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Lubiprostone targets prostanoid signaling and promotes ion transporter trafficking, mucus exocytosis and contractility

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Abstract

Background and Aim—Lubiprostone is a chloride channel activator in clinical use for the treatment of chronic constipation, but the mechanisms of action of the drug are poorly understood. The aim of this study was to determine whether lubiprostone exerts secretory effects in the intestine by membrane trafficking of ion transporters and associated machinery.

Methods—Immunolabeling and quantitative fluorescence intensity were used to examine lubiprostone-induced trafficking of the cystic fibrosis transmembrane conductance regulator (CFTR), sodium/potassium-coupled chloride co-transporter 1 (NKCC1), electrogenic sodium/bicarbonate co-transporter 1 (NBCe1), down-regulated in adenoma (DRA), putative anion transporter 1 (PAT1), sodium/proton exchanger 3 (NHE3), Ca²⁺ activated chloride channel 2 (CIC-2) serotonin and its transporter SERT, E prostanoid receptors EP4 and EP1, sodium/potassium ATPase (Na-K-ATPase) and protein kinase A (PKA). The effects of lubiprostone on mucus exocytosis in rat intestine and human rectosigmoid explants were also examined.

Results—Lubiprostone induced contraction of villi and proximal colonic *plicae* and membrane trafficking of transporters that was more pronounced in villus/surface cells compared to the crypt. Membrane trafficking was determined by: (1) increased membrane labeling for CFTR, PAT1, NKCC1, and NBCe1 and decreased membrane labeling for NHE3, DRA and CIC-2; (2) increased serotonin, SERT, EP4, EP1 and PKA labeling in enterochromaffin cells; (3) increased SERT, EP4, EP1, PKA and Na-K-ATPase in enterocytes; (4) and increased mucus exocytosis in goblet cells.

Conclusion—These data suggest that lubiprostone can target serotonergic, EP4/PKA and EP1 signaling in surface/villus regions; stimulate membrane trafficking of CFTR/NBCe1/NKCC1 in villus epithelia and PAT1/NBCe1/NKCC1 in colonic surface epithelia; suppress NHE3/DRA trafficking and fluid absorption; enhance mucus-mobilization and mucosal contractility.

Keywords

ion transporters; membrane trafficking; receptor signaling; cystic fibrosis conductance regulator

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Competing interests: None to declare.

Introduction

Lubiprostone is an FDA-approved prostaglandin E1 derivative in clinical use for the treatment of chronic constipation [1] and irritable bowel syndrome with constipation in adults [2]. Enhancement of luminal liquidity is a major factor in its efficacy in patients as the majority report bowel movement within 24 hours [1]. Lubiprostone also increases fasting gastric volume, delays gastric emptying, accelerates transit in the small intestine and all colonic segments [3], and has minor effects on colonic tone and compliance [4]. However, the underlying mechanisms responsible for these observations are not fully established. According to the manufacturer, lubiprostone acts locally within the gastrointestinal (GI) tract [5]. How therapeutic concentrations of the drug are achieved in the colon to stimulate bowel contractions is not clear.

Lubiprostone stimulates electrogenic Cl^- secretion in human and rodent intestinal epithelia [6-9]. There is evidence that the *E prostanoïd receptor 4/cAMP/protein kinase A/cystic fibrosis transmembrane conductance regulator (EP4/cAMP/PKA/CFTR)* pathway is involved in lubiprostone-induced Cl^- and HCO_3^- secretion [6, 9-11]. But cAMP/CFTR mediated secretion [6] cannot fully explain lubiprostone's mild diarrhea-inducing profile since it has been shown to be beneficial in CF patients where CFTR is dysfunctional [1-2, 12]. Of note, misoprostol, a prostaglandin E1 derivative ulcer-treating drug, promotes HCO_3^- and mucus secretion, induces CFTR-mediated fluid secretion in jejunum of healthy subjects, but also reduces Na^+/H^+ exchanger 3 (NHE3) mediated fluid absorption in CF patients in a CFTR-independent manner [13]. Since E1 prostaglandins regulate Na-K-ATPase and salt absorption through both cAMP and Ca^{2+} pathways [14], and cAMP and Ca^{2+} can inhibit (NHE3) mediated fluid absorption, a similar cAMP/ Ca^{2+} co-activation by lubiprostone cannot be excluded. Furthermore, it has been suggested that lubiprostone could activate an alternative Cl^- exit pathway, such as the voltage-gated ClC-2 channel [7-8, 15]. However, direct evidence linking lubiprostone to apical ClC-2 activity in the intestine has not been provided [6, 9]. Lubiprostone was reported to activate ClC-2 without affecting CFTR in human colon T84 cells [8]. However, two subsequent studies conducted in the same cell model reported the opposite: that lubiprostone activates CFTR without affecting ClC-2 [6, 9]. Furthermore, studies in kidney cells suggested that lubiprostone could activate ClC-2 at low (< 100nM) but CFTR at higher concentration. But these conclusions have similarly been challenged [9,15]. Thus, there remains a lack of consensus regarding lubiprostone's mechanism of action.

Second messenger dependent trafficking of transporters from subcellular endosomes to the plasma membrane is an important physiological mechanism underlying intestinal anion secretion and can be demonstrated in native tissues using indirect morphological approaches [16]. We hypothesized that lubiprostone's secretory effects [7, 10] could involve trafficking of intestinal ion transporters including CFTR, the basolateral $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter 1 (NKCC1), the electrogenic basolateral $\text{Na}^+/\text{HCO}_3^-$ co-transporter 1 (NBCe1), and the apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers PAT1 (a.k.a. Putative Anion Exchanger 1; SLC26A6) and/or DRA (a.k.a. Down-Regulated in Adenoma; SLC26A3). On the other hand, lubiprostone-induced inhibition of fluid absorption could be explained by trafficking of apical NHE3. Because of the lack of consensus on the role of ClC-2 in mediating lubiprostone's action, we examined the distribution of ClC-2 using three independent antibodies, and tested lubiprostone's influence on CFTR and ClC-2 membrane trafficking. We also assessed its effects on E prostanoïd receptors 4 and 1 (EP4 and EP1) because we identified both receptors in enterochromaffin (EC) cells in the intestine. Similar to naturally occurring prostaglandins lubiprostone exerts pro-secretory effects in small and large intestine through binding to prostanoïd receptors on epithelial cells to stimulate intracellular adenylyl cyclase and cAMP [6]. Finally, lubiprostone's effect on mucus exocytosis was examined because the amount

and composition of intestinal mucus can greatly influence both liquidity and transit of luminal contents, and nothing is known about lubiprostone's effects on mucus release. The results of this study using human and rat intestine suggest that lubiprostone has multiple targets; stimulating CFTR-*dependent* as well as CFTR- and ClC-2-*independent* anion secretion, suppressing fluid absorption, enhancing mucus secretion and promoting local mucosal contractility.

Materials and Methods

Human Tissue

Rectosigmoid biopsy specimens (provided by Dr. Anthony Bauer; University of Pittsburgh, PA) were obtained from healthy subjects after informed consent. The study was approved by the Institutional Review Board (IRB) of the University of Pittsburgh. Colonic biopsies (1.5 mm × 3 mm) were maintained in Dulbecco's modified Eagle medium and left untreated, or treated *ex vivo* with normal saline (pH 7.4) or 2.5 µg/ml or 7.5 µg/ml lubiprostone (mol. w. 390.46 g) in saline (Takeda Pharmaceutical North America, Deerfield, IL, USA) for 30 minutes at 37 °C in 5% CO₂/90% air atmosphere incubator. Tissues were fixed in 2% PFA for 1 hour.

Animals

The Institutional Animal Care and Use Committee of Yale University School of Medicine approved the study. Male Sprague-Dawley rats (200-250 g, Charles River Laboratories) were fasted overnight but allowed access to drinking water, anesthetized with Inactin (120 mg/kg i.p.), and euthanized (Inactin; 200 mg/kg, i.p.) at the end of the experiment.

Treatment of rat intestinal loops

Ligated jejunal, ileal and proximal colonic loops (~3 cm length) were instilled with 0.1 ml normal saline (pH 7.4, 37 °C) or 0.25 µg (0.64 nMol) lubiprostone in 0.1 ml saline (6.4 nMol/ml; i.e. 2.5 µg/ml) for 30 min.

Immunofluorescence labeling and histochemistry

Intestinal segments were isolated, fixed and processed for immunofluorescence and histochemical labeling. See Supplementary Methods for a detailed description and full list of primary antibodies.

Fluorescence image analysis and statistics

Immunolabeled sections were examined on a Nikon Eclipse E800 epifluorescent microscope equipped with a Hamamatsu Orca R2-C10600 digital camera. The acquisition parameters were standardized in relation to the highest intensity regions to avoid oversaturation of pixel intensity. Digital images (8 bits/channel; 1344 × 1024 pixels) were taken at 40× magnification and same exposure time. The images were digitally converted into a grey scale image for analysis. Densitometry analysis was performed as described before [3]. The *Record Measurements* tool of Adobe Photoshop CS4 Extended provided the pixel intensities (*mean gray value*) of the selected areas. For background correction, pixel intensity values from the lamina propria were subtracted. For apical membrane labeling, ~2.0 µm thick ribbons were traced centered on apical fluorescence. For lateral membrane labeling, ~1.5 µm thick ribbons were centered on lateral membrane fluorescence. For intracellular apical pole labeling in enterocytes, ~3.0 µm thick ribbons were traced. Fluorescence intensity (FI) levels were measured on digital images as before [16]: (1) FI levels for CFTR, ClC-2, DRA, PAT1, EP1, EP4, NBCe1, NKCC1, NHE3 and SERT over the intracellular apical pole, apical membrane, or lateral membrane, and over entire cells for 5-HT (2) FI levels in short

rows of mucosal cells were measured for Na-K-ATPase and PKA. Data from 4 to 12 selected areas were averaged in each image, 6 images were analyzed for each measurement group in one animal, and data were collected from three animals (n=3). The number of mucus-filled and empty goblet cells were counted from at least six 10× images of Alcian Blue/PAS stained tissue per animal. All values were presented as means ± SEM. Statistical significance between two individual measurement groups was determined by unpaired *t*-test. Differences among groups were determined using oneway ANOVA and the Tukey's *post hoc* method of multiple comparisons. The level of significance was set at $P < .05$.

Results

Contractile effects of lubiprostone

The mucosal layers in human and rodent colon are organized into ridges called *plicae circulares* which increase the surface area by ~ 3-fold. Small intestinal villi further increase surface area by ~10-fold. All lubiprostone-treated proximal colonic *plicae* (consisting of mucosal sheets and lamina propria) were contracted (see Figs. 1A, 4C-D; 5A-B). Area measurements revealed that the mucosa shrunk from 100% to 86.5%. The lamina propria shrunk to 44%, less than half of controls, and was the main contributing factor to *plica* shrinkage. These size reductions resulted in 22% increased gut lumen area (see graphs in Fig. 1A). Many lubiprostone treated villi in jejunum (Fig. 6A and D) and ileum were also contracted, exhibiting a “Christmas-tree” shape. Contracted and non-contracted villi exhibited similar anion transporter distribution profile. Contractions were not observed in untreated and saline-treated intestine.

Lubiprostone-induced trafficking of EP4 and EP1 receptors in rat intestine *in vivo*

Since lubiprostone-induced intestinal contractions have been shown to be EP1 and EP4-mediated [26], we investigated the drug's effect on the distribution of these receptors. In rat proximal colonic mucosa, both EP4 (Fig. 1A-C) and EP1 receptor-positive cells were detected by immunolabeling (Fig. 1D-E). Labeled cells included colonic enterocytes (also called colonocytes) and 5-HT-positive and 5-HT-negative enterochromaffin (EC) cells (Fig. 1C-E). In untreated and saline treated conditions, EP4 and EP1 labeling were predominantly intracellular (Fig. 1B, 1D-E) in both surface enterocytes and 5-HT-labeled EC cells. EP4 labeling was prominent in surface cells and low in crypt cells, whereas EP1 labeling was relatively even in surface and crypt cells (see graphs of Fig. 1F). Consistent with our findings, both EP4 and EP1 were detected in ileal EC cells in *Mastomys* [17]. EP4-positive cells were also detected in the lamina propria (Fig. 1B).

Lubiprostone increased EP4 labeling in proximal colonic surface mucosa (Fig. 1A-B). EP4 labeling shifted from intracellular stores to the apical membrane of enterocytes and cell membranes of EC cells (Fig. 1B). Membrane-localized EP4 *increased ~3.3-fold* in surface EC cells and *~3.6-fold* in surface enterocytes, and intracellular EP4 *decreased* in parallel (Fig. 1F). In 5-HT/EP4 double-labeled tissues, EP4 was recruited to the cell membrane of serotonergic (Fig. 1C) and non-serotonergic surface EC cells (not shown). In contrast, in crypts, EP4 localization and labeling intensity did not change significantly (Fig. 1F). After lubiprostone treatment, EP1 labeling remained intracellular. EP1 labeling intensity *increased ~1.7-fold* in surface EC cells and *~1.4-fold* in surface enterocytes (Fig. 1F). However, EP1 levels were not changed in crypt cells.

Lubiprostone-induced increase in serotonin and serotonin transporter labeling intensities in rat intestine

To determine whether lubiprostone specifically affects the EC cells, we examined the drug's effect on 5-HT levels in EC cells by measuring fluorescence intensities of 5-HT and its

transporter (SERT) labeling. These experiments also examined whether lubiprostone could potentially modulate intestinal serotonin, as several other constipation drugs [19]. Lubiprostone significantly increased 5-HT labeling in proximal colonic surface EC cells (Fig. 2A), and jejunal/ileal villus EC cells (not shown). In proximal colon, lubiprostone *increased* 5-HT FI **~1.36-fold** in surface EC cells, but only **~1.1-fold** in crypt EC cells (Fig. 2C). Lubiprostone also increased SERT labeling in proximal colon (Fig. 2B), jejunum and ileum (not shown). In proximal colon SERT FI *increased* **~2-fold** in the apical membrane of surface enterocytes, **~1.6-fold** in the cell membrane of SERT/5-HT double-labeled surface EC cells, and **~1.2-fold** in crypt EC cells (Fig. 2D). In summary, both 5-HT and SERT labeling were mainly increased in surface/villus regions by the drug, suggesting that lubiprostone may selectively target these epithelial regions.

Lubiprostone-induced membrane traffic of CFTR, DRA, PAT1, NKCC1, NBCe1 and NHE3 in rat intestine *in vivo*

Could lubiprostone selectively target surface/villus epithelia at the level of ion transporters as well? In the next series of experiments we examined how lubiprostone influences the trafficking of ion transporters involved in fluid secretion/absorption, by examining changes in the subcellular distribution of transporters. In rat proximal colon, lubiprostone decreased basolateral NKCC1 in *lower crypt* enterocytes and goblet cells to **78%**, but increased it in *upper crypt* enterocytes and goblet cells **~1.4-fold**, and in surface enterocytes (**~2.2-fold**) and goblet cells (**~1.3-fold**) (Fig. 3A, 3D, 4A-B). In *lower crypt* cells NBCe1 levels remained low, but lubiprostone increased basolateral NBCe1 in *upper crypt* enterocytes **~1.5-fold** and surface enterocytes **~2.3-fold** (Fig. 5A-B). In summary, in the rat proximal colon, the largest lubiprostone induced increases in both NKCC1 and NBCe1 membrane labeling occurred in surface cells.

In rat proximal colon, lubiprostone decreased apical CFTR in *lower crypt* cells to **71%**, but increased it in *upper crypt* cells **~1.45-fold** (Fig. 4A-B, 4E). CFTR was not detected in surface epithelia (Fig. 4A-B). In parallel, lubiprostone increased apical PAT1 **~1.8-fold** (Fig. 3A-B), but reduced apical DRA to **37%** (Fig. 3C) and NHE3 to **61%** (Fig. 5B) in surface enterocytes.

Lubiprostone induced similar ion transporter redistribution in rat jejunum (Figs. 6-7) and ileum (Fig. 8). In jejunum, lubiprostone (1) *reduced* apical membrane NHE3 of villus enterocytes to **65%** (Figs. 6C, 7A); (2) *increased* apical membrane CFTR of crypt enterocytes **~1.4-fold** and villus enterocytes **~3-fold** (Figs. 6A, 7B) and also increased apical PAT1 in villus enterocytes (not shown); (3) *increased* lateral membrane NBCe1 of villus enterocytes **~1.5-fold** (Figs. 6D-E, 7C); (4) *increased* enterocyte lateral membrane NKCC1 in crypt **~1.4-fold** and villus **~2.3-fold** (Figs. 6A-B, 7D); and (5) *increased* goblet cell lateral membrane NKCC1 in crypt by **~1.4-fold** and villus **~1.3-fold** (Fig. 7D). Both apical NHE3 reduction and basolateral NBCe1 increase were particularly robust in the upper villus (Fig. 6C-D). Intracellular NBCe1 partially co-localized with the early endosome marker EEA1 in saline- but not lubiprostone-treated jejunum (Fig. 6E), consistent with membrane traffic. In all tissues and for each ion transporter, intracellular FI changes were reciprocal to the respective membrane FI changes. The decrease in membrane label for NHE3 and DRA and the increased membrane label for CFTR, PAT1, NBCe1, and NKCC1 that were induced by drug were all most prominent in surface/villus regions, further supporting selectivity for lubiprostone targeting to these epithelial regions.

Lubiprostone-induced membrane traffic of CFTR and NKCC1 in human colon

In human colonic biopsy explants, treatment with lubiprostone for thirty minutes increased apical CFTR **~1.45-fold** in colonic crypts (Fig. 9A-B, and E). CFTR was not detected in

surface cells (not shown). Lubiprostone also increased basolateral NKCC1 in crypt enterocytes **~1.5 fold**, surface enterocytes **~2.4-fold**, crypt goblet cells **~1.5 fold** and surface goblet cells **~1.6-fold** (Fig. 9C-D, and F). NHE3 levels were low in untreated and lubiprostone-treated explants (data not shown). Membrane recruitment of CFTR and NKCC1 was accompanied by concomitant *decreased* intracellular labeling FI (Fig. 9E-F).

Lubiprostone-induced internalization of CIC-2 in rat and human intestinal tissues

We examined the intestinal localization of CIC-2 using three independent antibodies and tested lubiprostone's influence on CIC-2 membrane trafficking, because of the lack of consensus on these issues (see Introduction). Three CIC-2 antibodies (ACL-002, H-90, and YY9) produced similar labeling patterns in human tissues. In rat tissues, only the CIC-2 (ACL-002) antiserum produced labeling patterns similar to that in human tissues. In both rat proximal colon (Fig. 5C) and human colon tissues (Fig. 10) CIC-2 labeling was prominent in surface enterocytes, but not detected in crypt cells; goblet cell labeling was near background level. In untreated (not shown) and saline treated rat proximal colon (Fig. 5C) and untreated human colon tissue (Fig. 10) CIC-2 label was partially localized to the basolateral membrane and intracellular compartments of surface enterocytes. In saline treated proximal colon (graph in Fig. 5F) normalized lateral membrane CIC-2 (**1.0**) was **~2.5-fold** higher than intracellular CIC-2 (**0.41**). In untreated human colon surface cells (Fig. 10A-C), lateral membrane CIC-2 (**1.0**) was **~2 to 2.5-fold** higher (depending on the CIC-2 antibody used) than the corresponding intracellular CIC-2: **0.39** – CIC-2 (ACL-002), **0.52** – CIC-2 (H-90), and **0.49** – CIC-2 (YY9) (see graphs, Figure 10). Basolateral CIC-2 labeling in rat jejunal and ileal villus epithelia was weak (not shown). In summary, in agreement with earlier studies [18], CIC-2 labeling was partially basolateral and partially intracellular; strong in colonic surface and weak in small intestinal epithelia.

In contrast to untreated tissues, CIC-2 label was predominantly intracellular in surface epithelium in lubiprostone treated proximal rat colon (Fig. 5C) and human colon explants (Fig. 10A-C). In lubiprostone treated rat proximal colon (see graph in Fig. 5F) intracellular CIC-2 (**0.75**) was **~2-fold** higher than lateral membrane CIC-2 (**0.36**). In lubiprostone-treated human colon (graphs in Fig. 10A-C), intracellular CIC-2 (**0.80** – CIC-2 (ACL-002), **0.75** – CIC-2 (H-90), and **0.72** – CIC-2 (YY9) was **~3.4 to 2.4-fold** higher, compared to corresponding lateral membrane CIC-2 FI: **0.24** – CIC-2 (ACL-002), **0.28** – CIC-2 (H-90), and **0.30** – CIC-2 (YY9).

Lubiprostone-induced mucus exocytosis from goblet cells

The observation that lubiprostone induced recruitment of NKCC1 in goblet cells suggested the drug's involvement in stimulating activity of these mucus-producing cells. Thus, we tested lubiprostone's potential influence on mucus exocytosis. To assess the presence of mucus in goblet cells, tissue sections were labeled with a Mucin 2 antibody, and/or stained with Alcian blue and periodic acid-Schiff base (AB/PAS) or Alcian blue and Toluidin blue (AB/TB). In rat proximal colon, lubiprostone reduced the ratio of mucus-filled goblet cells from 78 % to 28% (Fig. 4C-D, F). Lubiprostone also induced prominent mucus exocytosis in rat jejunum (Fig. 6F), ileum (Fig. 8D) and human colon explant (Fig. 11).

Lubiprostone's effect on Na-K-ATPase, Protein Kinase A, and β -catenin labeling in rat proximal colon

Next we attempted to gather further evidence for the phenomenon that lubiprostone may preferentially target ion transporter trafficking in surface cells. We examined Na-K-ATPase labeling, since throughout the gut, fluid and electrolyte transport is driven primarily by Na-K-ATPase across the basolateral membrane. We also studied PKA labeling, since this

signaling pathway plays a major role in lubiprostone-induced Cl^- and HCO_3^- secretion (see Introduction). In untreated and saline treated rat proximal colon, Na-K-ATPase (Fig. 12A and D) and PKA labeling (Fig. 12B and E) were both most intense in upper crypts, where NKCC1 and NBCe1 levels were highest. Lubiprostone decreased Na-K-ATPase labeling in *lower crypt* cells to **61%**, but increased it in *upper crypt* cells **~ 1.2 -fold** and **~ 1.7 -fold** in surface cells (Fig. 12A and D). Lubiprostone decreased PKA labeling in *lower crypt* cells to **55%**, in *upper crypt* cells to **57%** (Fig. 12B and E), but increased it **~ 1.6 -fold** in surface cells (Fig. 12A and E). Lubiprostone also increased PKA labeling **~ 1.6 -fold** in 5-HT-labeled surface EC cells, without significantly changing PKA levels in crypt EC cells (Fig. 12B *inset*, and F). In summary, lubiprostone (in parallel with the labeling increase for NKCC1 and NBCe1) also induced the largest increase in Na-K-ATPase and PKA labeling intensity in surface cells. We examined p-catenin levels to further confirm this effect, since PKA activity is known to regulate β -catenin levels at cell borders. Indeed, lubiprostone selectively increased β -catenin labeling in surface cells (Fig. 12C).

Discussion

Overall, the results suggest that lubiprostone preferentially targets small intestinal villus and colonic surface epithelia, to activate fluid secretion machinery (Fig. 13). Specifically lubiprostone selectively increases NKCC1, NBCe1, PKA and Na-K-ATPase labeling intensities in villus/surface cells, and appears to signal EP4/PKA, EP1, and serotonergic pathways in these regions. Although previous studies demonstrated lubiprostone activation of EP4 receptors, the current study extend those observations by identifying EP4 and EP1 receptors in intestinal 5-HT-containing EC cells and effects of lubiprostone on these cells, particularly in villus/surface regions [6]. These observations suggest that at least part of the drug's action could perhaps be due to 5HT4 action in the colon. EP4 and EP1 have also been shown to coexist in serotonergic ileal EC cells [17]. Thus, lubiprostone may also exert some action by modulating serotonin levels [19]. We also found that lubiprostone possesses multiple cellular targets and transporters in the intestine. The data suggest that lubiprostone can *suppress* apical NHE3/DRA- and basolateral CIC-2 mediated fluid absorption; and *stimulate* (1) CFTR-dependent anion secretion in crypt and villus epithelia, (2) PAT1-dependent, but CFTR- and CIC-2-independent anion secretion in colonic surface epithelia, (3) mucus exocytosis from goblet cells, (4) transformation of condensed mucus to mobile expanded mucus via enhanced alkaline secretions, and (5) mixing/movement of luminal content via mucosal contractions. As a lumenally active agent, lubiprostone may transiently enhance a combination of events in intestinal segments of limited length. This profile may contribute to its constipation-alleviating effects, without disturbing nutrient absorption.

The therapeutic dose of lubiprostone for clinical use is 24 μg (61.4 nMol). About 100-fold less lubiprostone, 0.25 μg (0.64 nMol) was injected into ~ 3 -cm long ligated segments of the rat intestine (considering the two-magnitude difference between human and rat body weight). However, in human patients, lubiprostone is partially metabolized in the stomach, and then distributed to at least 100-fold longer intestinal segments with 10^4 to 10^5 -fold larger mucosal surface areas, compared to a ~ 3 cm ligated loop. Therefore, lubiprostone effects described here could represent effects induced by the drug at 10^2 - 10^3 fold higher *local* concentration compared to that of human intestine. The potential clinical implications of our findings will require further investigation, because one cannot reliably compare *local* lubiprostone concentrations in the intestine of patients *versus* those affecting ligated rat intestinal loops and human colon explants.

In untreated and saline treated tissues, CFTR (part apical, part intracellular) was localized to enterocytes of small intestinal crypts *and* villi and colonic crypts, as shown before [16]. CFTR was not detected in rat and human colon surface enterocytes, confirming earlier

reports [20]. As shown before in rat [16], NKCC1 present in enterocytes *and* goblet cells was largely basolateral in crypt cells, and mainly intracellular along small intestinal villus and colonic surface epithelia. The current study extended these findings to the human colon. NBCe1 (part basolateral, part intracellular) was present in small intestinal villus enterocytes and colonic crypt and surface enterocytes. NHE3 (mainly apical membrane and also intracellular) was localized to small intestinal villus and colonic surface enterocytes, as described [16].

The recruitment of NKCC1 to the basolateral membrane of small and large intestinal epithelial cells by lubiprostone is consistent with NKCC1-mediated Cl^- secretion [6-7, 10]. Increased apical CFTR in rat and human intestine is consistent with previous observations that lubiprostone induces apical recruitment of CFTR in cultured human intestinal T84 cells [9], and with the findings that CFTR_{inh}-172 suppressed lubiprostone-induced Cl^- transport, confirming its dependence on CFTR [6, 9-10]. The lubiprostone-induced NHE3 internalization is also consistent with the drug's anti-absorptive properties, and the concomitant DRA internalization supports the notion that NHE3 and DRA are functionally coupled [21]. Acting upon EP4/PKA-containing enterocytes, lubiprostone could activate the well-established inhibitory effect of cAMP/PKA signaling on salt and water absorption. Detection of CIC-2-type currents as the dominant anion conductance of enterocyte basolateral membrane [22] suggests that enterocytes extrude Cl^- through basolateral CIC-2 during fluid absorption. The lubiprostone-induced removal of CIC-2 from the basolateral membrane suggests that lubiprostone *suppresses*, rather than stimulates CIC-2 activity. This effect appears to be another anti-absorptive property of the drug. The basolateral localization of CIC-2 at steady state condition and its lubiprostone-induced internalization are at odds with prevailing hypotheses: that (1) CIC-2 is an apical channel and that (2) lubiprostone's pro-secretory actions would involve the activation of CIC-2 [8, 15].

Lubiprostone's "dual action", recruiting apical CFTR, and basolateral NKCC1/NBCe1 to the membrane, while internalizing apical NHE3 in small intestinal villus enterocytes is probably an EP4/PKA/cAMP mediated effect [6, 10]. The upper villus and colonic surface exhibit the most robust NBCe1 and NHE3 responses (see Figs. 5 and 6C-D) and both regions are also distinctly enriched in EP4 protein (present study) and mRNA [23]. These data suggest that the upper villus and the colonic surface epithelia may be a major target site for the drug.

The unstimulated colonic surface epithelium absorbs salt and fluid from the lumen: in this condition, apical NHE3 and basolateral CIC-2 activities promote lumen-to-serosa fluid flow (i.e. fluid absorption), while intracellular NKCC1 and NBCe1 gate serosa-to-lumen fluid flow (i.e. fluid secretion). The lubiprostone-induced membrane trafficking changes suggest a switch to fluid secretion: basolateral membrane recruitment of NKCC1 and NBCe1 allow serosa-to-lumen $\text{Cl}^-/\text{HCO}_3^-$ flow, while internalized NHE3 and CIC-2 limit lumen-to-serosa Na^+/Cl^- flow. As our results indicate, HCO_3^- can exit to the lumen through apical PAT1 (SLC26A6) anion exchanger, which does not require CFTR [24]. However, the apical channel for Cl^- exit is uncertain in the absence of apical CFTR and CIC-2 in colonic surface epithelia: here lubiprostone may activate apical Cl^- channels not identified in this study including calcium activated chloride channels [25].

Lubiprostone possesses EP1 and EP4 agonist properties [6, 10, 26]. Our findings indicate that intestinal villus/surface enterocytes and EC cells possess both receptors, and our data suggest that the drug acts upon these receptors. EP1 activation increases intracellular Ca^{2+} , whereas EP4 activation increases cytoplasmic cAMP [27]. cAMP/ Ca^{2+} co-activation elicits synergistic, but time-constrained biphasic secretory response: cAMP (forskolin) activation alone sustains NKCC1 activity over 4 hours, but forskolin/acetylcholine (Ca^{2+} agonist) co-stimulation promotes NKCC1 internalization and fluid secretion declines within 30-60 min

in human colon *ex vivo* [28]. In our lubiprostone-treated human colon explants, NKCC1 reverted to an intracellular localization *after 60 min* (data not shown) suggesting co-stimulation of cAMP/Ca²⁺ pathways. Similar to this scenario, it is possible that the lubiprostone-induced EP1 internalization (accompanied with a EP1 labeling intensity increase) observed after 30 min is a secondary event that follows a rapid EP1 activation. Furthermore, in rat jejunum, lubiprostone elicited a transporter trafficking profile and cell shrinkage similar to that induced by carbachol, but the lubiprostone-induced changes were less robust [16]. The physiological findings that both forskolin [6, 29] and carbachol [6] enhanced lubiprostone effects also implicate cAMP/Ca²⁺ co-activation as a mechanism of action for lubiprostone. Lubiprostone enhanced both carbachol [7] and forskolin effects [29], and its secretory response appeared biphasic with a peak and decline within 35 min [10] or 40 min [29]. The time-constrained biphasic secretory response characteristic of cAMP/Ca²⁺ (EP4/EP1) co-activation could explain lubiprostone's mild diarrhea-inducing effect.

Lubiprostone has been shown to stimulate HCO₃⁻ secretion in duodenum [10]. Recruitment of NBCe1 to the basolateral membrane of small and large intestinal enterocytes observed in this study suggests that a lubiprostone-induced HCO₃⁻ secretion may also occur in jejunum, ileum and colon. This effect is most likely CFTR-dependent, since CFTR_{inh}-172 has been shown before to decrease lubiprostone-induced HCO₃⁻ secretion [10]. However, colonic surface cells, where CFTR is not detectable, but NBCe1 is upregulated by lubiprostone, may be a source of CFTR-independent HCO₃⁻ secretion. The mucus exocytosis data and the increased basolateral NKCC1 in goblet cells indicate that lubiprostone can enhance intestinal mucus release, similar to the airway [29]. After the removal of mucus “plug”, colonic goblet cells may secrete HCO₃⁻ through uptake by the Ca²⁺ activated basolateral bestrophin-2 channel [25]. Polyanionic mucins, stored in granules with Ca²⁺ and protons screening the negative charges, expand 1000-fold when exocytosed [30]. HCO₃⁻ plays a key role unmasking the negative charges [31], promoting access of proteases to mucins; thus producing low-viscosity mucus. Lubiprostone's ability to load the lumen with low-viscosity mucus could contribute to its therapeutic effect [3]. In human subjects lubiprostone accelerates intestinal transit [3], but the same research group [4] could not replicate the effect in subjects who performed overnight colon cleansing. This mucus-depleting procedure could accelerate lubiprostone's degradation in proximal intestine, and prevent the drug from reaching distal intestinal segments.

Constipation and distal intestinal obstruction syndrome (DIOS) due to viscous accumulation of mucus are common in Cystic Fibrosis patients and are relieved by lubiprostone [12]. Two CFTR-independent mechanisms may play a role: (1) an inhibitory effect on NHE3-mediated fluid absorption [13]; and (2) lubiprostone's potential to alkalinize mucus independent of CFTR could directly target the “mucoviscidosis” in CF patients. Indeed, recent observations suggested that lubiprostone could promote mucus expansion in CF mice [32]. The drug could simultaneously stimulate alkaline secretions [10] and mucus release that may be beneficial for treating duodenal ulcer disease, under conditions where the alkaline response to acid is impaired.

Lubiprostone has been shown to induce EP1-mediated contraction in stomach longitudinal muscles, and EP4-mediated inhibition of contractions in colon circular muscles [26]. EP1-mediated Ca²⁺ mobilization contracts smooth muscle cells, whereas EP4-mediated cAMP increase induces relaxation. The lubiprostone-induced contraction of small intestinal villi and proximal colonic ridges observed in this study resembles the piston-like villus motility, that is experimentally inducible e.g. by increasing capillary fluid filtration [33].

In summary, by selectively targeting serotonergic, EP4/PKA and EP1 signaling in surface/villus regions, lubiprostone may (1) preferentially stimulate CFTR/NBCe1/NKCC1-

dependent anion secretion by villus epithelia and PAT1/NBCe1/NKCC1-dependent, but CFTR/CIC-2-*independent* anion secretion by colonic surface epithelia (2) suppress NHE3/DRA- mediated fluid absorption (3) augment mucus-mobilization and (4) promote bowel movement via enhanced mucosal contractility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Johanson JF, et al. Clinical trial: phase 2 study of lubiprostone for irritable bowel syndrome with constipation. *Aliment Pharmacol Ther.* 2008; 27(8):685–96. [PubMed: 18248656]
2. Drossman DA, et al. Clinical trial: lubiprostone in patients with constipation-associated irritable bowel syndrome--results of two randomized, placebo-controlled studies. *Aliment Pharmacol Ther.* 2009; 29(3):329–41. [PubMed: 19006537]
3. Camilleri M, et al. Effect of a selective chloride channel activator, lubiprostone, on gastrointestinal transit, gastric sensory, and motor functions in healthy volunteers. *Am J Physiol Gastrointest Liver Physiol.* 2006; 290(5):G942–7. [PubMed: 16603730]
4. Sweetser S, et al. Effect of a chloride channel activator, lubiprostone, on colonic sensory and motor functions in healthy subjects. *Am J Physiol Gastrointest Liver Physiol.* 2009; 296(2):G295–301. [PubMed: 19033530]
5. Product information Amitiza (lubiprostone). Sucampo Pharmaceuticals, I.a.T.P.A., Inc.; Bethesda, MD, and Deerfield, IL: 2008.
6. Bijvelds MJ, et al. Activation of intestinal Cl⁻ secretion by lubiprostone requires the cystic fibrosis transmembrane conductance regulator. *Gastroenterology.* 2009; 137(3):976–85. [PubMed: 19454284]
7. Fei G, et al. Stimulation of mucosal secretion by lubiprostone (SPI-0211) in guinea pig small intestine and colon. *Am J Physiol Gastrointest Liver Physiol.* 2009; 296(4):G823–32. [PubMed: 19179625]
8. Cuppoletti J, et al. SPI-0211 activates T84 cell chloride transport and recombinant human CIC-2 chloride currents. *Am J Physiol Cell Physiol.* 2004; 287(5):C1173–83. [PubMed: 15213059]
9. Ao M, et al. Lubiprostone Activates Cl⁻ Secretion via cAMP Signaling and Increases Membrane CFTR in the Human Colon Carcinoma Cell Line T84. *Dig Dis Sci.* 2010
10. Mizumori M, Akiba Y, Kaunitz JD. Lubiprostone stimulates duodenal bicarbonate secretion in rats. *Dig Dis Sci.* 2009; 54(10):2063–9. [PubMed: 19657734]
11. Sun X, et al. Lubiprostone reverses the inhibitory action of morphine on mucosal secretion in human small intestine. *Dig Dis Sci.* 2011; 56(2):330–8. [PubMed: 21181441]
12. O'Brien CE, Anderson PJ, Stowe CD. Use of the chloride channel activator lubiprostone for constipation in adults with cystic fibrosis: a case series. *Ann Pharmacother.* 2010; 44(3):577–81. [PubMed: 20179256]
13. Coates SW Jr, et al. Inhibition of neutral sodium absorption by a prostaglandin analogue in patients with cystic fibrosis. *Gastroenterology.* 2004; 127(1):65–72. [PubMed: 15236173]
14. Matlhagela K, Taub M. Regulation of the Na-K-ATPase beta(1)-subunit promoter by multiple prostaglandin-responsive elements. *Am J Physiol Renal Physiol.* 2006; 291(3):F635–46. [PubMed: 16478973]

15. Bao HF, et al. A synthetic prostone activates apical chloride channels in A6 epithelial cells. *Am J Physiol Gastrointest Liver Physiol*. 2008; 295(2):G234–51. [PubMed: 18511742]
16. Jakab RL, Collaco AM, Ameen NA. Physiological relevance of cell-specific distribution patterns of CFTR, NKCC1, NBCe1, and NHE3 along the crypt-villus axis in the intestine. *Am J Physiol Gastrointest Liver Physiol*. 2011; 300(1):G82–98. [PubMed: 21030607]
17. Kidd M, et al. Isolation, functional characterization, and transcriptome of *Mastomys* ileal enterochromaffin cells. *Am J Physiol Gastrointest Liver Physiol*. 2006; 291(5):G778–91. [PubMed: 16455786]
18. Lipecka J, et al. Distribution of ClC-2 chloride channel in rat and human epithelial tissues. *Am J Physiol Cell Physiol*. 2002; 282(4):C805–16. [PubMed: 11880269]
19. Hasler WL. Serotonin and the GI tract. *Curr Gastroenterol Rep*. 2009; 11(5):383–91. [PubMed: 19765366]
20. Trezise AEO, Buchwald M. In vivo cell-specific expression of the cystic fibrosis transmembrane conductance regulator. *Nature*. 1991; 353:434–436. [PubMed: 1716739]
21. Walker NM, et al. Down-regulated in Adenoma Cl/HCO₃ exchanger couples with Na/H exchanger 3 for NaCl absorption in murine small intestine. *Gastroenterology*. 2008; 135(5):1645–1653. [PubMed: 18930060]
22. Catalan M, et al. Basolateral ClC-2 chloride channels in surface colon epithelium: regulation by a direct effect of intracellular chloride. *Gastroenterology*. 2004; 126(4):1104–14. [PubMed: 15057749]
23. Morimoto K, et al. Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract. *Am J Physiol*. 1997; 272(3 Pt 1):G681–7. [PubMed: 9124591]
24. Tuo B, et al. Involvement of the anion exchanger SLC26A6 in prostaglandin E₂- but not forskolin-stimulated duodenal HCO₃⁻ secretion. *Gastroenterology*. 2006; 130(2):349–58. [PubMed: 16472591]
25. Yu K, et al. Bestrophin-2 mediates bicarbonate transport by goblet cells in mouse colon. *J Clin Invest*. 2010; 120(5):1722–35. [PubMed: 20407206]
26. Bassil AK, et al. Activation of prostaglandin EP receptors by lubiprostone in rat and human stomach and colon. *Br J Pharmacol*. 2008; 154(1):126–35. [PubMed: 18332851]
27. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Rev*. 1999; 79(4):1193–226. [PubMed: 10508233]
28. Reynolds A, et al. Dynamic and differential regulation of NKCC1 by calcium and cAMP in the native human colonic epithelium. *J Physiol*. 2007; 582(Pt 2):507–24. [PubMed: 17478539]
29. Joo NS, Wine JJ, Cuthbert AW. Lubiprostone stimulates secretion from tracheal submucosal glands of sheep, pigs, and humans. *Am J Physiol Lung Cell Mol Physiol*. 2009; 296(5):L811–24. [PubMed: 19233902]
30. Kesimer M, et al. Unpacking a gel-forming mucin: a view of MUC5B organization after granular release. *Am J Physiol Lung Cell Mol Physiol*. 2010; 298(1):L15–22. [PubMed: 19783639]
31. Garcia MA, Yang N, Quinton PM. Normal mouse intestinal mucus release requires cystic fibrosis transmembrane regulator-dependent bicarbonate secretion. *J Clin Invest*. 2009; 119(9):2613–22. [PubMed: 19726884]
32. De Lisle RC, Mueller R, Roach E. Lubiprostone ameliorates the cystic fibrosis mouse intestinal phenotype. *BMC Gastroenterol*. 2010; 10:107–118. [PubMed: 20843337]
33. Womack WA, et al. Villous motility: relationship to lymph flow and blood flow in the dog jejunum. *Gastroenterology*. 1988; 94(4):977–83. [PubMed: 3345896]

Figure 1. Lubiprostone-induced changes in EP4 and EP1 receptor localization in rat proximal colon

Rat proximal colonic loops (~3cm) were treated with 0.1 ml saline (left images) or 0.25 μ g lubiprostone in 0.1 ml saline (right images). (A) Cross sections of EP4 labeled proximal colon. Arrowheads point to the colonic plicae. Traced areas of immunolabeled cross-sections are shown. Bars represent the traced areas of mucosa (grey), lamina propria (LP; white), and gut lumen (black) in saline (S) and lubiprostone (L) treated colon. (B) Localization of EP4 receptors in crypt and surface cells of colon. High power micrographs show EP4-labeled surface enterocytes and enterochromaffin-like (EC) cells. Open arrowhead indicates intracellular labeling; arrowhead indicates apical membrane. Asterisks denote EP4-positive cells in lamina propria. (C) Colonic surface cells double labeled for EP4 (green) and serotonin (5-HT) (red). Open arrowhead indicates intracellular EP4 staining in surface enterocytes and open arrow denotes intracellular EP4 staining in 5-HT-labeled EC cells in saline treated tissue. Arrowhead indicates EP4 labeling at the apical membrane of surface enterocytes and arrow denotes EP4 labeling at the cell surface of 5-HT labeled EC cells in lubiprostone treated tissue. (D) Localization of EP1 receptors in EC cells in crypt (arrowheads) and surface epithelium (arrows). *High power insets*: Intracellular localization

of EP1 in EC cells. **(E)** EP1 (green)/5-HT (red) double labeled EC cells in crypt and surface epithelia. Open arrowheads denote low levels of EP1 labeling and arrowheads denote high levels of EP1 labeling. **(F)** *Top graph*: Normalized EP4 apical and intracellular fluorescence intensity (FI) in enterocytes (ENT) and 5-HT-labeled EC cells from saline or lubiprostone treated tissues. *Bottom graph*: Normalized EP1 FI in 5-HT-labeled EC cells from saline or lubiprostone treated tissues. * - significant difference from untreated group. Scalebars: B, 10 μm , insets in B, 5 μm ; C, 5 μm ; D, 50 μm , insets in D, 5 μm ; E, 5 μm .

Figure 2. Lubiprostone-induced increase in serotonin (5-HT) and serotonin transporter (SERT) immunolabeling intensity in rat proximal colon

Left images: saline treated tissue. *Right* images: lubiprostone (0.25 μ g) treated tissue. **(A)** distribution of 5-HT positive enterochromaffin (EC) cells (arrowhead). *High power inset*: 5-HT-labeled EC cell. **(B)** Localization of SERT (green); open arrowhead -intracellular label; arrowhead - apical label and 5-HT-labeled (red) EC cells (arrow - cell membrane). *High power insets*: SERT/5-HT double labeled EC cells. **(C)** Normalized 5-HT FI in EC cells treated with saline or lubiprostone. **(D)** Normalized cell membrane and intracellular SERT FI in enterocytes (ENT) and (5-HT-labeled) EC cells in saline or lubiprostone treated tissues. * - significant difference from untreated group. Scalebars: A, 100 μ m; insets in A, 5 μ m; B, 10 μ m; insets in B. 5 μ m.

Figure 3. Lubiprostone-induced redistribution of NKCC1, PAT1 (SLC26A6) and DRA (SLC26A3) in rat proximal colon

Rat proximal colon was treated with saline or lubiprostone (0.25 μ g). (A) Distribution of NKCC1 (green) and PAT1 (red) immunolabeling in mucosal sections of saline and lubiprostone treated tissue. *High power insets*: PAT1 labeled surface enterocytes (open arrowhead - intracellular label; arrowhead - apical membrane label). (B-C) High power images of DRA labeled (green) surface enterocytes in saline and lubiprostone treated human colon explant (B) and rat proximal colon (C). (Bracketed region of low power insets show location of immunolabeled cells) Open arrowhead - intracellular; arrowhead - apical membrane (D) Normalized NKCC1 fluorescence intensity (FI) at lateral membranes, subapical compartments of enterocytes (ENT) and goblet cells (GOB) in proximal colonic lower crypt, upper crypt, and surface epithelia treated with saline or lubiprostone. Normalized PAT1 (E) and DRA (F) FI at apical membranes and subapical compartments of enterocytes in rat proximal colonic surface epithelia treated with saline or lubiprostone. * - significant difference from untreated group. Scalebars: A, 25 μ m; insets in A, 5 μ m; B, 5 μ m; insets in B, 50 μ m; C, 5 μ m; insets in C, 25 μ m.

Figure 4. Lubiprostone-induced redistribution of CFTR and NKCC1, and mucus exocytosis in rat proximal colon
(A-B) CFTR (green) / NKCC1 (red) double labeled sections from saline **(A)** and lubiprostone (0.25 μ g) treated **(B)** rat proximal colon. *Left* images: CFTR (top) and CFTR/NKCC1 (bottom) in *lower* crypt (dotted line) and *upper* crypt. *Right* images: Distribution of CFTR/NKCC1 in surface and upper crypt. Open arrowhead - intracellular NKCC1 label; arrowhead - basolateral membrane NKCC1 label in surface enterocytes. Small arrows - apical CFTR in crypt cells. *High power insets*: NKCC1 localization in goblet cells (G); open arrowhead - intracellular label; arrowhead - lateral membrane label. **(C-D)** Alcian Blue/PAS staining in saline **(C)** and lubiprostone **(D)** treated tissue (arrow - mucus-filled goblet cell; open arrow - goblet cell depleted of mucus). *Low power insets*: Proximal colonic *plicae* - double-headed arrows indicate lamina propria width **(E)** Normalized apical membrane and subapical CFTR fluorescence intensity (FI) in saline or lubiprostone treated proximal colonic crypts **(F)** Percentage of mucus-filled goblet cells in saline or lubiprostone treated proximal colon * - significant difference from untreated group. Scalebars: A-B, left panels, 20 μ m; right panels, 10 μ m; C-D, 25 μ m; insets in A-B, 5 μ m.

Figure 5. Lubiprostone-induced contractility and redistribution of NHE3, NBCe1 and CIC-2 in rat proximal colon

Left: saline treated tissue; *right:* lubiprostone (0.25 μ g) treated tissue. (A) NBCe1 (green) and NHE3 (red) double labeled section. Small arrows; NBCe1 label in surface (s) and crypt (c). Double-headed arrows; lamina propria width. Long arrows; contraction of lubiprostone-treated colonic *plica* (B) *Top:* NHE3 (red) distribution in surface epithelium (arrowhead - apical membrane; open arrowhead - intracellular label); *Bottom:* NBCe1 (green) and NHE3 (red) double-labeled surface epithelium (open arrowhead - intracellular; arrowhead - lateral membrane label) (C) CIC-2 (ACL-002; green) distribution in proximal colonic crypt and surface epithelia. *Lower left:* high magnification image shows lateral membrane label (arrowhead) in saline treated surface enterocytes *Right:* intracellular label (open arrowhead) in lubiprostone-treated surface enterocytes. Quantification of normalized NHE3 (D), NBCe1 (E) and CIC-2 (F) fluorescence intensity (FI) at apical or lateral membranes, and subapical compartments of enterocytes in saline or lubiprostone-treated proximal colonic crypt and surface epithelia * - significant difference from untreated group. Scalebars: A, 50 μ m; B, 10 μ m; C upper left, 25 μ m; C, 10 μ m.

Figure 6. Lubiprostone-induced redistribution of CFTR, NHE3, NBCe1 and NKCC1, and mucus exocytosis in rat jejunum

Left: saline treated tissue; right lubiprostone (0.25 μ g) treated tissue. Double-headed arrows indicate crypt (c) - villus (v) orientation. (A) CFTR (green) labeled tissue. Small arrows point to CFTR High Expresser (CHE) cells. Upper right insets: Apical membrane (arrowhead) and subapical (open arrowhead) localization of CFTR in mid-villus enterocytes. Lower left insets: Crypt epithelium double labeled for CFTR (green) and NKCC1 (red). (B) Basolateral membrane (arrowheads) and intracellular (open arrowheads) localization of NKCC1 (red) in mid-villus enterocytes and goblet cells (G). High power insets: NKCC1 accumulation on lateral membranes (brackets) of lubiprostone treated enterocytes, and in vesicles in proximity to the lateral membrane in saline treated tissue. (C) NHE3 (red) distribution from crypts to villus tip (vtip). High power insets: Apical (arrowheads) and subapical (open arrowheads) localization of NHE3 in mid-villus enterocytes. (D) NBCe1 (green) labeled jejunal villi. (E) NBCe1 (green) and EEA1 (red) double labeled mid-villus enterocytes exhibiting NBCe1/EEA1 co-labeled subapical vesicles (yellow; open arrowheads) in saline treated tissue. High power insets: NBCe1-labeled enterocyte lateral membrane (brackets), and NBCe1-labeled vesicles near the lateral membrane (open

arrowheads) in saline treated tissue. **(F)** Alcian Blue (and counterstained with Toluidine Blue; TB) identifies mucus-filled goblet cells in saline treated (left; arrow) and mucus-depleted goblet cells (right; open arrow) in lubiprostone treated jejunal villi. Scalebars: A, C, D, 50 μm ; B, 10 μm ; E, 5 μm ; F, 100 μm ; insets in A and C, 5 μm ; insets in B and E, 1 μm .

Figure 7. Densitometry of normalized NHE3, CFTR, NBCe1 and NKCC1 fluorescence intensities (FI) at apical or lateral membranes, and subapical compartments of crypt and villus enterocytes and goblet cells in jejunum treated with saline or 0.25 µg lubiprostone for 30 min

Cells were analyzed from the middle third of crypts, and middle third of villi. * -significant difference from untreated group;

Figure 8. Lubiprostone-induced redistribution of CFTR, NHE3, NKCC1 and mucus exocytosis in rat ileum

Left: saline treated tissue; *right* images: lubiprostone (0.25 μg) treated tissue. **(A)** Localization of CFTR (green) in crypts (c) and villi (v). **(B)** High magnification images of CFTR (green) / NHE3 (red) double labeled villus epithelium. Arrowhead - apical membrane label; open arrowhead - intracellular localization; G - goblet cell. **(C)** Crypt-villus junction region double labeled for CFTR (green) and NKCC1 (red). Open arrowhead - intracellular NKCC1 label; arrowhead - basolateral NKCC1 label. **(D)** Alcian Blue/Toluidine Blue (TB) stained ileal tissue: arrow - goblet cell filled with mucus; open arrow - goblet cell depleted of mucus. Scalebars: A, 100 μm ; B, 5 μm ; C, 10 μm ; D, 100 μm .

Figure 9. Lubiprostone-induced redistribution of CFTR and NKCC1 in human colon *ex vivo* Cryosections from untreated (A and C) or 2.5 $\mu\text{g/ml}$ lubiprostone-treated (B and D) human colon were double labeled for (A-B) CFTR (green) and F-Actin or NKCC1 (red) *Insets*: bracketed areas enlarged (arrowhead - apical membrane label). (C-D) *Left*: Crypt epithelium double labeled for CFTR (green) and NKCC1 (red); the bracketed region is enlarged: open arrowhead - intracellular NKCC1 label; open arrow -intracellular CFTR label; arrowhead - basolateral membrane NKCC1 label; arrow -apical membrane CFTR label; G - goblet cell. *Right*: NKCC1 labeled surface epithelium (arrowhead - basolateral membrane; open arrowhead - intracellular). Normalized CFTR (E) and NKCC1 (F) fluorescence intensity (FI) at apical or lateral membranes, and subapical compartments of enterocytes and goblet cells in crypt and surface epithelia. * - significant difference from untreated group. Scalebars: A-B, 25 μm ; C-D, 10 μm ; insets, 5 μm .

Figure 10. Lubiprostone-induced internalization of CIC-2 in human colon *ex vivo*
Upper images: Control tissue. *Lower* images: lubiprostone (2.5 μ g/ml) treated tissue. CIC-2 distribution in surface epithelium labeled with (A) CIC-2 (ACL-002), (B) CIC-2 (H-90), or (C) CIC-2 (YY9) antibody (arrowhead - lateral membrane localization; open arrowhead - intracellular localization; G - goblet cell). Low power images (bottom panels) show the labeling patterns of the three CIC-2 antibodies in lubiprostone treated tissue. Normalized CIC-2 fluorescence intensity (FI) at lateral membranes and supapical compartments of surface enterocytes in untreated and lubiprostone-treated human colon explants labeled with three CIC-2 antibodies: CIC-2 (ACL-002); CIC-2 (H-90); CIC-2 (YY9). Cells in the central surface epithelium were analyzed. * - significant difference from untreated group. Scalebars: A-C, 10 μ m; low power panels - 100 μ m.

Figure 11. Effect of lubiprostone on mucus exocytosis in human colon *ex vivo*

Human colon tissue was untreated (**A-B**), or treated with 2.5 $\mu\text{g/ml}$ lubiprostone (**C-D**). Cryostat sections were immunolabeled with Mucin 2 antibodies, (green; **A, C**) or stained with Alcian Blue/PAS (**AB/PAS**; **B, D**). (**A**) Crypt goblet cells filled with Mucin 2-labeled mucus (arrowhead). (**B**) Alcian Blue/PAS-labeled mucus in surface and crypt goblet cells. High magnification image of bracketed region shows goblet cells (G) filled with mucus (arrowhead). (**C**) Crypt goblet cells depleted of Mucin 2-labeled mucus (open arrowhead). Mucin 2-labeled mucus is present in crypt lumen (arrow). (**D**) Surface and crypt goblet cells depleted of Alcian Blue/PAS labeled mucus (arrowhead). Scalebars: A, C, 25 μm ; B, D, 50 μm ; enlarged insets, 10 μm .

Figure 12. Lubiprostone-induced redistribution of Na-K-ATPase, protein kinase A, and β -catenin in rat proximal colon

Left: saline treated tissue; *right* lubiprostone (0.25 μ g) treated tissue. (A) Distribution of Na-K-ATPase (white) in surface, upper crypt (orange dotted line), and lower crypt (yellow dotted line) epithelia. Asterisks denote lumen of crypt base. (B) Distribution of protein kinase A (PKA) (white) and 5-HT-labeled enterochromaffin (EC) cells (red) in surface and crypt (dotted line) epithelia. *High power inset* 5-HT/PKA double labeled EC cell. (C) β -Catenin labeled (white) surface and crypt (dotted line) epithelia. (D) Normalized Na-K-ATPase fluorescence intensity (FI) in lower crypt, upper crypt, and surface epithelia. Normalized PKA FI in lower crypt, upper crypt, and surface epithelia (E), and 5-HT-labeled EC cells (F). * - significant difference from untreated group. Scalebars: A, 25 μ m; B-C, 100 μ m; inset, 5 μ m.

Figure 13. Schematic drawing summarizing lubiprostone's effects on ion transporter trafficking in small intestinal villus and proximal colonic surface enterocytes in the rat intestine
Schematic drawing illustrates directionality of subcellular trafficking of enterocyte ion transporters following lubiprostone (0.25 μ g; 30 min). Under control conditions (*left*), the apical transporters CFTR, PAT1 (SLC26A6) and NHE3 are localized to the apical membrane and subapical endosomes in villus enterocytes of jejunum (*top*). Similarly, the basolateral transporters CIC-2, NKCC1 and NBCe1 are on the basolateral membrane and in intracellular endosomes. In proximal colonic surface enterocytes (*bottom*) PAT1, DRA (SLC26A3) and NHE3 are distributed on the apical membrane and subapical endosomes, while CIC-2, NKCC1 and NBCe1 are partly basolateral and intracellular. Lubiprostone (*right*), recruited CFTR and PAT1 to the apical membranes of villus enterocytes (*top*) and NKCC1 and NBCe1 were recruited to the basolateral membrane (white arrows). In contrast, apical NHE3 and basolateral CIC-2 were both internalized (black arrows). Apical PAT1 and basolateral NKCC1 and NBCe1 in surface colonocytes (*bottom*), were recruited to the membrane (white arrows), whereas apical NHE3 and DRA, as well as basolateral CIC-2 were internalized (black arrows) following lubiprostone. Changes in ion transporter subcellular distribution were most prominent in small intestinal villus and proximal colonic surface epithelia, suggesting that lubiprostone may locally act to transiently activate fluid secretion while suppressing fluid absorption.