# Hydrophobic bile salts inhibit gallbladder smooth muscle function via stimulation of GPBAR1 receptors and activation of $K_{ATP}$ channels

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**Hydrophobic bile salts are thought to contribute to the disruption of gallbladder smooth muscle (GBSM) function that occurs in gallstone disease, but their mechanism of action is unknown. The current study was undertaken to determine how hydrophobic bile salts interact with GBSM, and how they reduce GBSM activity. The effect of hydrophobic bile salts on the activity of GBSM was measured by intracellular recording and calcium imaging using wholemount preparations from guinea pig and mouse gallbladder. RT-PCR and immunohistochemistry were used to evaluate expression of the G protein-coupled bile acid receptor, GPBAR1. Application of tauro-chenodeoxycholate (CDC, 50–100 $\mu$M) to *in situ* GBSM rapidly reduced spontaneous $Ca^{2+}$ flashes and action potentials, and caused a membrane hyperpolarization. Immunoreactivity and transcript for GPBAR1 were detected in gallbladder muscularis. The GPBAR1 agonist, tauro-lithocholic acid (LCA, 10 $\mu$M) mimicked the effect of CDC on GBSM. The actions of LCA were blocked by the protein kinase A (PKA) inhibitor, KT5720 (0.5–1.0 $\mu$M) and the $K_{ATP}$ channel blocker, glibenclamide (10 $\mu$M). Furthermore, LCA failed to disrupt GBSM activity in *Gpbar1*$^{-/-}$ mice. The findings of this study indicate that hydrophobic bile salts activate GPBAR1 on GBSM, and this leads to activation of the cyclic AMP–PKA pathway, and ultimately the opening of $K_{ATP}$ channels, thus hyperpolarizing the membrane and decreasing GBSM activity. This inhibitory effect of hydrophobic bile salt activation of GPBAR1 could be a contributing factor in the manifestation of gallstone disease.**

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**Abbreviations** CCK, cholecystokinin; CDC, tauro-chenodeoxycholic acid; CGRP, calcitonin gene-related peptide; GBSM, gallbladder smooth muscle; GPBAR1, G protein-coupled bile acid receptor; ICC, interstitial cells of Cajal; $K_{ATP}$, ATP-sensitive potassium channel; LCA, tauro-lithocholic acid; PKA, protein kinase A; VDCC, voltage-dependent calcium channel.

## Introduction

Decreased gallbladder contractility is a hallmark of both calculous and acalculous gallbladder disease and is associated with inflammation and gallstone formation. Diminished gallbladder emptying in response to a meal and/or cholecystokinin (CCK) infusion is observed in the vast majority of patients with cholesterol gallstones (Jazrawi *et al.* 1995; Shoda *et al.* 1995). These individuals also have increased fasting residual gallbladder volumes, as well as a decreased ejection fraction after a meal. Moreover, the interrelationship between bile stasis, inflammation and stone formation is still poorly understood.

Decreased contractility in gallstone disease may be caused by elevation of two main bile components, cholesterol and hydrophobic bile salts. Gallbladder smooth muscle (GBSM) strips from patients with cholesterol gallstones have impaired contractile responses to a variety of stimuli *in vitro* (Behar *et al.* 1989). When ground squirrels are fed a lithogenic (stone forming) diet rich in cholesterol and cholate, they exhibit a progressive increase in biliary cholesterol that

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is associated with a decrease in gallbladder contractility, cholesterol stone formation and cholecystitis (Xu & Shaffer, 1993). Gallbladders of C57L mice fed a lithogenic diet also have altered histology, are larger and have greater residual fasting volume (Wang *et al.* 1997; van Erpecum *et al.* 2006). Their bile shows cholesterol supersaturation, accumulation of mucin gel, and the formation of cholesterol crystals and gallstones. Cellular mechanisms responsible for the impaired GBSM activity are still unclear.

GBSM activity is characterized by rhythmic spontaneous action potentials that cause synchronized increases in intracellular [$Ca^{2+}$] called $Ca^{2+}$ flashes (Zhang *et al.* 1993; Balemba *et al.* 2006*b*). While voltage-dependent $Ca^{2+}$ channels (VDCCs) are mainly responsible for the fast depolarization component of the action potential, the repolarization involves various members of the $K^+$ channel family (Zhang *et al.* 1993). Coupling between interstitial cells of Cajal (ICC), known as the pacemaker cells of the GI tract, and GBSM is in part responsible for the synchronicity and rhythmicity of the GBSM activity (Lavoie *et al.* 2007). This spontaneous activity in GBSM, which underlies the maintenance of tone and gallbladder contractions, is disrupted by activation of the cAMP–PKA pathway (Zhang *et al.* 1994*b*). PKA-mediated suppression of gallbladder activity occurs primarily via the activation of ATP-sensitive $K^+$ ($K_{ATP}$) channels (Zhang *et al.* 1994*a*).

In addition to cholesterol, bile also contains both hydrophobic and hydrophilic bile salts referred to as 'bad' and 'good' bile salts, respectively, because of their relative effects on gallbladder function. Hydrophobic bile salts attenuate gallbladder contractility, an effect that has been directly related to the level of bile salt hydrophobicity (Xu *et al.* 1997). Using cannulated gallbladders *in vitro*, Rutishauser (1978) showed that hydrophobic bile salts inhibit gallbladder motor activity. Subsequently, Shaffer and colleagues demonstrated that hydrophobic bile salts cause a concentration-dependent inhibition of CCK-induced gallbladder muscle strip contractions (Xu *et al.* 1997).

The cellular mechanisms by which hydrophobic bile salts disrupt GBSM function have not been resolved, but in other tissues, hydrophobic bile salts relax smooth muscle (Bomzon & Ljubuncic, 2001; Dopico *et al.* 2002). Furthermore, hydrophobic bile salts appear to activate different $K^+$ channels in various cell types. For example, in murine small intestinal ICCs, they inhibit pacemaker currents by activating $K_{ATP}$ channels (Jun *et al.* 2005), whereas in rabbit mesenteric artery myocytes, they increase large conductance, $Ca^{2+}$-activated $K^+$ (BK) channel function (Dopico *et al.* 2002).

Specific bile acid receptors have been identified and they have been divided into two classes. The first class includes the nuclear bile acid receptors, farnesoid X receptor and pregnane X receptor, which control transcriptional level of enzymes involved in synthesis of bile acid. More recently a plasma membrane bile acid receptor called the G protein-coupled bile acid receptor (GPBAR1; also termed TGR5, M-Bar, or GPR131) has been characterized (Kawamata *et al.* 2003). It is a member of the rhodopsin-like family of G protein-coupled receptors that is ubiquitously expressed, but is most highly expressed in the gallbladder (Vassileva *et al.* 2006; Watanabe *et al.* 2006). GPBAR1 activation may be involved in gallstones disease because *Gpbar1*$^{-/-}$ mice do not form gallstones when fed a lithogenic diet (Vassileva *et al.* 2006). GPBAR1 activation by bile salts stimulates the Gs–cAMP–PKA pathway (Kawamata *et al.* 2003; Watanabe *et al.* 2006). Increased cAMP levels by $\beta$-receptor agonists, vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) induce gallbladder relaxation. Activation of the cAMP–PKA pathway mediates the inhibition of gallbladder contractility by activating $K_{ATP}$ channels, which, in turn, causes a membrane potential hyperpolarization of GBSM. We have previously shown in detail how CGRP, a smooth muscle relaxant, activates the opening of $K_{ATP}$ channels via the Gs–cAMP–PKA pathway (Zhang *et al.* 1994*a*; Morales *et al.* 2004). The aim of this study was to test the hypothesis that hydrophobic bile salts disrupt GBSM function by binding to the Gs-coupled receptor GPBAR1 and activating a cAMP-mediated opening of $K_{ATP}$ channels.

## Methods

### Animals

In this study, adult (female and male, 250–350 g) guinea pigs and C57BL/6J mice (male 7–15 weeks) were used. The *Gpbar1*$^{-/-}$ mice were generated directly into C57BL/6J genetic background by using C57BL/6J-derived ES cells and mating the chimeric males to C57BL/6J females (Vassileva *et al.* 2006, 2010).

The animals were anaesthesized with isoflurane and exsanguinated using protocols that were approved by the Institutional Animal Care and Use Committee of the University of Vermont. The abdominal cavity was opened and the gallbladder removed. Tissue was collected into ice-cold modified Krebs solution composed of (in mM): 121 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 $NaHPO_4$ and 8 glucose (pH 7.4).

Gallbladders were opened from fundus to the cystic duct, stretched and pinned mucosa side up on a Sylgard dish filled with ice-cold Krebs solution. In guinea pig preparations, the mucosal layer was removed with sharp forceps to enhance penetration of Fluo-4AM and visualization of the GBSM, whereas full-thickness

wholemounts were used for mice, which are thinner and removal of the mucosa was not necessary.

### Imaging and analysis of $Ca^{2+}$ events

Each segment of tissue was stretched and pinned between two pieces of Sylgard. Tissue was equilibrated in HEPES buffer containing (in mM): 110 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES and 5 glucose (pH 7.4) and then loaded for 1 h at room temperature in a HEPES buffer containing 10 $\mu$M fluo-4AM (Invitrogen) and 2.5 $\mu$g-ml$^{-1}$ pluronic acid (Invitrogen). These preparations were washed in HEPES for at least 30 min at room temperature to allow deesterification.

The preparations were then placed in a $Ca^{2+}$ imaging chamber, and superfused with aerated physiological saline solution (PSS) containing (in mM): 119 NaCl, 7.5 KCl, 1.6 $CaCl_2$, 1.2 $MgCl_2$, 23.8 $NaHCO_3$, 1.2 $NaH_2PO_4$, 0.023 EDTA and 11 glucose (pH 7.3) at 35–37°C. After a 15–20 min equilibration, $Ca^{2+}$ events were analysed using a Nikon TMD inverted microscope with a 60× water immersion lens attached to a Noran Oz laser confocal system. Movie files were acquired over periods of 20–30 s (30 frames per second). In some experiments, $Ca^{2+}$ events were visualized using an Andor iXon$^{EM}$+ 897 back-illuminated EMCCD camera attached to an inverted fluorescent Olympus IX70 microscope equipped with a 40× objective. Movies were analysed using SparkAN, a custom software written at the University of Vermont (A. D. Bonev).

### Intracellular recording

Wholemount sections of guinea pig gallbladder muscularis propria were stretched in a Sylgard covered recording chamber serosa side up on a Nikon TMD inverted microscope equipped with Hoffman modulation contrast optics (Modulation Optics, Greenvale, NY, USA) to visualize smooth muscle bundles. Tissue was continuously superfused with aerated (95% $O_2$–5% $CO_2$) Krebs solution at 35–37°C. Electrical activity was recorded using glass microelectrodes filled with 2 M KCl (resistance range of 70–150 MΩ) and a negative-capacity compensation amplifier (Axoclamp 2A, Molecular Devices, Sunnyvale, CA, USA). Membrane potential traces were recorded and analysed using PowerLab/4SP and Chart5 software packages (ADInstruments, Colorado Springs, CO, USA).

### Detection of *Gpbar1* mRNA in the gallbladder by RT-PCR

Total RNA was isolated from mouse and guinea pig gallbladder using RNAeasy Midi kit (Qiagen Inc., Valencia, CA, USA). For each reaction 2 $\mu$g of total RNA was combined with oligo(dT), dNTP, RNasin, and M-MLV RT enzyme and buffer (Promega, Madison, WI, USA). The PCR reaction was performed using the Hot Start IT taq with the following primer sets: mouse *Gpbar1*: forward (5′-TGGAAGTTTATGGCCTCCTG-3′) and reverse (5′-CCAACACAGCAAGAAGAGCA-3′) primers; guinea pig *Gpbar1*: forward (5′-TGGAAGTTTATGGCCTCCTG-3′) and reverse (5′-CCAACACAGCAAGAAGAGCA-3′) primers; mouse *Muc5ac*: forward (5′-GTCTGGCAGAAACAGTGGAG ATT-3′) and reverse (5′-TCGTGGCTTCTCACAGAA CTTG-3′) primers. The 208 bp mouse and 246 bp guinea pig *Gpbar1* products, and the 78 bp mouse *Muc5ac* were detected on an ethidium bromide-stained agarose gel and sequenced to confirm identity.

### Immunohistofluorescence to detect GPBAR1 localization in gallbladder tissue

Mouse and guinea pig gallbladders were fixed in 4% paraformaldehyde (0.1 M phosphate buffered saline (PBS), pH 7.4, overnight, 4°C). Gallbladders were cleared with dimethyl sulfoxide (DMSO), washed with PBS, and incubated in 30% sucrose PBS (overnight, 4°C). They were embedded in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA), sectioned (10 $\mu$m), and thaw-mounted onto slides. Sections were incubated in PBS containing 4–10% normal serum and 0.1–0.5% Triton X-100 (1 h, room temperature). Sections were incubated with GPBAR1 antibody (Poole *et al.* 2010), smooth muscle actin (Sigma-Aldrich, St Louis, MO, USA, 1:10,000) and GPBAR1 antibody preadsorbed with immunizing peptide (50 $\mu$M, overnight, 4°C). Slides were washed and incubated with secondary antibody conjugated to Cy3, Rhodamine Red X or FITC (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA, 1:200–500, for 1–3 h at room temperature). Mouse sections were mounted using ProlongGold (Invitrogen, Calsbad, CA, USA) and observed using a Zeiss LSM510 META confocal system. Guinea pig preparations were mounted using Citi-Fluor (Electron Microscopy Sciences Co., Ft Washington, PA, USA) and observed using an inverted Olympus IX-70 fluorescence microscope equipped with a MagnaFire CCD camera (Optronics, Goleta, CA, USA).

### Statistical analyses

Statistical analyses were done using Graph Pad Prism (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as means ± S.E.M., and $n$ refers to the number of preparation from different animals used. $P$ values $\leq 0.05$ were considered statistically significant (Student's $t$ test for

two samples or one-way ANOVA and Dunnett's multiple comparison test for more than two samples).

### Chemicals

Unless otherwise specified drugs used in this study including tauro-chenodeoxycholate, tauro-lithocholate, glibenclamide, KT-5720, pinacidil, and forskolin were from Sigma-Aldrich (St Louis, MO, USA).

## Results

### The hydrophobic bile salt tauro-chenodeoxycholate disrupts GBSM function

We have previously shown that GBSM activity is characterized by rhythmic firing of spontaneous action potentials that cause $Ca^{2+}$ flashes in all of the GBSM cells of a given bundle, which in turn stimulate tissue contraction (Zhang *et al.* 1993; Balemba *et al.* 2006*b*). We therefore used $Ca^{2+}$ imaging and intracellular recording techniques to evaluate the effect of the hydrophobic bile salts on the basal rhythmic activity in the guinea pig GBSM bundles.

In guinea pig, $Ca^{2+}$ flashes in GBSM cells were synchronized within a given bundle (Fig. 1*A*). The basal frequency of $Ca^{2+}$ flashes recorded in the current study was $0.22 \pm 0.02$ Hz ($n = 19$), which is similar to that previously reported (Balemba *et al.* 2006*b*). Addition of the hydrophobic bile salt tauro-chenodeoxycholate (CDC, 50–100 $\mu$M) to the bathing solution caused a rapid reduction of spontaneous $Ca^{2+}$ activity in GBSM, with elimination of flashes as soon as 2.5 min after the application of CDC to the bath.

When recording from GBSM with intracellular microelectrodes, the addition of CDC (100 $\mu$M) resulted in a loss of action potentials within 2.5 min of administration (Fig. 1*B*). The disruption of action potential activity was associated with a membrane hyperpolarization of $8.2 \pm 2.5$ mV (basal, $-44.8 \pm 4.2$ mV; CDC, $-53.0 \pm 6.1$ mV; $P < 0.05$, paired $t$ test; $n = 4$) (Fig. 1*C*). Washout reversed the effect of CDC at 50 and 100 $\mu$M within 5 min.

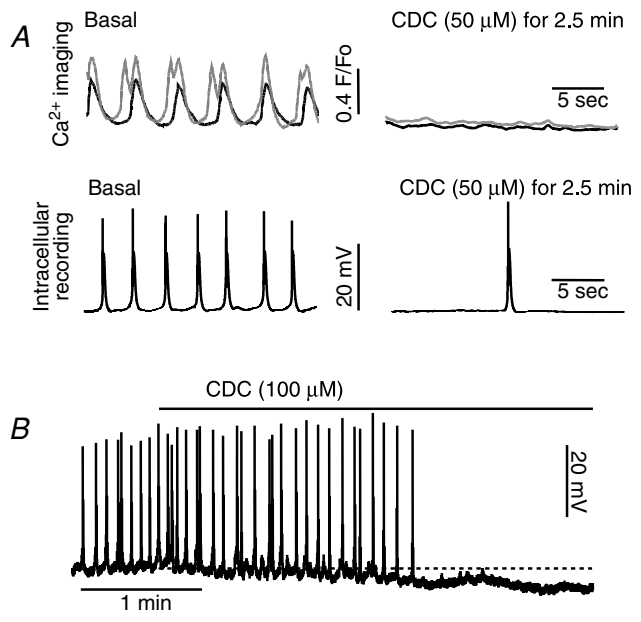


**Figure 1. The hydrophobic bile salt tauro-chenodeoxycholate (CDC) disrupts spontaneous activity in guinea pig GBSM**
*A*, the hydrophobic bile salt CDC (50–100 $\mu$M) rapidly reduces the rhythmic discharge of $Ca^{2+}$ flashes (top traces; two muscle cells in the same bundle are represented) and eliminates action potentials (bottom traces; recorded in a separate preparation). *B*, the intracellular recording demonstrates a membrane potential hyperpolarization that was induced by CDC (the dashed line indicates the resting membrane potential). Resting potentials were: *A*, −47.4 mV; *B*, −40.7 mV.

### GPBAR1 is expressed by GBSM

The rapid action of CDC on GBSM supports the concept that hydrophobic bile salts disrupt GBSM function via activation of a membrane receptor. Recent studies have shown that the membrane bile acid receptor GPBAR1 is highly expressed in the gallbladder, but it was thought to be primarily expressed by epithelial cells on the basis of *in situ* hybridization studies (Vassileva *et al.* 2006). Using mouse and guinea pig *Gpbar1* sequences from the GenBank and Ensembl databases, respectively, we designed species-specific primer sets that allowed us to amplify *Gpbar1* cDNA fragments. *Gpbar1* mRNA transcripts were expressed in the full thickness of the gallbladder as well as in the mucosal and muscularis layer of the tissue (Fig. 2*A* and *B*). In mouse, we used primers for *Muc5ac* (Wang *et al.* 2004), a gene expressed in gallbladder epithelial cells, to verify that the muscularis sample did not contain epithelial cells. *Muc5ac* mRNA transcripts were expressed in the full-thickness of the gallbladder as well as in the mucosal layer, but were absent from the muscularis layer. Since the *Gpbar1* sequence is located in a single exon, appropriated controls (DNase-treated and no RT samples) were amplified, and no band was amplified (data not shown).

An antibody against the human GPBAR1 was used for immunohistochemistry to confirm the presence of GPBAR1 in the mouse and guinea pig gallbladders and determine its cellular distribution. In cryostat cross-sections, intense immunoreactivity for GPBAR1 was detected in the epithelial layer, which corresponds to the previous *in situ* hybridization studies, as well as the muscularis propria (Fig. 2*C* and *D*). The presence of GPBAR1 in GBSM was confirmed by double label immunohistochemistry for smooth muscle actin (Fig. 2*D–F*). Collectively, these findings demonstrated the

presence of GPBAR1 transcript and immunoreactivity in the muscularis propria of the gallbladder, indicating that GBSM cells express GPBAR1.

### The GPBAR1 agonist lithocholic acid disrupts GBSM function

Lithocholic acid (LCA) has a high affinity for, and acts as an agonist of, GPBAR1 (Iguchi *et al.* 2009). We therefore tested whether LCA disrupts GBSM $Ca^{2+}$ transients and action potentials in a manner similar to that observed in response to CDC.

Addition of LCA (10 $\mu$M) to the perfusion solution caused a significant reduction in the frequency of GBSM $Ca^{2+}$ flashes (Fig. 3*A*). LCA decreased the frequency of $Ca^{2+}$ flashes from $0.22 \pm 0.04$ Hz to $0.05 \pm 0.01$ Hz within 5 min ($n = 9$; $P < 0.001$). The decrease in GBSM $Ca^{2+}$ flash frequency persisted for at least 20 min ($0.05 \pm 0.01$ Hz; $n = 7$; $P < 0.001$ at 20 min).

Intracellular recordings demonstrated that bath application of LCA (10 $\mu$M) elicits a similar effect on GBSM to that of CDC. LCA caused a rapid reduction of action potential frequency that was associated with a membrane potential hyperpolarization of $5.22 \pm 1.4$ mV (Fig. 3*B*) (basal, $-49.6 \pm 2.1$ mV; LCA $-54.9 \pm 1.9$ mV; $P < 0.05$, paired *t* test; $n = 4$). The hyperpolarization elicited by LCA was comparable to that caused by CDC ($P = 0.34$; unpaired *t* test). The response to 10 $\mu$M LCA partially recovered following washout.

### GPBAR1 activates the cAMP–PKA pathway in GBSM

GPBAR1 is known to act via stimulation of the cAMP–PKA pathway (Kawamata *et al.* 2003). Therefore, we tested the hypothesis that blocking this pathway would protect GBSM from the disruptive effects of hydrophobic bile salts.

The selective PKA inhibitor KT5720 (0.5–1.0 $\mu$M) was added to the bathing solution at least 30 min prior to LCA application. KT5720 (0.5–1 $\mu$M) alone did not have any detectable effect on the basal $Ca^{2+}$ flash activity in GBSM (PSS, $0.20 \pm 0.05$ Hz, $n = 4$; KT5720 after 30 min: $0.26 \pm 0.05$ Hz; $n = 6$; $P > 0.05$). In presence of KT5720, LCA (10 $\mu$M) failed to disrupt $Ca^{2+}$ flash activity in GBSM (Fig. 4*A*) (LCA plus KT5720, $0.20 \pm 0.05$ Hz; $n = 6$; $P > 0.05$ *vs.* KT5720). Frequencies remained unchanged even after prolonged exposure to both compounds ($0.20 \pm 0.07$ Hz after 15 min; $n = 5$; $P > 0.05$). The inhibition of $Ca^{2+}$ flashes in response to CDC was also attenuated by KT5720 ($n = 2$; data not shown).

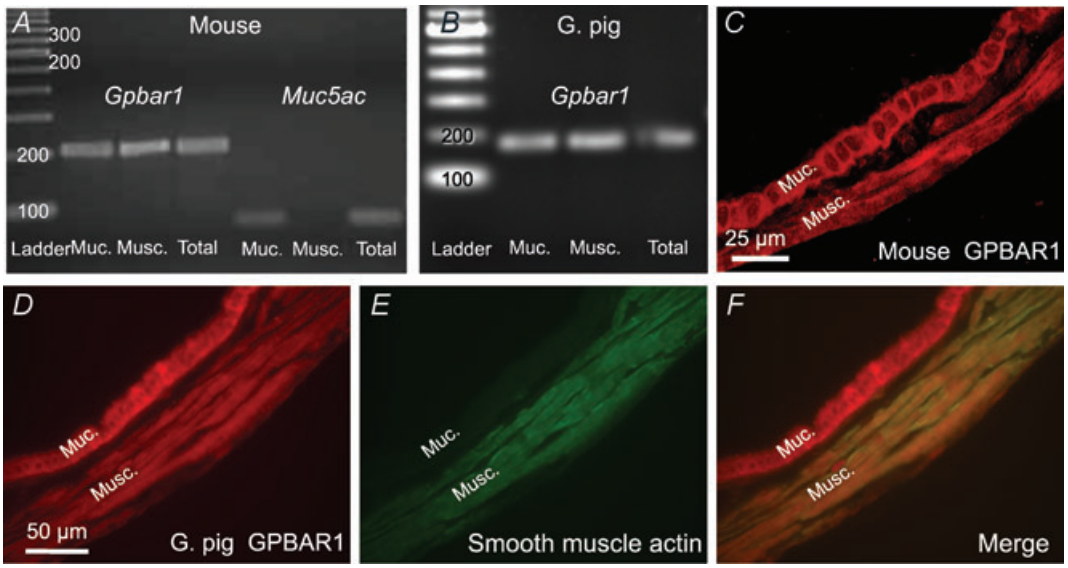


**Figure 2. The bile salt receptor GPBAR1 is expressed in mouse and guinea pig GBSM**
RT-PCR results demonstrating that the *Gpbar1* transcript is detectable in RNA extracted from mouse (*A*) and guinea pig (*B*) gallbladder muscularis, as well as RNA from the mucosa, suggesting that this receptor is present in GBSM. The predicted product size was 208 bp for the mouse and 246 bp for the guinea pig. In the mouse, the *Muc5ac* transcript (78 bp) was only detected in RNA from the mucosa indicating that the muscularis propria sample did not contain epithelial cells. Cross-sections of full-thickness mouse (*C*) and guinea pig (*D*) gallbladder show that GPBAR1 is expressed in mucosa and muscularis layer. GBSM cells that express smooth muscle actin (*E*) also express GPBAR1 (*F*) as shown using double immunostaining of guinea pig gallbladder. Muc., mucosal layer; musc., muscularis layer.

### Hydrophobic bile salt disruption of GBSM is inhibited by the $K_{ATP}$ blocker glibenclamide

We have previously demonstrated that stimulation of the cAMP–PKA pathway leads to an activation of a $K_{ATP}$ current in GBSM (Zhang *et al.* 1994*a*). Furthermore, calcitonin gene-related peptide (CGRP), which stimulates receptors that act via the cAMP–PKA pathway, activates the $K_{ATP}$ current in GBSM and suppresses action potentials with an accompanying hyperpolarization of the membrane potential (Zhang *et al.* 1994*b*). Therefore, if hydrophobic bile salts act on GPBAR1, which in turn activates the cAMP–PKA pathway, it would be expected to inhibit GBSM function by opening $K_{ATP}$ channels. We tested this hypothesis by applying LCA in the presence of the $K_{ATP}$ channel blocker glibenclamide.

In the presence of glibenclamide (10 $\mu$M), LCA (10 $\mu$M) failed to alter the frequency of $Ca^{2+}$ flashes (Fig. 4*B*) (glibenclamide: $0.32 \pm 0.06$ Hz; LCA plus glibenclamide: $0.25 \pm 0.05$ Hz; $n = 6$; $P > 0.05$). The flash frequency did not decrease during prolonged exposure to both compounds ($0.28 \pm 0.05$ Hz after 20 min; $n = 6$; $P > 0.05$). The inhibition of $Ca^{2+}$ flashes in response to CDC was also attenuated by glibenclamide ($n = 3$; data not shown).

### LCA failed to disrupt GBSM function in *Gpbar1*$^{-/-}$ mice

To investigate whether LCA disrupts GBSM function via activation of GPBAR1, we evaluated its effects on GBSM in preparations from mice that lack GPBAR1. These *Gpbar1*$^{-/-}$ mice have been characterized previously, and interestingly, they are less prone to developing gallstones when fed a lithogenic diet (Vassileva *et al.* 2006, 2010).

In control C57BL/6J mice, the basal rate of GBSM $Ca^{2+}$ flashes was very similar to that observed in guinea pig tissue. In these preparations, 10 $\mu$M LCA caused a rapid and persistent disruption of $Ca^{2+}$ flash activity (Fig. 5*A*) that was similar to the effect of LCA on $Ca^{2+}$ flashes in guinea pig GBSM. In these experiments involving mouse gallbladder whole mount preparations with intact mucosa, $Ca^{2+}$ waves were more predominant than in the guinea pig muscularis preparation. This was not unexpected because we had previously reported that $Ca^{2+}$ waves are more common in full-thickness preparations (Balemba *et al.* 2006*a*).

In *Gpbar1*$^{-/-}$ mice basal activity of the GBSM was comparable to that observed in C57BL/6J mice and the guinea pig. Unlike the responses to LCA in wild-type mice described above, addition of LCA (10 $\mu$M) to preparations

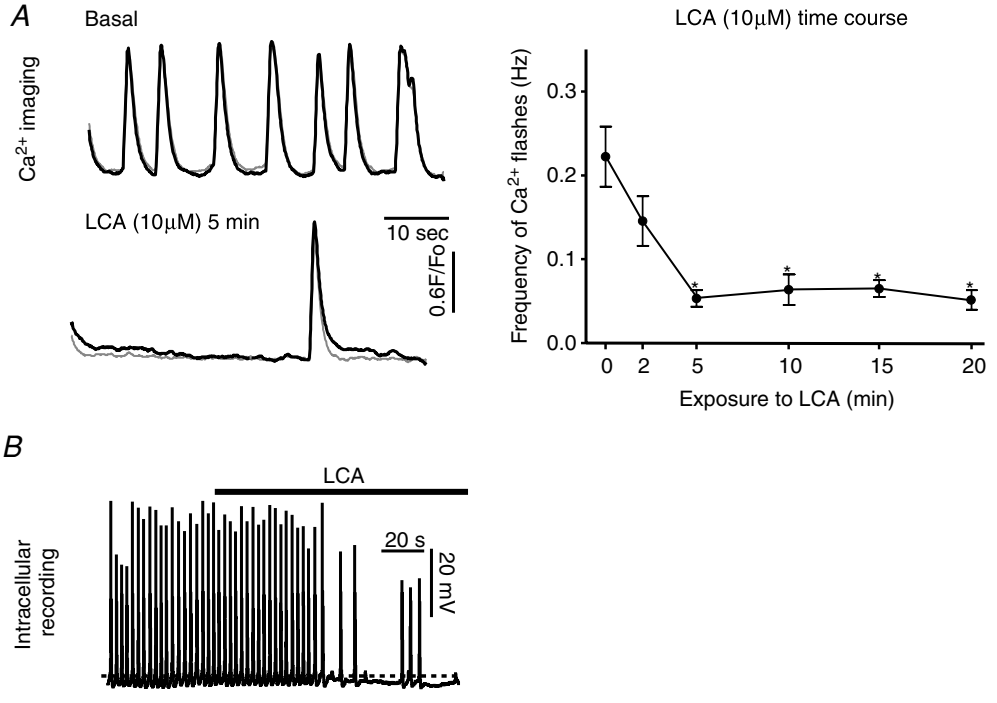


**Figure 3. The GPBAR1 agonist lithocholic acid (LCA) decreases GBSM activity**
*A*, application of LCA (10 $\mu$M) to the bath rapidly reduced the spontaneous $Ca^{2+}$ flashes in the GBSM (*A*). (Traces represent two separate cells in the same muscle bundle.) The effect of LCA was maintained during prolonged incubations (right panel). *B*, intracellular recording from guinea pig GBSM demonstrates that soon after application of LCA (10 $\mu$M), the membrane potential is hyperpolarized (the dashed lined denotes the resting membrane potential), and spontaneous action potential activity is dramatically reduced. *Significantly different from basal ($P < 0.001$). Resting potential: −46.6 mV. *n* values for data in the graph are 7–9.

from *Gpbar1*$^{-/-}$ mice failed to alter spontaneous $Ca^{2+}$ flash activity (Fig. 5; basal: $0.36 \pm 0.04$ Hz; LCA after 5 min: $0.32 \pm 0.06$ Hz; $n = 10$; $P > 0.05$). To verify that necessary components of the cAMP–PKA–$K_{ATP}$ pathway were present and functional in tissue from the *Gpbar1*$^{-/-}$ mice, we tested the effects of the PKA activator forskolin (10 $\mu$M) and the $K_{ATP}$ channel opener pinacidil (10 $\mu$M) on the basal activity of GBSM in the *Gpbar1*$^{-/-}$ mice. In preparations from *Gpbar1*$^{-/-}$ mice, both forskolin (10 $\mu$M) and pinacidil (10 $\mu$M) rapidly decreased the frequency of $Ca^{2+}$ flashes (Fig. 5*B*) (forskolin 5 min: $0.04 \pm 0.02$ Hz, $n = 5$, $P < 0.001$; pinacidil 5 min: $0.06 \pm 0.02$ Hz, $n = 4$, $P < 0.01$).

## Discussion

The present study was undertaken to investigate the effects of hydrophobic bile acids on spontaneous activity in gallbladder smooth muscle cells, and to elucidate the mechanisms by which these compounds decrease GBSM contractility. The findings of this study support the concept that hydrophobic bile acids disrupt GBSM function by activating a G protein-coupled receptor that leads to membrane hyperpolarization via the activation of a $K_{ATP}$ channel (Fig. 6). Evidence reported here in support of this include the following: (1) the membrane-bound bile acid receptor GPBAR1 is expressed by smooth muscle cells in the mouse and guinea pig gallbladder; (2) stimulation of GPBAR1 with CDC or LCA inhibited GBSM activity; (3) in the presence PKA or $K_{ATP}$ channel inhibitors, LCA failed to alter GBSM function; and (4) gallstone-resistant mice that do not express the GPBAR1 protein were not responsive to LCA.

### Bile acids inhibit GBSM activity via G protein-coupled GPBAR1 receptors

Bile acids are now recognized as signalling molecules that can exert effects non-genomically by activating the G protein-coupled receptor, GPBAR1/TGR5, and genomically by activating the farnesoid X receptor, a

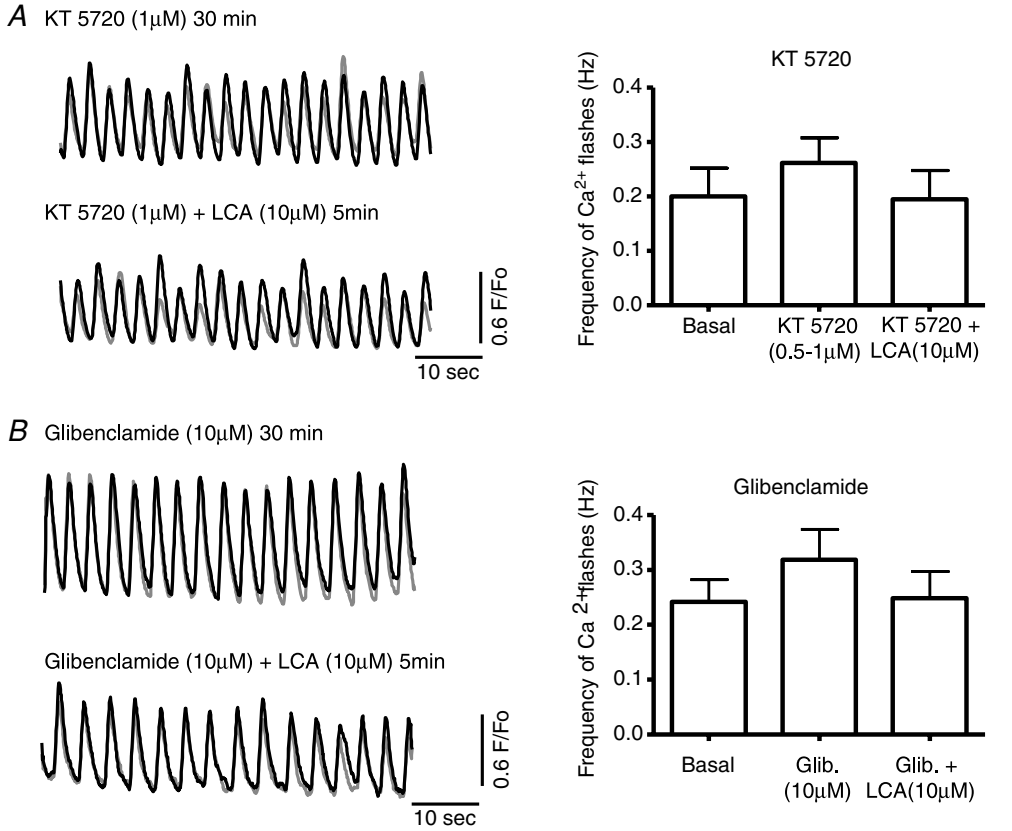


**Figure 4. Inhibition of protein kinase A (PKA) or $K_{ATP}$ channels protects guinea pig muscularis against adverse effects of LCA**
*A*, the addition to the bath of the PKA inhibitor KT5720 (0.5–1 $\mu$M) alone did not affect $Ca^{2+}$ flashes in guinea pig GBSM ($P > 0.05$; $n = 6$). However, in the presence of KT5720, LCA (10 $\mu$M) failed to inhibit GBSM function ($P > 0.05$; $n = 6$). *B*, the $K_{ATP}$ channel blocker glibenclamide (10 $\mu$M) blocked the effect of LCA (10 $\mu$M) on the frequency of $Ca^{2+}$ flashes ($P > 0.05$; $n = 6$).

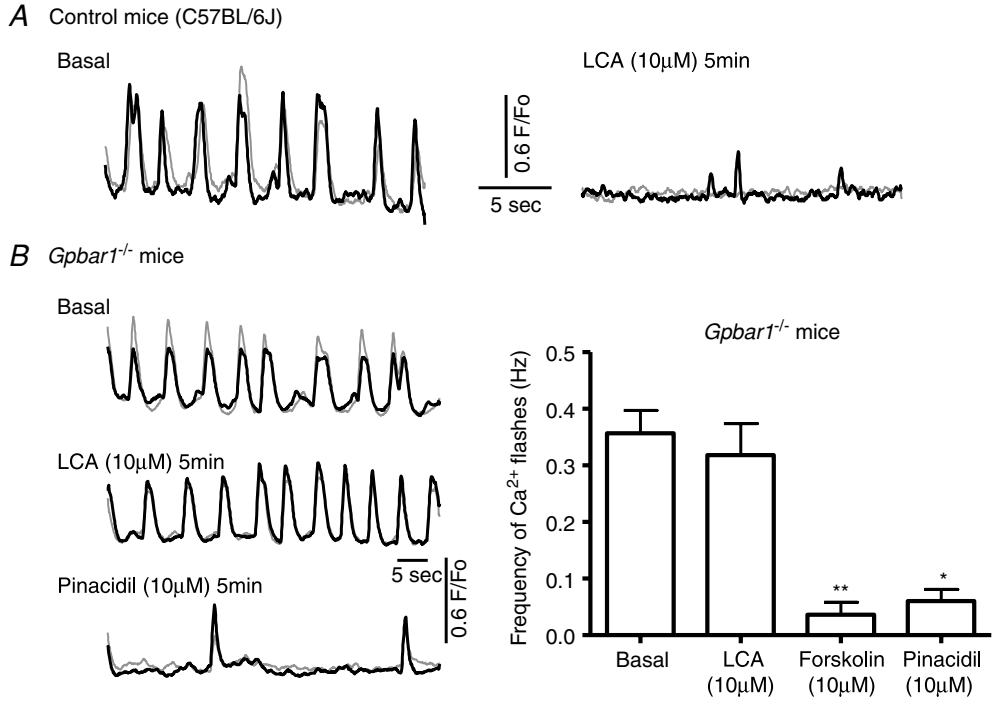


**Figure 5. In *Gpbar1* knockout mice, the rhythmic activity of GBSM is not affected by LCA**
*A*, basal activity of GBSM in C57BL/6J mice is comparable to the basal activity of guinea pig GBSM. In control mice, LCA (10 $\mu$M) rapidly reduced the frequency of $Ca^{2+}$ flashes ($n = 6$). *B*, in *Gpbar1*$^{-/-}$ mice, LCA (10 $\mu$M) did not alter the activity of $Ca^{2+}$ flashes ($P > 0.05$; $n = 11$). However GBSM activity was quickly disrupted by forskolin, an activator of the PKA pathway (**$P < 0.001$, $n = 5$), and pinacidil (10 $\mu$M), a $K_{ATP}$ channel opener (*$P < 0.01$; $n = 4$). These results support the hypothesis that LCA activates $K_{ATP}$ channels.

member of the nuclear hormone receptor superfamily (Nguyen & Bouscarel, 2008).

GPBAR1 is widely distributed, but it is expressed at particularly high concentrations in the gallbladder, where it was previously thought to be present primarily in epithelial cells (Vassileva *et al.* 2006; Keitel *et al.* 2009). The current study provides new evidence that GPBAR1 mRNA and protein are present in GBSM in addition to epithelial cells in mouse and guinea pig gallbladders. Tauro-chenodeoxycholate and LCA, a relatively selective GPBAR1 agonist present in the human bile, hyperpolarized GBSM cells and eliminated spontaneous action potential generation. The disruptive effect of LCA on GBSM activity occurred rapidly and in the absence of

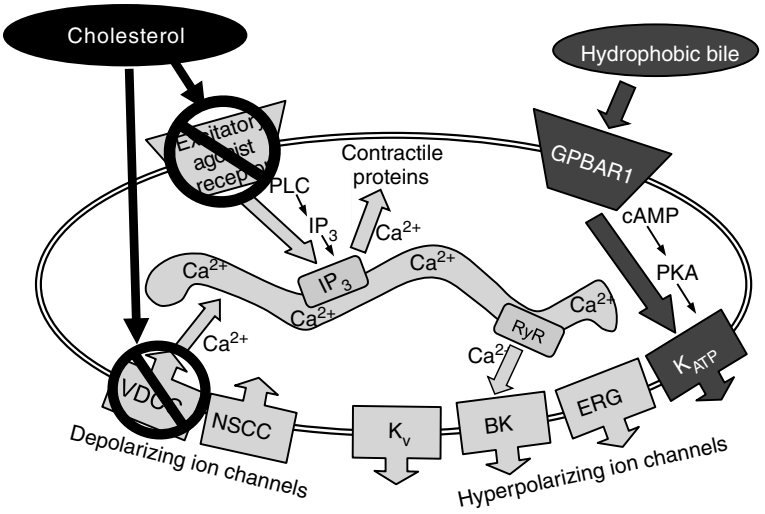


**Figure 6. Proposed pathway for the action of hydrophobic salts on GBSM activity**
Based on the results of this study, we proposed that activation of GPBAR1 on the plasma membrane of the GBSM by hydrophobic bile salts leads to a rapid activation of the cAMP–PKA pathway causing a hyperpolarization of the muscle cells due to the opening of the ATP-sensitive $K_{ATP}$ channels and a subsequent decrease in GBSM activity. In *Gpbar1*$^{-/-}$ mice, which do not express GPBAR1, GBSM activity was not affected by the hydrophobic bile salt LCA. Our data also support that cholesterol and bile salts are affecting GBSM activity via two distinct pathways. Previous investigations have demonstrated that cholesterol disrupts GBSM function by inhibiting voltage-dependent $Ca^{2+}$ channel (VDCC) activity, and by preventing activation of excitatory agonist receptors on GBSM.

the epithelial cells, supporting a direct activation of G protein-coupled receptors on the smooth muscle cells. Interestingly, LCA also mediated its action on GBSM in full-thickness preparations, with the epithelial layer exposed directly to the bathing solution, supporting the concept that 'intraluminal' bile could gain access to GBSM by penetrating the epithelial layer. In the full-thickness preparations, the basal $Ca^{2+}$ flash activity and the time course of LCA action were similar to those observed in muscularis preparations.

It is possible that GPBAR1 plays a role in the development of gallstone disease. Bile acids have been shown to decrease GBSM contractility in response to agonists or nerve stimulation, suggesting that they may contribute to the alterations in motor activity that are observed in gallstone disease. Also, *Gpbar1*$^{-/-}$ mice do not develop gallstones when fed a lithogenic diet that leads to cholesterol crystal and gallstone formation as well as cholecystitis in wild-type animals. Interestingly, the *Gpbar1*$^{-/-}$ mice develop normally and do exhibit altered levels of cholesterol, bile acids or any other bile constituents (Vassileva *et al.* 2006). Therefore, the protective effect of the gene deletion may involve the lack of a target for the bile acids in GBSM rather than, or in addition to, an effect on bile synthesis. Consistent with this concept, gallstone disease in humans is associated with elevated gallbladder *Gpbar1* mRNA levels (Keitel *et al.* 2009).

### $K_{ATP}$ channels and smooth muscle relaxation

In human gallbladder, $K_{ATP}$ channels can mediate GBSM relaxation (Bird *et al.* 2002). Interestingly the action of the smooth muscle relaxant CGRP, which has been well characterized in gallbladder and arterial smooth muscles, is known to activate the ATP-sensitive $K_{ATP}$ via PKA (Quayle *et al.* 1994; Zhang *et al.* 1994*a*). Activation of the cAMP–PKA pathway by other signalling molecules such as $\beta$-adrenergic receptor agonists and VIP also leads to muscle relaxation. The signal transduction mechanisms underlying the activation of the $K_{ATP}$ channels by GPBAR1 are similar to that described for CGRP. The GPBAR1 agonist, LCA, like CGRP, hyperpolarized smooth muscle cells by activation of the cAMP–PKA pathway, which leads to the opening of $K_{ATP}$ channels. Other channels may also be activated by the cAMP–PKA pathway in the GBSM. However, when applied alone, the $K_{ATP}$ channel inhibitor glibenclamide prevented the LCA-induced decrease of GBSM activity, supporting a critical role of $K_{ATP}$ channels. Physiologically, the hyperpolarization mediated by $K_{ATP}$ channel activation would greatly diminish the ability of VDCCs to open by driving the membrane potential to a level below their activation threshold. This is critical because the activity of these channels is fundamental for action potentials and associated $Ca^{2+}$ entry to occur (Balemba *et al.* 2006*b*), and for the maintenance of intracellular $Ca^{2+}$ stores (Morales *et al.* 2005).

### Cholesterol *vs.* bile acid in gallstone disease

Taken together these findings also indicate that while both cholesterol and hydrophobic bile acids have harmful effects on gallbladder contractility, their mechanisms of action are different (Fig. 6). The mechanism by which cholesterol interacts with GBSM is fairly well understood. Cholesterol accumulates in the plasma membrane of GBSM, predominantly in the caveolar regions, where it acts by decreasing membrane fluidity and by attenuating the function of CCK receptors, which are abundant in these regions (Jennings *et al.* 1999; Xiao *et al.* 2000, 2007). CGRP-induced hyperpolarizations are reduced by cholesterol enrichment, supporting changes in receptor–ligand binding and/or second messenger interactions. Cholesterol reduces the action potential firing in intact tissue by disrupting $Ca^{2+}$-mediated events via inhibition of $Ca^{2+}$ channel function. In isolated cells, $Ca^{2+}$ currents are reduced but the activity of the $K_{ATP}$ and voltage-activated $K^+$ channels are not affected by cholesterol (Jennings *et al.* 1999). In contrast, hydrophobic bile salts inhibit GBSM contractility via a hyperpolarization caused by activation of $K_{ATP}$ channels.

### Bile acid receptors and metabolic disease

In addition to their role on G protein-coupled receptor GPBAR1, bile acids can also signal through the activation of specific nuclear receptors (farnesoid X receptor, pregnane X receptor, and vitamin D receptor) and intracellular kinase (c-jun N-terminal kinase 1/2, AKT, and ERK 1/2) in the liver and gastrointestinal tract (Hylemon *et al.* 2009). Their role includes the regulation of bile acid, lipid and glucose metabolism, energy homeostasis, and insulin sensitivity.

In addition to their involvement in gallstone disease, bile acids and their receptors have been associated with a number of metabolic disorders including obesity and diabetes (Houten *et al.* 2006; Thomas *et al.* 2008; Lefebvre *et al.* 2009; Vassileva *et al.* 2010). For example, GPBAR1 activation induces the release of intestinal glucagon-like peptide, which in obese mice can improve liver and pancreatic functions as well as glucose tolerance (Thomas *et al.* 2009). Interestingly, although type II diabetic patients are likely candidates for gallbladder disorders, genetic variation on the *Gpbar1* gene is not associated with the increased risk of the metabolic disorder (Mussig *et al.* 2009).

The development of specific bile acid receptor ligands offers new avenues to prevent and treat lipid and glucose-related metabolic diseases such as obesity and type II diabetes. However, in light of the findings reported here, demonstrating a disruptive effect of GPBAR1 activation on GBSM function, it is quite possible that the development of gallstone disease could be an adverse effect of these compounds.

Our results suggest that blocking GBSM $K_{ATP}$ channels or increasing GBSM excitability could prevent gallbladder stasis and gallstone formation to hydrophobic bile salts. Sulfonylurea drugs inhibit $K_{ATP}$ channels in smooth muscle and pancreatic $\beta$ cells, and therefore would not be a viable approach. However, $K_{ATP}$ channels in smooth muscle and pancreatic $\beta$ cells have different structures: SUR2B/Kir6.2 and SUR1/Kir6.1, respectively. Therefore, identification of smooth muscle-specific $K_{ATP}$ channel antagonists may represent a possible approach. Conversely, gain-of-function polymorphisms of the SUR2B/Kir6.1 may increase the risk of gallbladder dysfunction. Nonetheless, our results provide a mechanistic basis of how hydrophobic bile salts influence gallbladder function, and thereby suggest therapeutic opportunities for intervention.

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## Author contributions

Conception and design of the experiments: B.L., G.V., C.U.C., M.T.N. and G.M.M.; collection of data: B.L., O.B.B., C.G., C.A.W.; analysis and interpretation of data: B.L., O.B.B., C.G., C.A.W., C.U.C., G.M.M.; drafting of the manuscript: B.L., G.M.M.; critical revision of the manuscript for important intellectual content: B.L., O.B.B., G.V., C.U.C., M.T.N., G.M.M.; obtained funding: C.U.C., G.M.M. All authors approved the final version of the manuscript.

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