Chenodeoxycholic Acid Requires Activation of EGFR, EPAC and Ca\textsuperscript{2+} to Stimulate CFTR-dependent Cl\textsuperscript{-} Secretion in Human Colonic T84 Cells

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Running head: CDCA activates EGFR, EPAC, and Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} secretion

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Abstract:

Bile acids are known to initiate intricate signaling events in a variety of tissues, primarily in the liver and gastrointestinal tract. Of the known bile acids, only the dihydroxy species, deoxycholic acid and chenodeoxycholic acid (CDCA), and their conjugates, activate processes that stimulate epithelial Cl⁻ secretion. We have previously published that CDCA acts in a rapid manner to stimulate colonic ion secretion via protein kinase A (PKA)-mediated activation of the dominant Cl⁻ channel, the cystic fibrosis transmembrane conductance regulator (CFTR) (AJP 305:C447-56, 2013); however, PKA signaling did not account for the entire CDCA response. Here we show that in human colonic T84 cells, CDCA’s induction of CFTR activity, measured as changes in short-circuit current (Isc), is dependent on epidermal growth factor receptor (EGFR) activation, and does not involve the bile acid receptors TGR5 or FXR. CDCA activation of Cl⁻ secretion does not require Src, mitogen activated protein kinases, or phosphoinositide-3 kinase downstream of EGFR, but does require an increase in cytosolic Ca²⁺. In addition to PKA signaling, we found that the CDCA response requires a novel involvement of the exchange protein directly activated by cAMP (EPAC). EPAC is a known hub for cAMP and Ca²⁺ cross talk. Downstream of EPAC, CDCA activates Rap2, and changes in [Ca²⁺], were dependent on both EPAC and EGFR activation. This study establishes the complexity of CDCA signaling in the colonic epithelium, and shows the contribution of EGFR, EPAC and Ca²⁺ in CDCA-induced activation of CFTR-dependent Cl⁻ secretion.

Key Words: CDCA, EGFR, Ca²⁺, EPAC, CFTR
Introduction:
Regulation of hepatic bile acid synthesis and efficient recycling of bile acids by ileal reabsorption and enterohepatic circulation are necessary to maintain the systemic bile acid pool. In a healthy individual, ~5% of bile acids enter the colon, some of which are passively reabsorbed and the rest eliminated in the feces (12, 32). Disease conditions that result in bile acid malabsorption, such as irritable bowel syndrome (IBS) or short bowel syndrome, lead to pathological concentrations of bile acids in the colonic lumen. In the colon, excess bile acids increase motility, and secretion of water and electrolytes, resulting in a secretory diarrhea. IBS related diarrhea is estimated to affect about 30 million people worldwide. Of these, ~10 million have bile acid-associated diarrhea (26). Although there is such a high prevalence of bile acid-associated diarrhea in the global population, and despite the accumulating literature on bile acid physiology, the signaling mechanism underlying bile acid stimulation of Cl⁻ secretion is not fully understood.

Physiologically, bile acids are essential for lipid digestion and absorption, but more recently their role as signaling molecules has been recognized. Bile acids are derived from cholesterol and are amphipathic in nature. Their hydrophobic properties allow them to pass through the plasma membrane to exert direct genomic effects via the bile acid-specific nuclear farnesoid X receptor (FXR). They can also act via the bile acid-specific membrane G-protein coupled receptor, TGR5. Interestingly, bile acids also activate other, non-bile acid-specific receptors, including the nuclear Vitamin D3 and pregnane X receptors, as well as the G-protein coupled muscarinic and sphingosine-phosphate receptors (18). Of note, taurine conjugated deoxycholic acid (TDCA) stimulates transactivation of the epidermal growth factor receptor (EGFR) via muscarinic receptors (16, 17), whereas unconjugated DCA can also activate EGFR without an intermediary transactivation step (34, 59). Thus bile acids modulate a variety of processes ranging from lipid handling to colorectal cancer (73). Despite the variety of receptors and effects elicited by bile acids, none of these receptors have been directly implicated in bile acid-induced Cl⁻ secretion and the resulting secretory diarrhea.

It is well documented that only the 7α-dihydroxy bile acids, chenodeoxycholic acid (CDCA) and DCA, exert pro-secretory effects (6, 25, 38, 68). DCA stimulation of Cl⁻ secretion has been shown to be Ca²⁺-dependent in both adult rabbit colonocytes (68) and human colonic T84 cells (23, 25). We previously demonstrated that in the rabbit colon, TDCA action is IP₃-Ca²⁺-protein kinase C (PKC)δ-, but not cAMP-, dependent (36, 68). Structure-activity studies of bile acid action show that CDCA, DCA, and their taurine conjugates primarily stimulate secretion when added to the basolateral (serosal) side of T84 cells (6, 38). Using mass spectrometry,
Keely et al. (38) reported that DCA, TDCA, and the 7β-dihydroxy bile acid, ursodeoxycholic acid (UDCA) showed greater uptake into cells from the basolateral side, although UDCA did not stimulate Cl⁻ secretion. In T84 cells, the cystic fibrosis transmembrane conductance regulator, CFTR, is the predominant apical Cl⁻ current, and while CIC-2 Cl⁻ channels and TMEM16A (42) have been implicated in some studies, they do not appear to contribute to transepithelial Cl⁻ currents in our studies ((7) and this paper). We recently explored the intracellular signaling mechanism underlying CDCA-stimulated Cl⁻ secretion in T84 cells, since CDCA had a greater response compared to DCA in our preparations (6). Our findings demonstrated that CDCA action required intact microtubules and CDCA acted via the protein kinase A (PKA) pathway to phosphorylate and stimulate CFTR. We also found that T84 cells express FXR, TGR5 (6), vitamin D3 receptor (unpublished data) and the broad-range, muscarinic M3 receptor (31). However, M3 receptors are not involved in the secretagogue action of bile acids as the inhibitor, atropine, neither altered CDCA- (6), nor DCA-stimulated Cl⁻ secretion (38).

An intriguing finding of our previous study was that the PKA inhibitor H89 rendered the CDCA response transient and did not completely block activation of Cl⁻ secretion, suggesting additional signaling pathways (6). The adenylyl cyclase activator forskolin, known to stimulate cAMP-PKA-dependent activation of CFTR, has recently been shown to also activate the exchange protein directly activated by cAMP (EPAC) to increase [Ca²⁺], and modify Cl⁻ transport in T84 cells (33, 62). Crosstalk between second messenger cascades, especially cAMP and Ca²⁺ has been studied in a variety of cell types. In T84 cells (15) and rat pancreatic acinar cells (67), Ca²⁺ and cAMP have a synergistic effect. In mouse macrophages (55) and in neuronal cells (40), bile acids acting via TGR5 increase both cAMP and [Ca²⁺], to alter function.

Therefore, we hypothesized that along with PKA signaling, EPAC, Ca²⁺ and other kinases may be contributing to the CDCA response. The objective of this study was to further investigate the role of a potential receptor-mediated pathway, and to elucidate the involvement of PKA-independent signaling cascades in CDCA-stimulated Cl⁻ transport in human colonic T84 cells. The data presented here suggest a role for EGFR, Ca²⁺, and EPAC, but not FXR, TGR5, ERK1/2, p38, PKC, and PI3K in CDCA’s action. These findings provide novel insights into the complexity of CDCA action on colonic Cl⁻ secretion.

Materials and Methods

Materials

Tissue culture media: Dulbecco’s Modified Eagle Medium (DMEM), Ham’s F-12 nutrient mixture, and bovine calf serum were purchased from Invitrogen (Carlsbad, CA). Transwell
inserts (24-well and 6-well), and culture dishes were purchased from Corning Inc. Life Sciences (Lowell, MA). Rat tail collagen, bovine serum albumin, chenodeoxycholic acid, forskolin, carbachol, CFTRinh-172, nystatin, ciprofloxacin, GW4064, AG1478, U73122, ESI-09, H89, PD98059, LY294002, pluronic F-127, wortmannin, and protease inhibitor cocktail (P8340), phosphatase inhibitor cocktails 2 & 3 were purchased from Sigma-Aldrich Corp (St. Louis, MO). The inhibitor 2-APB was bought from Santa Cruz Biotech Inc. (Santa Cruz, CA). H1152 was purchased from Enzo Life Sciences (Farmingdale, NY). GGTL298 was purchased from Tocris Bioscience (Pittsburgh, PA). The protein kinase C inhibitors rotellerin and chelerythrine were obtained from Millipore Calbiochem (Danvers, MA). Lipofectamine 2000 and Opti-MEM were purchased from Thermo Fisher Scientific Inc., while BAPTA-AM and Fura2-AM were purchased from Molecular Probes of Thermo Fisher Scientific Inc. (Hanover Park, IL). EGFR specific siRNA (HsEGFR 11) and negative control siRNA were synthesized by and purchased from Qiagen (Valencia, CA). Primary antibody to GAPDH was obtained from Novus Biologicals (Littleton, CO). SB203580, phospho-specific and non-phospho specific antibodies to EGFR (Y1068), ERK1/2 (T202/Y204), and p38 (T180/Y182) were purchased from Cell Signaling (Boston, MA). Human Phospho-Kinase Antibody Array was purchased from R&D systems (Minneapolis, MN). HitHunter Inositol (1,4,5)-Triphosphate assay was obtained from DiscoveRx (Fremont, CA). Unless otherwise specified, all other reagents were of analytical grade and were purchased from either Sigma-Aldrich Corp. (St. Louis, MO) or Fisher Scientific (Hanover Park, IL).

**Cell Culture**

Human colon carcinoma T84 cells were acquired from the American Type Culture Collection (Manassas, VA, USA) and were cultured in media containing equivalent amounts of DMEM and F-12, supplemented with 6% bovine calf serum, ampicillin (8μg/mL), penicillin (100 U/mL) and streptomycin (100μg/mL) as described previously (5). These cells are considered female as determined by RT-PCR amplification of the amelogenin gene (51). Cells (passages 39-50) were incubated in a humidified atmosphere of 95% O₂/ 5% CO₂ at 37°C. For Ussing chamber studies, cells were seeded at a density of ~250,000 cells per Transwell insert (6.5mm, 0.4μm pore size, 24-well plate) coated with rat-tail collagen. For immunoblot studies, cells were grown on 6-well Transwells (24.5mm, 0.4μm pore size) or 6-well plates at a density of 1.5x10⁶ cells per insert. T84 cells were serum starved overnight prior to immunoblot or Ussing chamber studies.
**siRNA Transfection**

T84 cells were plated in 6-well plates to ~50% confluency and transiently transfected with 100pmol of predesigned (Qiagen) scrambled siRNA (SCRsiRNA) or EGFR siRNA as described by Annaba et al. (4). Briefly, Opti-MEM medium was used to dilute the siRNA and Lipofectamine 2000 transfection reagent. The diluted siRNA and Lipofectamine 2000 were then combined in a 1:1 ratio and incubated for 5 minutes at room temperature to allow for the siRNA to be taken into the liposomes. After incubation, the siRNA-lipid complex was added to the cells for transfection. After 24 hours, Opti-MEM media containing the transfection reagent was removed and replaced with regular DMEM/F12 (composition listed above). After a further 48 hours (72 hours after transfection) cells were harvested for protein or used to measure CDCA-stimulated Cl⁻ transport by iodide efflux (described below).

**Electrophysiological Measurements of Short Circuit Current (Iₛₑ) in Ussing Chambers**

Electrophysiological measurements were performed as previously described (6). Briefly, T84 cells were cultured as described above, and transepithelial resistance (Rᵣ: Ω·cm²) was routinely monitored using an EVOM² voltohmmeter and a STX2 chopstick electrode (World Precision Instruments, Inc. Sarasota, FL). When Rᵣ reached values ≥ 1000 Ω·cm² (~14-21 days), inserts were mounted in Ussing chambers (area: 0.33 cm²; Physiologic Instruments, Inc., San Diego, CA) and bathed in 5mL (per reservoir) oxygenated buffer (95% O₂, 5% CO₂). The buffer consisted of (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 D-glucose, at 37°C. Short-circuit current (Iₛₑ; µA/cm²) and Rᵣ were measured over the course of the experiment, as previously described (6). For inhibitor studies, monolayers were pretreated for 30 minutes with bilateral addition of the inhibitor(s), or with 0.1% DMSO as vehicle control, unless otherwise stated. Following equilibration with the inhibitors, CDCA (500µM) was added to the basolateral reservoir. Forskolin (10µM) and carbachol (100µM) were then added sequentially as an index of cell responsiveness and viability. For measurements of apical Cl⁻ currents, we used the nystatin permeabilization method as described by Anderson and Welsh (2) and adapted by us (6). We established a Cl⁻ gradient by mounting monolayers in an apical buffer containing 115.4mM NaCl and a basolateral buffer where the NaCl was replaced with equimolar sodium gluconate. To account for potential Ca²⁺ chelation by gluconate, the concentration of CaCl₂ was increased to 2mM in the basolateral buffer. The monolayers were then permeabilized by addition of 200µg/ml nystatin to the basolateral surface. Once monolayers were equilibrated, they were treated with 0.1% DMSO or
10μM ESI-09 and stimulated with CDCA and forskolin. CFTRinh172 (10μM) was added in the end to assess the contribution of CFTR.

Iodide Effluxes

We used the previously established method of iodide efflux to measure Cl⁻ transport in transfected T84 cells (6, 10, 28); this method does not require the use of resistive monolayers. Transfection of siRNA is most effective at 72 hours post transfection (~7 days after plating), a time when the monolayers do not meet the minimal Rₜ needed for studies in Ussing chambers (Rₜ of ≥1000 Ω·cm²). At 72 hours post transfection, media was removed and cells were incubated with iodide containing buffer (in mM: 136 NaI, 3 KNO₃, 2 Ca(NO₃)₂, 11 glucose and 20 HEPES, pH 7.4) for 1 hour at room temperature in the dark. After iodide loading, the iodide-containing buffer was aspirated and wells were washed three times with iodide efflux buffer (same as iodide-containing buffer but NaNO₃ replaced NaI). Cells were then exposed to iodide efflux buffer (1mL) containing DMSO (0.1%), CDCA (500µM), or forskolin (10µM) after baseline efflux measurements. Efflux buffer collected at 2-minute intervals was saved and replaced with fresh efflux buffer. At the end of the experiment the wells were washed with PBS, and cells were harvested for protein and expression of EGFR was assessed by immunoblotting using an EGFR specific antibody. An iodide-sensitive electrode (Orion 96-53, Fisher Scientific) and a pH/mV meter were used to measure the amount of iodide in the buffer that was collected. Iodide concentration was calculated based on a standard curve as previously described (10) and depicted as the fold change in mean cumulative iodide efflux ± SEM relative to the starting point (28).

IP₃ measurements

Generation of IP₃ was measured using the HitHunter Inositol (1,4,5)-Triphosphate Assay. As per the manufacturer’s instructions, T84 cells were grown in a T25 flask (Corning) until confluency. Cells were then trypsinized and counted. Cells were brought to a final concentration of 2x10⁶ cells/mL in PBS (without CaCl₂ or MgCl₂) and were aliquoted into microcentrifuge tubes for agonist treatment for different time periods. Perchloric acid was added to stop the reaction. Samples and IP₃ standards were aliquoted into individual wells of a 96-well plate. IP₃ tracer (green) and IP₃ binding protein were added sequentially with a 5-minute incubation for each. The solutions were mixed using a microplate shaker at 650 RPM. The fluorescence polarization signal was read using the Tecan Infinite 200 Pro multimode reader (Tecan US Inc., Morrisville, NC) with the following settings as per manufacturer’s instructions: excitation filter fluorescein at
485nm, emission filter fluorescein at 530nm, and the dichroic fluorescein at 505nm. A standard curve was plotted using a 4 parameter best-fit analysis. With the DiscoveRx fluorescence polarization technology, IP₃ generated by the cells competes with the IP₃ tracer for the IP₃ binding protein. In principle, in the absence of additional IP₃, the tracer will tumble more slowly and result in a higher polarized signal. As IP₃ in the sample increases, the unbound IP₃ tracer will increase, making it tumble faster and thereby lower the polarized signal. Thus the polarization signal is inversely proportional to the IP₃ produced by the cells.

**Immunoblotting**

T84 cells were grown to confluency in 6-well plates and treated with various agonists and antagonists (see results for details). After treatments, cells were washed with cold PBS and lysed in a buffer containing in mM: 25 Tris-HCl (pH 7.4), 1 EDTA, 2 MgCl₂, 5 β-mercaptoethanol, 1 DTT, and protease and phosphatase inhibitor cocktail (10µl/1mL lysis buffer). The lysates were sonicated (Branson Sonifier Cell Disruptor Model 350; Fisher Scientific, Hanover Park, IL) for 30 seconds on ice and centrifuged at 2,000 x g for 10 minutes (4°C). The supernatant (post-nuclear, total cell lysate) was saved and protein concentrations were measured by the Bio-Rad Protein Assay protocol. Protein fractions (50µg unless specified otherwise) were separated by SDS-polyacrylamide gel (7.5% or 4-15%) electrophoresis and subjected to Western blotting protocol as described previously (6, 28). Blots were blocked with 5% milk or with bovine serum albumin (for phospho-specific antibodies) made in Tris-buffered Saline-Tween 20 (TBST; 50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween-20) for 1 hour at room temperature. Primary antibodies were made in a 1% blocking solution and incubated at 4°C overnight. Blots were washed with TBST (6x 5 minutes). Secondary antibodies conjugated to horseradish peroxidase (in 1% blocking solution) were incubated with the blots for 1 hour at room temperature and then washed with TBST (6x 5 minutes). Resulting signals were visualized with Pierce SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL). When needed, Pierce SuperSignal West Femto Chemiluminescent Substrate Kit was used. For blots where phosphorylated proteins were screened initially, the membranes were then stripped of the antibody in a buffer containing 100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl (pH 6.7) for 15 minutes, 55°C. The stripped blots were then treated as naïve blots, and subjected to blocking followed by exposure to primary antibodies for the unphosphorylated protein. Immunoblot bands were quantified by Image J software after scanning densitometry.
Phosphokinase Array

A Human Phospho-Kinase Antibody Array was performed according to the manufacturer’s instructions (R&D Systems). When T84 cells reached ~90% confluency in 100mm dishes, they were treated with either 500µM CDCA or 0.1% DMSO (as control) for 5 minutes to induce kinase phosphorylation. Cells were then washed with cold PBS and lysed with the lysis buffer provided. Cell lysates were centrifuged (14,000 x g, 5 minutes, 4°C) and the resulting supernatant was collected. Equal amounts of the supernatant were incubated overnight with the phospho-kinase array membranes. The membranes have “capture” antibodies that will bind to their respective proteins in the cell lysate. Similar to standard immunoblotting protocol (see above), membranes were washed and then exposed to a cocktail of detection antibodies. Signals were detected using streptavidin-HRP and chemiluminescent reagents provided by the manufacturer. The pixel density of each “spot” was determined using ImageJ, and duplicate spot signals were averaged. β catenin was used to normalize the kinase signals as its phosphorylation state remained unchanged. Normalized signals were compared between the DMSO and CDCA treated samples.

[Ca^{2+}], measurements

T84 cells were seeded on 2-cm² glass bottom dishes (MatTek Corporation, MA, USA) for 3 to 4 days. The cells were loaded with 5 µM Fura2-AM (Molecular Probes) in serum-free DMEM/F12 (+0.02% pluronic F-127) for 1 hour in a tissue culture incubator at 37ºC. To remove any extracellular or membrane bound Fura2-AM, dishes were washed three times with Krebs-Ringer-Hepes buffer (KRH) containing in mM: 120 NaCl, 5.4 KCl, 0.8 MgCl₂, 1 CaCl₂, 11.1 glucose and 20 Hepes, pH 7.4. Dishes were then filled with fresh KRH and equilibrated at room temperature for 15 minutes and mounted on a dual channel temperature controlled (Warner Instruments, Hamden, CT) platform of a fluorescence microscope (Olympus IX51, Olympus America, Center Valley, PA). Ca^{2+} agonists (e.g., CDCA) in KRH buffer were delivered to the cells by a perfusion and vacuum system (Warner Instruments). Ca^{2+} signals were captured using a Q-8 spectrofluorometer system (Photon Technology International; Edison, NJ), and were recorded as the change in fluorescence ratios (Exλ: 340/380 nm; Emλ: 505 nm) (14, 28, 68). The amount of [Ca^{2+}], was calculated based on a standard curve of fluorescence ratios to Ca^{2+} concentrations. To probe the influence of signaling pathways, cells were pretreated with signaling specific inhibitors during the final 30 minutes of Fura2-AM loading and the inhibitor was included in the KRH in subsequent steps of the procedure. Some of the experiments used
a Photon Technology International -Ratiomaster spectrofluorometer and Nikon TE200 inverted fluorescence microscope (Nikon, Melville, NY) with the same wavelength settings.

**Rap Activation Assay**

To assess CDCA activation of the small GTP-ase Rap2, Rap activation assays were performed, on T84 cells grown on 10cm dishes to 80-90% confluence, according to the manufacturer’s instructions (Cell Biolabs, Inc.). Cells were stimulated with 500µM CDCA (0-20 minutes) and then washed with PBS. Plates were then incubated on ice with 1X lysis buffer provided by the manufacturer. Samples were transferred to microcentrifuge tubes and centrifuged at 14,000 x g for 10 minutes at 4°C. The protein concentration of the supernatant was determined by the Bio-Rad Protein Assay as described above. An aliquot of 5mg of total lysate was then incubated with agarose beads conjugated to an activated Rap binding domain for 1 hour at 4°C. After incubation, the beads were pelleted by centrifugation for 30 seconds at 14,000 x g at 4°C and supernatant was removed. Beads bound to activated Rap were then washed with 1X lysis buffer (3x) for 30 seconds at 14,000 x g. After the final wash all excess lysis buffer was aspirated and beads were resuspended in 2X loading dye and boiled for 5 minutes. Samples were then subjected to SDS-PAGE and immunoblotting with Rap2 antibody (1:500 antibody dilution in 1% milk) as described above.

To test efficacy of the assay, untreated cells were harvested for protein in the same manner described above. The protein concentration of the total lysate was measured, and 1.5mg of protein was aliquoted into two separate tubes with 0.5M EDTA (20µL). Either 10µL of 10mM GTPγS (positive control) or 100mM GDP (negative control) were added and the tubes were incubated for 30 minutes at 30°C. The reaction was stopped by addition of 65µL of MgCl₂. Lysates were then incubated with the agarose beads as described above for the CDCA-treated samples and immunoblotted for Rap2.

**Statistical analysis**

Data from at least three individual experiments were analyzed and presented as mean ± SEM. Statistical significance was determined using unpaired Student’s t-test or 1-way ANOVA with multiple comparisons post tests by Dunnett’s or Tukey’s as specified in the figure legends. Only values of p<0.05 were considered statistically significant and are denoted with an * or # or $.
Results

CDCA action does not involve TGR5 and FXR

We previously reported that CDCA (500µM) causes a rapid (< 5 minutes) increase in Cl’ secretion in T84 cells via CFTR and that these cells express transcripts for the bile acid-specific receptors TGR5 and FXR, and protein for TGR5 (6). While T84 cells largely express CFTR as the dominant Cl’ channel, they also express the Ca2+-activated Cl’ channel TMEM16A (23). However, CDCA-induced Cl’ secretion was not inhibited by the TMEM16A antagonist AO1 (ΔIsc, μA/cm2: CDCA: 7.9±1.4; +AO1: 10.2±2.5; n=7, p=0.44). Because CDCA has a rapid effect on Cl’ secretion we hypothesized involvement of a plasma membrane receptor; more specifically, that TGR5, and not FXR, may be involved. Currently there are no available antagonists to either receptor, so specific agonists were used to compare activation of Cl’ secretion to that of CDCA. GW4064 is an FXR agonist with an EC50 of 15nM (45) and has been used at micromolar concentrations in a variety of cell types (41, 63), including 5µM GW4064 in T84 cells (50). As per our prediction that CDCA may be acting via a membrane receptor, it was not surprising that the nuclear receptor FXR agonist GW4064 (5µM) did not affect Cl’ secretion compared to vehicle DMSO as control (Table 1). We next examined if the TGR5 specific activators, ciprofloxacin (19) and the monohydroxy bile acid lithocholic acid (LCA), would stimulate Isc. Ciprofloxacin was determined to be a TGR5 agonist by in silico screening (19), based on its ability to stimulate cAMP production only in those cell types that express TGR5 (EC50 8µM-10µM). As shown in Table 1, while CDCA significantly increased Cl’ secretion, ciprofloxacin (20µM) had no effect. Furthermore, we have recently shown that LCA, the most potent bile acid agonist of TGR5, also does not increase secretion (1-500µM; (6)), but does alter cAMP-dependent Isc in T84 cells (5). These results suggest that neither the transmembrane receptor, TGR5, nor the nuclear receptor, FXR, are involved in CDCA-induced Cl’ secretion.

EGFR plays a role in CDCA activation of CFTR

In T84 cells, both the Gαs-linked vasoactive intestinal peptide (VIP) receptor (8) and the Gαq linked muscarinic receptor (47, 65) transactivate EGFR to alter Cl’ secretion. Therefore, we determined if EGFR mediated CDCA’s activation of Cl’ secretion. T84 cells were pretreated with the EGFR inhibitor AG1478 (1µM), to block autophosphorylation, and then stimulated with CDCA. AG1478 significantly inhibited (58%) CDCA’s increase in Isc (Figure 1A). Concomitantly, the representative blot shows that CDCA increased EGFR autophosphorylation of tyrosine 1068 in a time-dependent manner (Figure 1B; fold of t=0: 2: 2.8 ± 1.1, 5: 2.6 ± 1.4, 10: 1.9 ± 0.5; n=4), and this phosphorylation was reduced by AG1478 (Figure 1C; blot representative of n=4).
AG1478 also inhibited carbachol-stimulated EGFR phosphorylation, used as a positive control (Figure 1C). Since transactivation of EGFR may occur via cleavage of EGFR ligands by matrix metalloproteinases (MMPs) (17), we examined the inhibition of MMPs by batimastat (20µM). As shown in Table 2, batimastat significantly reduced the CDCA response by 68% (p=0.04). Of note, bilateral addition of EGF (16.3nM), a potent ligand of EGFR, did not increase $I_{sc}$ (in µA/cm²: -0.08 ± 0.08; n=4), nor did it significantly alter CDCA’s response (in µA/cm²: CDCA: 19.3 ± 1.7, EGF+CDCA 15.7 ± 1.2; p=0.13, n=4). These data suggest that CDCA requires transactivation of EGFR via MMPs, but does not appear to involve EGF as a ligand.

To validate that EGFR plays a role in CDCA action, we used siRNA to knockdown EGFR expression. As shown in Figure 1D, EGFR siRNA, but not scrambled control siRNA (SCRsiRNA), decreased EGFR protein expression by 56% (n≥6, p=0.02). To confirm whether knockdown of EGFR affected CDCA-induced $I_{sc}$ transport, we performed iodide efflux assays. Figure 1D shows the CDCA-stimulated cumulative iodide efflux in cells transfected for 72 hours with EGFRsiRNA or control SCRsiRNA. CDCA significantly increased iodide efflux in cells transfected with SCRsiRNA (solid black line) compared to DMSO-treated cells transfected with SCRsiRNA or EGFRsiRNA. However, in cells transfected with EGFR specific siRNA, the CDCA response was decreased significantly at 12 minutes compared to the CDCA response in SCRsiRNA cells (p<0.05) (dotted black line), confirming EGFR’s role in CDCA-induced $I_{sc}$ transport. It is important to note that the CDCA-induced iodide efflux in control non-transfected T84 cells is attenuated by 84% with 10µM CFTRinh172 pretreatment (rate of iodide efflux normalized to basal efflux: control: 0.43±0.2; CDCA: 0.73±0.08; CDCA+CFTRinh172: 0.48±0.2; n=3).

**Src, MAPKs, and PI3K signaling are not involved in CDCA-Induced $I_{sc}$ secretion**

As EGFR signals via a multitude of pathways, we assessed the activation of several kinases simultaneously by performing a human phospho-kinase array. The array includes 43 different kinases, with several that are well-known downstream mediators of EGFR action. The kinases include Src kinase, the mitogen activated protein kinases (MAPKs) extracellular signal related kinases 1/2 (ERK 1/2) and p38, and protein kinase B (AKT). As shown in Figure 2A, CDCA (500µM, 5 minutes) elicited a strong increase in EGFR and p38 phosphorylation and a small increase in ERK 1/2 phosphorylation, but did not alter phosphorylation of Src or AKT. The phospho-kinase array confirmed the increase in EGFR phosphorylation shown in Figure 1.

To explore the array results further, we investigated the effects of kinase-specific inhibitors on CDCA-induced $I_{sc}$ current in Ussing chambers, and performed immunoblot studies.
using phospho-specific and total kinase-specific antibodies. As suggested by the phospho-
kinase array, pretreatment of T84 cells with the Src inhibitor PP2 (10µM) did not affect CDCA’s
increase of $I_{sc}$ (Table 2; n=3, p>0.05). Furthermore, as predicted by the array, Figure 2B shows
that CDCA caused a time dependent and robust increase in p38 phosphorylation with a
significant increase at 10 minutes compared to t=0’ (fold of t=0: 2’: 9.3 ± 5.8, 5’: 10.3 ± 6.0, 10’:
8.2 ± 1.9; n=4). The pharmacological p38 antagonist, SB203580 (10µM), has been extensively
used in T84 cells to block p38 signaling (20, 21). SB203580 acts by blocking p38 catalytic
activity at the ATP binding site and does not inhibit the phosphorylation of p38 by its kinase
MKK. Surprisingly 10µM SB203580, a concentration known to inhibit p38 in T84 cells, had no
effect on CDCA stimulation of $I_{sc}$ (Figure 2C).

The CDCA increase in ERK 1/2 phosphorylation indicated by the phospho-kinase array
was confirmed in a time course using phospho-specific and total antibodies to ERK 1/2. CDCA
caused a transient increase in ERK 1/2 activation, with a significant increase at 5 minutes
compared to t=0 (Figure 3A; fold of t=0’: EGF: 2.4 ± 0.7; CDCA 2’: 2.1 ± 0.5; 5’: 3.6 ± 0.6; 10’:
2.5 ± 1.0; n=3). In the MAPK signaling cascades, the proximal step to ERK 1/2 activation is
phosphorylation of ERK 1/2 by the MAPK kinase, MEK. Both the CDCA-stimulated and EGF-
stimulated (positive control) phosphorylation of ERK 1/2 was inhibited by the MEK inhibitor
PD98059 (Figure 3B), confirming activity of the inhibitor. Most striking was the fact that despite
the efficacy of the MEK inhibitor on CDCA-induced ERK 1/2 phosphorylation, there was no
effect of PD98059 on CDCA-stimulated Cl‘ transport (Figure 3C). The literature (30, 72)
suggests that ERK 1/2 and p38 kinases can cross talk, such that when one pathway is inhibited,
the other can compensate. To investigate if this occurs in CDCA-induced $I_{sc}$, T84 cells were
pretreated with inhibitors of both pathways simultaneously. However, the combination had no
effect on CDCA action (Figure 3D). So although CDCA increases activation of these kinases,
they are not necessary for Cl‘ secretion.

Since Src, ERK 1/2, and p38 kinases did not appear to be involved in CDCA’s secretory
action, we investigated the involvement of phosphoinositide-3 kinase (PI3K) as another
downstream effector of EGFR activation. Using wortmannin and LY294002 as PI3K inhibitors,
Bertelsen et al. (8) demonstrated that cAMP agonists stimulate PI3K via transactivation of
EGFR, and from this implied that AKT was involved. Since CDCA similarly activates the cAMP
cascade, we used these inhibitors to determine if CDCA action involves PI3K. Interestingly, we
found that wortmannin (500nM) significantly attenuated the CDCA response by 55% (Figure
4A). However, pretreatment with LY294002 (20µM-100µM) had no effect on CDCA action
(Figure 4B). PI3K is commonly associated with activation of AKT, therefore we examined the
effects of the AKT inhibitor MK2206 (1μM). Not surprisingly inhibition of AKT did not alter the
CDCA response (Figure 4C). These data suggest that CDCA does not act via the PI3K-AKT
signaling cascade.

**Ca2+ signaling is necessary for CDCA action**

Several studies, including ours in the rabbit colon (36, 68) and others in T84 cells (23, 25)
demonstrated that TDCA stimulates Ca2+-dependent Cl⁻ transport. To determine if CDCA-
induced Cl⁻ secretion is also Ca2+-dependent, we first explored if phospholipase C (PLC) activity
is required for the CDCA response. Inhibition of PLC by U73122 (10μM) attenuated CDCA -
induced Iₛₑｃ. U73122 also inhibited action of the muscarinic Gαₛ-coupled receptor agonist
carbachol (CCH; Figure 5A). Activation of PLC converts PIP₂ to diacyl glycerol and IP₃, and the
former can stimulate the PKC cascade. We had previously shown PKCδ involvement in bile acid
action in the rabbit colon using rottlerin and a PKCδ-specific inhibitor peptide (36). Therefore,
we pretreated T84 cells with rottlerin (10μM) and assessed the CDCA response. In contrast to
rabbit colonocytes, rottlerin had no effect on CDCA-stimulated Iₛₑ𝐜 in T84 cells (Table 2; p>0.05).
To determine if non-PKCδ isoforms, may be involved, we pretreated the cells with varying
concentrations of the general PKC inhibitor chelerythrine. Interestingly, chelerythrine also had
no significant effect on CDCA-induced Iₛₑᶜ (Figure 5B). Since activation of PLC leads to
production of IP₃, we stimulated T84 cells with CDCA. Interestingly, we found that CDCA
significantly increases IP₃ compared to vehicle control (Figure 5C). Thus, PLC, but not PKC,
activity is necessary for CDCA action.

Once IP₃ is produced, it can bind to its receptor on the endoplasmic reticular membrane
to release Ca²⁺ from intracellular stores. To assess whether this Ca²⁺ release is necessary for
CDCA action, we inhibited the IP₃ receptor with 2-APB (50μM) and found that the CDCA
response was significantly attenuated, implying a role for IP₃ receptor activation (Figure 5D).
Changes in free cytosolic Ca²⁺ ([Ca²⁺]ᵢ) in response to CDCA were measured using Fura2. As
shown in Figure 5E, CDCA increased [Ca²⁺]ᵢ as compared to basal levels. Furthermore, this
increase in [Ca²⁺]ᵢ is significantly decreased by removal of extracellular Ca²⁺, (0mM [Ca²⁺]ₒ)_(Fig.
5E). Additionally, chelation of free [Ca²⁺] by pretreatment with BAPTA-AM reduced the
CDCA-induced [Ca²⁺]ᵢ and the CDCA-induced Iₛₑᶜ (Figure 5E & F respectively). While the CDCA
response in the presence of 1mM [Ca²⁺]ₒ +BAPTA-AM was not different from basal values, the
CDCA response in the absence of [Ca²⁺]ₒ was significantly increased compared to basal values
and compared to 1mM [Ca²⁺]ₒ +BAPTA-AM. Interestingly, inhibition of EGFR by AG1478
significantly decreased the CDCA-induced changes in [Ca²⁺]ᵢ by 45% (Figure 5G). The data in
this section confirm that Ca\textsuperscript{2+} signaling plays a role in CDCA stimulation of Cl\textsuperscript{−} secretion in T84 cells.

**EPAC mediates CDCA-stimulation of I\textsubscript{sc}**

We next examined how Ca\textsuperscript{2+} signaling relates to our earlier findings that cAMP-PKA signaling is involved in CDCA stimulation of Cl\textsuperscript{−} transport. As Figure 5F shows that [Ca\textsuperscript{2+}]\text{\textsubscript{i}} accounts for 79% of the response, we re-examined the magnitude of the PKA contribution. Figure 6 of our earlier report (AJP 305:C447-56, 2013; (6)), showed that H89 caused the CDCA response to become transient and decreased the plateau phase; this decrease measured at a single time point, 30 minutes after CDCA addition, was 94%. Recently we found that LCA had a similar inhibitory effect, causing the CDCA response to become transient (5), and we reported these results as area under the curve (AUC) rather than at a single time point. Therefore we performed additional measurements of the CDCA responses ± H89 (30µM) and calculated the AUC.

Pretreatment with H89 resulted in a 62% inhibition of the entire CDCA response (%AUC: CDCA: 100; +30µM H89: 38.3 ± 8.8; n≥8, p<0.0001), suggesting that PKA is only partially responsible for CDCA’s stimulation of Cl\textsuperscript{−} secretion via CFTR.

Not all cAMP signaling occurs via PKA, and cAMP is known to activate the guanine nucleotide exchange factor, EPAC, which can also increase [Ca\textsuperscript{2+}]. EPAC activates the small G-proteins, Rap1 and 2, leading to downstream signals that modulate ion transport (33, 62). Thus, a potential candidate for linking the cAMP (6) and Ca\textsuperscript{2+} cascades (Figure 5) is EPAC. To investigate the potential role of EPAC, we examined the effects of the inhibitor ESI-09 (10µM), which specifically competes with EPAC’s cAMP binding site. As shown in Figures 6A and C, ESI-09 significantly attenuated CDCA-stimulated I\textsubscript{sc} by 70% and also inhibited the effect of the cAMP activator forskolin (Figure 6A and C). In contrast, ESI-09 did not inhibit Cl\textsuperscript{−} secretion by the muscarinic agonist carbachol (Figure 6B and D), known to act via a G\textsubscript{q}, non-cAMP cascade. Importantly, we find that combined inhibition of PKA (by 30µM H89) and EPAC (by 10µM ESI-09) blocks the CDCA response more than either inhibitor alone both when the data is quantified as AUC (%AUC: CDCA: 100; +30µM H89 +10µM ESI-09: 11.1 ± 4.5; n=7, p<0.0001), and as peak I\textsubscript{sc} (% I\textsubscript{sc}: CDCA: 100; +H89 +ESI-09: -5±25; n=7, p=0.001). These results verify that ESI-09 inhibits a cAMP-dependent pathway and that CDCA acts via cAMP to activate both PKA and EPAC.

To assess the ionic basis of EPAC involvement, we examined its effects on CDCA activation of apical membrane Cl\textsuperscript{−} currents. Monolayers were subjected to an apical to basolateral Cl\textsuperscript{−} gradient, where the basolateral membrane was permeabilized with nystatin. In
our Ussing chamber preparation, the recording electrode is in the basolateral medium with the
reference electrode in the apical medium. Thus, changes in Cl⁻ entry across the apical
membrane are recorded as a negative current. However, for ease of reading we have inverted
the I_{sc} tracings of monolayers treated with ESI-09 in gray or DMSO in black (Figure 7A). We
have previously established that the apical Cl⁻ current activated by CDCA is due to CFTR, as
CFTRinh172 attenuated the current (6). Figure 7A (black line) shows a representative tracing
where basolateral permeabilization caused a slow and steady increase in the current due to the
Cl⁻ gradient. The addition of CDCA caused a more robust increase. Addition of forskolin further
increased the current in a transient manner, and increases in current were inhibited by
CFTRinh172. Figure 7A (gray line) also demonstrated that the CDCA response was significantly
attenuated to baseline levels by ESI-09 (p<0.0001; Figure 7B). Additionally, the representative
tracing (Figure 7A) shows that inhibition of EPAC by ESI-09 stimulates a small transient Cl⁻
current that is significantly reduced to baseline by CFTRinh172 (n=6; p=0.02; Figure 7B). These
data, taken with the results in intact monolayers, establish EPAC’s role in CDCA’s activation of
Cl⁻ transport via CFTR.

EPAC signaling can lead to a sequential activation of Rap1 and rho-associated protein
kinase (ROCK). EPAC activators have been shown to stimulate Rap1 and ROCK and increase
epithelial K⁺ transport, which contributes to the driving force for Cl⁻ secretion (62). Interestingly,
neither inhibition of Rap1 by GGTI298 nor of ROCK by H1152 had any effect on the CDCA
response (Table 3; p>0.05 for both inhibitors compared to CDCA alone). EPAC can also
activate Rap2, which, in turn, leads to an increase in [Ca^{2+}]. (61). As there are no readily
available inhibitors of Rap2, we determined if CDCA increased GTP-Rap2 by activated Rap
pull-down assays. Figure 8A shows a representative immunoblot of Rap2 after stimulation with
CDCA, in which, CDCA increases GTP-bound Rap2 at 10 minutes. Because activation of Rap2
leads to Ca^{2+} mobilization, we next determined if pretreatment with ESI-09 would affect CDCA-
induced changes in [Ca^{2+}]. In control cells, CDCA caused an increase of 22.4±3.2nM from basal
[Ca^{2+}] levels. This was significantly reduced by 67% in the presence of ESI-09 (Figure 8B).
Interestingly, also shown in Figure 8B, 2-APB decreased CDCA-stimulated Δ[Ca^{2+}], by 63%.
While the combined addition of ESI-09 and 2-APB reduced the CDCA-stimulated Δ[Ca^{2+}], by
89%, this was not statistically different from the response to the inhibitors individually. These
data suggest that EPAC, most likely via the activation of Rap2, is involved in CDCA action on
[Ca^{2+}] and Cl⁻ secretion.
Discussion

The actions of bile acids as hormone-like molecules, and not just as agents for increasing lipid solubility, are becoming increasingly relevant. As regulators, bile acids have been shown to activate nuclear receptors (FXR, PXR, VDR), G protein-coupled receptors (TGR5 and M3R), as well as multiple signaling pathways in the gastrointestinal tract (18). Several studies have established the role of the 7α-dihydroxy bile acids (CDCA and DCA) in the stimulation of fluid and electrolyte secretion in the colon. In this study, we continued our investigations on the mechanisms underlying CDCA’s stimulation of Cl⁻ secretion via CFTR. In addition to our previous findings on the role of PKA (6), the present study demonstrates that CDCA’s secretory effect is also dependent on EGFR, EPAC, and Ca²⁺ signaling.

A majority of bile acid signaling has been attributed to the activation of the bile acid specific receptors, FXR and TGR5. FXR activation largely regulates bile acid synthesis in order to maintain the bile acid pool (27). Previous studies in T84 cells have shown that activation of FXR by GW4064 for 24 hours lead to an inhibition of forskolin- and carbachol-stimulated $I_{sc}$ responses, inhibition of Na⁺/K⁺-ATPase activity and a reduction in CFTR expression (50). Interestingly, CDCA is the most potent natural agonist of FXR, but we show that acute (20 minute) activation of FXR with GW4064 does not have any effect on Cl⁻ secretion (Table 1). In addition, we recently reported that prolonged treatment with GW4064 (24 hours) did not alter forskolin-induced $I_{sc}$ responses in our preparations of T84 cells (5). This suggests that the immediate effect of CDCA or forskolin on CFTR-mediated $I_{sc}$ does not involve FXR activation.

CDCA acts in a rapid, sustained, and side-dependent manner to increase $I_{sc}$ in T84 cells, suggesting the involvement of a membrane bound receptor, rather than a nuclear receptor (6). While TGR5 is currently considered to be a key player in the interaction of bile acids, metabolism, and insulin signaling (29, 44), its role in Cl⁻ secretion varies by cell type. The report that activation of TGR5 in cholangiocytes resulted in stimulation of CFTR in wild type, but not in TGR5 knockout mice (39), led us to hypothesize that it would mediate CDCA’s activation of CFTR in T84 cells. Currently, there are no available antagonists to TGR5. Thus, studies use TGR5 agonists to assess its involvement. Ciprofloxacin was previously identified as a potent TGR5 agonist by *in silico* screenings and docking studies (19). However, when we stimulate T84 cells with ciprofloxacin there is no increase in $I_{sc}$ (Table 1). Additionally, LCA, the most potent bile acid activator of TGR5, does not increase $I_{sc}$ (5). Interestingly, in a study done by Ward et al. (69), activation of TGR5 by its synthetic agonist INT-777, or by LCA, in the rat colon, reduced the basal current and inhibited Ca²⁺-dependent Cl⁻ secretion. Furthermore, we find that in T84 cells, LCA inhibits cAMP-dependent Cl⁻ secretion (5). This further supports the idea that
while TGR5 may regulate Cl− transport in cholangiocytes, its agonists do not stimulate Cl− secretion in T84 cells. Additionally, in hepatocytes conjugated bile acids have been shown to act via the G-protein coupled sphingosine 1 phosphate receptor 2, however this receptor has not been shown to be expressed in intestinal cells (43) and is therefore an unlikely candidate for initiating CDCA action in T84 cells.

Several studies have shown that bile acids can activate other membrane and nuclear receptors with broad specificities. Raufman and colleagues have published that secondary bile acids, specifically TDCA, activate the M3 muscarinic receptor. This initiates a signal transduction pathway involving MMP 7, cleavage of HB-EGF, and transactivation of EGFR to induce colonic epithelial cell proliferation (16, 17). We had previously shown that inhibition of muscarinic receptors by atropine had no effect on CDCA-induced $I_{sc}$ (6). Although muscarinic receptors are not involved, CDCA signaling is dependent on EGFR autophosphorylation of tyrosine 1068 (Figure 1). Batimastat attenuated the CDCA response suggesting the involvement of MMPs and cleavage of an EGFR ligand (Table 2). However, while EGF alone stimulates Cl− secretion in the rabbit colon (14), it did not alter the CDCA response in T84 cells. This suggests that CDCA’s activation of EGFR does not involve EGF per se, but may involve a related but yet to be identified ligand, such as TGFα or HB-EGF. EGFR can also be transactivated by Src kinase (3, 71), however this is not the case with CDCA since inhibition of Src did not affect the CDCA response (Table 2).

Since activation of MMPs often require Ca$^{2+}$ mobilization (22, 57, 74), it is possible that increases in [Ca$^{2+}$]i by CDCA can activate MMPs and lead to cleavage of an EGFR ligand. Transactivation of EGFR is accomplished by ligand binding, autophosphorylation of specific residues, and receptor dimerization (53). AG1478 blocks EGFR’s autophosphorylation activity; thus, the decrease in CDCA-induced EGFR phosphorylation of tyrosine 1068 by AG1478 (Figure 1C) could imply that CDCA causes EGFR autophosphorylation and dimerization. Furthermore, data of AG1478 shown in Figure 1A and Figure 5G respectively suggest that this autophosphorylation is critical for CDCA action on $I_{sc}$ and [Ca$^{2+}$]i. EGFR has several phosphorylation sites including tyrosine 992, 1045, 1068 (studied here), 1148, and 1173 that lie within its autophosphorylation domain. Specific phosphorylation of these sites might enable the docking of EGFR adaptor proteins. Furthermore, EGFR has additional phosphorylation sites within the kinase domain that regulate downstream signaling cascades. For example, phosphorylation of tyrosines 992, 1101, and 1148 lead to activation of PLCγ, PI3K/AKT, and MAPKs, respectively (53). Although we ruled out involvement of MAPKs and PI3K/AKT activation in CDCA-induced Cl− secretion (Figures 3-4), CDCA may activate PLCγ in an EGFR-
dependent manner (Figure 5G), and thus CDCA could also increase phosphorylation of tyrosine

Although bile acids are capable of binding directly to plasma membrane-bound receptors, the more hydrophobic bile acids, such as DCA and CDCA, are capable of incorporating into the plasma membrane and affecting intracellular signaling. For example, in primary rat hepatocytes, DCA-induced EGFR phosphorylation lead to downstream signaling via MAPKs, which was sensitive to AG1478 inhibition of EGFR activation. Interestingly, when neutralizing antibodies to either EGF or TGFα were used to block activation of EGFR, DCA was still able to induce EGFR phosphorylation. This suggested that DCA was acting in a ligand-independent manner, potentially through modulation of membrane dynamics (59). Furthermore, in HCT11 colon carcinoma cells, DCA caused phosphorylation of EGFR in a ligand-independent manner and induced apoptosis. Investigation of DCA’s effect on membrane dynamics revealed a decrease in membrane fluidity, an increase in cholesterol, and a redistribution of caveolin-1 (34). These authors also reported that the cholesterol sequestrant, methyl-β-cyclodextrin (MβCD), a tool used to show involvement of cholesterol-containing lipid rafts in signaling cascades, inhibited DCA-induced apoptosis. However, when MβCD was washed out of the preparations, there was less of an inhibition of DCA action, suggesting that MβCD was acting as a DCA and cholesterol sequestrant (34). We similarly found MβCD to be an ineffective tool in studying CDCA action. Thus, while treatment of T84 cells with MβCD reduced the CDCA response, washing of the monolayers to remove MβCD restored CDCA’s secretory response (data not shown). Nevertheless, it is conceivable that CDCA, which has a similar hydrophobic profile to DCA, could be altering EGFR in a ligand-independent manner via direct membrane perturbations. This will be worthy of future exploration in light of the well-known differences in apical and basolateral membrane lipid composition, which may account for the sidedness of bile acid action (58).

Bile acids have been shown to activate a variety of kinases downstream of EGFR activation (35, 37, 60, 70). Although high doses of DCA cause increases in $I_{sc}$ in 5 minutes (38), Keating et al. reported that 24 hour-exposure to low doses (50μM) of DCA inhibited forskolin and carbachol-stimulated $I_{sc}$, which was mediated by EGFR, but did not involve p38 and ERK 1/2 (37). Our results (Figures 1-3) and the findings of Keating et al. (37) demonstrate that ERK 1/2 and p38 kinases are unrelated to bile acid regulation of Cl⁻ secretion. Furthermore, Src was not found to be involved in CDCA action (Table 2).

Our results share many but not all features of the cascade reported by Bertelsen et al. (8). In that study, VIP increased ERK 1/2 phosphorylation and VIP-stimulated $I_{sc}$ was inhibited
by the EGFR inhibitor, AG1478, but not by the MEK inhibitor, PD98059 (8). Our results in
Figures 3A-C and 4A parallel these findings. However, our studies with respect to the
involvement of PI3K differ: while LY294002 had no effect on CDCA stimulated Isc, Bertelsen et
al. showed an inhibition of VIP-stimulated Isc (8). Whereas, Bertelsen et al. (8) suggested the
involvement of AKT, we demonstrated that AKT also does not play a role in CDCA-induced Cl−
secretion (Figure 4). Our results with wortmannin are intriguing in that we observed inhibition
only at a dose of 500nM (100nM had no effect; data not shown). This implies that other target
kinases for wortmannin, such as myosin light chain kinase, may be involved (13, 52, 64).

Thus far we have ruled out involvement of the known bile acid specific receptors, the
MAPKs (ERK 1/2 and p38), Src, and PI3K-AKT, but have implicated the contribution of EGFR.
Additionally, Figure 5 provides strong evidence supporting the involvement of Ca2+ signaling in
CDCA activation of Cl− secretion. While several studies have investigated TDCA regulated Cl−
secretion and cell signaling, the few studies on CDCA report only ion transport changes (9, 49,
54). The findings on TDCA action, show that TDCA activates K+ and Cl− conductances via an
IP3-dependent increase of [Ca2+]i in T84 cells (23-25). From studies in rabbit colon, TDCA’s
effect on Cl− secretion is age-dependent (56, 68), is mediated by [Ca2+]i and IP3 (68), and
requires PKCδ activation (36). What has become evident is that there is structural specificity to
the signaling initiated by the dihydroxy bile acids. Thus, while the rise in [Ca2+]i by TDCA was
independent of [Ca2+]o in both T84 cells and rabbit colon (23, 68), we find that CDCA partially
requires [Ca2+]o for the increase in [Ca2+]i (Figure 5E). Furthermore, while TDCA action in the
rabbit colon is dependent on PKC (36), our results show that CDCA action in T84 cells is PKC-
independent (Table 2 and Figure 5B). Interestingly, similar to TDCA’s activation of PLC and IP3
in the rabbit colon (68), CDCA action in T84 cells is also dependent on PLC activation, IP3
production and [Ca2+]i (Figure 5). Additionally, the ability of AG1478 to decrease CDCA-induced
changes in [Ca2+]i suggests that EGFR may be a route by which CDCA alters [Ca2+]i (Figure 5G).
As discussed above, EGFR has long been shown to be an activator of Ca2+ signaling via
induction of tyrosine phosphorylation of PLCγ (46, 48).

Clearly CDCA’s effect on transepithelial Isc in T84 cells requires the contribution of
several signaling pathways, leading us to investigate potential points of crosstalk. Transporters
that contribute to Cl− secretion have long been shown to be regulated by Ca2+ and cAMP. Thus,
in T84 cells, cAMP and Ca2+ agonists individually act on different basolateral K+ channels to
increase the driving force for Cl− transport, and when added together have a synergistic effect
(15, 66). As cAMP (6) and Ca2+ (Figure 5) are the major contributors to CDCA action, a prime
candidate bridging these two second messenger cascades is EPAC. EPAC is a guanine
nucleotide-exchange factor that is activated by binding of cAMP to the cyclic nucleotide binding
domain in the regulatory region of the protein. Binding of cAMP causes a conformational change
that allows for association with Rap proteins, exchange of GDP for GTP, and Rap activation
(11). Hoque et al. (33) established the role of EPAC in forskolin-stimulated Cl⁻ transport in T84
cells by showing that knockdown of EPAC reduced the forskolin response, with the remaining
response being inhibited by the PKA inhibitor H89 (33). Additionally, in wild-type cells, the
forskolin response was sensitive to BAPTA chelation of Ca²⁺, however BAPTA had no effect on
the forskolin response in EPAC knockdown cells, suggesting EPAC activation was involved in
Ca²⁺ signaling. We have reported that CDCA action can be inhibited by H89 and that in
basolaterally permeabilized T84 monolayers, the CDCA-activated apical Cl⁻ current is
dependent on CFTR (6). Here we show for the first time that CDCA-induced Iₗc is also
dependent on EPAC (Figure 6). Furthermore, inhibition of both PKA and EPAC pathways
simultaneously completely blocked the CDCA-induced transepithelial current. Elucidation of the
ionic contribution of EPAC demonstrated that EPAC plays a direct role in CDCA-stimulation of
apical CFTR Cl⁻ currents (Figure 7). EPAC activation by forskolin has also been implicated in
regulation of the driving force for Cl⁻ secretion by increasing surface expression and activation of
the KCNN4 K⁺ channel in a Rap1-ROCK-dependent manner (62). However, inhibitors of Rap1
or ROCK did not alter CDCA action (Table 3). This was not surprising since we previously
showed that CDCA does not activate K⁺ conductances (6).

Our evidence suggests PKA [(6); this paper], EPAC (Figure 6), and Ca²⁺ (Figure 5) are
all necessary for CDCA action and that EPAC may be contributing to CDCA-induced Ca²⁺
responses. Activation of Rap2 by EPAC has been shown to activate PLCε and lead to changes
in [Ca²⁺], as transfection of a Rap2 mutant reduced adrenaline stimulated IP₃ production and
increases in [Ca²⁺], in HEK-293 cells (61). Similarly, we find that CDCA increases activation of
Rap2 and inhibition of EPAC significantly reduces CDCA’s effect on [Ca²⁺] (Figure 8). Both of
these observations suggest that CDCA is acting in a Rap2-dependent manner downstream of
EPAC. Additionally, while inhibition of the IP₃R also decreased CDCA’s effect on [Ca²⁺],
combined addition of 2-APB and ESI-09 did not significantly decrease the response compared
to addition of the inhibitors individually. This suggests that these signaling proteins are
converging on a single pathway. Because the combined inhibition was not complete, it can be
postulated that the residual CDCA-induced [Ca²⁺] response may be due to a 2-APB insensitive
Ca²⁺ uptake pathway. Furthermore, preliminary studies suggest that inhibition of cAMP signaling
by H89 or ESI-09 alter CDCA-induced EGFR phosphorylation (data not shown; n=2). The facts
that ESI-09 did not affect carbachol action, inhibited CDCA-stimulated [Ca²⁺], and that the
combined addition of ESI-09 and H89 blocked CDCA induced Isc, suggest that CDCA is utilizing cAMP to activate both the PKA and EPAC pathways to modulate EGFR signaling and increase Cl− secretion. Clearly, there is also a role for the transactivation of EGFR, both as a potential target of cAMP action, and as a contributor to Ca2+ signaling. Taken with the evidence that MMPs are required for the complete CDCA response (Table 2), we propose that activation of the EPAC-Rap2-PLCε-Ca2+ pathway stimulate Ca2+-dependent MMPs, which lead to transactivation of EGFR. Because inhibition of EGFR also decreases the CDCA-induced Δ[Ca2+]i (Figure 5G), we believe that activation of EGFR leads to stimulation of PLCγ by tyrosine phosphorylation, and a further increase in [Ca2+]i, contributing to Cl− secretion by CFTR. Our data that 2-APB and ESI-09 are not additive would fit with such a proposed cascade, since EPAC activation is upstream of both PLCε and PLCγ activation.

Although Cl− secretion is a major contributor to secretory diarrhea, diarrhea is also the consequence of a decrease in absorptive processes. Interestingly, a recent study by Pallagi-Kunstar et al. demonstrated that bile acids, specifically CDCA, inhibit Na+/H+ exchangers and Cl−/HCO3− exchangers in a Ca2+-dependent manner, contributing to the development of diarrhea (54). This finding substantiated the study by Alrefai et al. in Caco-2 cells, where TDCA and glycol-CDCA inhibited Cl-/OH− exchange in a Ca2+-, PKCβ-, and PI3K-dependent manner (1). Taking our current and earlier (6) findings, with those of Pallagi-Kunstar et al. (54) and Alrefai et al. (1), it is probable that when there is a pathological rise of luminal bile acid concentrations, signaling cascades are initiated to inhibit electrolyte absorption (1, 54) and to activate electrolyte secretion (6), leading to bile acid-induced diarrhea.

In summary, we have shown that in human colonic T84 monolayers, in which CFTR is the major contributor to transepithelial lsc, the the 7α-dihydroxy bile acid CDCA initiates a complex signaling mechanism leading to Cl− secretion (Figure 9). Activation of Cl− transport by CDCA does not appear to involve any of the well-characterized, traditional bile acid receptors, but does involve EGFR phosphorylation, suggesting either a role for direct membrane perturbation or of an as yet unidentified bile acid receptor. CDCA action also does not require Src kinase, MAPK or PI3K signaling downstream of EGFR. In conjunction with the involvement of cAMP and PKA signaling (6), we show the novel contribution of EPAC in CDCA-induced Cl− secretion. We postulate a mechanism (Figure 9) where CDCA stimulates production of cAMP, activating PKA and EPAC, both of which are required for the Cl− secretory response. In addition, CDCA may transactivate EGFR in a cAMP-dependent manner (Figure 9, line 1), or by membrane perturbations (Figure 9, line 3). CDCA stimulation also leads to activation of PLC (likely PLCγ by EGFR and PLCε by EPAC), IP3 production and Ca2+ release from intracellular
stores, which are required for CDCA stimulation of Cl− secretion. EGFR, EPAC-Rap2, and extracellular Ca2+ all contribute to the CDCA-stimulated rise in [Ca2+]i. The findings presented here add to our knowledge of bile acid physiology and identify new targets, such as the EPAC pathway, to alleviate bile acid induced diarrheal symptoms.

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References


**Figure Legends**

**Figure 1:** The role of EGFR in CDCA-induced Cl⁻ transport. A. Representative tracing of Isc (µA/cm²) induced by the dihydroxy bile acid CDCA. T84 monolayers were mounted in Ussing chambers and pretreated bilaterally with the EGFR antagonist, AG1478 (1µM), or 0.1% DMSO for 30 minutes before basolateral addition of CDCA (500µM). The right panel is a quantification of the changes in Isc by CDCA ± AG1478; n≥8; p=0.0005. B. Representative western blot of EGFR phosphorylation (p-EGFR, Y1068) and total EGFR (175kDa) after T84 cells were stimulated with 500µM CDCA (0-10 minutes); n=4. C. Representative western blot of EGFR Y1068 phosphorylation by CDCA, or carbachol (CCH, 100µM) as a positive control, ± AG1478 pretreatment. Vehicle is the effect of AG1478 alone with stimulation by DMSO; n=4. Densitometry ratios of pEGFR:total EGFR were normalized to vehicle (AG1478 pretreatment then stimulation with DMSO) and are presented below the western blots. D. T84 cells transfected with scrambled (SCR; control) or EGFR specific siRNA for 72 hours were immunoblotted for EGFR protein (top part of blot) and GAPDH (lower part of blot); dotted arrow indicates where the membrane was cut and reassembled for the figure (representative of n≥6). E. Cl⁻ transport was assessed by the iodide efflux assay. Tracing shows the average cumulative iodide efflux (fold increase over t=0 minutes) from SCR (solid lines) or EGFR (dotted lines) siRNA transfected cells stimulated with CDCA (black lines) or 0.1% DMSO (gray lines); n=5. *= p<0.05 to DMSO control; #: p<0.05 to treatment +EGFRsiRNA.

**Figure 2:** EGFR-related kinases in CDCA action. A. Human phospho-kinase array. Densitometric analysis of the (7/43) kinases activated by 500µM CDCA or 0.1% DMSO for 5 minutes. Results were quantified as the phosphorylated density of the specific kinases normalized to β catenin whose phosphorylation remained unchanged. B. Representative western blot shows p38 phosphorylation (43 kDa) in T84 cells treated with CDCA (0-10 minutes); n=4. C. Quantification of the ΔIsc measured in monolayers pretreated bilaterally with 0.1% DMSO or 10µM SB203580 (p38 inhibitor) for 30 minutes and then stimulated with CDCA; n≥7, p=0.26.

**Figure 3:** The role of MAPKs in CDCA action. A. Representative western blot of phosphorylated and total ERK 1/2 protein (42kDa) expression after treatment with 500µM CDCA (0-10 minutes) or 16.3nM EGF; n=3. B. T84 cells were pretreated with the MEK inhibitor PD98059 (20µM) for 30 minutes, then stimulated with EGF or CDCA for 5 minutes and immunoblotted for phosphorylated and total ERK 1/2 (representative western blot; n=3). C.
Change in $I_{sc}$ by CDCA with or without 30-minute pretreatment of PD98059 ($n=3$, $p>0.05$). D. Effect of 30-minute pretreatment with a combination of PD98059 and the p38 inhibitor SB203580 on CDCA-induced $I_{sc}$ ($n=5$, $p>0.05$).

**Figure 4: Effect of antagonists to PI3K and AKT on CDCA-induced $I_{sc}$**. A. Quantification of $\Delta I_{sc}$ measured in monolayers pretreated bilaterally with the PI3K inhibitor wortmannin (500nM) or 0.1% DMSO then stimulated with basolateral addition of 500µM CDCA; $n=8$, $p=0.009$. B. Effect of 20-100µM LY294002 pretreatment on CDCA-induced $I_{sc}$; $n\geq4$. C. Representative $I_{sc}$ tracing of T84 monolayers pretreated bilaterally with 0.1% DMSO or the AKT inhibitor MK2206 (1µM) for 30 minutes then stimulated with sequential addition of CDCA, forskolin (FSK, 10µM) and carbachol (CCH, 100µM). Right panel is the CDCA-induced $\Delta I_{sc}$ ± MK2206; $n=4$, $p=0.58$.

**Figure 5: CDCA and Ca$^{2+}$ signaling in T84 cells**. A. Quantification of $\Delta I_{sc}$ when monolayers were pretreated bilaterally with the PLC inhibitor U73122 (10µM) for 30 minutes and then stimulated with basolateral addition of 500µM CDCA or carbachol (CCH, 100µM); $n=5$, $p=0.0001$ for CDCA and carbachol compared to inhibitor. B. Effect of 2-10µM chelerythrine pretreatment on CDCA-induced $I_{sc}$; $n\geq4$. C. Measurement of IP$_3$ production after cells were stimulated with CDCA or 0.1% DMSO (control); $n=3$, $p=0.001$ for 10 minutes compared to control. D. CDCA-induced $\Delta I_{sc}$ ± bilateral pretreatment (30 minutes) with 50µM 2-APB (IP$_3$R inhibitor); $n=3$, $p=0.002$. E. Quantification of [Ca$^{2+}$]$_i$ in response to CDCA in the presence of 1mM extracellular Ca$^{2+}$ ([Ca$^{2+}$]$_o$), 0mM [Ca$^{2+}$]$_o$, and 1mM [Ca$^{2+}$]$_o$ + 30 minute pretreatment with the Ca$^{2+}$ chelator BAPTA-AM (20µM). For all conditions $n\geq3$; * = $p<0.05$ compared to basal [Ca$^{2+}$]$_i$, # = $p<0.05$ compared to CDCA in the presence of 1mM [Ca$^{2+}$]$_o$, and $\$ = $p<0.05$ between CDCA in the presence of 0mM [Ca$^{2+}$]$_o$ compared to CDCA in the presence of 1mM [Ca$^{2+}$]$_o$ + BAPTA-AM as determined by 1-way ANOVA with Dunnett’s multiple comparison post-test. F. The effect of 30-minute bilateral pretreatment of BAPTA-AM on CDCA-induced changes in $I_{sc}$; $n=4$, $p=0.002$. G. Quantification of CDCA-induced $\Delta [Ca^{2+}]_{i}$ ± bilateral pretreatment (30 minutes) with 1µM AG1478; $n=3$, * denotes $p=0.03$.

**Figure 6: The role of EPAC in cAMP and Ca$^{2+}$-stimulated $I_{sc}$**. A. Representative tracing of $I_{sc}$ ± pretreatment (30 minutes) with the EPAC inhibitor ESI-09 (10µM, gray line), followed by basolateral addition of 500µM CDCA and 10µM forskolin (FSK). B. Representative tracing of carbachol (CCH, 100µM)-induced $I_{sc}$ ± ESI-09 pretreatment. C. Quantification of peak $I_{sc}$
response to CDCA and FSK ± ESI-09 (n=6, p≤0.001 for CDCA and FSK compared to inhibitor).

D. Carbachol-induced ΔI_{sc} ± bilateral pretreatment (30 minutes) with ESI-09; n=6, p>0.05.

**Figure 7: Effect of ESI-09 on apical Cl⁻ currents.** A. Representative I_{sc} tracings of apical Cl⁻ currents across nystatin permeabilized monolayers. Nystatin (200µg/mL) was added to the basolateral (BLM) chamber of monolayers mounted in an apical to basolateral Cl⁻ gradient. Monolayers were then treated DMSO (black line) or 10µM ESI-09 (gray line) and stimulated with 500µM CDCA, and subsequent addition of forskolin (FSK, 10µM). Finally, CFTRinh172 (CFTRinh, 10µM) was added to the apical solution. B. Quantification of ΔI_{sc} in response to vehicle (0.1% DMSO), ESI-09, ESI-09 +CFTRinh172 (ESI-09+CFTRinh), CDCA, and CDCA+ESI-09; n≥3 for all conditions, *=p<0.05 compared to vehicle, #=p<0.05 compared to inhibitor.

**Figure 8: The role of Rap2 in CDCA action.** A. Left panel: Representative western blot of Rap2 (24kDa) after T84 cells were stimulated with 500µM CDCA (0-20 minutes); n=3. Protein was harvested and incubated with RalGDS-RBD agarose beads that pull down GTP-bound Rap and then immunoblotted for Rap2; Right panel: Unstimulated cell lysates were incubated with either 0.1mM GTP (positive control) or 1mM GDP (negative control) and subjected to pull down assays and immunoblotting. B. Quantification of CDCA-induced Δ[Ca^{2+}] ± pretreatment (30 minutes) with 10µM ESI-09; (n=5), 50µM 2-APB (n=3), or with a combination of ESI-09 and 2-APB (n=3); 1-way ANOVA with a Tukey’s multiple comparisons post-test was performed; * denotes p<0.05 compared to CDCA alone; the effect of the individual inhibitors were not significantly (NS) different from the combined inhibition.

**Figure 9. Summary of CDCA action in T84 cells.** CDCA requires EGFR activation (see Figure 1) and activation of cAMP signaling, including PKA (6) and EPAC (see Figures 6 and 7). While PKA phosphorylates CFTR (6), activation of EPAC leads to signaling via Rap2, and an increase in Ca^{2+} mobilization (see Figure 8). The cAMP pathways may be leading to the transactivation of EGFR (dotted line 1), which further contributes to increases in intracellular Ca^{2+} (see Figure 5G). Signaling via PKA, EPAC, and EGFR are all required for CDCA-induced Cl⁻ secretion via CFTR. CDCA may be initiating this signaling cascade through an unidentified bile acid receptor that may be activating PLC directly (dotted line 2), or via membrane perturbations (dotted line 3).
Tables

Table 1: Effects of Bile Acid Receptor Agonists

<table>
<thead>
<tr>
<th>Agonist (n)</th>
<th>DMSO 0.1% (8)</th>
<th>CDCA 500μM (4)</th>
<th>GW4064 5μM (3)</th>
<th>Ciprofloxacin 20μM (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔI(sc) ± SEM</td>
<td>1.5±0.3</td>
<td>24.3±6.6*</td>
<td>1.5±1.5</td>
<td>-3.4±4.8</td>
</tr>
</tbody>
</table>

Values are mean ΔI(sc) (μA/cm²) ± SEM; n = number of experiments. Agonists were added basolaterally at the indicated concentrations and I(sc) measured 30 minutes later. Ciprofloxacin was used as an agonist of TGR5 and GW4064 was used as an agonist of FXR. ΔI(sc) for each agonist was compared to DMSO as control and significance (p<0.05) is indicated by an *.

Table 2: Effect of Enzyme Inhibitors

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MMP</th>
<th>Src</th>
<th>PKCδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (n)</td>
<td>CDCA</td>
<td>+20μM Batimastat (3)</td>
<td>CDCA +10μM PP2 (3)</td>
</tr>
<tr>
<td>ΔI(sc) ± SEM</td>
<td>12.1 ± 1.4</td>
<td>*3.9 ± 2.6</td>
<td>20.7 ± 2.9</td>
</tr>
</tbody>
</table>

Values are mean ΔI(sc) (μA/cm²) ± SEM; n = number of experiments. Inhibitors to specified enzymes were used to assess their involvement in CDCA-induced I(sc) and were added bilaterally at the stated concentrations 30 minutes before addition of 500μM CDCA. Batimastat was used as an inhibitor of MMPs, PP2 as an inhibitor of Src kinase, and Rottlerin as an inhibitor of PKCδ. *= p<0.05 compared to CDCA alone.

Table 3: Effect of Inhibitors of EPAC Pathway Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Rap1</th>
<th>ROCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (n)</td>
<td>CDCA</td>
<td>+10μM GGTI298 (4)</td>
</tr>
<tr>
<td>ΔI(sc) ± SEM</td>
<td>8.3 ± 1.8</td>
<td>8.9 ± 1.1</td>
</tr>
</tbody>
</table>

Values are mean ΔI(sc) (μA/cm²) ± SEM; n = number of experiments. Inhibitors to specified EPAC effectors were used to assess their involvement in CDCA-induced I(sc) and were added bilaterally at the stated concentrations 30 minutes before addition of 500μM CDCA. GGTI298 was used as an inhibitor of Rap1, and H1152 as an inhibitor of ROCK.
Figure 1

A

Graph showing the change in $I_{sc}$ (μA/cm²) over time (mins) in response to Control and +1μM AG1478 treatments. The graph indicates a significant increase in $I_{sc}$ with the +1μM AG1478 treatment compared to the Control.

B

Western blot analysis showing the expression of p-EGFR (phosphorylated EGFR) and Total EGFR at 175 kDa. The blot includes lanes for 0', 2', 5', and 10' after 500μM CDCA treatment.

C

Western blot analysis showing the expression of p-EGFR (phosphorylated EGFR) and Total EGFR at 175 kDa for different treatments: CDCA, CCH, Vehicle, CDCA, and CCH. The pEGFR:total EGFR ratio is provided for each condition.
Figure 1 (cont)

D

E

Time (min)

Fold increase

EGFR siRNA

SCR siRNA

EGFR 175 kDa

GAPDH 36 kDa

Ratio EGFR:GAPDH ± SEM

0.87 ± 0.11

0.38 ± 0.13

DMSO CONsi

DMSO EGFRsi

CDCA CONsi

CDCA EGFRsi

#
Figure 2

A

Phospho Density/β Catenin

<table>
<thead>
<tr>
<th>Protein</th>
<th>DMSO</th>
<th>CDCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Catenin</td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>EGFR</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>Src</td>
<td>600</td>
<td>800</td>
</tr>
<tr>
<td>p38α</td>
<td>1000</td>
<td>1200</td>
</tr>
<tr>
<td>ERK 1/2</td>
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<td>70</td>
</tr>
<tr>
<td>AKT S473</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>AKT T308</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

B

500μM CDCA

C

ΔI_{sc} (μA/cm²)

<table>
<thead>
<tr>
<th>Condition</th>
<th>ΔI_{sc}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDCA</td>
<td>10.5</td>
</tr>
<tr>
<td>+10μM SB203580</td>
<td>5.5</td>
</tr>
</tbody>
</table>
Figure 3

A) Western blot analysis of p-ERK and Total ERK in response to EGF (16.3 nM) and CDCA (500 μM) for 0', 2', 5', and 10' time points.

B) Western blot analysis of p-ERK and Total ERK in the presence of PD98059 (20 μM) and DMSO, CDCA 5', and EGF.

C) Bar graph showing the change in ΔIsc (μA/cm²) for CDCA and CDCA + 20 μM PD98059.

D) Bar graph showing the change in ΔIsc (μA/cm²) for CDCA and CDCA + SB+PD.
**Figure 4**

A. 

- $\Delta I_{sc}$ ($\mu A/cm^2$)
- Treatment: CDCA, +500nM Wortmannin

B. 

- $\Delta I_{sc}$ ($\mu A/cm^2$)
- Treatment: CDCA, +20μM, +50μM, +100μM LY294002

C. 

- $I_{sc}$ ($\mu A/cm^2$)
- Time (mins)
- Treatments: Control, +1μM MK2206
- Events: CDCA, FSK, CCH

**Legend:**
- Control
- +1μM MK2206
Figure 5

A. PLC Inhibition

B. PKC Inhibition

C. IP₃R Inhibition

- CDCA
- +50μM 2-APB
Figure 5 (cont)

E

$\Delta [Ca^{2+}_i]$ (nM)

- Basal
- CDCA 500µM

[F] $\Delta I_{sc}$ (µA/cm²)

- CDCA
- +20µM BAPTA-AM

G

$\Delta [Ca^{2+}_i]$ (nM)

- CDCA
- +1µM AG1478
Figure 6

(A) Graph showing the change in current density (I_sc) over time (in minutes) for control and +10μM ESI-09 conditions. Arrows indicate the addition of CDCA and FSK.

(B) Graph showing a different set of data with a time axis ranging from 82.5 to 85 minutes, indicating a response to CCH.

(C) Bar graph showing changes in I_sc for CDCA, CDCA+ESI-09, FSK, and FSK+ESI-09. Asterisks denote significant differences.

(D) Bar graph showing changes in I_sc for CCH and CCH+ESI-09.
Figure 7

A

$\Delta I_{sc} (\mu A/cm^2)$

Time (mins)

0 10 20 30 40 50 60 70 80

- +10μM ESI-09
- Control

BLM Nystatin DMSO/ESI-09 CDCA

FSK CFTRinh

B

$\Delta I_{sc} (\mu A/cm^2)$

- Vehicle
- 10μM ESI-09
- ESI-09+CFTRinh
- CDCA
- CDCA+ESI-09

* #
Figure 8

A

GTP-Rap2
24kDa

500μM CDCA  0min  5min  10min  20min  GTP  GDP

RalGDS-RBD

B

ΔCa^{2+} (nM)

CDCA  +10μM ESI09  +50μM 2-APB  +ESI-09 +2-APB

* NS

0  15  30  45