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RESEARCH ARTICLE | *Hormones, Neurotransmitters, Growth Factors, Receptors, and Signaling*

Bile acids drive colonic secretion of glucagon-like-peptide 1 and peptide-YY in rodents

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Christiansen CB, Trammell SA, Wewer Albrechtsen NJ, Schoonjans K, Albrechtsen R, Gillum MP, Kuhre RE, Holst JJ. Bile acids drive colonic secretion of glucagon-like-peptide 1 and peptide-YY in rodents. *Am J Physiol Gastrointest Liver Physiol* 316: G574–G584, 2019. First published February 15, 2019; doi:10.1152/ajpgi.00010.2019.—A large number of glucagon-like-peptide-1 (GLP-1)- and peptide-YY (PYY)-producing L cells are located in the colon, but little is known about their contribution to whole body metabolism. Since bile acids (BAs) increase GLP-1 and PYY release, and since BAs spill over from the ileum to the colon, we decided to investigate the ability of BAs to stimulate colonic GLP-1 and PYY secretion. Using isolated perfused rat/mouse colon as well as stimulation of the rat colon in vivo, we demonstrate that BAs significantly enhance secretion of GLP-1 and PYY from the colon with average increases of 3.5- and 2.9-fold, respectively. Furthermore, we find that responses depend on BA absorption followed by basolateral activation of the BA-receptor Takeda-G protein-coupled-receptor 5. Surprisingly, the apical sodium-dependent BA transporter, which serves to absorb conjugated BAs, was not required for colonic conjugated BA absorption or conjugated BA-induced peptide secretion. In conclusion, we demonstrate that BAs represent a major physiological stimulus for colonic L-cell secretion.

NEW & NOTEWORTHY By the use of isolated perfused rodent colon preparations we show that bile acids are potent and direct promoters of colonic glucagon-like-peptide 1 and peptide-YY secretion. The study provides convincing evidence that basolateral Takeda-G protein-coupled-receptor 5 activation is mediating the effects of bile acids in the colon and thus add to the existing literature described for L cells in the ileum.

bile acids; colon; glucagon-like-peptide 1; peptide-YY; TGR5

INTRODUCTION

The endocrine glucagon-like peptide-1 (GLP-1)- and peptide-YY (PYY)-positive L cells are found along the length of the gut, with increasing density toward the colon (16, 18, 20, 55). The colonic L-cells release biologically active GLP-1 (14), but only little is known about the impact of colonic-derived peptide hormones on whole body metabolism. The best characterized stimuli for L-cell secretion in vivo are nutrients (including carbohydrates, proteins, and fats) (11, 17, 33, 43, 45, 53), but as they are absorbed in the most proximal part of the small intestine, only limited amounts reach the colonic L cells. Like the small intestine, the colon expresses the sodium glucose-cotransporter 1 (SGLT-1), the primary transporter responsible for intestinal glucose absorption, and can absorb glucose resulting in GLP-1 secretion. However, the colonic absorption rate and secretory responses are dramatically reduced compared with what is found for the ileum or the jejunum (32, 58), and glucose does, therefore, not appear to be a major stimuli for GLP-1 and PYY secretion from the colon. Other possible colonic stimuli include bile acids (BAs), since they are among the few substances that, in spite of small intestinal uptake, are found in the colon (4). Recent in vitro as well as in vivo studies have already pointed to BAs as potent stimulators of GLP-1 and PYY secretion (1, 2, 8, 19, 23, 34, 42, 46, 50, 51), but exactly where in the intestine BAs particularly act to stimulate L cell secretion is unknown. Once absorbed, BAs are returned to the liver trough the enterohepatic circulation (13, 38), where they interact with the nuclear farnesoid X receptor (FXR), which regulates hepatic BA synthesis and also appears to be involved in lipid and glucose metabolism (29, 35, 66). Additionally, BAs stimulate the Takeda-G protein-coupled-receptor 5 (TGR5; or G protein-bile-acid-receptor-1), which activates $G\alpha_s$-signaling increasing intracellular cAMP levels (27, 28, 36, 60). TGR5 is the central candidate receptor proposed to be involved in BA-induced GLP-1 secretion from the small intestine (8, 34, 46, 63), and although TGR5 mRNA is found in the mouse colon (5, 26, 52),

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it is not known whether TGR5 is expressed on colonic L cells and involved in secretion here.

In the present study, we investigated whether BAs stimulate colonic hormone secretion, and if so, by what mechanisms. To do so, we isolated and perfused colons from rats and TGR5 receptor knockout mice to assess the importance of colonic BA delivery and finally tested if the findings ex vivo could be recapitulated in vivo.

METHODS

Ethical approval. Handling of the donor animals was performed in accordance with international accepted guidelines (National Institutes of Health Publication No. 8023) and with permission from the Danish Animal Experiments Inspectorate (License No. 2013-15-2934-00833).

Chemicals. The following BAs were obtained from Sigma-Aldrich: sodium cholate (CA; cat. no. C1254), sodium glycol-cholate (GCA; cat. no. G7132), sodium tauro-cholate (TCA; cat. no. 86339), sodium deoxycholate (DCA; cat. no. D6750), sodium glyco-deoxycholate (GDCA; cat. no. G9910), sodium tauro-deoxycholate (TDCA; cat. no. T0875), sodium chenodeoxycholate (CDCA; cat. no. C8261), sodium glycol-chenodeoxycholate (GCDCA; cat. no. G0759), sodium tauro-chenodeoxycholate (TCDCA; cat.no T6260), sodium glycol-urso-deoxycholate (GUDCA; cat. no. 06863). Sodium urso-deoxycholate was purchased from Santa Cruz Biotechnology (UDCA; cat. no. 222407) and sodium tauro-urso-deoxycholate from Millipore (TUDCA; cat. no. 580549). The remaining reagents were purchased from the flowing: Cholesevelam (Cholestagel; Genzyme Biosurgery), TGR5 agonist (codename no. tauro-RO5527239, kindly provided by Dr. Christopher Ullmer, Roche Holding), sodium-dependent BA transporter inhibitor (IBAT inhibitor; GlaxoSmithKline Pharma), bombesin (positive control; cat. no. H-2155; Bachem), DMSO (cat. no. 67–68–5; Sigma-Aldrich), dextran T-70 (Pharmacosmos; cat. no. 40014), bovine serum albumin (cat. no. 1.12018.0500; Merck), and 3-isobutyl-1-methylxanthine (cat. no. I5879; Sigma-Aldrich).

Animals. Male Wistar rats (Janvier, Saint Berthevin Cedex, France) weighing between 250 and 300 g and 16-wk-old TGR5 knockout ($^{-/-}$) mice (2 males and 2 females) and wild-type littermates (3 males and 2 females) were used as donors. TGR5$^{+/+}$ and TGR5$^{-/-}$ were generated from heterozygous ($^{+/-}$) mice (60), which were transferred from Switzerland and bred in-house at the University of Copenhagen. Animals followed a 12:12-h light-dark cycle with free access to standard chow and drinking water. Rats were used for experiments after at least 1 wk of acclimatization and were anaesthetized with a subcutaneous injection of hypnorm/midazolam (0.0158 mg fentanyl citrate + 0.5 mg fluanisone + 0.25 mg midazolam/100 g) before surgery. Mice were anesthetized with an intraperitoneal injection of ketamine-xylazine (90 mg/kg ketamine; Ketaminol Vet.; MSD Animal Health, Madison, NJ) + 10 mg/kg xylazine (Rompun Vet; Bayer Animal Health, Leverkusen, Germany) before surgery.

Isolation of rat/mouse colon. After induction of surgical anesthesia, a midline incision was made exposing the abdominal cavity. The colonic vascular supply was isolated from the remaining circulation as described in Ref. 12. In brief, the arterial supply to the small intestine, cecum, spleen, stomach, and kidneys was ligated to prevent perfusion of these organs. The celiac artery partly perfusing the pancreas was ligated too. The colon was transected immediately after the cecum and again just proximal to the entry of the inferior mesenteric artery leaving a segment of on average 10.9 ± 1.0 cm (rats) and 5.6 ± 0.4 cm (mice), which was used for perfusion. A tube was inserted into the most proximal colon, thus establishing a route for luminal stimulations, and allowing removal of colonic content by gently flushing with a prewarmed saline (0.9% NaCl solution) infusion. A catheter was inserted into the abdominal aorta and perfusion was started immediately. A draining catheter was then inserted into vena portae allowing collection of venous effluent. Finally, the animal was euthanized by cutting the diaphragm, while the colon was kept artificially alive.

Perfusion protocol. After the operation, the colon was perfused in situ using equipment dedicated for rodent organ perfusion (Hugo Sachs Elektronik, March-Hugstetten, Germany) (12). Experiments were initiated after a 30-min equilibrium period to ensure that hormone secretion had stabilized and that any impact from anesthesia had vanished. In the rat, flow rate of vascular perfusion buffer was kept constant at 3 ml/min while luminal saline was infused at 0.15 ml/min throughout the experiment, except when test substances were administered luminally. In the mouse, the vascular perfusion flow was kept constant at 1.5 ml/min, while saline was infused luminally at 0.025 ml/min. Luminally applied test substances were infused for 15 min at an initial flow rate of 0.25 ml/min in the rat and at 0.1 ml/min in the mouse to remove luminal saline. After 3 min, flow rate was reduced to 0.15 ml/min (rat) and 0.025 ml/min (mouse) for the remaining stimulation period. After luminal stimulations were completed, saline was infused luminally at 0.25 ml/min (rat) and 0.1 ml/min (mouse) for 3 min to remove test substances. Vascularly applied test substances were infused at a flow rate of 0.15 and 0.075 ml/min in rat and mouse, respectively, via a three-way stop cock using a syringe infusion pump. The perfusion buffer consisted of a modified Krebs-Ringer bicarbonate buffer, containing in addition 5% dextran T-70, 0.1% bovine serum albumin, 10 μM 3-isobutyl-1-methylxanthine, 3.5 mM glucose, and 5 mM pyruvate, fumarate, and glutamate. Perfusion buffer was heated to 37°C and continuously gassed throughout the experiment with 95% O_2-5% CO_2 to achieve pH 7.4 and a high oxygen partial pressure. Effluent samples were collected by a fraction collector at 1-min intervals, stored on ice within few minutes and subsequently kept at −20°C until analysis. Perfusion pressure, vascular output, and respiration (calculated from the partial pressures of CO_2 of arterial and venous perfusion medium samples) were monitored throughout the experiments to assess colonic condition.

In vivo protocol. Male Wistar rats (~300 g; Janvier) were fasted overnight (from 2200) and used for experiments the following day between 0800 and 1400 (total fast of 10–16 h). The rats were anesthetized as described above, and, once unconscious, a midline incision was made exposing their abdominal cavity. A plastic tube was inserted into the most proximal part of the colon and secured by a tight ligation, thereby establishing a route for colonic luminal stimulation but also serving to prevent leakage of test substances. Retrograde flow through the tubing was prevented by keeping the syringe used for administration attached. A needle was inserted into the abdominal vena cava, and blood samples (300 μl) were drawn at −10, 0, 2, 5, 10, 15, and 25 min and transferred into prechilled EDTA-coated Eppendorf tubes. Test substances (2 ml) were administered immediately before *sample 0* was drawn (10 s). After collection, samples were instantly put on ice and centrifuged at 2,800 rpm for 10 min (4°C) within 30 min. Subsequently, plasma was transferred into fresh Eppendorf tubes and kept on ice until stored at −20°C (within 1 h). Rats were randomly divided into three test groups: one group received 2 ml of a mixture of nine different BAs consisting of both conjugated and unconjugated BAs (CA, GCA, TCA, DCA, GDCA, TDCA, CDCA, GCDCA, and TCDCA: 1 mM each; total concentration of 9 mM), another group received 2 ml of a mixture of ursodeoxy cholic acid forms (UDCA, TUDCA, and GUDCA: 3 mM each; total concentration of 9 mM), and the third group received 2 ml of a 50% (wt/vol) glucose solution (3 g/kg body wt). Rats from the same cage received different treatments, so fasting times were equal between groups. Based on plasma glucose and peptide secretion, the difference in fasting hours did not affect the study outcome.

Sample analysis. Effluent perfusion samples were analyzed by in-house radioimmunoassays: total GLP-1 was measured using antiserum no. 89390, directed against the COOH terminus of the GLP-1 molecule and validated in (44), while total PYY immunoreactivity (i.e., the sum of PYY 1–36 + PYY 3–36) was measured with a porcine antiserum (cat. no T-4093; Bachem) validated in Ref. 62.

Total BA concentrations (in perfusion effluents and rat plasma) were determined using an enzymatic assay (cat. no. STA-631; Cell Biolabs). For the in vivo study, plasma glucose was measured immediately after blood samples were drawn by a handheld glucometer (Accu-check). Plasma total GLP-1 (cat. no. 10-1278-01; Mercodia;) and plasma insulin (cat no. 10-1250-01; Mercodia) were measured by sandwich ELISAs. For each commercial assay the protocol provided by the manufacturer was followed.

Individual BA quantitation. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (now Merck KGaA, Darmstadt, Germany) at highest purity. Conjugated and nonconjugated murichолic acid (MCA) alpha, beta, and gamma were obtained from Steraloids (Newport, RI).

Perfusion buffer and effluent perfusion samples at time points 26–32 min (BA infusion fraction) and 60–64 min (BA + IBAT infusion fraction) were thawed at ambient temperature with constant shaking at 300 rpm using an Eppendorf Thermomixer. After thawing, all samples were placed in wet ice. Time points within each fraction were combined in equal volumes (5 μl each) and diluted 1/152nd using perfusion buffer. The dilution was determined from total BA concentrations (method described above) to ensure BA concentrations were within the linear range of the assay. A standard curve was composed by combining 10 μl of standard (0.15, 0.3, 0.5, 1.5, 3, 5, 10, 15, and 25 μM of CA, CDCA, DCA, GCA, GCDCA, GDCA, GUDCA, LCA, MCA alpha, MCA beta, MCA gamma, TCA, TCDCA, TDCA, tauro-MCA alpha (TMCA alpha), TMCA beta, TMCA gamma, TLCA, TUDCA, and UDCA) with 10 μl of internal standard solution (0.3 μM of TLCA-d_4, TDCA-d_4, DCA-d_6, CA-d_5, TCA-d_4, TUDCA-d_4, TDCA-d_4, LCA-d_5, and CDCA-d_4) and 120 μl of perfusion buffer. Standard quality controls (QCs) were composed in triplicate in the same manner as standards at concentrations of 0.25, 2.5, and 12.5 μM. An internal standard blank containing liquid chromatography-mass spectrometry (LC-MS) grade water instead of standard was also composed in the same manner. Samples were adulterated with the same amount of internal standard as above. Five-hundred twenty-five microliters of ice-chilled LC-MS grade methanol was added to all standards and samples. All were vortexed briefly (~3 s, top speed of 1,850 rpm) immediately after addition. All were placed in an Eppendorf thermomixer and shaken at 1,400 rpm and 22°C for 10 min. Samples were centrifuged for 10 min at 22°C and 20,817 *g* to clarify supernatants. Supernatants were dried via speed vacuum for 2 h.

Dried samples were suspended in 50 μl of LC-MS grade 5 mM ammonium acetate/acetonitrile [95/5% (vol/vol)] containing 0.1% LC-MS grade formic acid (Thermo Fisher Scientific, Roskilde, DK). Samples were centrifuged as above and transferred to autosampler vials (cat. no. AR0–3920–12, Phenomenex, Værløse, Denmark). Ten microliters of each sample were combined in one vial to form a QC-pooled sample. Samples were randomized and placed in a Dionex Ultimate 3,000 autosampler without temperature control. The QC-pooled sample as well as the QC standards were injected (15 μl) at equal intervals throughout the queue. BAs were detected in negative ion mode using a Bruker Impact II quadrupole coupled to time of flight (Q-TOF) mass spectrometer (BRUKER DALTONIK Life Sciences, Bremen, Germany) after reverse phase separation over a Phenomenex PS C18 column (2.1 × 100 mm, 1.7 μm; Phenomenex). BAs were quantified using the internal standard normalized standard curve for all except CDCA, which was quantified based on the ratio of the area of CDCA in sample compared with the area of CDCA D4. The limit of quantification was estimated based on the lowest concentration included in the standard curve (0.15 μM).

Immunohistochemistry. Colonic tissue samples were collected from male Wistar rats (~300 g; Janvier) and fixed in paraformaldehyde. Tissue samples were used for double immunofluorescence staining of GLP-1 and the TGR5 receptor as described in detail elsewhere (34).

Statistical analysis. Hormone secretion from isolated perfused colon is presented as total output (fmol/min; effluent concentration × perfusion flow; means ± SE). To compare treatments within experiments, baseline subtracted outputs are presented. Statistical analysis of responses was performed by comparing mean basal output (10-min consecutive observations) with mean output during stimulation using a paired *t*-test. For vascular stimulations 10-min observations starting 5 min into the infusion period were used, while 10-min observations starting 15 min into the infusion period were used for luminal stimulations. Baseline subtracted total outputs are compared by a paired *t*-test except for the comparison of outputs from $TGR5^{-/-}$ versus $TGR5^{+/+}$ where an unpaired *t*-test was used. Absorption data are also presented as means ± SE and as baseline subtracted mean values, but the statistical analysis was done by a one-way ANOVA for repeated measurements followed by Tukey's multiple comparison test. In vivo data are presented as means ± SE and as incremental (baseline subtracted) areas under the curve (AUCs) (minutes × concentration) calculated over the entire course of the experiments. Statistical significance was tested by comparing the total AUCs (iAUCs) by one-way ANOVA for repeated measurements followed by Tukey's multiple comparison test, except for the analysis of BA absorption, which was analyzed by an unpaired *t*-test since the glucose group was not included, leaving only two groups to be tested. All statistics were performed using GraphPad Prism 7. $P < 0.05$ were considered significant.

RESULTS

Colonic BA absorption elicits GLP-1 and PYY secretion. We first stimulated the isolated perfused rat colon with a mixture of BAs (CA, GCA, TCA, DCA, GDCA, TDCA, CDCA, GCDCA, and TCDCA) representing a combination of conjugated and unconjugated BAs with varying agonist potency to TGR5 to mimic in vivo conditions. Intra-arterial (total concentration: 0.1 mM) and luminal (total concentration: 9 mM) BA administration resulted in markedly increased GLP-1 and PYY secretion (GLP-1: 2.2-fold increase, $P = 0.001$; PYY: 2.2-fold increase, $P = 0.0499$; $n = 6$; Fig. 1*A*) (GLP-1: 4.2-fold increase, $P = 0.003$; PYY: 4.3-fold increase, $P = 0.01$; $n = 6$; Fig. 1*B*). In another line of experiments, intraluminal administration of same BA mixture failed to stimulate secretory responses when administered together with the BA sequestrant cholesevelam (GLP-1: $P = 0.51$; PYY: $P = 0.25$), while subsequent luminal BA administration without cholesevelam stimulated secretion as seen in the first experiment (GLP-1: 2.5-fold increase, $P = 0.004$; BA + cholesevelam vs. BA − cholesevelam, $P = 0.004$; PYY: 2.0-fold increase, $P = 0.006$; BA + cholesevelam vs. BA − cholesevelam, $P = 0.03$; Fig. 1*C*). In all experiments, intra-arterial administration of 10 nM bombesin was included as a positive control at the end of protocol and resulted in robust GLP-1 and PYY responses in all experiments (Fig. 1, *A–C*). Total concentration of BAs was measured in perfusion effluents, directly reflecting the BA absorption rate. As expected, no BA absorption occurred in presence of the BA sequestrant cholesevelam, Fig. 1*D* (baseline = 16.50 ± 1.02 nmol/min vs. infusion = 19.41 ± 2.70 nmol/min, $P = 0.55$; $n = 6$), whereas BAs were efficiently absorbed when subsequently administered alone (baseline = 17.88 ± 2.16 nmol/min vs. infusion = 84.15 ± 5.79 nmol/min, $P = 0.005$; $n = 6$). Together, our data support that BAs stimulate GLP-1 and PYY secretion from the rat colon.

Basolateral TGR5 activation is essential for BA-mediated colonic GLP-1 and PYY secretion. Next, we stimulated the perfused rat colon with a poorly absorbable TGR5-specific agonist (tauro-RO5527239). Luminally administered tauro-

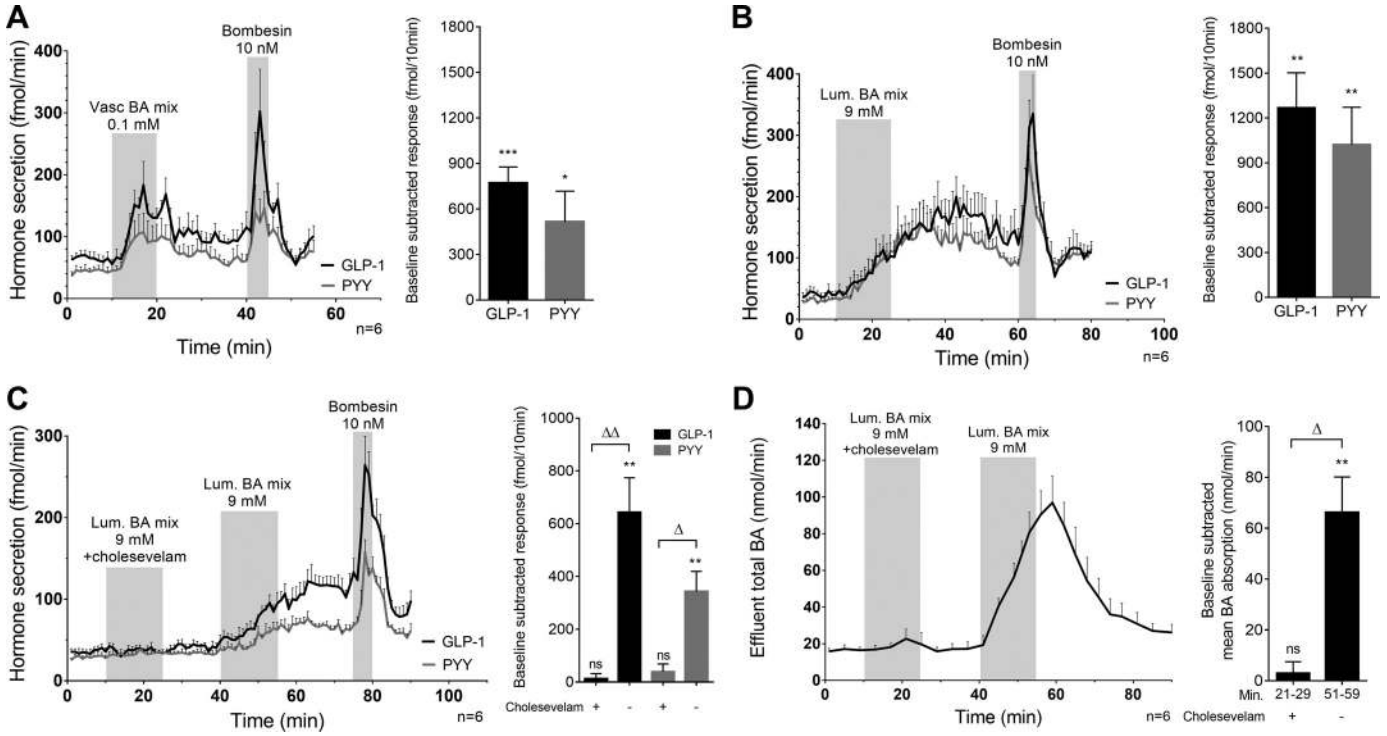

Fig. 1. Glucagon-like-peptide-1 (GLP-1) and peptide-YY (PYY) secretion (fmol/min) from isolated perfused rat colon. Values are means ± SE and baseline subtracted total outputs ± SE; $n = 6$ in each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant increase from baseline. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, significant difference between baseline subtracted outputs. *A*: intra-arterial stimulation with a 0.1-mM bile acid (BA) mixture solution (sodium cholate, sodium glycol-cholate, sodium tauro-cholate, sodium deoxycholate, sodium glyco-deoxycholate, sodium tauro-deoxycholate, sodium chenodeoxycholate, sodium glycol-chenodeoxycholate, and sodium tauro-chenodeoxycholate). *B*: luminal bile acid mixture stimulation, 1 mM each = total concentration 9 mM. *C*: BAs infused luminally with and without the bile acid sequestrant cholesevelam. *A–C*: 10 nM bombesin (intra-arterially) were included at the end of each experiment as a positive control. *D*: total bile acid absorption measured as the presence of BAs in perfusion effluents (nmol/min) with and without cholesevelam, means ± SE.

RO5527239 had no impact on GLP-1 or PYY secretion (GLP-1: 1.0-fold increase, $P = 0.73$; PYY: 1.1-fold increase, $P = 0.054$; $n = 6$), whereas vascular stimulation resulted in instant and robust GLP-1 and PYY responses (GLP-1: 3.1-fold increase, $P = 0.003$; PYY: 2.7-fold increase, $P = 0.005$; $n = 6$; Fig. 2*A*) Furthermore, two conjugated BAs, TUDCA and TDCA, were infused intra-arterially to the perfused colon. TUDCA is a poor TGR5 receptor agonist and neither affected GLP-1 nor PYY secretion (GLP-1: $P = 0.80$; PYY: $P = 0.22$; $n = 6$), while TDCA (a potent TGR5 receptor agonist) increased secretion of both hormones robustly, Fig. 2*B* (GLP-1: 3.3-fold increase, $P = 0.008$; PYY: 3.6-fold increase, $P = 0.003$; $n = 6$). To test dependence of TGR5 signaling for colonic GLP-1 and PYY secretion, isolated colon preparations from TGR5 knockout mice as well as their wild-type littermates were perfused and stimulated with the BA mixture. Intra-arterial (total concentration: 0.1 mM) as well as luminal (total concentration: 9 mM) BA stimulation had no impact on GLP-1 secretion in TGR5 knockout mice (vascular: 0.8-fold increase, $P = 0.19$; luminal: 1.7-fold increase, $P = 0.08$; $n = 4$; Fig. 2*Ca*). A small increase in PYY secretion was, however, seen during luminal BA stimulation in TGR5 knockouts but not during vascular BA stimulation (vascular: 1.0-fold increase, $P = 0.778$; luminal: 2.0-fold increase, $P = 0.003$; $n = 4$; Fig. 2*Cb*). In contrast, the same BA mixture dramatically increased secretion of both hormones in wild-type control mice both from the vascular and luminal route (GLP-1: vascular stimulation, 8.0-fold increase, $P = 0.03$, luminal stimulation: 3.0-fold increase, $P = 0.003$; PYY: vascular stimulation: 4.2-fold increase, $P = 0.04$, luminal stimulation: 3.0-fold increase, $P = 0.0001$; $n = 5$). These responses were significantly different from those observed in TGR5 knockouts (GLP-1: vascular responses control vs. knockout, $P = 0.018$, luminal responses control vs. knockout, $P = 0.002$; PYY: vascular responses control vs. knockout, $P = 0.022$, luminal responses control vs. knockout, $P < 0.0001$; $n = 4–5$; Fig. 2, *Ca* and *Cb*). Moreover, immunohistochemical data show that TGR5 expression in the rat colon colocalizes with the colonic GLP-1-producing L cells (Fig. 2*D*; $n = 4$).

Conjugated BAs induce GLP-1 and PYY secretion independent of IBAT. In the terminal ileum conjugated BAs are absorbed by IBAT (or ABST). Low expression of IBAT is also found in the colon, so to study the fate of conjugated BAs in the colon, we infused three conjugated BAs, TDCA, GDCA, and GCDCA (1 mM each), into the colonic lumen with and without an IBAT inhibitor. When administered alone, the conjugated BAs significantly increased GLP-1 and PYY secretion (GLP-1: 2.4-fold increase, $P = 0.0001$; PYY: 2.4-fold increase $P = 0.006$, $n = 6$), and this was also the case when infused together with the transport inhibitor (GLP-1: 2.0-fold increase, $P = 0.003$; PYY; 1.5-fold increase, $P = 0.002$; $n = 6$; Fig. 3*A*). BA absorption, measured as the presence of total BAs in perfusion effluents, indicated that inhibition of IBAT transport reduced uptake of conjugated BAs by only 16.0% compared with BA absorption without IBAT inhibition (Fig. 3*B*). To investigate the role of possible bacterial deconjugation

in the colon, the individual BAs as well as their deconjugated forms were measured in effluents by LC-MS. The deconjugated version of the BAs (DCA and CDCA) were present in only low amounts in perfusion effluents (DCA_{26-30} = 5.4 ± 1.5 nmol/min; DCA_{60-64} = 3.9 ± 1.2 nmol/min; $CDCA_{26-30}$ = 6.9 ± 4.8 nmol/min; $CDCA_{60-64}$ = 2.7 ± 1.2 nmol/min; n = 6; Fig. 3, *Ca* and *Cb*). In contrast, GDCA and GCDCA were present in amounts of 26.1 ± 4.2 nmol/min and 33.6 ± 5.4 nmol/min, respectively, when infused alone, and although levels were lower when infused together with the IBAT inhibitor (GDCA = 14.1 ± 2.7 nmol/min; GCDCA = 17.7 ± 3.6 nmol/min), the responses were not significantly different (Fig. 3, *Cc* and *Cd*). Finally, IBAT inhibition did not significantly impact TDCA absorption ($TDCA_{26-30}$ = 10.9 ± 4.4 nmol/min; $TDCA_{60-64}$ = 14.1 ± 7.8 nmol/min, n = 6; Fig. 3*Ce*).

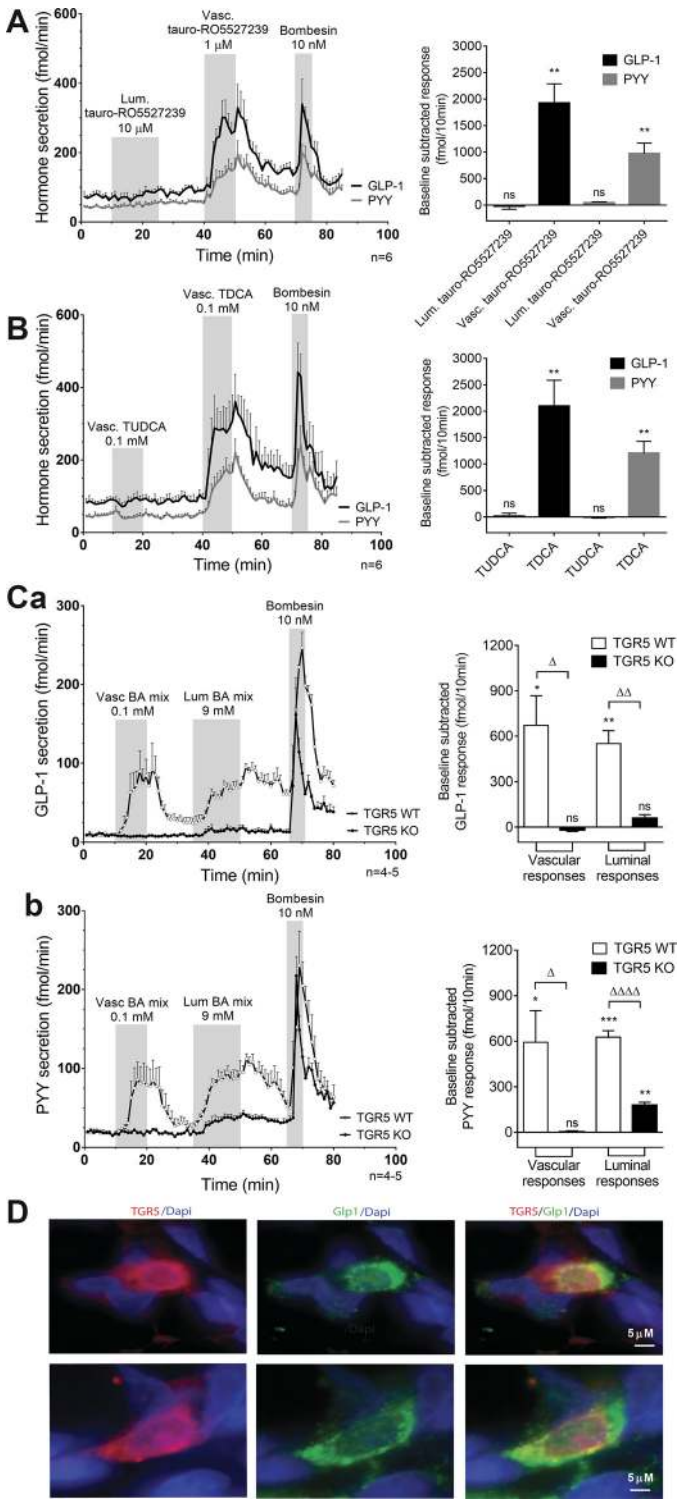

Fig. 2. Glucagon-like-peptide-1 (GLP-1) and peptide-YY (PYY) secretion (fmol/min) from isolated perfused rat colon and Takeda-G protein-coupled-receptor 5 [TGR5($^{-/-}$)/($^{+/+}$)] mouse colon. Values are means ± SE and baseline subtracted total outputs ± SE. *P < 0.05, **P < 0.01, ***P < 0.001, significant increase from baseline. ΔP < 0.05, ΔΔP < 0.01, ΔΔΔΔP < 0.0001, significant difference between baseline subtracted outputs. *A*: colonic (rat) luminal (10 μM) and vascular (1 μM) stimulation with a poorly absorbable TGR5 specific agonist (tauro-RO5527239) (n = 6). *B*: infusion of 0.1 mM sodium tauro-urso-deoxycholate (TUDCA) and sodium tauro-deoxycholate (TDCA) into the vascular supply of the rat colon (n = 6). *Ca*: GLP-1 secretion from TGR5 knockout mice ($^{-/-}$; KO) and wild-type littermates ($^{+/+}$; WT) upon vascular (0.1 mM) and luminal (9 mM) bile acid (BA) stimulation. *Cb*: PYY secretion from TGR5 knockout mice ($^{-/-}$) and wild-type littermates ($^{+/+}$) upon vascular (0.1 mM) and luminal (9 mM) BA stimulation [n(KO) = 4, n(WT) = 5]. *A–C*: 10 nM bombesin (intra-arterially) were included at the end of each experiment as a positive control. *D*: immunohistochemistry showing colocalization of GLP-1 and TGR5 in rat colon [TGR5: red; GLP-1: green; bar = 5 μM (×900); n = 4].

BAs stimulate GLP-1 secretion from the colon in vivo. To support perfusion findings using another physiologically relevant model, the complex BA mixture was administered to the colonic lumen in vivo in anesthetized rats. Plasma glucose concentrations did not differ significantly between treatment groups ($iAUC_{BA\ mix}$ vs. $iAUC_{urso\ mix}$, P = 0.528; $iAUC_{BA\ mix}$ vs. $iAUC_{glucose}$, P = 0.471; $iAUC_{urso\ mix}$ vs. $iAUC_{glucose}$, P = 0.087; n = 7–8 in each group; Fig. 4, *Aa* and *Ab*). The total BA concentration in plasma did not increase after intraluminal BA administration, and no significant difference in plasma concentrations was observed between groups ($iAUC_{BA\ mix}$ vs. $iAUC_{urso\ mix}$, P = 0.393; n = 7–8 in each group; Fig. 4, *Ba* and *Bb*). Plasma total GLP-1 concentration was significantly higher upon BA stimulation compared with the two other treatment groups ($iAUC_{BA\ mix}$ vs. $iAUC_{urso\ mix}$, P = 0.021; $iAUC_{BA\ mix}$ vs. $iAUC_{glucose}$, P = 0.007; $iAUC_{urso\ mix}$ vs. $iAUC_{glucose}$, P = 0.933; n = 7–8 in each group; Fig. 4, *Ca* and *Cb*). Plasma insulin secretion was not significantly different between groups but tended to increase upon stimulation with the UDCA and the complex BA mixture ($iAUC_{BA\ mix}$ vs. $iAUC_{urso\ mix}$, P = 0.377; $iAUC_{BA\ mix}$ vs. $iAUC_{glucose}$, P = 0.784; $iAUC_{urso\ mix}$ vs. $iAUC_{glucose}$, P = 0.129; n = 7–8 in each group; Fig. 4, *Da* and *Db*).

DISCUSSION

This study investigated whether the colon might act as an endocrine organ and, in significant amounts, release the products of one of its most abundant endocrine cells, the L cell, into the circulation. Since BAs act as powerful stimuli for L-cell secretion in the distal half of the small intestine (34), and because there is a significant spillover of BAs from the ileum to the colon, we hypothesized that BAs may be a potent stimulator of colonic secretion. To test our hypothesis, we investigated the effects of a complex mixture of BAs on colonic GLP-1 and PYY secretion. Secretion of both hormones

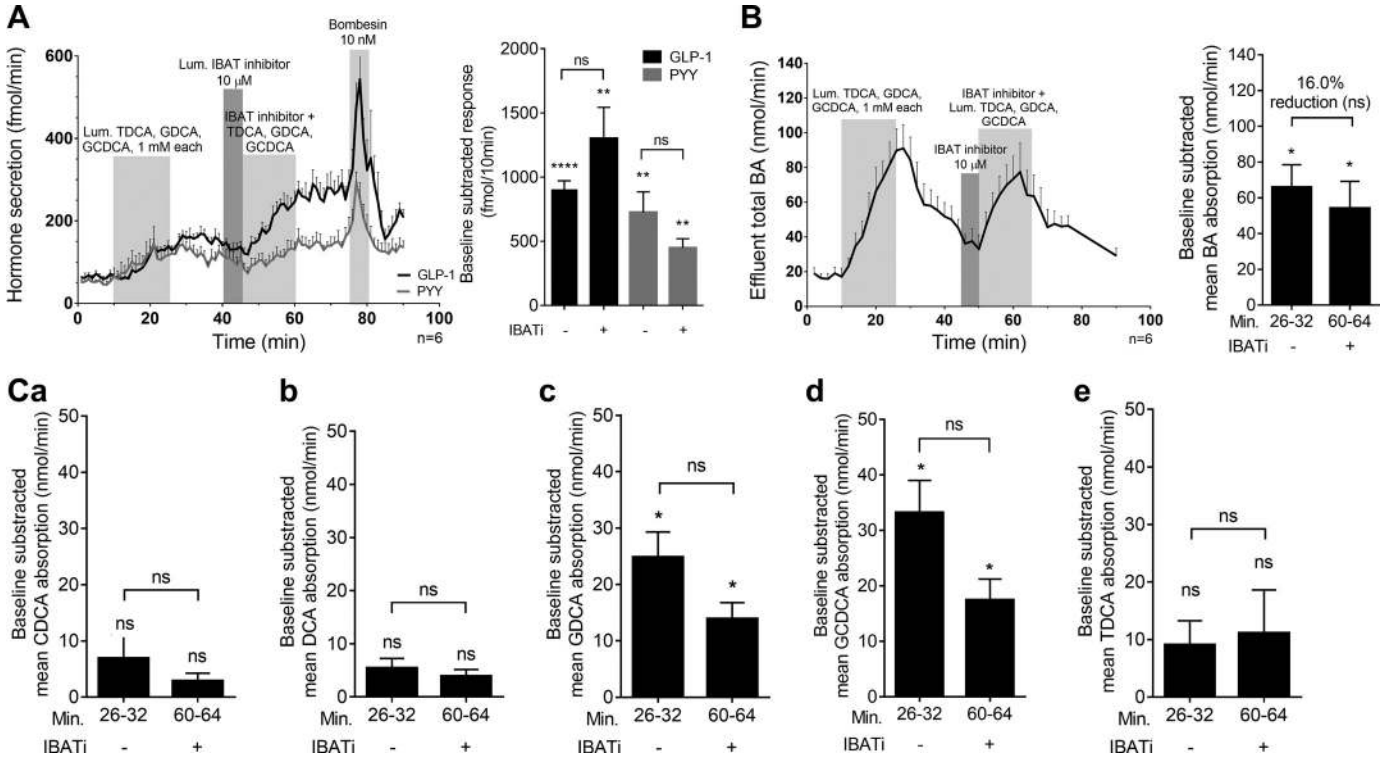

Fig. 3. *A*: glucagon-like-peptide-1 (GLP-1) and peptide-YY (PYY) secretion (fmol/min) from isolated perfused rat colon, means ± SE and baseline subtracted total outputs ± SE. Sodium tauro-deoxycholate (TDCA), sodium glyco-deoxycholate (GDCA), and sodium glycol-chenodeoxycholate (GCDCA) were infused with and without an apical sodium-dependent bile acid transporter (IBAT) inhibitor (IBATi) into the lumen of the rat colon. Ten nanomoles of bombesin (intra-arterially) were included at the end as a positive control. *B*: total bile acid absorption rate measured as the presence of BAs in perfusion effluents (nmol/min) with and without inhibition of IBAT activity. Values are means ± SE. *C*: absorption rate of sodium chenodeoxycholate (CDCA; *Ca*), sodium deoxycholate (DCA; *Cb*), GDCA (*Cc*), GCDCA (*Cd*), and TDCA (*Ce*) with and without inhibition of IBAT activity measured in perfusion effluents (nmol/min) by mass spectrometry. Values are means ± SE; $n = 6$ in each group. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, significant increase from baseline.

was markedly increased upon both intra-arterial and intraluminal BA stimulation. Furthermore, we demonstrate that responses rely on BA absorption followed by basolateral TGR5 activation. Interestingly, we also found that conjugated BAs induce peptide secretion independently of IBAT. The present results are summarized in Fig. 5 and are consistent with previous animal and human studies as will be discussed in the following but are significant as we demonstrate the isolated direct effect of BAs on colonic endocrine secretion. This was possible due to the use of our isolated perfused colon preparation, which allows for the mechanistic evaluation of a targeted sensory system without disturbing the organ's natural blood flow, intrinsic neural supply, and cell polarity. Thereby, investigation of the integrated communication between different cell types of an organ is possible, which is optimal for studying the dynamics of secretion.

In cell lines transiently transfected with TGR5 cDNA some BAs activate human and rat TGR5 (7, 34), and in isolated primary cell cultures from mice these BAs increase GLP-1 and PYY release (8), suggesting that TGR5 mediates the response. Here, we demonstrate that the importance of TGR5 also applies for colonic hormone responses (Fig. 2), and we show that the TGR5 receptor co-localizes with colonic L cells, supporting that BAs stimulate endocrine secretion from the colon by a direct mechanism. Furthermore, since no impact on secretion was observed upon luminal stimulation with a specific water soluble and poorly absorbable TGR5 receptor agonist (tauro-RO5527239), while markedly increased GLP-1 and PYY responses were observed upon intra-arterial stimulation, our functional data suggest that the TGR5 receptor is located on the basolateral side of the colonic L cell. This is consistent with a previous study by Ullmer and colleagues (63), which demonstrated that the same TGR5-specific agonist stimulated GLP-1 and PYY secretion from mice when administered systemically but not luminally. In line with this, inhibition of BA absorption by resin drugs with BA-sequestering properties (such as cholesevelam) led to an acute blockage of GLP-1 secretion (9, 25); however, prolonged resin administration is also found to increase GLP-1 secretion (22, 26) and improve glucose tolerance (41), which may be due to increased colonic BA delivery and thus colonic TGR5 activation. Absent peptide responses to the poor TGR5 receptor ligand, TUDCA (28, 34), further support the importance of the receptor, which again is similar to a human study where no gut hormone responses were seen upon oral ingestion of UDCA, whereas the primary BA, CDCA, increased secretion of GLP-1, PYY, neurotensin, C-peptide, and glucagon (42). Finally, GLP-1 responses to intra-arterial as well as intraluminal BA stimulation remained unchanged in TGR5 knockouts (opposite to $TGR5^{+/+}$ mice), clearly demonstrating that TGR5 is essential for BA-mediated GLP-1 secretion from the colon. Regarding PYY, responses were similar to those observed for GLP-1, except that luminal BA stimulation in TGR5 knockouts resulted in a small increase in PYY secretion. However, compared with the response in

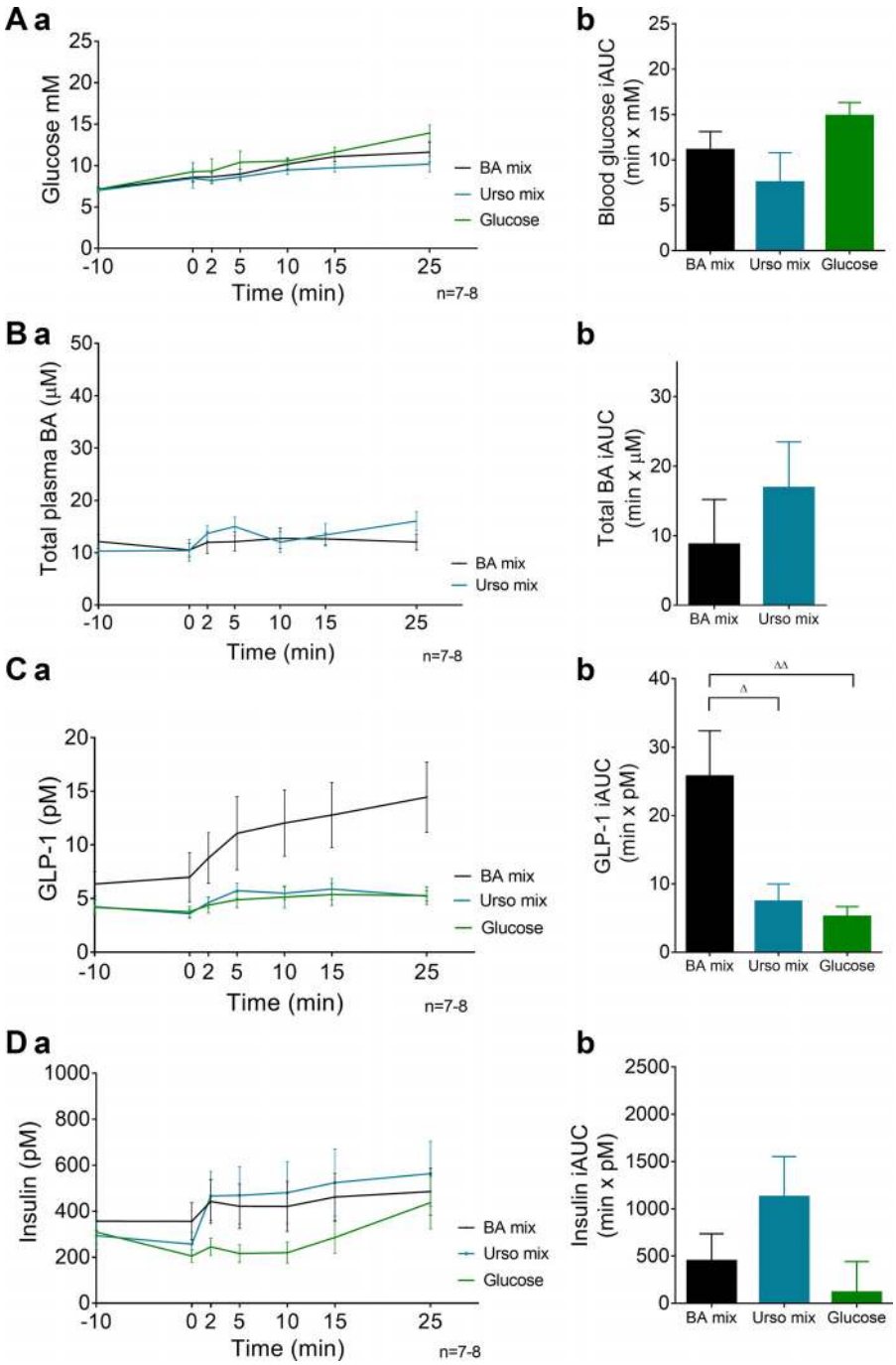

Fig. 4. Rat colons stimulated with one of the following 3 test substances in vivo: *1*) bile acid (BA) mix (black): mixture of 9 bile acids (sodium cholate, sodium glycol-cholate, sodium tauro-cholate, sodium deoxycholate, sodium glyco-deoxycholate, sodium tauro-deoxycholate, sodium chenodeoxycholate, sodium glycol-chenodeoxycholate, and sodium tauro-chenodeoxycholate, 9 mM); *2*) urso mix (blue): mixture of 3 ursodeoxy cholic acids (sodium urso-deoxycholate, sodium tauro-urso-deoxycholate, sodium glycol-urso-deoxycholate, 9 mM); and *3*) glucose (green): a 50% (wt/vol) glucose solution. Data are presented as means ± SE and baseline (mean of time point −10 and 0) subtracted total area under the curve (iAUC) ± SE (min × concentration); $n = 7–8$ in each group. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, significant difference between iAUCs. *Aa* and *Ab*: plasma glucose concentrations (mM). *Ba* and *Bb*: Total BA concentration in plasma (μM). *Ca* and *Cb*: plasma total glucagon-like-peptide-1 (GLP-1) concentrations (pM). *Da* and *Db*: plasma insulin concentration (pM). *n*(BA mix) = 8; *n*(urso mix) = 7; *n*(glucose) = 8.

TGR5$^{+/+}$ mice the increase appears insignificant, and may soley be a side effect caused by distention of the colon when luminal flow rate was increased or perhaps might be a secondary effect of the physicochemical properties of BAs.

Previously, we have shown that the conjugated BA TDCA stimulates peptide secretion from the distal small intestine by an IBAT-dependent mechanism and that unconjugated BAs stimulate secretion by an IBAT-independent mechanism (34). In the colon, IBAT expression is very low (34, 37) and the transporter may therefore not play a major role for colonic absorption of conjugated BAs. However, the three conjugated BAs, GDCA, GCDCA, and TDCA increased GLP-1 and PYY secretion both with and without inhibition of the conjugated BA transporter (IBAT) (Fig. 3). In these experiments, absorption was also measured and showed that IBAT inhibition resulted in a minor reduction in total BA absorption of only 16%. Apparently, this reduction was not enough to impact GLP-1 and PYY output, as peptide responses were not different between the two stimulations. This can be explained by the EC_{50} values of GDCA ($EC_{50} = 0.453$ μM), GCDCA ($EC_{50} > 1$ μM), and TDCA ($EC_{50} = 0.530$ μM) to TGR5 (34), since the effluent BA concentrations during IBAT inhibition reached levels far beyond the EC_{50} values, just as they did without IBAT inhibition. It is unlikely that we did not block the

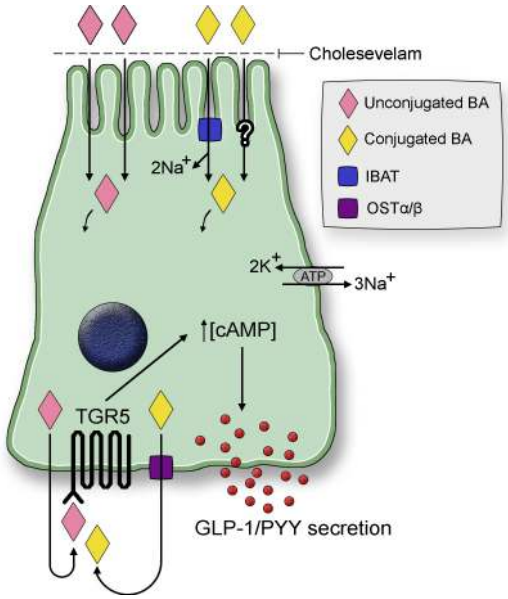

Fig. 5. Bile acids (BAs) that escape absorption in the ileum spill over to the colon where absorption also takes place, thus completing the enterohepatic circulation (total gut reabsorption capacity ~95%). Unconjugated BAs passively diffuse across the apical membrane, most likely facilitated by bacterial deconjugation of conjugated BAs. In the colon, a small fraction of conjugated BAs are actively absorbed by Na^+ coupled transport through apical sodium-dependent-bile-acid-transporter (IBAT) but perhaps also via an unknown mechanism. Irrespectively of absorption mechanism, conjugated and unconjugated BAs bind and activate Takeda-G protein-coupled-receptor 5 (TGR5) on the basolateral L cell membrane. TGR5 signaling results in increased intracellular cAMP levels, which eventually stimulates colonic glucagon-like-peptide-1 (GLP-1) and peptide-YY (PYY) secretion. OST, organic solute transporter.

transporters completely, since same concentration (10 μM) of the inhibitor previously was used to completely block the transporters in the distal small intestine, where BA absorption as well as peptide release was completely inhibited, in spite of the much greater expression of the transporter (34). Also, it does not seem plausible that our perfused preparation was suffering from any damages, since a leaky epithelium would make it impossible to completely block absorption with cholesevelam and impossible to prevent the poorly absorbable TGR5 agonist from being absorbed and stimulate secretion. However, it does not explain how the conjugated BAs were absorbed, and therefore, we tested whether the conjugated BAs had been deconjugated before absorption by measuring which specific BAs were actually absorbed. The deconjugated CDCA and DCA levels were relatively low throughout the experiments, and no sign of bacterial dconjugation (increase in CDCA and DCA during IBAT inhibition) was observed. On the contrary, TDCA and especially GDCA and GCDCA levels in perfusion effluents increased during infusions, and their absorption was not different with and without inhibition of IBAT-mediated transportation. Eventually, it needs to be mentioned that the perfusion setup may not be the best model for investigating bacterial modifications as the colonic lumen is emptied before experiments, whereby a large part of the bacteria most likely will be lost. Our findings suggest that conjugated BAs that escape absorption in the terminal ileum are passively absorbed in the colon, both in their intact conjugated form, but most likely also after bacterial dehydroxylation and de-conjugation, which is a known effect of the colonic bacteria (13, 38). The low expression of IBAT transporters in the colon may account for a small fraction of active conjugated BA absorption (absorbing BAs against their gradient) and could possibly be important for the high total BA reabsorption rate (~95%) in the gut.

Through the use of isolated perfused rat and mouse colon, we have demonstrated BAs as direct stimulators of colonic GLP-1 and PYY secretion. To support the relevance of our findings and to understand whether this activation would impact plasma levels of these hormones in vivo, we stimulated the colon of anesthetized rats with luminal BAs (Fig. 4). One group of animals received glucose, but considering the poor colonic glucose absorption capacity and the mechanisms behind glucose induced GLP-1 secretion (32, 33, 58), glucose is not an ideal colonic stimulus. However, responses obtained with glucose illustrate the power and relevance of BAs, as BA-induced GLP-1 responses were significantly greater than responses to glucose. Since BAs stimulate secretion upon absorption, it is not unlikely that BAs impact secretion from cells outside site of delivery (in this case the colon) once they enter the systemic circulation. However, this would require that some BAs escape retention in the liver in significant amounts, which in the present study did not seem to be the case, as plasma BA levels did not increase upon colonic BA administration. The total BA concentration in the portal vein (3, 6, 15) is estimated to be four to five times higher than that measured in peripheral veins (where concentrations are within the lower micromolar range) but much less concentrated than luminal levels (4–12 mM in response to food intake; Refs. 56, 57, 64). Accordingly, the BA concentrations measured directly in perfusion effluents (before retention in the liver) were found to reach ~30 μM (equaling almost 100 nmol/min, Fig. 1), whereas the in vivo data would be consistent with near complete BA uptake by the liver. The dependence of BA absorption on BA-induced secretion is in line with the relatively smaller magnitude of GLP-1 secretion obtained in humans upon proximal BA delivery (25) compared with studies investigating distal BA delivery either by rectal infusions (a rather unphysiological stimulus) (1, 2) or by oral delivery to gastric bypass operated individuals where the upper part of the gut is surgically shunted away from intestinal continuity (42). The significantly larger responses reported here, suggest that response size depends on site of stimulation and absorption as also pointed out by Nielsen et al. (42). Finally, BAs do not directly impact secretion of pancreas-derived hormones (34), but insulin secretion was measured, since BAs have been reported to increase both insulin and glucagon secretion when ingested by humans (25, 42) or when administered intraluminal into the upper small intestine in rats (34). However, no difference in insulin secretion was observed between groups in the present study, most likely due to the fact that GLP-1 stimulated insulin secretion is glucose dependent (39), and plasma glucose levels remained fairly low throughout experiments.

Although it has been known for decades that the colonic mucosa harbors a significant number of the GLP-1- and PYY-producing L cells (16, 18, 20, 55), secretagogues of colonic endocrine secretion are much less investigated compared with those of the small intestine (11, 17, 33, 43, 45, 53). However, cell number and actual level of hormone release do not necessarily correlate, since secretion, in particular, also depends on

the expression pattern of a given molecular sensory machinery (59), as exemplified here with BA-mediated secretion. Nutrient stimuli such as glucose (32, 50, 51) and peptones (products of partial hydrolyzed proteins; perfusion data from our laboratory, unpublished) have a minor impact on peptide secretion from the colon, consistent with their low prevalence in the distal gut in vivo. Endocrine routes for distal gut stimulation, including a proximal-distal hormonal loop, have repeatedly gained interest as this would contribute to an explanation of the early postprandial rise in plasma GLP-1 (43, 61). Thus, in our hands glucose-dependent insulinotropic polypeptide (1 nM), but not cholecystokinin (1 nM), is able to induce GLP-1 and PYY secretion from the isolated perfused rat colon (unpublished data) in agreement with a previous study (48) but in contrast to others (31, 49). However, the physiological relevance of high concentrations of duodenal hormones is questionable (24), while the neuropeptide vasoactive intestinal peptide (10 nM), stimulating cAMP accumulation in enterocytes, also seems to be without effect on endocrine secretion from the perfused rat colon (unpublished data). Another currently hot topic is the impact of colonic fermentation products (short chain fatty acids) on GLP-1 and PYY secretion, and recently, we showed that acetate and butyrate increased peptide secretion from the colon once metabolized in the L cells (12). In humans, however, data are more ambiguous as several different outcomes of colonic fermentation on hormone release have been reported (10, 21, 47, 54, 65). Compared with the impact of fermentation products and the other just mentioned stimuli, which in our hands had remarkable little or no impact on colonic secretion, BAs appear to induce much more robust endocrine responses from the colon. This raises the question about the timing of the response, since transfer of BA from the small to the large intestine does not occur in the early postprandial phase, suggesting that a colonic release of these hormones does not contribute to the typical postprandial response. Indeed, meal-induced GLP-1 responses were completely unaffected by total colectomy in humans (40). Instead, the colonic release may be related to the delayed arrival of nutrients to the colon or may even account for the low amounts of circulating peptides measurable in plasma during fasting. Considering the slow colonic transit time, BAs may constitute a late but long-term colonic L-cell stimuli. Irrespectively, the colon may hereby contribute to dampening of appetite and upper gastrointestinal motility or perhaps exert local protective actions on the colonic mucosa, as clearly demonstrated in models of chemotherapy induced mucositis (30).

In conclusion, we find that BAs are potent and direct promoters of colonic gut peptide secretion and may represent the most important physiological stimulus for colonic secretion of GLP-1 and PYY.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.B.C., R.E.K., and J.J.H. conceived and designed research; C.B.C. and R.E.K. performed experiments; C.B.C., S.A.J.T., and N.J.W.A. analyzed data; C.B.C., S.A.J.T., N.J.W.A., R.A., M.P.G., R.E.K., and J.J.H. interpreted results of experiments; C.B.C. prepared figures; C.B.C. and J.J.H. drafted manuscript; C.B.C., S.A.J.T., N.J.W.A., K.S., R.A., M.P.G., R.E.K., and J.J.H. edited and revised manuscript; C.B.C., S.A.J.T., N.J.W.A., K.S., R.A., M.P.G., R.E.K., and J.J.H. approved final version of manuscript.

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