The G Protein-Coupled Bile Acid Receptor, TGR5, Stimulates Gallbladder Filling

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TGR5 is a G protein-coupled bile acid receptor present in brown adipose tissue and intestine, where its agonism increases energy expenditure and lowers blood glucose. Thus, it is an attractive drug target for treating human metabolic disease. However, TGR5 is also highly expressed in gallbladder, where its functions are less well characterized. Here, we demonstrate that TGR5 stimulates the filling of the gallbladder with bile. Gallbladder volume was increased in wild-type but not $Tgr5^{-/-}$ mice by administration of either the naturally occurring TGR5 agonist, lithocholic acid, or the synthetic TGR5 agonist, INT-777. These effects were independent of fibroblast growth factor 15, an enteric hormone previously shown to stimulate gallbladder filling. *Ex vivo* analyses using gallbladder tissue showed that TGR5 activation increased cAMP concentrations and caused smooth muscle relaxation in a TGR5-dependent manner. These data reveal a novel, gallbladder-intrinsic mechanism for regulating gallbladder contractility. They further suggest that TGR5 agonists should be assessed for effects on human gallbladder as they are developed for treating metabolic disease. (*Molecular Endocrinology* 25: 1066–1071, 2011)

Bile, which is composed primarily of bile acids, cholesterol, and phospholipids, is produced by the liver and stored in the gallbladder. In response to the ingestion of food, the hormone cholecystokinin (CCK) is secreted from enteroendocrine cells in the duodenum to promote gallbladder contraction and the release of bile into the small intestine, where it facilitates the absorption of lipophilic nutrients. The effect of CCK on gallbladder motility is opposed by fibroblast growth factor (FGF)15/19, a hormone that is released from the ileum 1–2 h after a meal to cause gallbladder relaxation (1, 2). FGF15 is the mouse ortholog of human FGF19. Mice lacking either FGF15 or its coreceptors, FGF receptor 4 and β-Klotho, have gallbladders containing less bile than those of wild-type mice (3, 4). Thus, the alternate release of CCK and FGF15/19 controls the cycle of gallbladder emptying and filling. Notably, impairments in gallbladder motility are associated with the development of gallstone disease (5).

TGR5 (also known as G protein-coupled bile acid receptor 1 and membrane-type bile acid receptor) is a G protein-coupled cell-surface receptor that is activated by both primary and secondary bile acids with a rank order efficacy of lithocholic acid (LCA) ~ deoxycholic acid > chenodeoxycholic acid (CDCA) > cholic acid (CA) (6, 7). TGR5 is expressed in macrophages, where its agonism suppresses phagocytosis and lipopolysaccharide-stimulated cytokine production (6). TGR5 is also expressed in brown adipose tissue and in enteroendocrine cells in the intestine, where its pharmacologic activation induces energy expenditure and improves insulin sensitivity in rodent models of obesity (8, 9). Interestingly, TGR5 is expressed in gallbladder at much higher levels than in other tissues (10). Within the gallbladder, TGR5 localizes to the apical membrane of the epithelium (10, 11). In studies performed with human gallbladder tissue, TGR5 activation by bile acids rapidly increased cAMP levels and decreased intracellular chloride concentrations, suggesting a role for TGR5 in promoting chloride and fluid secretion in gallbladder epithelial cells (11). Mice lacking TGR5

Abbreviations: CA, Cholic acid; CCK, cholecystokinin; CDCA, chenodeoxycholic acid; DMSO, dimethylsulfoxide; FGF, fibroblast growth factor; LCA, lithocholic acid; qPCR, quantitative PCR; tβMCA, tauro-β-muricholic acid.

($Tgr5^{-/-}$) are viable and develop normally but have a decreased bile acid pool size (10, 12). The $Tgr5^{-/-}$ mice are also refractory to the formation of cholesterol gallstones caused by a high-fat "lithogenic" diet containing CA (10). This may be due in part to an increase in hepatic expression of cholesterol 7α-hydroxylase (CYP7A1), which catalyzes the rate-limiting step in the conversion of cholesterol to bile acids. Based on these findings, it was proposed that TGR5 antagonism might prove useful for treating or preventing gallstone formation (10).

Although the pharmacologic effects of activating TGR5 in macrophage, brown adipose tissue, and intestine have been studied (8, 9), little is known about the physiologic actions of TGR5. Given the abundant expression of TGR5 in gallbladder, we have analyzed its function in this tissue. We demonstrate that TGR5 agonism in gallbladder causes smooth muscle relaxation and promotes gallbladder filling.

Results and Discussion

Because TGR5 is abundantly expressed in the gallbladder (10, 11), we analyzed bile acid parameters in wild-type and $Tgr5^{-/-}$ mice fed a regular chow diet. Consistent with a previous report (12), bile acid pool size was significantly smaller in both male and female $Tgr5^{-/-}$ mice (Fig. 1A).
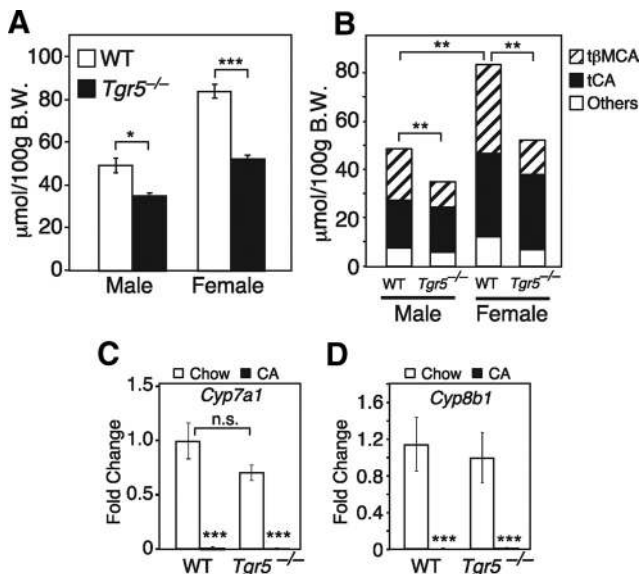
This decrease in bile acid pool size was accompanied by a decrease in the fraction of tauro-β-muricholic acid (tβMCA) in the pool (Fig. 1B). Although *Cyp7a1* mRNA levels trended lower in $Tgr5^{-/-}$ mice (Fig. 1C), which may have contributed to the decrease in bile acid pool size, this decreased expression did not reach statistical significance. *Cyp8b1* expression (Fig. 1D) and, consequently, tauro-CA levels (Fig. 1B) were unchanged in the $Tgr5^{-/-}$ mice. Furthermore, negative feedback regulation of *Cyp7a1* and *Cyp8b1* transcription was intact in $Tgr5^{-/-}$ mice, because CA administration repressed mRNA levels of these genes (Fig. 1, C and D). *Cyp27a1* expression levels were also unchanged in the $Tgr5^{-/-}$ mice (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Thus, the basis for the change in the tβMCA composition of the bile acid pool remains unresolved.

While measuring the bile acid pool size, we noted a trend toward reduced gallbladder volume in $Tgr5^{-/-}$ mice. This difference was markedly enhanced by administration of CA in the diet for 2 wk (Fig. 2A). Under these conditions, gallbladder volume increased approximately 3-fold in wild-type mice but did not change significantly in $Tgr5^{-/-}$ mice. To assess whether acute TGR5 activa-
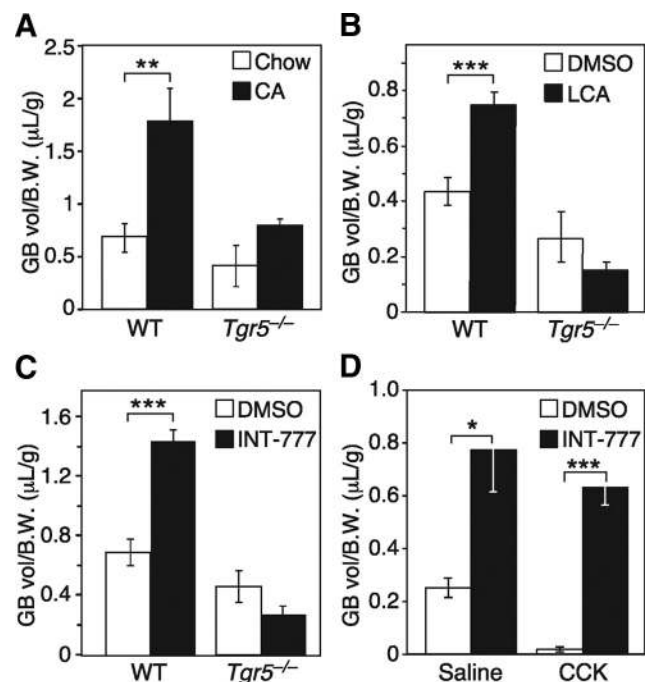

FIG. 1. Bile parameters in $Tgr5^{-/-}$ mice. A, Bile acid pool size was measured in male and female wild-type (WT) and $Tgr5^{-/-}$ mice (n = 6/group). B, Bile acid composition, including tβMCA and tauro-CA (tCA), was measured by liquid chromatography/mass spectrometry in WT and $Tgr5^{-/-}$ male and female mice (n = 6/group). Note statistical significance refers to changes in tβMCA levels. C and D, *Cyp7a1* and *Cyp8b1* mRNA levels were measured by qPCR in liver from WT and $Tgr5^{-/-}$ mice fed with chow or 0.2% CA diet for 2 wk (n = 4–5/ group). All data are the mean ± SEM. *, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.001$. B.W., Body weight; n.s., not significant.

FIG. 2. TGR5 agonism increases gallbladder (GB) volume. A–C, Gallbladder volume was measured in male wild-type (WT) and $Tgr5^{-/-}$ mice treated for: 2 wk with 0.2% CA in the diet (n = 4–5/group) (A), 30 min with LCA (60 mg/kg) or DMSO control by ip injection (n = 5/group) (B), and 30 min INT-777 (60 mg/kg) or DMSO control by ip injection (n = 5–6/group) (C). D, Male mice were treated with CCK (200 ng/kg) or saline control and INT-777 (60 mg/kg) or DMSO control by ip injection (n = 5/ group). Gallbladder volumes were normalized to body weight (B.W.). All data are the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
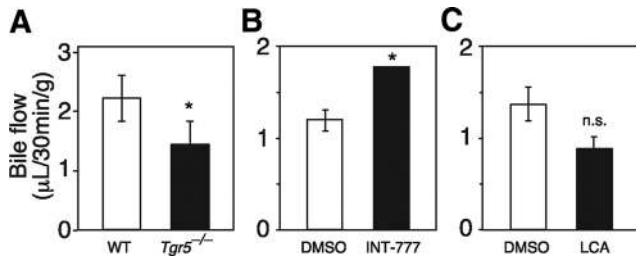
FIG. 3. TGR5 regulates bile flow. A, Bile flow rate was measured in male wild-type (WT) and *Tgr5*$^{-/-}$ mice (n = 6–8/group). B and C, Bile flow rates were measured in male mice 15 min after ip injection of LCA (60 mg/kg) or DMSO control (n = 4/group) (B) or INT-777 (60 mg/kg) or DMSO control (n = 5–6/group) (C). Bile flow rates were normalized to body weight. All data are the mean ± SEM. *, $P < 0.01$. n.s., Not significant.

tion increases gallbladder volume, mice were injected with either the naturally occurring TGR5 agonist, LCA, or the synthetic TGR5 agonist, INT-777 (13), and gallbladder volume was measured 30 min later. Both agonists significantly increased gallbladder volume in wild-type but not *Tgr5*$^{-/-}$ mice (Fig. 2, B and C). Importantly, the effects of INT-777 on gallbladder filling occurred without changing the concentration of CCK (Supplemental Fig. 2). In fact, INT-777 treatment completely reversed CCK-induced contraction of the gallbladder and stimulated gallbladder filling to levels similar to that observed in the absence of CCK (Fig. 2D). Thus, TGR5 activation stimulates gallbladder filling through a rapid and CCK-independent mechanism.

We next examined whether the TGR5-mediated increase in gallbladder volume was due to increased bile flow from the liver. *Tgr5*$^{-/-}$ mice had significantly reduced bile flow (Fig. 3A). Conversely, INT-777 treatment increased bile flow (Fig. 3B). These results indicate that TGR5 directly regulates bile flow, a finding consistent with its expression in cholangiocytes (14, 15). Interestingly, LCA, which is well known to cause cholestasis, decreased bile flow (Fig. 3C) under conditions in which it increased gallbladder volume in a TGR5-dependent manner (Fig. 2B). We conclude that TGR5 activation stimulates bile flow, but that this increase is not required for TGR5-mediated gallbladder filling.

To test whether TGR5 has direct effects on gallbladder smooth muscle, we performed *ex vivo* tensiometry experiments with gallbladders isolated from wild-type and *Tgr5*$^{-/-}$ mice. Both LCA and INT-777 markedly relaxed gallbladders from wild-type but not *Tgr5*$^{-/-}$ mice (Fig. 4, A and B). In contrast, forskolin, which induces cAMP levels through a TGR5-independent mechanism, relaxed both wild-type and *Tgr5*$^{-/-}$ gallbladders (Fig. 4, A and B). INT-777 treatment significantly increased cAMP concentrations in gallbladder tissue from wild-type but not *Tgr5*$^{-/-}$ mice (Fig. 4C). Similarly, LCA treatment also increased cAMP

concentrations in gallbladder tissue (Supplemental Fig. 3). We conclude that TGR5 acts directly on gallbladder to cause smooth muscle relaxation via induction of cAMP levels. Lastly, we tested for cross talk between the TGR5 and FGF15/19 pathways in gallbladder filling. INT-777 administration increased gallbladder volume in *Fgf15*$^{-/-}$ mice (Fig. 5A), suggesting that TGR5 may act downstream of FGF15 in a common signaling pathway.

In summary, we describe an important role for TGR5 in stimulating gallbladder smooth muscle relaxation and filling. It was previously shown that *Tgr5*$^{-/-}$ mice were resistant to lithogenic diet-induced cholesterol gallstone formation, which was attributed to an increase in *Cyp7a1* expression and a corresponding reduction in the cholesterol saturation index of the bile (10). Although it remains unclear whether TGR5's effect on gallbladder volume contributes to cholesterol gallstone formation, decreased gallbladder motility such as that seen in the *Tgr5*$^{-/-}$ mice is generally associated with an increase in gallstones (16). Notably, TGR5 is an attractive drug target for treating obesity and type 2 diabetes, because TGR5 agonism stimulates energy expenditure and lowers serum glucose (8, 9). Because TGR5 is also highly expressed in human gallbladder (11), it will be important to assess the impact of its activation on human gallbladder function because TGR5 agonists are developed as drugs.

We also provide evidence that TGR5 may act in concert with FGF15/19 in signaling the gallbladder to fill. Consistent with this hypothesis, both TGR5 agonists and FGF15/19 increase cAMP concentrations in gallbladder (Fig. 4C and Ref. 1). Based on our findings, we suggest a two-pronged "distal/proximal" signaling mechanism for controlling gallbladder filling. FGF15/19, which is induced by bile acids in the ileum, represents a distal hormonal signal that effectively "primes" the gallbladder for refilling after a meal. The presence of TGR5 in gallbladder provides a reinforcing proximal mechanism for detecting local bile acid concentrations and adjusting gallbladder volume accordingly. In this regard, it is intriguing that TGR5 induces chloride secretion from the gallbladder epithelium via the cystic fibrosis transmembrane conductance regulator (17), which may contribute to gallbladder filling. Future studies will be directed at further defining the relationship between TGR5 and FGF15/19 and understanding their respective roles in regulating gallbladder motility.

We note that while this manuscript was in preparation, Lavoie *et al.* (18) reported that TGR5 is expressed in the gallbladder smooth muscle, where its activation causes relaxation via a cAMP-protein kinase A-K$_{ATP}$ channel pathway. Their *ex vivo* findings are consistent with and complementary to our *in vivo* and *ex vivo* studies.
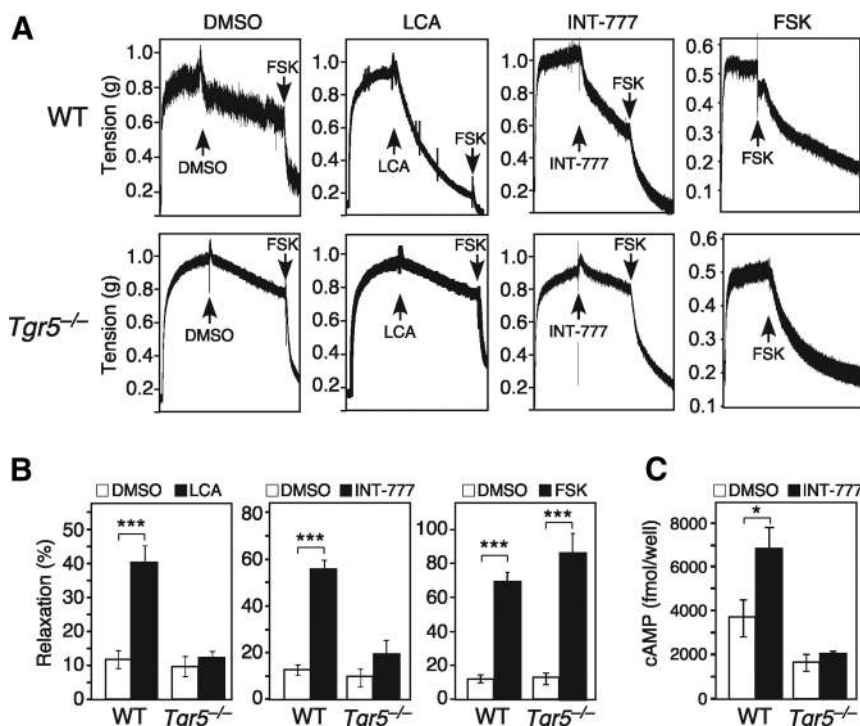
FIG. 4. TGR5 agonism relaxes gallbladder smooth muscle *ex vivo*. A, *Ex vivo* tensiometry was performed using gallbladder tissue from female wild-type (WT) (*top panels*) or $Tgr5^{-/-}$ mice (*lower panels*). Gallbladders were precontracted with 1 nM CCK for 20 min, washed, and subsequently treated with either vehicle (DMSO), LCA (10 μM), INT-777 (10 μM), or forskolin (FSK) (2 nM) for 20 min. DMSO, LCA, and INT-777 treatments were followed by a second wash and treatment with forskolin (2 nM) for 20 min to ensure integrity of the tissue. Representative tension tracings are shown with treatment times indicated by *arrows*. B, Quantification of gallbladder relaxation using tissue derived from WT or $Tgr5^{-/-}$ mice treated with vehicle (DMSO), LCA, INT-777, or forskolin as in A. Data are the mean $\pm$ SEM of experiments performed in quadruplicate and are expressed as percent relaxation of CCK-induced contraction. ***, $P < 0.001$. C, Intracellular cAMP concentrations were measured in gallbladder tissue from WT or $Tgr5^{-/-}$ mice treated *ex vivo* with vehicle (DMSO) (*open bars*) or INT-777 (25 μM) (*closed bars*) for 15 min. Experiments repeated in male mice produced similar results. Data are the mean $\pm$ SEM of assays performed six to seven times. *, $P < 0.01$.

Materials and Methods

Animal procedures

All experiments were performed with the approval of the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center, which assures that all animal use adheres to federal regulations as published in the
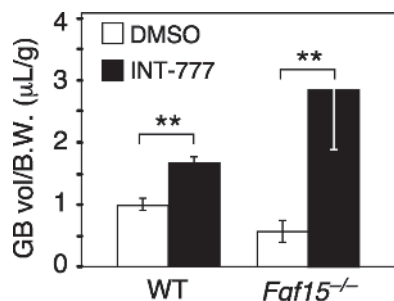

FIG. 5. Cross talk between TGR5 and FGF15 pathways. A, Gallbladder (GB) volume was measured 30 min after ip injection of INT-777 (60 mg/kg) or vehicle (DMSO) into male wild-type (WT) or and $Fgf15^{-/-}$ mice (n = 4–6/group). Gallbladder volumes were normalized to body weight (B.W.). All data are the mean $\pm$ SEM. *, $P < 0.01$.

Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, the Public Health Service Policy, and the U.S. Government Principles Regarding the Care and Use of Animals. $Tgr5^{-/-}$ and $Fgf15^{-/-}$ mice were described previously (10, 19). Experiments were performed in both male and female aged-matched mice as noted in figure legends. Mice were fed global irradiated rodent chow (TD.2916, Harlan Teklad, Madison, WI) supplemented with or without 0.2% CA *ad libitum*. For measurement of bile acid pool size and composition, mice were fasted 4 h before euthanasia. For *in vivo* gallbladder volume assays, mice were fasted starting at midnight and 14–18 h later treated with CCK, TGR5 agonists, or vehicle control by ip injection. Gallbladders were removed and volume measured 30 min after treatment.

Bile acid analysis

For measurement of bile acid pool size, bile acids were extracted and quantified as previously described (20). Briefly, the liver, gallbladder, and entire small intestine were collected, spiked with CDCA-D4, homogenized, and extracted with ethanol under reflux. Samples were filtered through no. 2 Whatman paper before an aliquot of the extract was dried under a nitrogen stream and redissolved in methanol for liquid chromatography/mass spectrometry analysis. Bile acids were qualified by ionization in negative ion mode. Selective ion monitoring was used to detect the conjugated and unconjugated bile acids. Quantification was performed based on peak areas using external calibration curves of standards prepared in methanol. CDCA-D4 was used to calculate the recovery of bile acids after extraction relative to a blank control.

Bile flow measurements

Mice were fasted for 12 h and then injected with vehicle, LCA, or INT-777 as described in *Results and Discussion*. After 15 min, mice were anesthetized by ip injection of avertin. The abdomen was opened, the cystic bile duct ligated, and a polyethylene-10 catheter inserted at the distal end of the common bile duct before the branching point to the gallbladder and liver. The surgery was performed at 37 C. Hepatic bile was collected for 30 min. The volume of the bile was measured and normalized to time and body weight of the mice.

RT-quantitative PCR (qPCR) analysis

Total RNA was extracted from liver and intestine using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). After deoxyribonuclease I (Roche, Indianapolis, IN) treatment, RNA was reverse transcribed into cDNA using the SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Primers for each gene were designed and validated as previously described (21). Primer

sequences include: *Cyp7a1* forward 5′agcaactaaacaacctgccag-tacta3′ and reverse 5′gtccggatattcaaggatgca3′, *Cyp8b1* forward 5′gccttcaagtatgatcggttcct3′ and reverse 5′gatcttcttgcccgacttg-taga3′, and *Cyp27a1* forward 5′gcctcacctatgggatcttca3′ and reverse 5′tcaaagcctgacgcagatg3′. RT-qPCR contained 25 ng of cDNA, 150 nM of each primer, and 5 μl of SYBR GreenER PCR Master Mix (Invitrogen) and were carried out in triplicate using an Applied Biosystems Prism 7900 HT instrument (Applied Biosystems, Carlsbad, CA). Relative mRNA levels were calculated by the comparative threshold cycle method using cyclophilin as the internal control.

Ex vivo tensiometry measurements

Gallbladders were removed from mice anesthetized by halothane. Cross-sections of the gallbladder were cut in the shape of a ring and the right and left lateral aspects of the gallbladder tethered with 0.2-mm pins. Gallbladder sections were mounted in organ baths containing 8 ml of physiological saline solution (120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 20.4 mM $NaHCO_3$, 1.6 mM $CaCl_2$, and 10 mM dextrose) at 37 C continuously bubbled with O_2:CO_2 (95:5%). Tension was measured with Grass FT03 force displacement transducers and a Powerlab 8SP digital recorder (ADInstruments, Colorado Springs, CO). Resting strip tension was adjusted to between 0.15 and 0.2 g. Experiments were started after a 1-h stabilization period. Tension was measured in response to CCK-8 (Phoenix Pharmaceuticals, Inc., Burlingame, CA), LCA, INT-777, forskolin, or dimethylsulfoxide (DMSO) control.

Gallbladder cAMP concentration measurements

Gallbladders from wild-type or $Tgr5^{-/-}$ mice were removed, drained, and cut into three to four pieces in ice-cold PBS. Gallbladder sections were subsequently placed in six-well plates and incubated at 37 C in William's E medium as described (17) in the presence of INT-777 or vehicle control. 3-Isobutyl-1-methylxanthine was included in all media. cAMP was extracted using 0.1 N HCl, and intracellular cAMP levels were determined using a cAMP EIA kit (GE Healthcare, Piscataway, NJ).

Statistical analysis

Results are expressed as mean ($\pm$SEM). For comparison between two groups, the unpaired two-tailed Student's *t* test was performed. One-way ANOVA followed by the Newman-Keuls procedure was used to compare more than two groups. All tests were performed using the software program GraphPad Prism 5 (GraphPad, La Jolla, CA). *P* values less than 0.05 were considered statistically significant.

Acknowledgments

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