretin-stimulated cAMP levels (Figure 7A) and Cl$^-$ efflux (Figure 7B) compared with their corresponding values of large BDL cholangiocytes. Small cholangiocytes from BDL GABA-treated rats *de novo* respond to secretin with increased cAMP levels (Figure 7A) and Cl$^-$ efflux (Figure 7B).

Intravenous infusion of secretin increased bile flow and bicarbonate secretion of BDI rats (Table 2).[4,32,38,39] In BDL rats treated with GABA, secretin-stimulated bicarbonate rich choleresis was lower (although significant) compared with its corresponding values of BDI rats treated with NaCl (Table 2). The smaller but significant increase in secretin-stimulated choleresis (observed in BDL GABA-treated rats) is likely attributable to small proliferating cholangiocytes, which *de novo* respond to secretin in this model of large cholangiocyte damage.

Discussion

The findings of this study relate to the heterogeneous effects of GABA on the apoptotic, proliferative, and secretory functions of small and large cholangiocytes in cholestatic BDL rats. Chronic administration of GABA to BDL rats: (i) induced apoptosis of large cholangiocytes; (ii) reduced large cholangiocyte proliferation and IBDM by down-regulation of cAMP signaling; and (iii) decreased AC8 expression and reduced secretin-stimulated choleresis in large cholangiocytes. After GABA administration, small cholangiocytes: (i) were resistant to GABA-induced biliary apoptosis and *de novo* proliferate leading to an increased number of small ducts; (ii) displayed membrane translocation and phosphorylation of Ca^{2+}-dependent PKCβII; and (iii) *de novo* express SR, CFTR, Cl$^-$/HCO$_3^-$ AE2 and AC8, and secrete water and electrolytes in response to secretin. During damage of large cholangiocytes, small ducts replenish the intrahepatic biliary tree by amplification of both Ca^{2+}-dependent signaling and the acquisition of large cholangiocyte phenotypes.

We first demonstrated that both small and large cholangiocytes express GABA$_A$, GABA$_B$, and GABA$_C$ receptors at similar levels; the expression of the GABA receptors in small and large cholangiocytes did not change with GABA administration. These findings suggest that the heterogeneous effects of GABA on small and large cholangiocyte function are not attributable to the differential expression of these receptors in the two cell types. In our *in vivo* model it is difficult to pinpoint the specific receptors involved in GABA modulation of small and large cholangiocyte functions. Likely, these actions are mediated by all three GABA receptor subtypes. Our concept is supported by our previous *in vitro* studies[21] in human cholangiocarcinoma cells showing that blocking of GABA$_A$, GABA$_B$, and GABA$_C$ receptors (by specific receptor antagonist) prevents GABA inhibition of cholangiocarcinoma proliferation.

Importantly, GABA administration induces large cholangiocyte damage only in rats with extrahepatic cholestasis, which can be explained based on the fact that: (i) GABA damages large ducts due to sensitization from obstructive cholestasis and consequent biliary/seric accumulation[40]; (ii) GABA damages only proliferating large cholangiocytes, and this is consistent with our previous findings in cholangiocarcinoma cells[21]; and (iii) GABA metabolism is dysregulated during liver damage induced by cholestasis.[41]
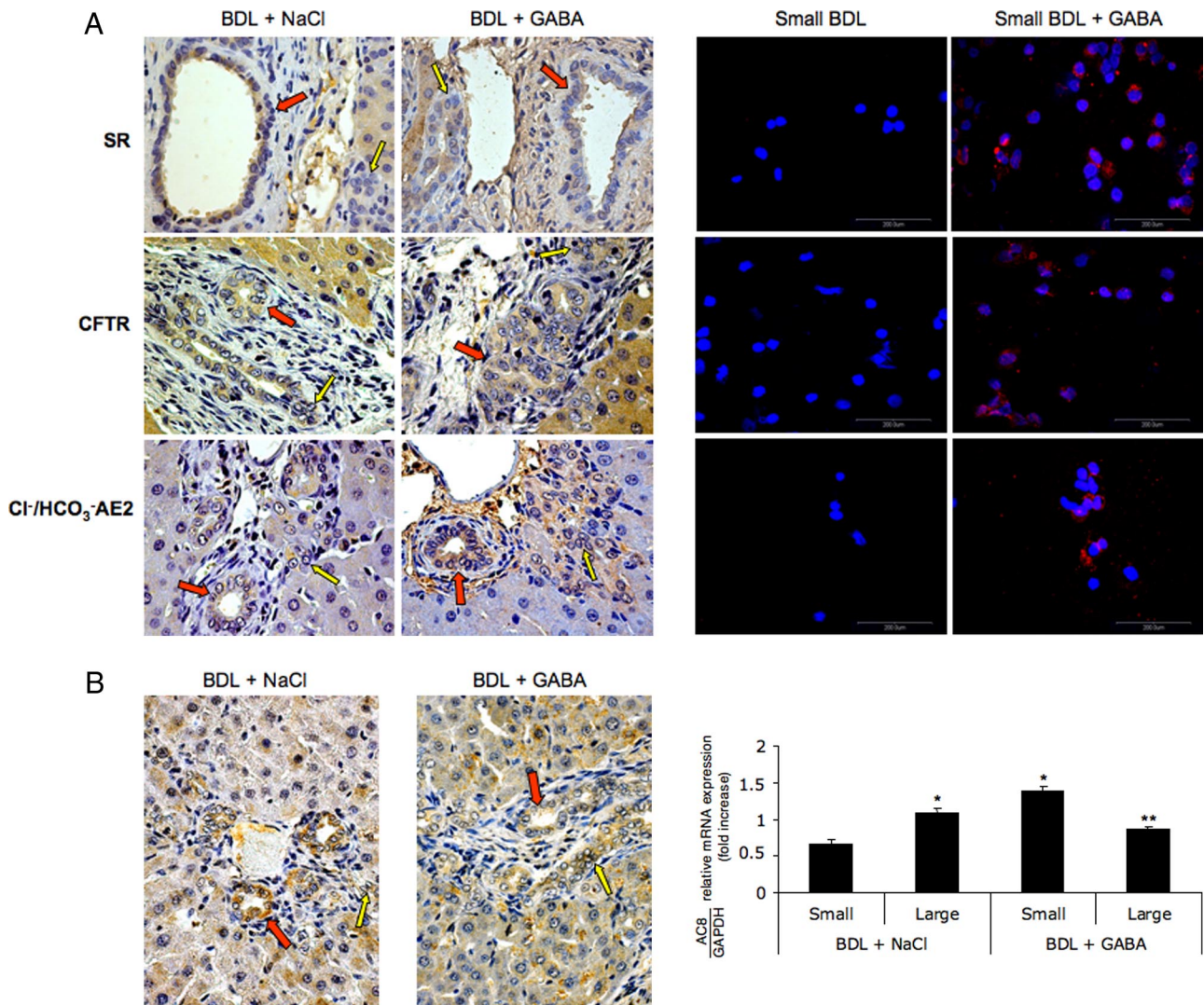
Figure 6. **A:** Representative immunohistochemistry for SR, CFTR, Cl^-/HCO_3^- AE 2 and immunofluorescence in liver sections (**left panel**) and immunofluorescence in freshly isolated small cholangiocytes (**right panel**) from BDL rats treated with NaCl or GABA for 1 week. In liver sections, the protein for SR, CFTR, and Cl^-/HCO_3^- AE2 was expressed only by large (**red arrows**) bile ducts from BDL rats; no expression for SR, CFTR, and Cl^-/HCO_3^- AE2 was seen in small (**yellow arrows**) bile ducts. In BDL rats treated with GABA, small bile ducts (**yellow arrows**) express *de novo* SR, CFTR, and Cl^-/HCO_3^- AE2. Original magnification, $\times 40$. By immunofluorescence, small BDL cholangiocytes do not express SR, CFTR, and Cl^-/HCO_3^- AE2. After administration of GABA to BDL rats, small cholangiocytes express *de novo* SR, CFTR, and Cl^-/HCO_3^- AE2. Scale bar = 200 μm. **B:** Expression of AC8 was evaluated by immunohistochemistry in liver sections and real-time PCR in freshly isolated cholangiocytes from BDL rats treated with NaCl or GABA for 1 week. In liver sections from BDL rats treated with GABA, the expression of AC8 in liver sections seemed lower in large ducts (**red arrows**) and present *de novo* in small bile ducts (**yellow arrows**, **left panel**). By real-time PCR we demonstrated that: (i) AC8 was present in large and at lower levels in small BDL cholangiocytes; and (ii) AC8 mRNA expression decreased in large cholangiocytes and significantly increased in small cholangiocytes from BDL rats treated with GABA (**right panel**). Data are mean $\pm$ SEM of 3 experiments. *$*P < 0.05$ versus small cholangiocytes from BDL rats treated with NaCl for 1 week. *$**P < 0.05$ versus large cholangiocytes from BDL rats treated with NaCl for 1 week.

A number of speculations and studies support the view that small cholangiocytes are more resistant than large cholangiocytes to hepatic injury/toxins.[8,42] For example, the anti-apoptotic protein bcl-2 is expressed at higher levels by ductules and small bile ducts in normal human liver and human liver with cirrhosis and focal nodular hyperplasia.[43] The higher resistance of small cholangiocytes to injury/toxins may be attributable to their primordial undifferentiated nature, whereas large cAMP-dependent cholangiocytes (more differentiated) are more susceptible to injury. Indeed, the presence of a larger nucleus and a smaller cytoplasm in small cholangiocytes[44] suggests the undifferentiated primitive nature of small bile ducts.[42,44] On the other hand, large cholangio-

cytes (displaying a larger cytoplasmic area)[42,44] are more differentiated/senescent cells and more likely more susceptible to damage. However, these points are speculative, and further studies are necessary to support this view.[42,44]

The PKC signaling pathway modulates cell resistance to apoptosis in a number of systems.[45,46] For example, the expression of PKCβI confers resistance to tumor necrosis factor-α and paclitaxel-induced apoptosis in HT-29 colon carcinoma cells.[45] In this regard, the activation of GABA receptors stimulates PKC activity in hippocampal and cultured spinal neurons.[47,48] Although our data do not demonstrate that the activation of IP_3/Ca^{2+}-dependent PKC signaling is the key factor for the higher

A

B
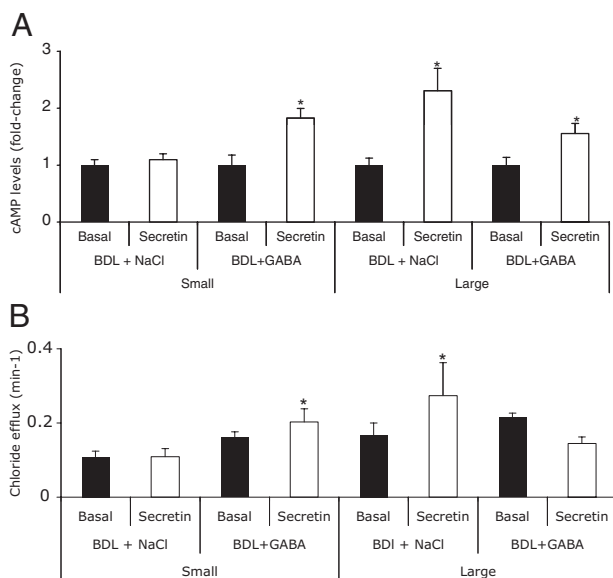
Figure 7. Measurement of basal and secretin-stimulated (**A**) cAMP levels and (**B**) Cl⁻ efflux in small and large cholangiocytes from BDL rats treated with NaCl or GABA for 1 week. **A:** Secretin increased cAMP levels of large (but not small) BDL cholangiocytes compared with their corresponding basal values. In BDL rats treated with GABA, secretin did not increase cAMP levels in large cholangiocytes, but significantly increased cAMP levels of purified small cholangiocytes compared with small cholangiocytes from BDL rats treated with NaCl. Data are mean ± SEM of six experiments. *$P < 0.05$ versus the corresponding basal value. **B:** Secretin increased Cl⁻ efflux of large (but not small) BDL cholangiocytes. In large cholangiocytes from BDL rats treated with GABA, secretin did not increase Cl⁻ efflux. In small cholangiocytes from BDL GABA-treated rats, secretin induced a *de novo* increase in Cl⁻ efflux. Data are mean ± SEM of 6 experiments. *$P < 0.05$ versus the corresponding basal value.

Figure 8. Working model of GABA-induced changes in small and large biliary proliferation/damage. **Right:** GABA stimulates a down-regulation of large cholangiocyte proliferation that results in the down-regulation of the functional capacity of cholangiocytes (ie, SR->cAMP->PKA->RK1/2 signaling mechanisms) and subsequently activates apoptosis. **Left:** GABA stimulates the proliferation of small cholangiocytes via the activation of calcium->AC8->PKCβII->ERK1/2-dependent signaling. In addition, GABA stimulates a phenotypic switch in small cholangiocytes resulting in the *de novo* expression of SR and the downstream cAMP->ERK1/2 signaling mechanisms. Also, GABA stimulates the *de novo* expression of CFTR and Cl⁻/HCO₃⁻ exchanger, which a partners in cholangiocyte functional activity. AC indicates adenylyl cyclase; cAMP, cyclic adenosine 3′,5′-monophosphate; ERK1/2, extracellular signal-regulated kinase1/2; GABA, γ-aminobutyric acid; PKA, protein kinase A; PKC, protein kinase C; SR, secretin receptor.

resistance of small cholangiocytes to GABA, they do suggest that activation of this signaling pathway is important for the activation of a "small bile duct compartment" to replenish the biliary epithelium during the damage of large bile ducts.[14,15] The IP₃/Ca²⁺-dependent signaling pathway and its cross talk with cAMP signaling are important regulators of small cholangiocyte functions.[14] We hypothesize that the trigger for the *de novo* activation of Ca²⁺/PKC-dependent proliferation of small cholangiocytes is attributable to the down-regulation of cAMP-dependent signaling in large cholangiocytes damaged by GABA treatment resulting in the absence of required functional capacity, which must be replenished by the activation of the small cholangiocyte compartment. In support of our hypothesis, studies have shown that GABA activity mediating cytosolic Ca²⁺ increases in developing

neurons is triggered by changes (activation or inhibition) in cAMP-dependent signal transduction.[49]

The *de novo* acquisition of proliferative and secretory phenotypes of large cholangiocytes by small cholangiocytes is likely attributable to the IP₃/Ca²⁺-dependent activation of AC8, a Ca²⁺-dependent AC isoform that plays a key role in the proliferative and secretory functions of large bile ducts.[17] Nine AC isoforms exist in mammalian cells.[50] All mammalian ACs are activated by GTP-bound stimulatory G protein α subunit (Gαₛ).[50] Whereas activation of Gαₛ increases AC-mediated cAMP synthesis, inhibitory G protein (Gαᵢ) inhibits all AC isoforms except AC₂ and AC₄.[50] The effects of [Ca²⁺]ᵢ are very diverse depending on the AC isoform.[50] The activities of AC₁, AC₃, and AC₈ are positively regulated by [Ca²⁺]ᵢ and calmodulin, whereas AC₅ and AC₆ are negatively modulated by [Ca²⁺]ᵢ.[50] Several studies demonstrate the role of AC in the regulation of cholangiocyte functions.[13,17,51] Changes in the activity of Gαₛ and Gαᵢ protein subunits

Table 2.   Measurement of Basal and Secretin-Stimulated Bile Flow and Bicarbonate Secretion

Treatment	Bile flow (μl/min/kg BW) (basal)	Bile flow (μl/min/kg BW) (secretin)	Bicarbonate secretion (μEq/min/kg BW) (basal)	Bicarbonate secretion (μEq/min/kg BW) (secretin)
BDI + NaCl	120.3 ± 13.0	186.3 ± 17.3*	5.2 ± 0.6	11.3 ± 1.5†
BDI + GABA	105.7 ± 8.3	139.5 ± 11.1*‡	4.1 ± 0.5	6.7 ± 0.5†§

BDI indicates bile duct ligation. Data are mean ± SEM of 5 experiments.
*$P < 0.05$ versus its corresponding value of basal bile flow.
†$P < 0.05$ versus its corresponding value of basal bicarbonate secretion.
‡$P < 0.05$ versus secretin-stimulated bile flow in BDI rats.
§$P < 0.05$ versus secretin-stimulated bicarbonate secretion in BDI rats.

are associated with alteration of cholangiocyte growth of BDL rats.[51] Increased cholangiocyte cAMP levels prevent bile duct damage by total vagotomy.[13] Also, cross talk between the IP_3/Ca^{2+}/PKC pathway and AC plays a key role in the regulation of cholangiocyte functions. Gastrin inhibits cholangiocyte proliferation through activation of Ca^{2+}-dependent PKCα.[7] The D2 dopaminergic agonist, quinelorane, inhibits secretin-stimulated ductal secretion of BDL rats by activation of the Ca^{2+}-dependent PKCγ, which leads to decreased PKA activity.[32] The $\alpha-1$ adrenergic receptor, phenylephrine, increases secretin-stimulated ductal secretion by activation of PKCα and PKCβII.[33]

In summary, we have developed a novel *in vivo* model characterized by impaired function of large cAMP-dependent cholangiocytes (Figure 8). In this model, small cholangiocyte function is regulated by both the activation of IP_3/Ca^{2+}-dependent PKC signaling and the acquisition of large cholangiocyte functional markers to compensate for the damage of large ducts (Figure 8). In pathological conditions in which large cAMP-dependent bile ducts are damaged (eg, such as cystic fibrosis),[5] the *de novo* activation of the IP_3/Ca^{2+}-/PKC-dependent small cholangiocyte compartment may be important compensatory mechanism for the replenishment of the biliary epithelium.

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