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Lubiprostone is a Non-Selective Activator of cAMP-Gated Ion Channels and Chloride Channel Protein 2 (Clc-2) Has a Minor Role in its Prosecretory Effect in Intestinal Epithelial Cells

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ABSTRACT

Loss of prosecretory Cl^- channel cystic fibrosis transmembrane conductance regulator (CFTR) activity is considered the key cause of gastrointestinal disorders in cystic fibrosis, including constipation and meconium ileus. Chloride channel protein 2 (Clc-2) is proposed as an alternative Cl^- channel in intestinal epithelia that can compensate for CFTR loss-of-function. Lubiprostone is a Food and Drug Administration-approved drug with Clc-2 activation as its presumed mechanism of action. However, relative contribution of Clc-2 in intestinal Cl^- secretion and the mechanism of action of lubiprostone remain controversial due to lack of selective Clc-2 inhibitors. Using recently identified selective Clc-2 inhibitor AK-42, we characterized the roles of Clc-2 in Cl^- secretion in human intestinal epithelial T84 cells. Clc-2 inhibitor AK-42 had minimal (15%) inhibitory effect on secretory short-circuit current (I_{sc}) induced by cAMP agonists, where subsequently applied CFTR inhibitor ($CFTR_{inh}$-172) caused 2- to 3-fold greater inhibition. Similarly, AK-42 inhibited lubiprostone-induced secretory I_{sc} by 20%, whereas $CFTR_{inh}$-172 caused 2- to 3-fold greater inhibition. In addition to increasing CFTR and Clc-2-mediated apical Cl^- conductance, lubiprostone increased basolateral membrane K^+ conductance, which was completely reversed by cAMP-activated K^+ channel inhibitor $BaCl_2$. All components of lubiprostone-induced secretion (Clc-2, CFTR, and K^+ channels) were inhibited by ~65% with the extracellular Ca^{2+}-sensing receptor (CaSR) activator cinacalcet that stimulates cAMP hydrolysis. Lastly, E-type prostanoid receptor 4 (EP4) prostaglandin receptor inhibitor GW627368 pretreatment inhibited lubiprostone-induced secretion by 40% without any effect on forskolin response. Our findings suggest that Clc-2 has a minor role in cAMP-induced intestinal Cl^- secretion; and lubiprostone is not a selective Clc-2 activator, but a general activator of cAMP-gated ion channels in human intestinal epithelial cells.

SIGNIFICANCE STATEMENT

Cl^- channel Clc-2 activation is the proposed mechanism of action of the Food and Drug Administration-approved constipation drug lubiprostone. Using first-in-class selective Clc-2 inhibitor AK-42, we showed that Clc-2 has minor contribution in intestinal Cl^- secretion induced by lubiprostone and cAMP agonists. We also found that lubiprostone is a general activator of cAMP-gated ion channels in human intestinal epithelial cells via EP4 receptors. Our findings clarify the roles of Clc-2 in intestinal Cl^- secretion and elucidate the mechanism of action of approved-drug lubiprostone.

Introduction

Intestinal epithelial Cl^- secretion promotes luminal hydration and plays key roles in maintenance of gut health (Rao, 2019). Cystic fibrosis transmembrane conductance regulator (CFTR) is a major prosecretory Cl^- channel in intestinal epithelia. Loss of CFTR function is thought to be the key pathology causing gastrointestinal complications of cystic fibrosis (CF), such as constipation, meconium ileus, and distal intestinal obstruction syndrome (Kelly and Buxbaum, 2015). Clc-2 is a Cl^- channel co-expressed with CFTR in various tissues commonly affected by CF, including epithelial cells of intestine, liver, pancreas and airways (Thiemann et al., 1992; Lipecka et al., 2002). Clc-2 activity is regulated by various factors including cAMP and protein kinase A (Wang et al., 2017). Due to its overlapping expression with CFTR in several epithelia, Clc-2 was proposed as an alternative cAMP-activated Cl^- channel that can rescue CF phenotype in various organs, including the lungs and gut (Schwiebert et al., 1998).

The US Food and Drug Administration-approved drug lubiprostone was developed as a "Clc-2 activator" and was shown to have efficacy in various forms of constipation, such as chronic idiopathic constipation and opioid-induced constipation (Wilson et al., 2015). Based on its mechanism of action of Clc-2 activation, lubiprostone was proposed as a treatment that can alleviate CF-associated constipation. However, in CF subjects, lubiprostone had no effect on stool firmness or bowel movement frequency (O'Brien et al., 2011), which goes against the proposed role of Clc-2 and/or mechanism of action

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ABBREVIATIONS: CaSR, extracellular Ca^{2+}-sensing receptor; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; $CFTR_{inh}$, CFTR inhibitor; Clc-2, chloride channel protein 2; I_{SC}, short-circuit current; NKCC1, Na^+-K^+-Cl^- cotransporter 1.

of lubiprostone. Although lubiprostone increases Cl^- secretion in rodent and human intestinal epithelia, there is no consensus regarding the ion channels mediating this effect. Some studies have suggested that lubiprostone is a direct Clc-2 activator (Cuppoletti et al., 2004; Bao et al., 2008), whereas others have shown that the effect of lubiprostone is CFTR-dependent (Bijvelds et al., 2009). These contradicting results in the earlier studies are in part due to lack of selective Clc-2 inhibitors.

Using the recently described selective small molecule Clc-2 inhibitor AK-42 (Koster et al., 2020), here we characterized the roles of Clc-2 on Cl^- transport in human intestinal epithelial T84 cells, which natively express Clc-2 and CFTR (Cuppoletti et al., 2014). We also investigated the relative contributions of other ion channels/transporters involved in intestinal Cl^- secretion by utilizing pharmacological inhibitors $CFTR_{inh}$-172 (for CFTR), $BaCl_2$ (for K^+ channels), and bumetanide (for Na^+-K^+-Cl^- cotransporter 1 [NKCC1]).

Methods

Chemicals. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except GW627368 (MedChemExpress, Monmouth Junction, NJ, USA), RO1138452 (Fisher Scientific, Hampton, NH, USA), and $CFTR_{inh}$-172 (MedChemExpress). AK-42 was synthesized and purified as described (Koster et al., 2020). $BaCl_2$, cholera toxin, and vasoactive intestinal peptide were dissolved in distilled water; other compounds were dissolved in DMSO. For all experiments, the final DMSO concentration was 0.2%–0.4% in the Ussing chambers. Equal concentration of DMSO was added to controls in all experiments. Our group has extensive experience in using DMSO as a solvent for small molecules and showed that up to 1% DMSO does not affect ion transport in cell models (Oak et al., 2021).

Cell Culture. T84 (CCL-248, American Type Culture Collection) and Fischer rat thyroid cells stably expressing human wild-type CFTR (cells were obtained from UCSF Cystic Fibrosis Drug Discovery Core Center) and cultured as described (Oak et al., 2021). Cells were grown on inserts (12 mm diameter, 0.4 μm polyester membrane; Corning Life Sciences, Tewksbury, MA, USA) at 37°C in 5% CO_2/95% air and used for short-circuit current experiments 5–7 days after plating.

Short-Circuit Current Measurements. Cells were mounted in Ussing chambers, with each hemichamber containing bicarbonate-buffered Ringer's solution (pH 7.4, in mM: 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$). Secretagogues, cinacalcet, and prostaglandin receptor inhibitors were added to both apical and basolateral bathing solutions. The ion channel/transporter inhibitors were added only to apical ($CFTR_{inh}$-172 and AK-42) or basolateral ($BaCl_2$ and bumetanide) chambers. The solutions were aerated with 95% O_2/5% CO_2 and maintained at 37°C during experiments. Short-circuit current was measured using an EVC4000 multichannel voltage clamp (World Precision Instruments, Sarasota, FL, USA) via Ag/AgCl electrodes and 3 M KCl agar bridges.

In some experiments to measure apical Cl^- conductance, the basolateral membrane was permeabilized with 500 μg/ml of amphotericin B for 30 minutes, and a 60 mM of basolateral-to-apical Cl^- gradient was applied. For these experiments, Ringer's was the basolateral bathing solution (120 mM NaCl), and the apical solution contained 60 mM of NaCl and 60 mM of sodium gluconate. To measure basolateral membrane K^+ conductance, the apical membrane was permeabilized with 20 μM of amphotericin B for 30 minutes, and apical-to-basolateral potassium gradient was applied. The apical solution (pH 7.4) contained in mM: 142.5 K-gluconate, 1 $CaCl_2$, 1 $MgCl_2$, 0.43 KH_2PO_4, 0.35 Na_2HPO_4, 10 HEPES, and 10 D-glucose. In the basolateral solution (pH 7.4), 142.5 mM of K-gluconate was replaced by 5.5 mM of K-gluconate and 137 mM N-methylglucamine.

Statistical Analysis. A priori power analysis was not used; however, minimum n = 5–6 replicates were used in each experiment to verify the reproducibility of the results. Paired measures were analyzed by two-tailed paired Student's t test, and unpaired measures were analyzed by two-tailed unpaired Student's t test. In all analyses, P value < 0.05 was considered as statistically significant.

Results

CFTR Inhibition Partially Reduces Forskolin-Induced Secretory Current in T84 Cells. Since CFTR is considered as the major apical membrane Cl^- channel in intestinal epithelial cells, we first tested the effects of CFTR inhibitor ($CFTR_{inh}$-172) on forskolin-induced short-circuit current (I_{sc}) change in T84 cells. As shown before (Oak et al., 2021), $CFTR_{inh}$-172 (10 μM) partially inhibited the forskolin response (Fig. 1A, left), even with basolateral membrane permeabilization and 60 mM basolateral-to-apical Cl^- gradient (Fig. 1A, right). As previously described (Oak et al., 2021; Ma et al., 2002), 10 μM of $CFTR_{inh}$-172 completely inhibited forskolin response in Fischer rat thyroid cells cells transfected with CFTR (Fig. 1B), suggesting complete CFTR inhibition at this concentration.

cAMP-induced Cl^- secretion in intestinal epithelia is mediated by coordinated actions of basolateral membrane ion channels/transporters (K^+ channels and NKCC1) and apical membrane Cl^- channels. In the basolateral membrane, NKCC1 is the main Cl^- entry pathway to the cells, and K^+ recycling through K^+ channels promotes apical membrane Cl^- secretion by maintaining negative intracellular potential (Das et al., 2018). Consistent with this model, both NKCC1 inhibitor bumetanide and cAMP-activated K^+ channel inhibitor $BaCl_2$ completely inhibited the forskolin-induced I_{sc} change (Fig. 1C). Collectively, these results suggest contributions of

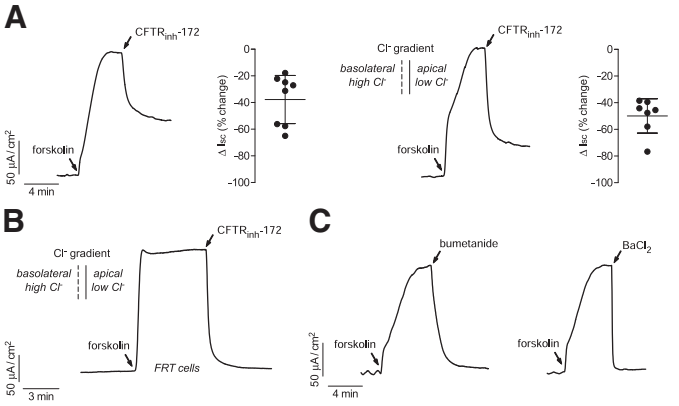

Fig. 1. CFTR inhibition partially reduces forskolin-induced short-circuit current (I_{sc}) in T84 cells. (A) I_{sc} traces showing responses to forksolin (10 μM) and $CFTR_{inh}$-172 (10 μM, apical) in intact T84 cells (*left*) and in T84 cells with basolateral membrane permeabilization (500 μg/ml amphotericin B) and 60 mM basolateral-to-apical Cl^- gradient (*right*). Summary data for $CFTR_{inh}$-172 response (percent inhibition of forskolin effect) in each experiment are shown as dot-plots next to the traces. Mean ± S.D., n = 7-8 per experiment. (B) I_{sc} trace showing that $CFTR_{inh}$-172 (10 μM, apical) completely inhibits forskolin (10 μM)-induced Cl^- secretion in Fischer rat thyroid cells cells transfected with CFTR (representative of 6 experiments). Basolateral membrane was permeabilized with amphotericin B and 60 mM basolateral-to-apical Cl^- gradient was applied as described above. (C) I_{sc} traces showing effects of bumetanide (100 μM, basolateral) *(left)* and $BaCl_2$ (5 mM, basolateral) *(right)* on forskolin responses in intact T84 cells (representative of 6 experiments per condition).

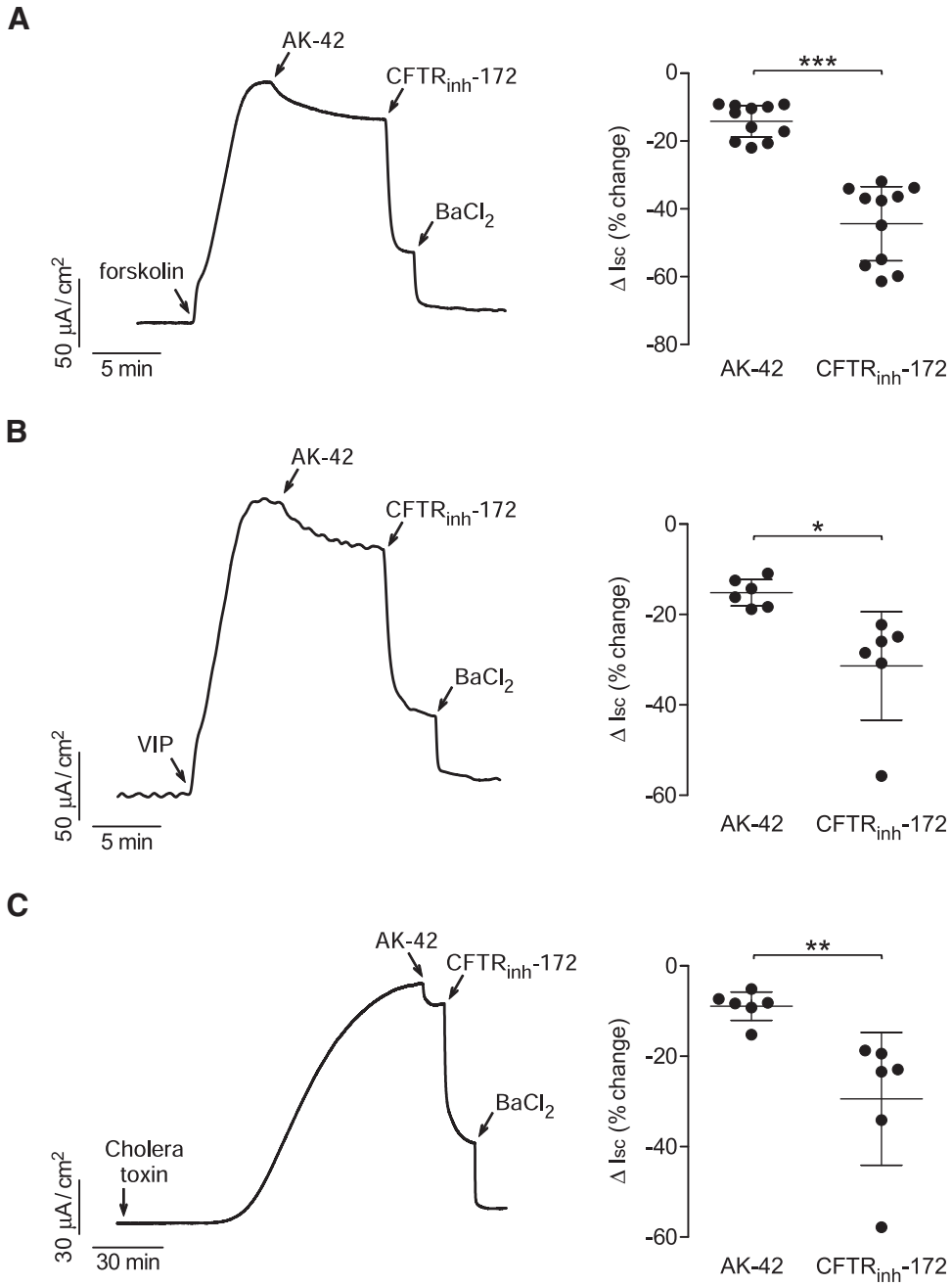

Fig. 2. cAMP agonists-induced short-circuit currents (I_{sc}) are mediated by apical membrane CFTR and Clc-2 Cl^- channels, and basolateral membrane K^+ channels in T84 cells. (A) *(left)* I_{sc} trace showing responses to forksolin (10 μM), AK-42 (10 μM, apical), $CFTR_{inh}$-172 (10 μM, apical) and $BaCl_2$ (5 mM, basolateral). (*right*) Summary data for AK-42 and $CFTR_{inh}$-172 responses (percent inhibition of forskolin effect). (B) I_{sc} trace and summary data as in A with vasoactive intestinal peptide (VIP, 10 nM) as agonist. (C) I_{sc} trace and summary data as in A with cholera toxin (1 μg/mL) as agonist. Mean ± S.D., n = 6–11 per experiment, Student's t test (paired), $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

non-CFTR apical membrane Cl^- channel(s) and/or basolateral membrane K^+ channels in forskolin-induced I_{sc} in T84 cells.

cAMP-Induced Secretory I_{sc} in T84 Cells is Mediated by Apical Membrane CFTR and Clc-2 Cl^- Channels and Basolateral Membrane K^+ Channels. Clc-2 is another Cl^- channel expressed in intestinal epithelia and human intestinal T84 cells (Lipecka et al., 2002; Cuppoletti et al., 2014). Selective Clc-2 inhibitor AK-42 had no effect on baseline I_{sc}; however, it had a modest (10%–20%) inhibitory effect on forskolin-induced I_{sc} in T84 cells (Fig. 2A). In this setting, subsequent $CFTR_{inh}$-172 treatment had 2- to 3-fold higher inhibitory effect compared with AK-42, albeit still incomplete inhibition of forskolin effect. The residual forskolin response was inhibited by K^+ channel inhibitor $BaCl_2$ treatment. Similar results showing 2- to 3-fold greater inhibitory effect of $CFTR_{inh}$-172 compared with AK-42 and inhibition of residual current with $BaCl_2$ were found when T84 cells were treated with other cAMP agonists vasoactive intestinal peptide (VIP, Fig. 2B) and cholera toxin (Fig. 2C). These results suggest that Clc-2 has a minor role in cAMP-induced

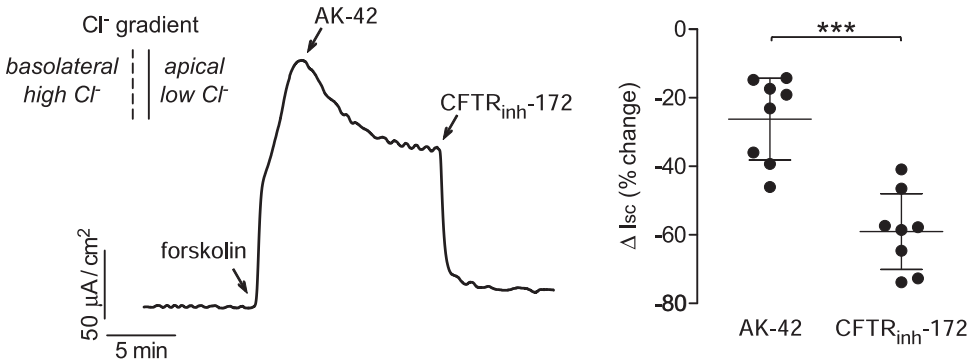

Fig. 3. Clc-2 is a minor contributor of forskolin-induced apical membrane Cl^- conductance in T84 cells. Short-circuit current (I_{sc}) trace showing responses to forksolin (10 μM), AK-42 (10 μM, apical) and $CFTR_{inh}$-172 (10 μM, apical) in T84 cells with basolateral membrane permeabilization (500 μg/ml amphotericin B) and 60 mM basolateral-to-apical Cl^- gradient. Summary data for AK-42 and $CFTR_{inh}$-172 responses (percent inhibition of forskolin effect) are shown on the right. Mean ± S.D., $n = 8$ experiments, Student's t test (paired), $^{***}P < 0.001$.

secretory I_{sc} in T84 cells, which is collectively mediated by apical membrane Cl^- channels (CFTR and Clc-2) and basolateral membrane K^+ channels.

Clc-2 is a Minor Contributor of Apical Membrane Cl^- Conductance in T84 Cells. To directly investigate the relative contributions of Clc-2 and CFTR on cAMP-induced apical membrane Cl^- conductance; experiments were done with basolateral membrane permeabilization and 60 mM basolateral-to-apical Cl^- gradient. Similar to non-permeabilized cells, in this setting $CFTR_{inh}$-172 had 2- to 3-fold greater inhibitory effect on forskolin-induced Cl^- secretion compared with AK-42, with essentially complete inhibition seen after AK-42 and $CFTR_{inh}$-172 treatments (Fig. 3). These results suggest that CFTR and Clc-2 are the main cAMP-activated apical membrane Cl^- channels in T84 cells, where Clc-2 is the minor contributor.

Clc-2 has a Minor Role in Lubiprostone-Induced Secretion in T84 Cells. We next tested the effects of AK-42 and $CFTR_{inh}$-172 on "Clc-2 activator" lubiprostone-induced I_{sc} in T84 cells. Similar to forskolin, lubiprostone caused a large secretory current, which was minimally (~15%) inhibited by AK-42 with 2- to 3-fold greater inhibitory effect of subsequently applied $CFTR_{inh}$-172 (Fig. 4A) and the residual I_{sc} was

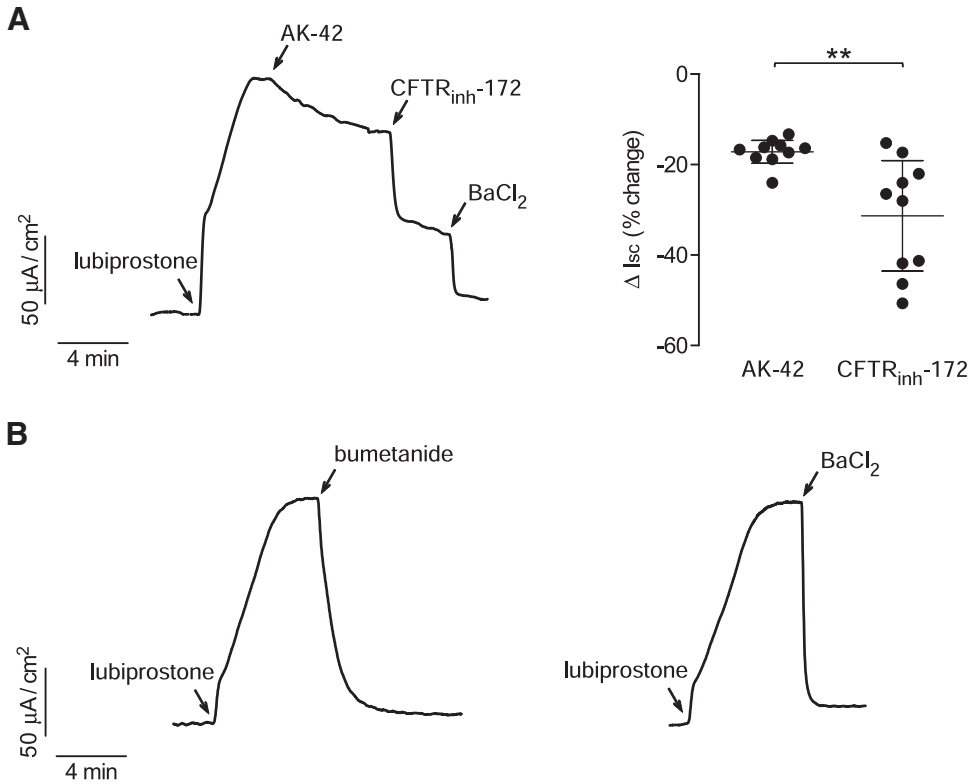

Fig. 4. Lubiprostone-induced short-circuit current (I_{sc}) is mediated by apical membrane CFTR and Clc-2 Cl^- channels, and basolateral membrane K^+ channels in T84 cells. (A) I_{sc} trace showing responses to lubiprostone (1 μM), AK-42 (10 μM, apical), $CFTR_{inh}$-172 (10 μM, apical) and $BaCl_2$ (5 mM, basolateral). Summary data for AK-42 and $CFTR_{inh}$-172 responses (percent inhibition of lubiprostone effect) are shown on the right. Mean ± S.D., $n = 10$ experiments, Student's t test (paired), $^{**}P < 0.01$. (B) I_{sc} traces showing effects of bumetanide (100 μM, basolateral) *(left)* and $BaCl_2$ (5 mM, basolateral) *(right)* on lubiprostone responses in T84 cells (representative of six experiments per each condition).

inhibited by K^+ channel inhibitor $BaCl_2$. As found for forskolin, lubiprostone-induced I_{sc} was completely inhibited by bumetanide and $BaCl_2$ (Fig. 4B).

Lubiprostone-Induced Secretory I_{sc} is Dependent on Activation of Apical Membrane Cl^- Channels and Basolateral Membrane K^+ Channels. Next, basolateral membrane permeabilization and basolateral-to-apical Cl^- gradient was used to selectively investigate the effects of lubiprostone on apical membrane Cl^- conductance. Compared with AK-42, $CFTR_{inh}$-172 had ~2-fold greater inhibitory effect on lubiprostone-induced apical membrane Cl^- secretion with essentially complete inhibition seen after AK-42 and $CFTR_{inh}$-172 treatments (Fig. 5A). Since lubiprostone-induced secretory I_{sc} in non-permeabilized T84 cells was dependent on the activity of basolateral K^+ channels, we directly investigated the effects of lubiprostone on basolateral membrane K^+ conductance by selective apical membrane permeabilization and apical-to-basolateral K^+ gradient (Rufo et al., 1997). In this setting, lubiprostone caused secretory K^+ current, which was completely inhibited by $BaCl_2$ treatment (Fig. 5B).

Lubiprostone Activates CFTR, Clc-2, and K^+ Channels Even at Low Concentrations. An earlier study suggested that lubiprostone might be a selective Clc-2 activator at low (<100 nM) concentrations and activate CFTR only at higher concentrations (Bao et al., 2008). To test whether lubiprostone is a selective Clc-2 activator in that setting, we studied effects of AK-42 and $CFTR_{inh}$-172 in the presence of lower concentrations of lubiprostone. Compared with AK-42, $CFTR_{inh}$-172 had at least threefold greater inhibitory effect on I_{sc} induced by lower (3–100 nM) concentrations of lubiprostone, with inhibition of residual I_{sc} by $BaCl_2$ (Fig. 6). These results suggest that lubiprostone is a general activator of cAMP-gated ion channels, including apical membrane CFTR and Clc-2 Cl^- channels and basolateral membrane K^+ channels, even at low concentrations. Interestingly, the AK-42 had relatively less inhibitory effect on Cl^- secretion induced by lower lubiprostone concentrations (mean±S.D.: 2.5±1.1% at 3 nM, 6.6±2.8% at 10 nM, 8.1±2.0% at 100 nM and 17.2±2.9% at 1 μM (see Fig. 4A for the last one), whereas $CFTR_{inh}$-172 had similar inhibitory effects (20%–25%) at all lubiprostone concentrations. These findings suggest that the lubiprostone might have different saturation and activation curves for Clc-2 and CFTR in T84 cells.

CaSR Activator Cinacalcet Inhibits All Components of Lubiprostone-Induced Secretory I_{sc}. Since lubiprostone appears to activate structurally dissimilar cAMP-gated ion channels (Clc-2, CFTR and K^+ channels) even at very low concentrations, its effect can potentially be explained by cellular cAMP elevation, which was demonstrated in T84 cells by earlier studies (Bijvelds et al., 2009; Ao et al., 2011). We recently showed that CaSR activator cinacalcet inhibits cAMP-mediated activation of CFTR and basolateral K^+ channels in T84 cells by increasing cAMP hydrolysis (Oak et al., 2021). Similarly, cinacalcet pretreatment inhibited all components of lubiprostone-induced absolute I_{sc} change in T84 cells, including Clc-2, CFTR, and K^+ channels (Fig. 7A and B). These results provide further functional evidence that lubiprostone is not a specific Clc-2 activator, but is a non-selective activator of cAMP-gated ion channels in intestinal epithelial cells via cAMP elevation. CaSR activation equally inhibited Clc-2 and CFTR activities as suggested by similar percent inhibitory effects of AK-42 and CFTRinh-172 in the presence and absence of cinacalcet (Fig. 7C).

EP4 Inhibition Reduces Lubiprostone-Induced Secretory I_{sc}. Lubiprostone is a prostaglandin derivative (Cuppoletti et al., 2004), and cAMP elevation is the known signaling pathway for IP, DP1, EP2, and EP4 prostaglandin receptors expressed in intestinal epithelia (Moreno, 2017). By using selective pharmacological inhibitors, we tested the roles of these receptors in prosecretory effect of lubiprostone. Pretreatment of T84 cells with IP inhibitor RO1138452 (Bley et al., 2006), DP1 inhibitor BWA868C (Shichijo et al., 2003), or EP2 inhibitor AH6809 (Woodward, 1995) had no effect on lubiprostone-induced secretory I_{sc} (Fig. 8A), whereas 10 μM of EP4 inhibitor GW627368 (Wilson et al., 2006) pretreatment reduced lubiprostone-induced I_{sc} by 40% in T84 cells (Fig. 8B), without any effects on forskolin-induced I_{sc} (Fig. 8C). To further study the competitive EP4 antagonist GW627368 effect, we studied lubiprostone concentration-response in the presence of submaximal (1 μM) GW627368. Consistent with competitive antagonism, GW627368 shifted lubiprostone concentration-response curve to the right (Fig. 9A and B) as indicated by ~20-fold increased EC_{50} (6.9 nM in control versus 135.6 nM in GW627368 group) and unchanged maximal lubiprostone response (Fig. 9C). These results suggest that

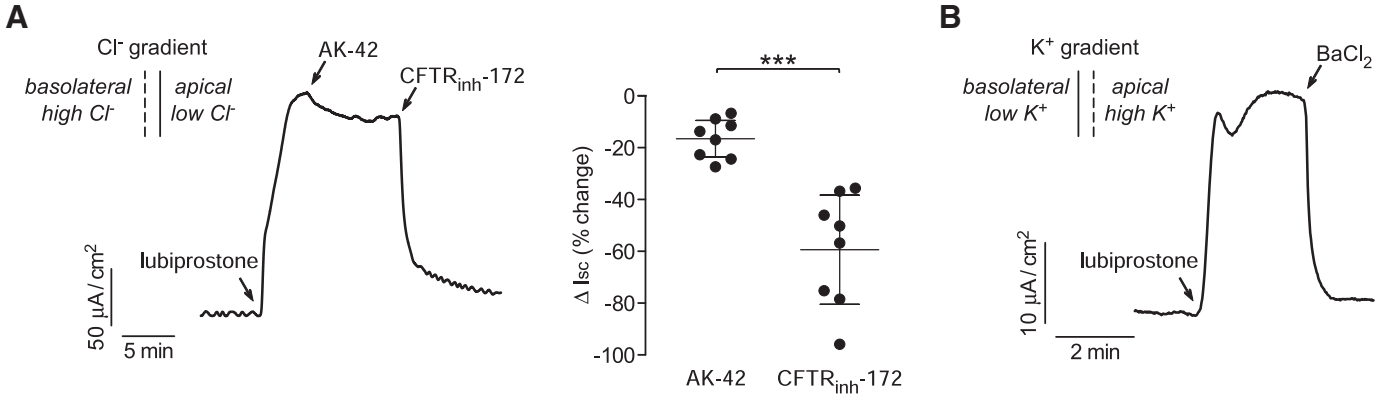

Fig. 5. Clc-2 is a minor contributor of lubiprostone-induced apical membrane Cl^- conductance and lubiprostone activates basolateral membrane K^+ channels in T84 cells. (A) *(left)* I_{sc} trace showing responses to lubiprostone (1 μM), AK-42 (10 μM, apical) and $CFTR_{inh}$-172 (10 μM, apical) with basolateral membrane permeabilization (500 μg/ml amphotericin B) and 60 mM basolateral-to-apical Cl^- gradient. *(right)* Summary data for AK-42 and $CFTR_{inh}$-172 responses (percent inhibition of lubiprostone effect). Mean ± S.D., $n = 8$ experiments, Student's t test (paired), $^{***}P < 0.001$. (B) I_{sc} trace showing responses to lubiprostone (1 μM) and $BaCl_2$ (5 mM, basolateral) with apical membrane permeabilization (20 μM amphotericin B) and apical-to-basolateral K^+ gradient (representative of 6 experiments).

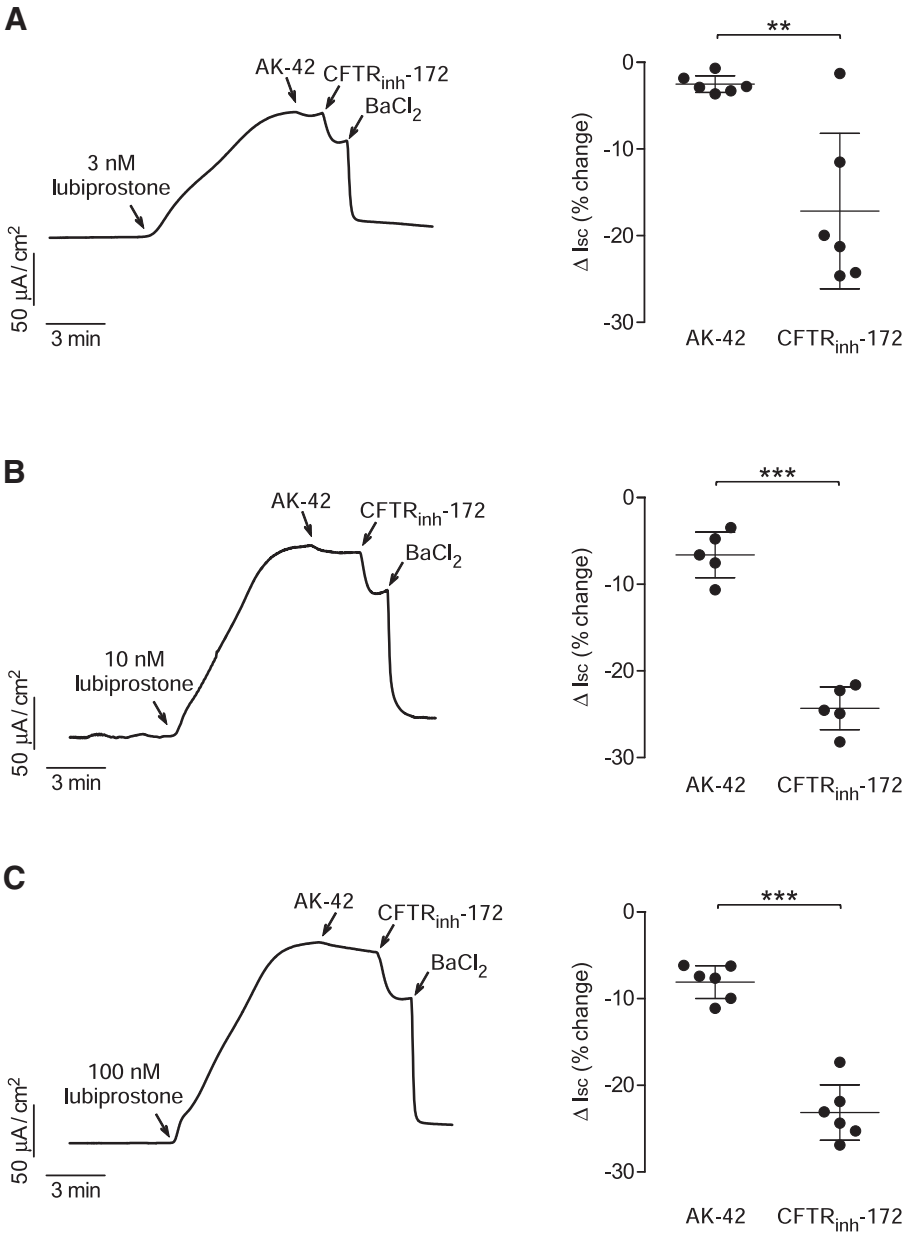

Fig. 6. Clc-2 is a minor contributor of lubiprostone-induced secretory short-circuit current (I_{sc}) even at low concentrations in T84 cells. (A) *(left)* I_{sc} trace showing responses to lubiprostone (3 nM), AK-42 (10 μM, apical), $CFTR_{inh}$-172 (10 μM, apical) and $BaCl_2$ (5 mM, basolateral). *(right)* Summary data for AK-42 and $CFTR_{inh}$-172 responses (percent inhibition of lubiprostone effect). (B) I_{sc} trace and summary data as in A with 10 nM lubiprostone. (C) I_{sc} trace and summary data as in A with 100 nM lubiprostone. Mean ± S.D., n = 5–6 experiments per concentration, Student's t test (paired), $^{**}P < 0.01$, $^{***}P < 0.001$.

lubiprostone exerts a prosecretory effect through activation of EP4 receptors (see Fig. 10 for proposed mechanisms).

Discussion

Here we provide functional evidence that lubiprostone is a non-selective activator cAMP-gated ion channels including CFTR, Clc-2 and K^+ channels in human intestinal epithelial cells. Although lubiprostone stimulates Cl^- secretion in rodent and human intestinal epithelia and has clinical efficacy in constipation, our findings suggest that it is not a selective Clc-2 activator contrary to what has been proposed. By using selective Clc-2 inhibitor AK-42, here we found that Clc-2 is only a minor contributor of Cl^- secretion induced by lubiprostone as well as cAMP agonists in human intestinal epithelial cells, where CFTR and basolateral membrane K^+ channels have predominant roles.

Constipation affects 15% of the general population and up to 47% of CF subjects (van der Doef et al., 2010). Linaclotide, plecanatide, and lubiprostone are the Food and Drug Administration-approved prosecretory drugs for constipation whose mechanism of action involves Cl^- channel activation. Linaclotide and plecanatide are the agonists of the guanylate cyclase C receptor that activate CFTR via cGMP elevation in enterocytes (Bharucha et al., 2017). As shown here, the prosecretory effect of lubiprostone is also dependent on CFTR activation. Although all these approved drugs had efficacy in constipation in the general population, they are predicted to have minimal efficacy in CF where CFTR is defective. This idea is also suggested by the lack

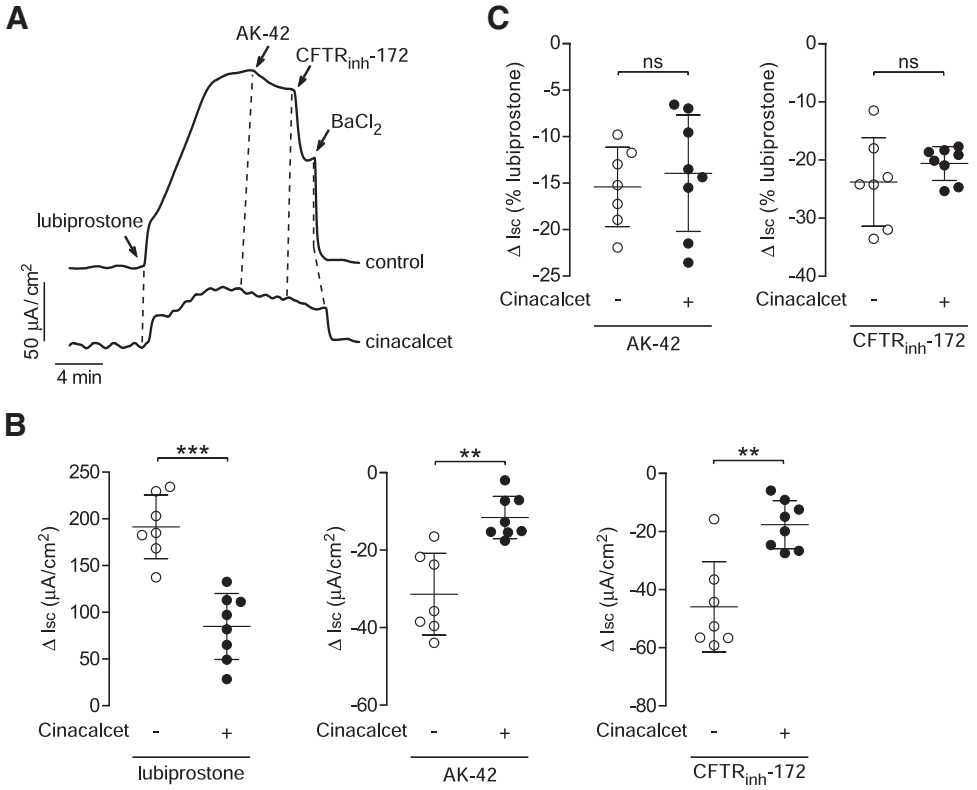

Fig. 7. CaSR agonist cinacalcet inhibits all components of lubiprostone-induced secretory short-circuit current (I_{sc}) in T84 cells. (A) I_{sc} traces showing responses to lubiprostone (1 μM), AK-42 (10 μM, apical), $CFTR_{inh}$-172 (10 μM, apical) and $BaCl_2$ (5 mM, basolateral) with and without cinacalcet (30 μM) pretreatment for 20 minutes. (B) Summary data for absolute lubiprostone, AK-42 and $CFTR_{inh}$-172 responses. (C) Summary data for AK-42 and $CFTR_{inh}$-172 responses as % of lubiprostone effect. Mean ± S.D., n = 7-8 experiments per group, Student's t test (unpaired), $^{**}P < 0.01$, $^{***}P < 0.001$, ns: not significant.

of efficacy of lubiprostone on stool firmness or spontaneous bowel movement frequency in a small series of CF subjects (O'Brien, 2011). Development of CFTR modulators has revolutionized management of CF, particularly for pulmonary disease (Lopes-Pacheco, 2020). For instance, triple combination therapy has shown impressive efficacy in pulmonary symptoms, weight gain, and quality of life (Middleton et al., 2019). However, recent studies in CF subjects showed ongoing high burden of constipation despite CFTR modulator therapy (Moshiree et al., 2021) and lack of effect of CFTR modulators on gut transit and water content (Ng, 2021). These studies suggest that there is an unmet clinical need for effective treatments for CF-associated constipation and related disorders. Inhibition of intestinal Cl^- absorption offers an alternative approach to increase stool hydration in intestinal hyposecretory conditions in CF including constipation, meconium ileus, and distal intestinal obstruction syndrome. We recently showed that inhibitors of intestinal Cl^-/HCO_3^- exchangers (SLC26A3 and SLC26A6) have efficacy in CF mouse models of intestinal fluid absorption and constipation (Cil et al., 2021; Haggie et al., 2018; Lee et al., 2019). Since SLC26A3 and SLC26A6 inhibitors work independent of CFTR, they can potentially have efficacy in gastrointestinal manifestations of CF regardless of the mutation type.

Our findings here are in disagreement with some earlies studies. Lubiprostone was reported to induce whole cell Cl^- currents in Clc-2-transfected cells but not in CFTR-transfected cells (Cuppoletti et al., 2004). Another study similarly showed that lubiprostone-induced whole cell Cl^- currents are abolished in T84 cells with Clc-2 knockdown, but not CFTR knockdown (Cuppoletti et al., 2014). The conflicting findings between these studies and the current study can potentially be explained by compensatory phenotypic changes in transfected cell models and knockdown studies. In addition, various prior studies in native cell line models and intestine are in agreement with our findings here. In A6 cells, single-channel recordings showed that lubiprostone activates two separate Cl^- channels and the electrical properties of these channels are consistent with Clc-2 and CFTR (Bao et al., 2008). In intestinal tissue, lubiprostone-induced Cl^- secretion was abolished in *Cftr* knockout mice and CF subjects suggesting key role of CFTR in clinical effect of lubiprostone (Bijvelds et al., 2009). Although we showed here that Clc-2 and CFTR collectively mediate apical membrane Cl^- conductance in human intestinal T84 cells, Clc-2 and CFTR co-localization was not demonstrated consistently in the gastrointestinal tract. In mice and rats, Clc-2 is mainly expressed in the basolateral membrane of intestinal epithelial cells (Lipecka et al., 2002; Catalán et al., 2012). *Clcn2* knockout in mice has no effect on cAMP-induced Cl^- secretion (Zdebik et al., 2004), whereas it greatly impairs electroneutral NaCl absorption in the colon (Catalán et al., 2012). In the human colon, Clc-2 expression was shown predominantly in surface epithelia (the site for Cl^- absorption), with very little expression in crypts, where Cl^- secretion mainly occurs (Lipecka et al., 2002; Jakab et al., 2012). Based on our findings here and these earlier studies, Clc-2 does not appear to have a major role in intestinal Cl^- secretion, and CFTR is the major cAMP-gated prosecretory intestinal Cl^- channel in mice (Zdebik et al., 2004) and humans (Bijvelds et al., 2009).

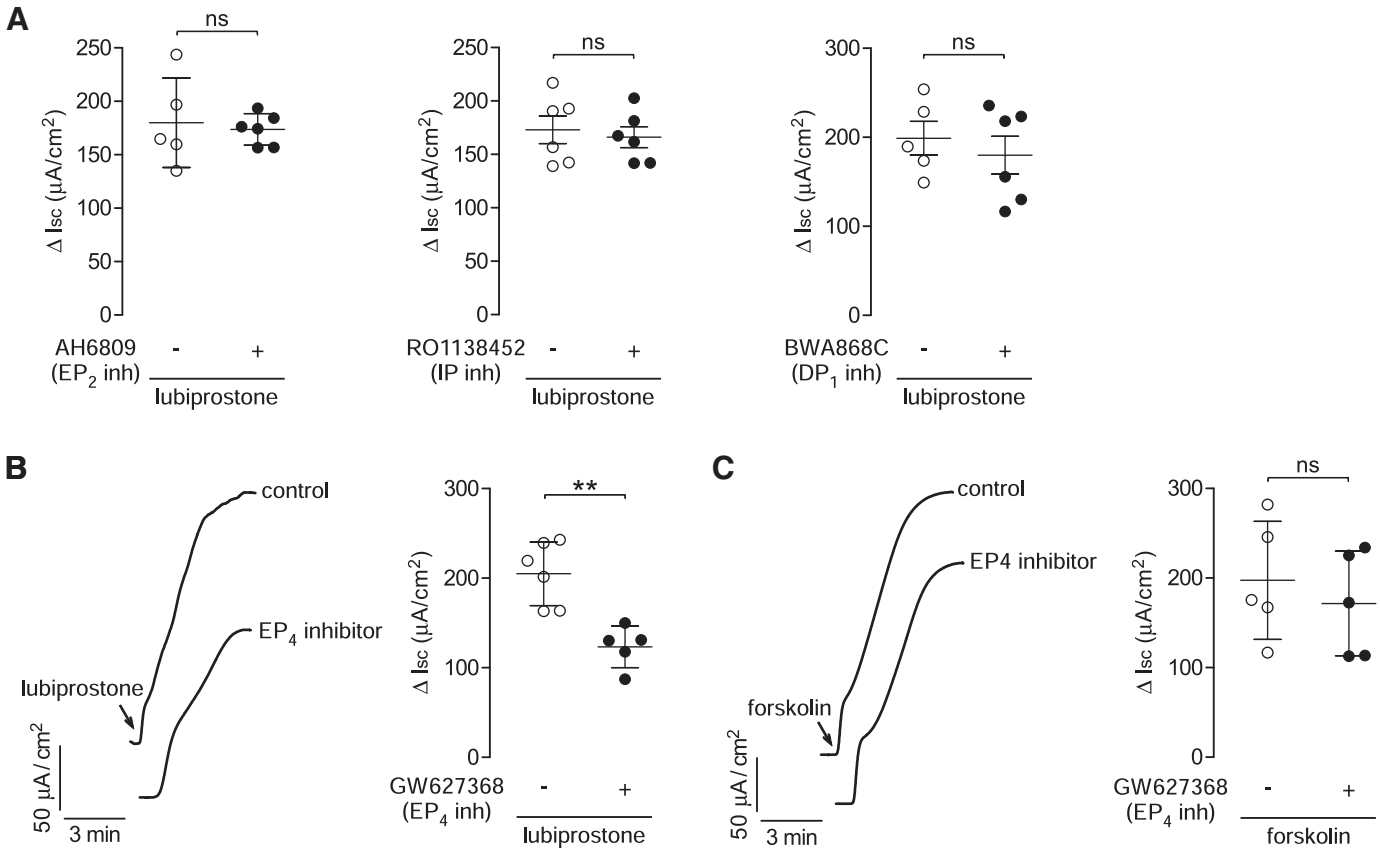

Fig. 8. The prosecretory effect of lubiprostone is mediated through EP4 receptors in T84 cells. (A) Maximal short-circuit current (I_{sc}) changes induced by lubiprostone (1 μM) with and without 20-minute pretreatment with EP2 receptor inhibitor AH6809 (10 μM, *left*), IP receptor inhibitor RO1138452 (1 μM, *middle*), or DP1 receptor inhibitor BWA868C (10 μM, *right*). (B) I_{sc} traces (*left*) and summary data (*right*) showing responses to lubiprostone (1 μM) with and without maximal EP4 receptor inhibitor GW627368 (10 μM) pretreatment for 20 minutes. (C) I_{sc} traces (*left*) and summary data (*right*) showing responses to forskolin (10 μM) with and without EP4 receptor inhibitor GW627368 (10 μM) pretreatment. Mean ± S.D., n = 5–6 experiments per group, Student's t test (unpaired), $^{**}P < 0.01$, ns: not significant.

Clc-2 inhibitor AK-42 is a valuable research tool to study roles of Clc-2 in various tissues, however it is unclear whether Clc-2 inhibitors will be viable therapeutic candidates. Clc-2 has widespread expression in many tissues and humans with *CLCN2* loss-of-function mutations develop leukoencephalopathy (Depienne et al., 2013). Based on its mild antisecretory effects in the presence of cAMP agonists, Clc-2 inhibitors might be beneficial for treatment of cAMP-mediated secretory diarrheas, such as cholera. However, considering the minor contribution of Clc-2 in cAMP-induced Cl^- secretion in human intestinal epithelial cells as shown here, the efficacy of Clc-2 inhibitors in diarrhea is likely going to be minimal. For

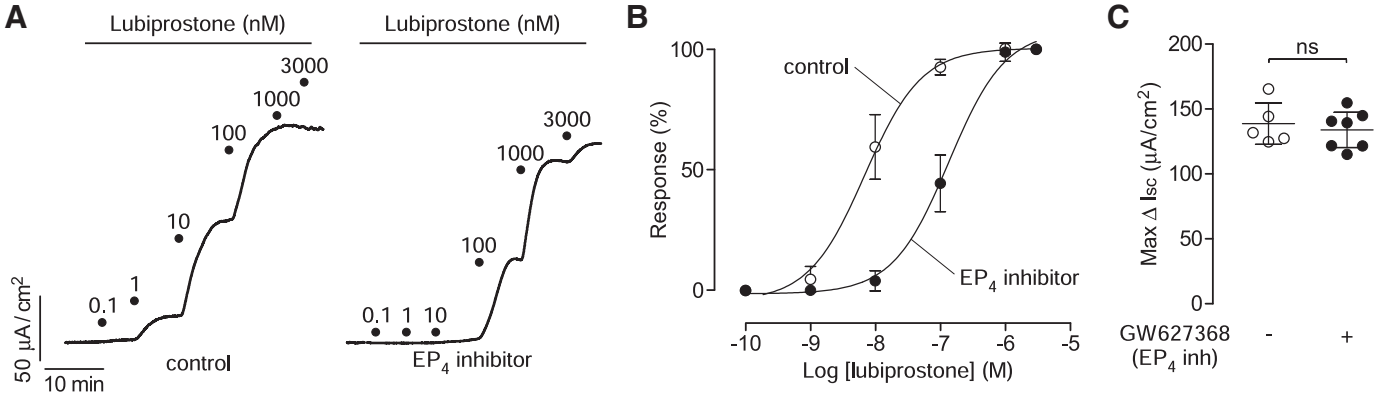

Fig. 9. Lubiprostone is an agonist of EP4 receptors in T84 cells. (A) Representative short-circuit current (I_{sc}) traces for lubiprostone concentration-response in control cells (*left*) and in the presence of submaximal (1 μM) GW627368 (*right*). (B) Deduced lubiprostone concentration-response curves in the absence and presence of submaximal GW627368. (C) Maximal lubiprostone-induced I_{sc} changes in the absence and presence of submaximal GW627368. Mean ± S.D., n = 5–7 experiments per group, Student's t test (unpaired), ns: not significant.

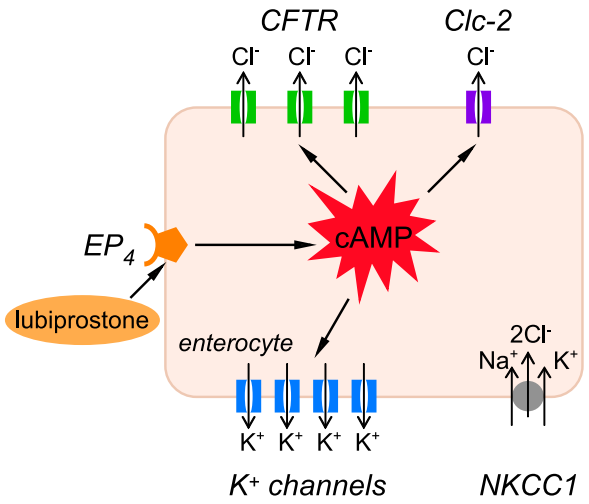

Fig. 10. Mechanisms of lubiprostone-induced Cl^- secretion in intestinal epithelial cells. See text for explanations.

cholera, various CFTR inhibitors are in development (Cil et al., 2017) and can potentially have greater efficacy than Clc-2 inhibitors since CFTR is the major intestinal cAMP-gated Cl^- channel. However, a recent randomized clinical trial in cholera patients showed lack of efficacy of the CFTR inhibitor iOWH032 (NCT04150250). In cholera, elevated cellular cAMP levels increase CFTR-mediated Cl^- secretion and reduce NHE3-mediated Na^+ absorption and both of these processes contribute in the intestinal fluid losses. Thus, inhibition of CFTR alone might potentially have limited efficacy in reducing diarrhea since it does not affect fluid absorption. We recently showed that CaSR activator cinacalcet inhibits CFTR-mediated Cl^- secretion and stimulates NHE3-mediated Na^+ absorption by promoting cAMP hydrolysis (Oak et al., 2021). For cholera and other cyclic nucleotide-mediated secretory diarrheas, CaSR activators, such as cinacalcet, can potentially have greater efficacy than Clc-2 or CFTR inhibitors by targeting the root cause of diarrhea (cAMP elevation).

Eicosanoids are important regulators of intestinal fluid and electrolyte transport. There are four G_s-coupled eicosanoid receptors expressed in intestinal epithelial cells: EP2, EP4, DP1, and IP (Moreno, 2017). Certain inflammatory conditions, such as Salmonella infection, induce Prostaglandin E2 synthesis, which stimulates cAMP-activated Cl^- secretion via EP4 and EP2 receptors (Bertelsen et al., 2003; Mosa et al., 2008). Prostaglandin D2 also stimulates cAMP-activated intestinal Cl^- secretion via DP1 receptors (Medani et al., 2015). Some earlier studies showed that EP4 antagonists can inhibit antisecretory effect of prostaglandin analog lubiprostone (Ao et al., 2011). Similarly, we found here that EP4 receptor inhibitor GW627368 pretreatment inhibits lubiprostone-induced Cl^- secretion without any effect on adenylate cyclase activator forskolin response. We also showed that other G_s-coupled intestinal eicosanoid receptors (EP2, DP1, and IP) have no role in lubiprostone effect. These results suggest that the prosecretory effects of lubiprostone in human intestinal cells are mainly mediated by EP4 receptor activation, similar to what was shown in ovine airways (Cuthbert, 2011) and recombinant cell models (Norimatsu et al., 2012).

Although $CFTR_{inh}$-172 is the most commonly used pharmacological CFTR inhibitor, some studies have suggested that it can have off-target effects like most pharmacological inhibitors. Cuppoletti et al. showed that $CFTR_{inh}$-172 inhibits forskolin-induced recombinant whole cell Cl^- currents equipotently in human embryonic kidney 293 cells transfected with Clc-2 or CFTR suggesting that it might be inhibiting Clc-2 in addition to CFTR (Cuppoletti et al., 2014). Here, we found that $CFTR_{inh}$-172 comparably inhibits forskolin-induced secretory I_{sc} when used alone (Fig. 1A. left) or after Clc-2 inhibitor AK-42 (Fig. 2A) in non-permeabilized T84 cells (37.8±18.7% versus 44.3±11.5%, respectively, mean ± S.D., $P = 0.35$, unpaired t test). Similarly, $CFTR_{inh}$-172 inhibits forskolin-induced secretory I_{sc} comparably when used alone (Fig. 1A, right) or after Clc-2 inhibitor AK-42 (Fig. 3) in T84 cells with basolateral membrane permeabilization (49.9±13.4% versus 59±11.5, respectively, mean ± S.D., $P = 0.18$, unpaired t test). Also, in majority of the experiments here (Figs. 2-7), we added $CFTR_{inh}$-172 after Clc-2 was already completely inhibited by 10 μM AK-42. These collectively suggest that $CFTR_{inh}$-172 effect observed here is not dependent on Clc-2 inhibition. The inconsistency between the previous (Cuppoletti et al., 2014) and the current study regarding $CFTR_{inh}$-172 effect on Clc-2 might be due to differences in cell types, since the earlier study used recombinant human embryonic kidney 293 cells, which are known to natively express several other Cl^- channels (Zhu et al., 1998) which might be affected by $CFTR_{inh}$-172. Regarding off-target effects, another study showed that although $CFTR_{inh}$-172 does not affect Ca^{2+}-activated Cl^- channel currents at 10 μM, it can inhibit volume-sensitive outwardly rectifying Cl^- conductance at concentrations higher than 5 μM (Melis et al., 2014). Thus, the effect of 10 μM $CFTR_{inh}$-172 observed here might in part be due to inhibition of additional Cl^- channels, such as volume-sensitive Cl^- channels, which is a limitation of the current study.

In conclusion, we showed that Clc-2 is a minor contributor of cAMP-activated Cl^- secretion in human intestinal epithelial cells, and lubiprostone is not a selective Clc-2 activator, but is a non-selective activator of cAMP-gated ion channels via EP4 receptors. Our findings also show that Clc-2 inhibitor AK-42 is a useful physiologic tool to study the roles of Clc-2 in different tissues.

Authorship Contributions

Participated in research design: Cil.
Conducted experiments: Oak, Chu, Yottasan, Chhetri.
Performed data analysis: Oak, Chu, Cil.
Wrote or contributed to the writing of the manuscript: Cil.

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