Lithocholic acid attenuates cAMP-dependent Cl⁻ secretion in human colonic epithelial T84 cells

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Ao M, Domingue JC, Khan N, Javed F, Osmani K, Sarathy J, Rao MC. Lithocholic acid attenuates cAMP-dependent Cl⁻ secretion in human colonic epithelial T84 cells. Am J Physiol Cell Physiol 310: C1010–C1023, 2016. First published April 13, 2016; doi:10.1152/ajpcell.00350.2015.—Bile acids (BAs) play a complex role in colonic fluid secretion. We showed that dihydroxy BAs, but not the monohydroxy BA lithocholic acid (LCA), stimulate Cl⁻ secretion in human colonic T84 cells (Ao M, Sarathy J, Domingue J, Alrefai WA, Rao MC, Am J Physiol Cell Physiol 305: C447–C456, 2013). In this study, we explored the effect of LCA on the action of other secretagogues in T84 cells. While LCA (50 μM, 15 min) dramatically (>90%) inhibited FSK-stimulated short-circuit current (Isc), it did not alter carbachol-stimulated Isc. LCA did not alter basal Isc, transepithelial resistance, cell viability, or cytotoxicity. LCA’s inhibitory effect was dose dependent, acted faster from the apical membrane, rapid, and not immediately reversible. LCA also prevented the Isc stimulated by the cAMP-dependent secretagogues 8-bromo-cAMP, lubiprostone, or chenodeoxycholic acid (CDCA). The LCA inhibitory effect was BA specific, since CDCA, cholic acid, or taurodeoxycholic acid did not alter FSK or carbachol action. While LCA alone had no effect on intracellular cAMP concentration ([cAMP]), it decreased FSK-stimulated [cAMP] by 90%. Although LCA caused a small increase in intracellular Ca²⁺ concentration ([Ca²⁺]), chelation by BAPTA-AM did not reverse LCA’s effect on Isc. LCA action does not appear to involve known BA receptors, farnesoid X receptor, vitamin D receptor, muscarinic acetylcholine receptor 5. LCA significantly increased ERK1/2 phosphorylation, which was completely abolished by the MEK inhibitor PD-98059. Surprisingly PD-98059 did not reverse LCA’s effect on Isc. Finally, although LCA had no effect on basal Isc, nystatin permeabilization studies showed that LCA both stimulates an apical cystic fibrosis transmembrane conductance regulator Cl⁻ current and inhibits a basolateral K⁺ current. In summary, 50 μM LCA greatly inhibits cAMP-stimulated Cl⁻ secretion, making low doses of LCA of potential therapeutic interest for diarrheal diseases.

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IN THE LAST COUPLE OF DECADES, bile acids have gained increasing importance as signaling molecules in the intestine, over and above their classical roles in aiding lipid digestion and absorption. In most mammals, including humans, the liver synthesizes the trihydroxy (positions 3, 7, and 12) bile acid cholic acid (CA), and the dihydroxy bile acid (positions 3 and 7) chenodeoxycholic acid (CDCA), from cholesterol. These primary bile acids are conjugated to either taurine or glycine and enter the intestinal lumen where they facilitate fat and fatsoluble vitamin absorption. Bacteria in the intestine deconjugate the amino acid side chains from the various bile acids (34). Following deconjugation, the primary bile acids are dehydroxylated by bacterial hydrolases at the 7α position to yield the secondary bile acids: dihydroxy, deoxycholic acid (DCA from CA) and monohydroxy, lithocholic acid (LCA from CDCA). Another secondary bile acid, normally present in trace amounts in bile (76), is ursodeoxycholic acid (UDCA; the β-epimer of CDCA), which is primarily synthesized by bacterial epimerization of CDCA (34). Over 95% of bile acids are reabsorbed in the distal ileum via the apical Na⁺-dependent bile acid transporter and recycled to the liver. Malfunctioning of one or more of the transporters and signaling proteins underlying this intricately regulated process can lead to disease (3). For example, ileal bile acid malabsorption leads to excess bile acids in the colon where it is well established that DCA and CDCA, but not UDCA, CA, or LCA, stimulate Cl⁻ secretion leading to fluid loss and diarrhea (4, 9, 23, 39, 41, 58, 75).

Under normal physiological conditions, the bulk of the di- and trihydroxy bile acids are reabsorbed via enterohepatic circulation. However, LCA is poorly reabsorbed and enters the colon where it can be sulfated, resulting in its excretion in the feces (34, 35). In the colon, LCA affects cell proliferation in a variety of animal and cell models (14), and excess LCA is known to cause DNA damage and promote cancer (50). The expanding role of bile acids has led to intense investigations on the mechanisms by which the primary and secondary bile acids elicit their biological effects. At least three mechanisms have been implicated in bile acid action: 1) specific nuclear receptors to elicit genomic responses; 2) membrane receptors to elicit rapid responses; and 3) membrane perturbations altering membrane lipid/protein interactions and leading to signaling cascades. The major nuclear receptor for bile acids is the farnesoid X receptor (FXR) with a rank order of potency of CDCA > LCA > DCA > CA, and its activation and regulation intricately control bile acid homeostasis in the gut-liver axis (24). LCA is also a potent ligand for the vitamin D receptor (VDR), and its activation leads to the induction of the cytochrome P-450 monooxygenase CYP3A, which results in the detoxification of LCA (50). Interestingly, VDR can serve both as a membrane-bound receptor where it is often associated with caveolin, causing rapid effects, or can translocate to the nucleus to alter genomic function (55).

As is the case for many cholesterol-derived ligands, specific membrane-associated receptors have also been identified for bile acids. In the H508 colon cancer cell line, Cheng and Raufman established that the taurine and glycine conjugates of
both LCA and DCA transactivate epidermal growth factor receptor (EGFR) via M3 muscarinic receptors to stimulate cell proliferation (14). More recently, the cloning and identification of a bile acid-specific G_{α}α_{i}-coupled membrane receptor (TGR5) has revolutionized the thinking of bile acid biology (30). TGR5 has a rank order of potency of LCA > DCA > CDCA > CA (40). It is widely distributed in many species and found in a variety of human tissues, including in the intestine, endocrine glands, adipocytes, muscle, spleen, and lymph nodes (30). Activation of TGR5 results in an increase in adenosine 3',5'-cyclic monophosphate (cAMP) and activation of protein kinase A (PKA) and the exchange protein activated by cAMP (6, 44, 61). In the intestine, TGR5 is localized to enteroendocrine, neuronal, and smooth muscle cells, activating glucagon-like peptide release (73), serotonin release (2), and causing membrane hyperpolarization (45), respectively. TGR5 is also expressed in rat colonocytes where it regulates cholinergic activation of electrolyte secretion (77). Interestingly, LCA is the most potent bile acid ligand of TGR5, and recent evidence implies this bile acid to have a protective role in inflammation (81) as opposed to its well-documented involvement in colon carcinogenesis (63). Because bile acids are cholesterol derivatives, it is conceivable that they can affect cellular processes by causing surface membrane perturbations, and such roles have been demonstrated for LCA in cholestasis (51, 83) and DCA in apoptosis (38).

We recently demonstrated that CDCA acts via an adenylate cyclase/PKA-dependent mechanism to stimulate Cl− secretion via cystic fibrosis transmembrane conductance regulator (CFTR) in the human colon carcinoma cell line T84 (4). Furthermore, at increasing concentrations, LCA did not stimulate Cl− secretion (4). Recent work of Keely and collaborators suggested that LCA may have a species-specific modulatory role on Cl− secretion. Thus, they reported that LCA inhibits both basal- and carbachol (CCH)-stimulated I_{sc} in rat distal colonic mucosa, stripped of underlying muscle within 10 min of addition (77). In contrast, in both stripped human colonic preparations and in T84 cells (42), they found that pretreatment with LCA (50–250 μM) for 15 min potentiated the action of CCH.

In the present study, we investigated the dose-dependent effect and sidedness of LCA action on cAMP-mediated Cl− secretion. To delineate the signaling mechanisms involved in LCA’s antisercretory action, we assessed the involvement of major bile acid receptors, measured intracellular cAMP concentration ([cAMP]_{i}), intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}), and protein kinase activation, and the ionic basis of LCA’s action. Here, we report the intriguing antisercretory effect of LCA on cAMP signaling, highlighting a possible beneficial effect of a bile acid, hitherto known largely for its deleterious actions such as in cholestasis.

MATERIALS AND METHODS

Materials. T84 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Dulbecco’s modified Eagle medium (DMEM), Ham’s F-12 nutrient mixture, and bovine calf serum were obtained from Invitrogen (Carlsbad, CA). LCA, tauroliothocholic acid (TLCA), CDCA, CFTRinh172, forskolin (FSK), CCH, 8-bromo-cAMP (8-Br-cAMP), nystatin, protease inhibitor cocktail, and phosphatase inhibitor cocktail 2 were purchased from Sigma-Aldrich (St. Louis, MO). Fura 2-AM was purchased from Life Technologies (Carlsbad, CA). Rabbit monoclonal ERK1/2 and phospho-ERK1/2 antibodies were from Cell Signaling (Boston, MA). The following kits were purchased for various assays: FITC Annexin V Apoptosis Detection Kit 1 BD Biosciences (Franklin Lakes, NJ); Pierce lactate dehydrogenase (LDH) assay kit from Life Technologies; HitHunter cAMP HS+ kit for measurement of cAMP from DiscoverRx (Fremont, CA); and human phosphokinase array kit from R&D Systems (Minneapolis, MN). Unless otherwise specified, all other reagents were of analytical grade and were purchased from either Sigma Aldrich or Fisher Scientific (Hanover Park, IL).

Cell culture and viability measurements. T84 cells were grown in DMEM-F-12 medium (1:1) containing 6% bovine calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and ampicillin (8 μg/ml). The cells were incubated in a humidified atmosphere of 5% CO2 at 37°C. T84 cells were used in passages 39–50 for these studies. Based on RT-PCR analysis of amelogenin expression, these cells are considered “female” (67).

Cellular viability and apoptosis were studied by flow cytometry (BD Accuri Flow cytometer, San Jose, CA) following staining with propidium iodide (PI) and FITC-labeled annexin V (Apoptosis Detection Kit I), respectively. T84 cells were grown to confluence on six-well plates and incubated at 37°C with different doses of LCA (1 h or overnight); etoposide (10 μM overnight) was used as a positive control for apoptosis. After LCA or etoposide exposure, cells were labeled for 15 min with FITC-annexin V at room temperature (RT) in the dark, and PI as a standard viability probe and subjected to flow cytometry as per the manufacturer’s instructions.

Cell necrosis was determined by measuring LDH release using a Pierce LDH assay kit. Briefly, T84 cells (~4,000 cells/well) were grown in 96-well plates overnight and treated with varying doses of LCA. The LDH in the media was quantified by a coupled enzymatic reaction, and the formation of the red formazan product was measured spectrophotometrically as absorbance at 490 nm. Maximal LDH release was measured after cells were treated with 10× lysis buffer.

Electrophysiological measurements. Ussing chamber measurements were performed as described previously (3). Briefly, T84 cells were seeded at a density of ~250,000 cells/insert in 6.5-mm 0.4-μm pore size Transwell tissue culture inserts (Corning 3413; Corning Life Sciences, Lowell, MA), coated with mouse tail collagen (Sigma). When trans epithelial electrical resistance (R_{TE}) reached values of ~950 Ω-cm², the inserts were mounted in Ussing chambers (area: 0.33 cm²; Physiologic Instruments, San Diego, CA) and bathed with oxygenated (95% O₂-5% CO₂) buffer (5 ml/reservoir) of the following composition (in mM): 115.4 NaCl, 5.4 KCl, 1.2 CaCl₂, 1.2 MgCl₂, 21.0 NaHCO₃, 0.6 NaH₂PO₄, 2.4 Na₂HPO₄, pH 7.4, and 10 n-glucose, at 37°C. Transmural short-circuit current (I_{sc}; μA/cm²) and R_{TE} were monitored throughout the experiment using an automatic voltage-clamp apparatus (VCC-MC6; Physiologic Instruments) as described earlier (78). Unless otherwise indicated, LCA (50 μM) was added to both the apical and basolateral (bilaterally) reservoirs. In all experiments, when used, FSK (10 μM) was added to the basolateral solution. At the end of each experimental run, CCH (100 μM) was added to the basolateral solution, and the resultant rapid transient rise in I_{sc} was used as an index of tissue viability. For nystatin experiments, cells were exposed to 200 μg/ml nystatin for 15 min either on the apical or basolateral surface. When the basolateral membrane (BLM) was permeabilized, a Cl− gradient was established by replacing NaCl in the basolateral reservoir with equimolar sodium gluconate, and the concentration of CaCl₂ was increased from 1.2 to 2 mM to account for chelation of Ca^{2+} by gluconate (49). When the apical membrane (AM) was permeabilized, a K+ gradient was created by substituting NaCl with KCl in the apical reservoir (29). Following the nystatin equilibration period, bile acids were added either to one side or bilaterally.

**Intracellular cAMP measurements.** [cAMP], was measured using the HitHunter cAMP HS+ kit based on enzyme fragment complementation. This technology uses two fragments of *Escherichia coli*.
chemiluminescent detection reagents were applied, and signals were detected with antibodies. Next, streptavidin-horseradish peroxidase and antibodies provided by the manufacturer. The membranes were incubated with antibody/lysis mix overnight according to the manufacturer’s instructions. The signals were read on a luminometer (SpectraMax M5e; Molecular Devices, Sunnyvale, CA) at 1 s/well. The cAMP concentrations of samples were calculated from the curve generated using standards provided in the kit.

Filtered cAMP measurements. T84 cells were plated on a 2-cm2 glass bottom dish (MatTek) for 3–4 days. The cells were loaded with 5 μM fura-2 AM in serum-free DMEM-F-12 for 1 h in a tissue culture incubator at 37°C and washed three times with Krebs-Ringer-HEPES medium (KRH) containing (in mM): 120 NaCl, 5.4 KCl, 0.8 MgCl2, 1 CaCl2, 11.1 glucose, and 20 HEPES, pH 7.4. The dish with 1 ml of fresh KRH was then set up on a dual-channel temperature controller (Warner Instruments, Hamden, CT) connected platform of a fluorescence microscope (Olympus IX51; Olympus America, Center Valley, PA). Secretagogues in KRH buffer were delivered to the cells by a perfusion and vacuum system (Warner Instruments). Ca2+ signals were captured using a Q-8 spectrofluorometer system (Photon Technology International, Edison, NJ). We used a calcium calibration buffer kit (Molecular Probes, Waltham, MA) to plot a standard curve.

Briefly, T84 cells were grown in 100-mm dishes to 90% confluence. After incubation with 0.01% DMSO or 50 μM LCA for 15 min. Cells were then sequentially incubated with antibody/lysis mix (RT for 1 h), ED- and CL-substrate (RT overnight) according to the manufacturer’s instructions. The signals were read on a luminometer reader (SpectraMax M5e; Molecular Devices, Sunnyvale, CA) at 1 s/well. The cAMP concentrations of samples were calculated from the curve generated using standards provided in the kit.

RESULTS

LCA attenuates responses to only cAMP-dependent secretagogues in T84 cell monolayers. FSK activates adenylate cyclase and cAMP production and is a well-established stimulator of cAMP-mediated Cl− secretion via CFTR (15, 18). As shown in Fig. 1, bilateral exposure to 50 μM LCA 15 min before FSK (10 μM) treatment (42) drastically inhibited the effects of FSK (ΔIsc in μA/cm2: FSK, 45.6 ± 6.4; LCA + FSK, 3.4 ± 0.2; n = 5). Control monolayers received DMSO 15 min before FSK addition. Exposure of naïve T84 monolayers to DMSO or LCA alone neither altered basal Isc (ΔIsc in μA/cm2: DMSO: ± 0.6, n = 16; LCA: ± 2.2 ± 0.8, n = 9; Fig. 1A) nor basal resistance (Rs, Ω·cm2: ± 1,000; ΔRs: DMSO, −24.8 ± 16.9, n = 16; LCA, 7.9 ± 29.8, n = 9). Addition of LCA 15 min before FSK either to both, or only to the apical or basolateral chambers caused similar inhibitions that were not statistically different (ΔIsc in μA/cm2: FSK, 45.6 ± 6.4; FSK + A-LCA, 8.3 ± 3.3; FSK + B-LCA, 7 ± 0.6; FSK + AB-LCA, 3.4 ± 0.2; n = 3; Fig. 1B). LCA (50–500 μM) for 1 h had no effect on cytotoxicity as measured by LDH release (control, 2.5 ± 1.8%; 50 μM LCA,

β-galactosidase: a large enzyme acceptor (EA) and a small peptide acceptor (PA). Secretagogues in KRH buffer were delivered to the cells by a perfusion and vacuum system (Warner Instruments). Ca2+ changes elicted by (DMSO or LCA) + FSK. AM, apical; BLM, basolateral; AB, apical + basolateral. *P < 0.05 in ΔIsc compared with DMSO + FSK; n = 3.

Fig. 1. Effect of lithocholic acid (LCA) on forskolin (FSK)-stimulated short-circuit current (Isc, μA/cm2) in T84 cells. A: representative tracing of Isc showing the effects of LCA vs. DMSO. T84 cell monolayers were incubated with bilateral DMSO (0.1%) or LCA (50 μM) for 15 min before the subsequent sequential basolateral addition of FSK (10 μM) and carbachol (CCH, 100 μM). B: bar graph showing quantification of the Isc changes elicited by (DMSO or LCA) + FSK. AM, apical; BLM, basolateral; AB, apical + basolateral. *P < 0.05 in ΔIsc compared with DMSO + FSK; n = 3.
2.3 ± 1.2%; 100 μM LCA, 1.2 ± 0.2%; 250 μM LCA, 0.85 ± 0.1%; 500 μM LCA, 0.02 ± 0.01%; n = 6). Similarly and as shown in Fig. 2, A and E–H, exposure to 50–500 μM LCA had no effect on apoptosis at 1 h (control, 5.1 ± 0.2%; 50 μM LCA, 4.8 ± 0.1%; 100 μM LCA, 5.6 ± 0.2%; 250 μM LCA, 5.5 ± 0.2%; 500 μM LCA, 5.5 ± 0.2%; n = 4). Overnight exposure to LCA (Fig. 2, B and C) also did not affect apoptosis (control, 4.9 ± 0.8%; LCA, 6.1 ± 0.3%; n = 4). In contrast, overnight exposure to 10 μM etoposide (Fig. 2D) caused a fourfold increase in apoptosis (20.7 ± 1.0%; n = 4). Thus, the inhibitory effect of LCA on FSK action is not due to increased cytotoxicity or apoptosis.

We have previously shown that lubiprostone, a prostaglandin E4 receptor agonist (5), and CDCA (4) also stimulate CFTR-mediated Cl− secretion via PKA in T84 cells. Therefore, we examined if LCA would affect lubiprostone- or CDCA-stimulated Isc. As shown in Fig. 3, A and B, LCA inhibited lubiprostone-stimulated Isc by 95% (250 nM, n = 4; Fig. 3A) and CDCA-stimulated Isc by 60% (500 μM, n = 3; Fig. 3B). Because the CDCA response often manifests as a peak and a plateau phase (4), the data have been represented as area under the curve to capture the entirety of the CDCA response. To determine if LCA is acting at a point distal to cAMP production, we examined the effects of LCA on 8-Br-cAMP action. As shown in Fig. 3C, LCA also inhibited 8-Br-cAMP (100 μM)-mediated Isc by 82%. Finally, we examined the effects of LCA on Ca2+-dependent secretagogues by adding CCH (100 μM) to DMSO-treated or to LCA (50 μM)-pretreated monolayers and measured Isc (n = 5). As shown in Fig. 3D, and in contrast to the findings of Kelly et al. (42), LCA did not change CCH-stimulated Isc in our T84 preparations. Therefore, for the remainder of the study, we probed the nature of the inhibition of cAMP-stimulated Isc by LCA, using FSK as the cAMP-dependent secretagogue.

Dose, time, sidedness, and specificity of LCA action. LCA action is dose dependent, the dose-response curve best fits an exponential relationship, and the data are depicted as log concentrations on the x-axis (Fig. 4A). The IC50 for bilateral addition was 6.6 μM. LCA’s inhibition is rapid (data not shown), and we examined if LCA was also capable of inhibiting Isc when added after 10 μM FSK had its peak effect. As shown in Fig. 4B, LCA also caused a rapid inhibition of FSK-stimulated current. As in the case of pretreatment with LCA, there was no difference in the maximal inhibition seen with apical, basolateral, or bilateral addition (data not shown). However, the rates of inhibition were different (Fig. 4C), and the half-time (t0.5) for apical addition was 2.6 min, for basolateral addition 4.6 min, and for bilateral addition 2.3 min.

LCA’s action is not reversible in the short term, since FSK failed to increase Isc even 30 min after LCA was removed from the buffer (Fig. 4, B and D). However, there is a time-dependent recovery of FSK-induced Isc after LCA removal (1 h, 33%; 2 h, 55%, and overnight, 80% recovery; data not shown). In all of the above experimental protocols, the monolayers remained viable since CCH (100 μM) added at the end of each experimental regimen caused a robust increase in Isc, as shown in the representative tracings of Fig. 4, B and D. It must be noted that the CCH response after FSK (Figs. 1A, 4B, and 4D) is consistently much greater than when added to naive tissue (Fig. 3D). We believe this is due to an increase in the driving force (CFTR activity in terms of channel openings and numbers) created by the cAMP-dependent secretagogues and have observed it in our earlier studies with lubiprostone and CDCA (4, 5).

Fig. 2. Effect of LCA on cell viability and apoptosis. T84 cells were grown to confluency and were incubated with DMSO [0.1%; 1 h (A); 24 h (B)], LCA (50 μM, 24 h (C); 50–500 μM, 1 h (E–H), or 10 μM etoposide for 24 h (D)). The cells were then analyzed using flow cytometry after staining with propidium iodine (PI) and FITC-labeled annexin V. Representative plot of n = 4.
To determine if other bile acids had a similar inhibitory effect as LCA, we tested the actions of CA, CDCA, and tauro-DCA (TDC) on FSK-stimulated $I_{sc}$. As shown in Fig. 5A, at 50 μM, neither CA ($n = 5$), CDCA ($n = 5$), nor TDC ($n = 5$) altered the $I_{sc}$ responses to FSK. In contrast, the tauro-conjugated LCA (TLCA, 50 μM, bilateral addition, 15 min) significantly inhibited FSK’s effect, but to a lesser extent than LCA ($\Delta I_{sc}$ in μA/cm²: FSK, 46 ± 4; TLCA + FSK, 32 ± 3; $n = 9$; Fig. 5B). In a separate series of experiments ($n \geq 4$) we compared the sidedness of TLCA action. Addition of TLCA bilaterally or to the basolateral side alone caused a significant 37% inhibition, but addition to the apical side had no effect (Fig. 5C). Because maximal inhibitory effects were observed with unconjugated LCA, we focused the remaining studies on its actions.

Mechanisms underlying LCA action. LCA has been demonstrated to elicit its biological effects on colonic epithelia through at least four different receptor mechanisms: the nuclear receptors, FXR (48) and VDR (48), and the G protein-coupled receptors, M3 muscarinic receptor (14) and TGR5, the bile acid-specific receptor (30). Although T84 cells possess FXR (4) and VDR (unpublished observations) transcripts, neither...
treatment with the FXR agonist GW-4064 (ΔIsc in μA/cm²: 15 min; DMSO + FSK, 41 ± 10, n = 4; GW-4064 + FSK, 44 ± 8, n = 3; 24 h; DMSO + FSK, 42 ± 3, n = 5; GW-4064 + FSK, 48 ± 8, n = 4) nor exposure (15 min) to the vitamin D₃ receptor agonist calcitriol (100 nM; ΔIsc in μA/cm²: DMSO + FSK, 29 ± 2, n = 4; calcitriol + FSK, 32 ± 3, n = 5) altered basal Isc or FSK-stimulated Isc. LCA is one of the most potent activators of TGR5, and, in a variety of tissues (40), activation of TGR5 increases intracellular cAMP. To examine if TGR5 plays a role in LCA action, we first determined if LCA altered the phosphodiesterase inhibitor IBMX (100 μM, 30 min pretreatment) did not alter LCA’s action on FSK-induced Isc (ΔIsc in μA/cm²: IBMX + LCA + FSK, 0.7 ± 0.3 vs. LCA + FSK, 3.4 ± 0.2; n = 3). It is noteworthy that LCA causes an equally robust >90% inhibition of FSK-stimulated [cAMP]; and of FSK-stimulated Isc. Furthermore, in intestinal cells, TGR5-induced increases in cAMP should lead to activation of CFTR and Cl⁻ secretion. However, in our studies, LCA has the opposite effect, inhibiting cAMP-stimulated Cl⁻ secretion. This suggests TGR5 is not involved in LCA action, and the rapidity of the response to LCA implies that perhaps genomic effects via nuclear receptors are also not involved in LCA’s antisecretory effects.

Cheng and Raufman have shown that LCA acts via muscarinic M3 receptors to transactivate EGFR and increase intracellular Ca²⁺ (14). As shown in Fig. 7A, the muscarinic receptor inhibitor atropine did not reverse LCA’s action. Similarly, the EGFR inhibitor AG-1478 (1 μM, 30 min) did not significantly change LCA’s inhibition (ΔIsc in μA/cm²: FSK, 18.2 ± 0.75; LCA + FSK, 1.3 ± 0.28; AG-1478 + LCA + FSK, 0.78 ± 0.15; n ≥ 3). However, LCA caused a modest, but significant, increase in [Ca²⁺], (43.2 ± 1.5 nM from 33.5 ± 1.9 nM, n = 9). Interestingly, this increase was partially inhibited by BAPTA-AM, but was not decreased in the absence of extracellular Ca²⁺ (Fig. 7B). Ionomycin (3 μM) caused a robust increase in [Ca²⁺], (73.1 ± 11.4 nM, n = 6) as expected. These ranges of [Ca²⁺] are similar to those reported by Wong et al. (79). FSK did not increase [Ca²⁺] (n = 10; data not shown). Our results with FSK are similar to those of Yue et al. (84); other authors have reported an increase in [Ca²⁺]; in response to FSK (36) in T84 cells. Regardless of the increase in [Ca²⁺], the action of LCA on FSK-stimulated Isc was not inhibited by BAPTA (Fig. 7A). Finally, in preliminary experiments, we explored whether disruption of lipid rafts would affect the action of LCA. Overnight treatment of T84 cells with...
LCA’s inhibition of FSK-induced 

cationic system. Bar graph shows 

Inhibitory effect of LCA on ERK1/2 phosphorylation in T84 cells treated with DMSO, LCA (50 μM, dose causing maximal inhibition of CFTR) inhibited LCA and FSK responses by 78 and 85%, respectively (data not shown). In LCA-pretreated monolayers, the magnitude of the FSK-induced current was decreased (ΔIsc in μA/cm2; LCA + FSK, 29 ± 8; slope, –7.7 ± 1.8; n = 4; Fig. 9). These data suggest that LCA has an effect on an apical Cl– current and that this is most likely CFTR mediated. In support of this finding, LCA had no effect on CFTR null cells, since we had reported that HEK-293 cells do not express endogenous CFTR, and LCA had no effect on Cl– transport as measured by iodide efflux in these cells (27).

Next, we permeabilized the AM in the presence of a K+ gradient to examine the role, if any, of K+ channels in the BLM. Permeabilization of the AM with nystatin, in the pres-
T84 cells grown on Transwell inserts were mounted in Ussing chambers in the presence of an apical-to-basolateral Cl\(^{-}\) gradient. A: representative tracing of \(I_{sc}\) showing LCA’s effect on FSK after BLM was permeablized by nystatin (200 \(\mu g/ml\)). Please note that, for consistency of representation with other plots, these tracings have been inverted. B: bar graph showing the quantification of the \(I_{sc}\) changes elicited by LCA, and by FSK pretreated with DMSO or LCA. *P < 0.05 in \(\Delta I_{sc}\) compared with DMSO + FSK; \(n = 4\).

Fig. 9. Effect of LCA on FSK-stimulated \(I_{sc}\) in BLM permeablized T84 cells. T84 cells grown on Transwell inserts were mounted in Ussing chambers in the presence of an apical-to-basolateral Cl\(^{-}\) gradient. A: representative tracing of \(I_{sc}\) showing LCA’s effect on FSK after BLM was permeablized by nystatin (200 \(\mu g/ml\)). Please note that, for consistency of representation with other plots, these tracings have been inverted. B: bar graph showing the quantification of the \(I_{sc}\) changes elicited by LCA, and by FSK pretreated with DMSO or LCA. *P < 0.05 in \(\Delta I_{sc}\) compared with DMSO + FSK; \(n = 4\).

These cover the gamut from causing hepatic cholestasis to being both a promoter of cancerous growth and demonstrating antiproliferative functions (17, 47). Of all the naturally occurring bile acids, LCA is most poorly absorbed via enterohepatic recycling, and the bulk of it transits into the colon. With increasing evidence that bile acids serve a wider biological function as signaling molecules, an understanding of the regulatory role of LCA in the colon is of relevance.

It is well recognized that primary (CDCA) and secondary (DCA) dihydroxy bile acids regulate epithelial ion transport processes and, in particular, stimulate colonic Cl\(^{-}\) secretion (4, 31, 64, 75). In contrast, neither the \(\beta\)-epimer of CDCA (UDCA) the primary trihydroxy bile acid (CA), nor the secondary monohydroxy bile acid (LCA) (4, 41) directly activates Cl\(^{-}\) secretion. However, recently Kelly et al. (42) demonstrated that acute administration of UDCA (15 min) inhibited both FSK and CCH-stimulated Cl\(^{-}\) secretion in T84 cells by decreasing the activity and not expression of basolateral Na\(^{+}\)-K\(^{+}\)-ATPase and K\(^{+}\) channels. In the mouse colon, non-metabolizable UDCA attenuated the FSK and CCH secretory responses (42). The role of LCA is less clear, and Keely’s group reported LCA modulating secretagogue-stimulated Cl\(^{-}\) transport in a species-specific manner, potentiating CCH’s secretory response in T84 cells and human and mouse colon (42) while inhibiting it in the rat colon (77). Given the importance of LCA as a metabolite in the colon, in this paper, we focused on exploring further the cellular and ionic basis of the modulatory role of LCA in Cl\(^{-}\) secretion using T84 cells as a model system.

We first confirmed our earlier findings (4) and those of others (41) that LCA, at 50 or 500 \(\mu M\), did not directly alter net transepithelial \(I_{sc}\) in T84 cells. However, in contrast to the report of Kelly et al. (42), LCA did not have any effect on CCH-stimulated \(I_{sc}\) in our cells (Fig. 3D), but LCA proved to be a potent inhibitor of Cl\(^{-}\) secretion stimulated by cAMP-dependent secretagogues. Preincubation with LCA prevented the stimulation of \(I_{sc}\) by lubiprostone, FSK, CDCA, and 8-Br-cAMP. The results with 8-Br-cAMP suggest that LCA’s action is post-cAMP production, although an additional role in cAMP production cannot be ruled out. Furthermore, the dose-dependent action of LCA was rapid and equally important; LCA was capable of rapidly inhibiting FSK-stimulated \(I_{sc}\). The effects of LCA were clearly specific to this monohydroxy bile acid, since neither CA, CDCA, nor TDC altered FSK-stimulated \(I_{sc}\). The effects of LCA were clearly specific to this monohydroxy bile acid, since neither CA, CDCA, nor TDC altered FSK-stimulated \(I_{sc}\) (Fig. 5A). Not surprisingly, TLCA also did not elevate \(I_{sc}\) but inhibited FSK-stimulated \(I_{sc}\) (Fig. 5B). The physiological relevance of our observation is underscored by the facts that LCA acted at \(\leq 50\ \mu M\), and at these concentrations LCA did not affect cell viability and was not cytotoxic.

Our results in describing an antisecretory role for LCA need to be examined in context of those reported by Keely and collaborators (42). Similar to our findings, they reported that LCA attenuated \(I_{sc}\) (77) in the rat colon, which they ascribed to the presence of TGR5 receptors on the colonic epithelial cells. However, in the rat, LCA attenuated CCH- but not cAMP-stimulated Cl\(^{-}\) secretion, whereas in our study the reverse is true. Other similarities rest in the antisecretory actions of LCA (present study) and UDCA (39). Not surprisingly, the conjugated forms of these bile acids are more effective on the basolateral side, most likely due to the presence of BLM permeabilization studies suggest that LCA activates an AM Cl\(^{-}\) permeability, most likely CFTR, and inhibits a BLM K\(^{+}\) channel. It is unlikely that the BLM channel is KCNN4, since CCH was able to activate K\(^{+}\) permeability even in the presence of LCA, but not in the presence of clortrimazole.

**DISCUSSION**

The secondary bile acid, LCA, makes up about 1–4% of the bile acid pool and has been ascribed a variety of functions.


receptors and transporters (78). Furthermore, both UDCA and LCA act rapidly and appear to inhibit basolateral K\(^+\) conductances. However, unlike UDCA, LCA does not alter CCH-mediated Cl\(^-\) secretion, and its effects on FSK are not reversible in the short term (30 min after removal) but require many hours (18 h after removal for 80% recovery).

In contrast to their findings in rat, the same group of investigators demonstrated that LCA potentiated CCH-stimulated I\(_{sc}\) both in T84 cells and human colonic tissue, and implied a similar effect in mice (42). While the authors do not address the differences between their findings in rats, mice, and humans, species-specific differences in the actions of humoral agents are well documented. The segmental and species specificities are most likely due to critical differences in the content and distribution of membrane proteins, lipids, proteolipids, and lipid-regulating enzymes, and this has been well documented (60). Despite the aforementioned similarities, overall our observations in T84 cells and those of the Keely group (42, 77) underscore the inherent variability even in stable cell lines. One possible explanation is the source and passage number of the cells; as detailed in METHODS, our cells were obtained from ATCC, and we used them between passages 39 and 50. The source of the cells in the Keely papers was not listed but is presumed to be derived from the original cultures of K. Dharmasathaphorn via K. E. Barrett at the University of California San Diego. Such differences in responses in T84 cells have been reported earlier; for example, while we observed a distinct heat-stable E. coli enterotoxin (STa) and cGMP responsiveness (66), the group of Forte et al. (32) found that their T84 cells did not respond to STa. Likewise, Bijvelds (8) and we (5) demonstrated that lubiprostone uses the cAMP-CFTR pathway to activate Cl\(^-\) secretion in T84 cells, whereas Cuppoletti et al. (16) reported that lubiprostone employs a Ca\(^{2+}\)-CIC-2 pathway. In T84 cells, Hoque et al. (36) showed that FSK caused sustained elevation of [Ca\(^{2+}\)], while Yue et al. reported that it has no effect on [Ca\(^{2+}\)] (84).

An examination of the ionic basis of LCA’s antisecretory actions provided interesting results. Bilateral addition of LCA to BLM-permeabilized monolayers showed a slow increase in an apical Cl\(^-\) current. This current was inhibited by CFTRinh172. Subsequent addition of FSK caused a further small, albeit rapid, increase in this Cl\(^-\) current that was also inhibitable by CFTRinh172. Not surprisingly, this current change was significantly lower than that seen with FSK alone. The LCA results were surprising, since we saw no evidence of a change in net I\(_{sc}\) and suggested that LCA was simultaneously activating an opposing current that nullified these changes in AM I\(_{sc}\). A likely mechanism would be an inhibition of basolateral cation conductances, and it is well established that the secretagogue action of cAMP and Ca\(^{2+}\) involves the activation of distinct K\(^+\) channels (59, 74). In T84 cells, Devor (20, 22, 33), Tabcharani (70, 71), and Sheikh (68) demonstrated that the K\(^+\) channels involved in cAMP- and Ca\(^{2+}\)-mediated secretion are different; cAMP action involves the KCNE3/ KCNQ1 channel, and Ca\(^{2+}\) activates the KCNN4 and KCNMA1 (MaxiK) channels. Our results corroborate these findings since the KCNN4-activated K\(^+\) channel inhibitor clotrimazole affects only the marked and transient increase in BLM K\(^+\) current caused by CCH but not that caused by LCA.

The rapidity of LCA’s action implies a membrane event while its ability to act from both membranes suggests either a membrane permeation event followed by the activation of an intracellular receptor or direct membrane perturbation events. We explored and considered different candidate receptors, based on LCA’s action in a variety of biological processes. A possible membrane receptor mechanism for LCA action is transactivation of EGFR via muscarinic M3 receptors as demonstrated in H508 colonic cells (14). We also observe transactivation of the EGFR pathway as a step in CDCA-stimulated I\(_{sc}\) in T84 cells (25), and others have reported it as a mechanism for CCH (52) and FSK (7) action in these cells. However, neither atropine (Fig. 7A) nor AG-1478 (see RESULTS) attenuated the Cl\(^-\) secretion.
ated LCA inhibition of FSK-stimulated $I_{sc}$, thereby ruling out M3-EGFR signaling as a pathway for LCA action. If TGR5, the other membrane receptor, is involved as suggested by Ward et al. in the rat colon (77), then based on its canonical signaling pathway, one would predict that LCA will increase cell cAMP. As shown in Fig. 6, LCA alone had no effect on [cAMP]. Such a finding would also have to be reconciled with the observation that LCA inhibits 8-Br-cAMP-stimulated $I_{sc}$. The preferential action of the more hydrophilic-conjugated TLCA from the basolateral side suggests that this conjugated bile acid may be acting via a yet to be identified basolateral receptor.

Although the rapidity of action of LCA may preclude the classical notion of nuclear steroid receptors, we explored whether activation of FXR with GW-4064 had an effect on basal or secretagogue-stimulated $I_{sc}$. A 24-h pretreatment with GW-4064 (53) did not alter basal $I_{sc}$, and, as shown in RESULTS, neither a 15-min nor a 24-h preexposure to GW-4064 altered FSK-stimulated $I_{sc}$. The VDR is another intriguing candidate, since LCA has shown to be a potent ligand (50). As mentioned in the Introduction, VDR can serve both as a membrane-associated receptor causing rapid effects or can translocate to the nucleus to alter genomic function. Modeling studies attribute this to the flexibility of the vitamin D$_3$ molecule wherein the cis conformation preferentially binds to VDR on the membrane and the trans conformation preferentially binds to the nuclear VDR. In intestinal epithelia it has been localized both to the AM in the colon (80) and to the BLM in the small intestine (54). T84 cells possess VDR transcripts (unpublished observations), and pretreatment with the VDR agonist (calcitriol, 0.1 μM, 15 min) had no effect on FSK-stimulated $I_{sc}$. Although the results with calcitriol suggest that VDR may not be involved, it remains to be determined via modeling approaches whether LCA can represent another ligand conformation for the membrane VDR site. LCA has been shown previously to conformationally mimic acetylcholine for the M3 binding site (62). Our data suggest that LCA appears to act faster when added to the apical side than to the basolateral side ($t_{0.5}$ to achieve maximal inhibition: apical, 2.6 min and basolateral, 4.6 min; Fig. 4C), although eventually maximal inhibition is achieved from both sides. Although hypothetical, it is tempting to postulate that, since T84 cells are colonc in origin, LCA acting rapidly via VDR-like receptors near the AM may account for its preferential sidedness of action.

It is also possible that LCA may be acting by causing perturbations in the membrane that could directly alter the activity of membrane transporters such as CFTR and K$^+$ channels and/or trigger signaling cascades such as EGFR and/or alter the microdomain dynamics. It is well established that the lipid and protein composition of AM and BLM of epithelia are distinct, and, if LCA is acting as a membrane perturbant, this may account for the time-dependent differences in sidedness of action. Roles have been implicated for LCA in increasing cholesterol incorporation in the hepatocyte canalicular membrane (82, 83) and in altering phospholipid and sphingolipid homeostasis in LCA-induced cholestasis (51). Similarly, DCA-induced apoptosis has been linked to membrane perturbations, resulting in increased cholesterol accumulation, decreased fluidity, and caveolin-1 redistribution in membrane microdomains (38). Neither the hydrophilic CA nor UDCA caused membrane perturbations, and these authors did not examine the effects of the more hydrophilic LCA. These authors used the cholesterol sequestrant MβCD to delinate lipid raft involvement. Interestingly, MβCD in the presence of DCA reduced the latter’s action on apoptosis by 40%, but rapid washing out of MβCD resulted in only a 15% reduction of DCA’s effect (38). Thus, being derivatives of cholesterol, bile acids can also be sequestered by MβCD and should be taken into consideration in using this probe to delineate bile acid action. With this caveat, if LCA causes membrane perturbation, and sequesters cholesterol as suggested above (38, 82, 83), then MβCD treatment should block LCA action. However, our preliminary results indicate that overnight incubation with MβCD had no effect on LCA’s inhibition of FSK-induced $I_{sc}$. Although this does not completely rule out a role for LCA as a membrane perturbant, lipid rafts are probably not involved in LCA action. Collectively, these results leave us with the possibility that LCA may be acting in a cholesterol-independent fashion via an unknown receptor.

CFTR is often found to exist in microdomains where it complexes with transporters such as the epithelial sodium channel, solute carrier family 26 transporters, and multidrug resistance proteins via PDZ domain-containing scaffolding proteins. Of these, a transporter pertinent to our observations on LCA may be the multidrug resistance-associated protein 4, which regulates cAMP levels in the microdomain around CFTR (see below). A recent study opens up exciting possibilities as to how LCA may act locally in the AM to alter CFTR dynamics. Abu-Arish et al. (1) reported that CFTR is localized to different membrane domains (e.g., minimally, a cholesterol-dependent slowly diffusing domain and a fast-diffusing domain) and which are regulated differentially. Thus a perturbation of any of these domains by LCA could alter CFTR function. In fact, the faster rate of inhibition we see with apical vs. basolateral addition of LCA may be because LCA is causing rapid changes at the AM CFTR microdomain. Considering the pleiotropic actions of CFTR, an examination of the “CFTR-ome” (28) may be the focus of future studies.

In terms of intracellular signaling, as shown in Fig. 6, LCA alone had no effect on [cAMP], but inhibited the FSK-stimulated rise in [cAMP], by >95% (a 28-fold difference). Our values of [cAMP], are in keeping with those reported by other laboratories, with the caveat that there may be variability with passage number, time course, etc. (57, 65). For example, Rogers et al. (65) reported a 16-fold increase in cAMP (up to 160 pmol/mg protein) caused by 10 μM FSK in 2 min, and we had previously reported that 10 μM FSK treatment for 20 min increases [cAMP] to 429 ± 105 pmol/mg protein (4). Additionally, a submaximal dose of FSK (1 μM) caused an increase of ~250 pmol/mg protein after 8 min in 3T3-CFTR cells (37).

Furthermore, it is well known that a number of secretagogues, including FSK, at submaximal concentrations stimulate greater increases in $I_{sc}$ than cAMP. The classic characterization studies of T84 cells by Dharmasathaporn and colleagues (12) showed that, at a submaximal concentration of 10$^{-9}$ M, vasoactive intestinal peptide stimulated [cAMP], by 31 pmol/mg protein (10% of the maximal cAMP generated) and $I_{sc}$ by 14 μA (20% of the maximal current). We (5) and others (37) have also shown that FSK increases cAMP concentration far above that required for maximal Cl$^-$ transport. Thus, although FSK (10 μM) and lubiprostone (250 nM) caused similar maximal increases in $I_{sc}$ (~80 μA/cm$^2$), FSK caused a 10-fold higher increase in cAMP compared with...
Lubiprostone (5). Furthermore, in 3T3 fibroblasts transfected with CFTR, FSK caused maximal Cl$^-$/H11002 transport at doses (≈1 μM) lower than the 10 μM needed to cause maximal increases in cAMP concentration (37). Extrapolating from these observations, we can predict that the cAMP generated by LCA + FSK should have evoked a current bigger than that observed (3 μA). This suggests that LCA’s effects on $I_{sc}$ are not only due to an inhibition of cAMP generation, a conclusion that is supported by LCA inhibiting the actions of 8-Br-cAMP as well.

This still begs the question of whether LCA inhibits FSK-stimulated cAMP by inhibiting synthesis, stimulating degradation, or efflux. It is unlikely to be increasing phosphodiesterase activity, since IBMX failed to reverse the LCA effect. The multidrug resistance-associated protein MRP4 is known to be part of the CFTR complex, and inhibition of MRP4 prevents cAMP efflux and leads to augmentation of CFTR activity in response to low concentrations of adenosine (46). Higher concentrations of adenosine generate sufficient cAMP and are not affected by MRP4 activity. It is possible that LCA is increasing MRP4 activity, augmenting the efflux of cAMP out of the cell. T84 cells possess MRP2, -3, and -4 transcript and protein for MRP4. Addition of the MRP inhibitor MK-571 augmented CDCa stimulation of $I_{sc}$ (26). However, we think it is unlikely that LCA is acting via MRP4 for the following reason. While LCA does not alter transepithelial $I_{sc}$, it increases an apical Cl$^-$/H11002 current in nystatin-permeabilized cells, and, in a preliminary experiment, MK571 failed to reverse LCA action (data not shown).

In terms of inhibiting cyclases to decrease [cAMP], T84 cells appear to express all isoforms of adenylyl cyclase (43), and there are multiple ways in which adenylyl cyclase can be inhibited. Space limitations preclude us from discussing all of the options so a few are provided. Thus, it is conceivable that LCA could be activating a hitherto unidentified Goi-coupled G protein-coupled receptor (10), or by triggering βγ inhibition of cyclase (72) or by activating nitric oxide (NO). Casellas et al. (13) showed that bile acids increase NO release in humans, whereas Spirili et al. (69) showed that proinflammatory cytokines release NO in cholangiocytes to inhibit adenylyl cyclase and HCO$_3^-$ and Cl$^-$ secretion leading to ductular cholestasis. While it remains to be determined if LCA is acting directly on NO production, it is unlikely that LCA is acting via cytokines. Our unpublished observations (19) indicate that, at 50 μM, LCA does not alter cytokine production in T84 cells.

Another putative route for LCA action is via [Ca$^{2+}$/H11001$^+$], as shown for its action on chief cells acting via the M3 muscarinic receptor (62). In addition, activation of the bile acid receptor TGR5 by its specific agonist INT-777 increases [Ca$^{2+}$/H11001$^+$], in enteroendocrine cells (73) and in primary macrophages (56). In T84 cells, although LCA has a modest effect on increasing [Ca$^{2+}$/H11001$^+$], this cascade does not appear critical to its transport role, since BAPTA partially blocked the rise in [Ca$^{2+}$/H11001$^+$]; but failed to prevent the inhibitory action of LCA (Fig. 7A).

An initial phosphokinase array screening showed that LCA increased the phosphorylation of ERK1/2 (4.7-fold) and p38 (1.4-fold). We pursued the role of ERK by demonstrating that the marked stimulation of ERK phosphorylation was completely inhibited by the MEK inhibitor PD-98059. Surprisingly, this inhibitor did not alter the functional inhibitory action of LCA on FSK-mediated $I_{sc}$. It is likely that LCA acts via ERK to activate other biological functions. Likewise, there was no effect of SB-203580 on LCA action, ruling out the involvement of the p38 kinase pathway. Detailed analyses of the other signaling pathways are currently being pursued.

Despite our extensive efforts, the signal transduction mechanisms by which LCA acts still remain elusive. Our mechanistic studies have, however, ruled out some obvious processes. The responses to LCA are not due to apoptosis or cytotoxicity, since the tissues remain responsive to CCH after LCA administration. LCA is most likely not acting via TGR5 and the canonical increase in [cAMP]; in fact, it may be acting by partially decreasing FSK-stimulated [cAMP]. It is also unlikely that LCA is acting via the M3 muscarinic receptor, transactivating EGFR, or via FXR or VDR. The data that LCA-induced apical currents are inhibited by the specific CFTRinh172, and its inhibition of BLM K$^+$ currents does not affect CCH action, suggest that, if LCA were acting as a membrane perturbant, it must be highly localized to some spatial domains in both AM and BLM. This is plausible considering that CFTR is known to exist in signaling complexes (CFTR-ome) with kinases and other transporters and the recent report (1) that CFTR is localized to differentially regulated and separate membrane domains. This opens up exciting possibilities as to how LCA may act locally in the AM to alter CFTR dynamics and likewise at the BLM to alter K$^+$ channel activity. It is also probable that LCA is acting by an as yet unidentified receptor or a yet to be defined intracellular pathway. In terms of intracellular signaling, while LCA increases [Ca$^{2+}$/H11001$^+$]; and intracellular kinases such as ERK1/2, neither of these is involved in its ability to block FSK-induced $I_{sc}$.

These results are interesting from a physiological perspective, and their extrapolation to potential therapeutic strategies needs to be viewed in context of the other effects of LCA. LCA has been shown to have both beneficial and deleterious effects that depend on the concentrations, cell and tissue type, and species. Thus, higher concentrations of LCA cause cholestasis (51, 82, 83) and have been implicated in cancer (50). In contrast, LCA has antiproliferative effects in tumor growth (17, 47). With this caveat, the low dose of LCA (≤50 μM), its rapid action, ability to act from the apical surface, potent inhibition of all AMP-dependent secretagogues, and the fact that colonic bacteria generate LCA from CDCA and UDCA make further examination of this pathway an attractive potential antidepressant strategy.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

REFERENCES


