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A comparison of linaclotide and lubiprostone dosing regimens on ion transport responses in human colonic mucosa

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Abstract

Linaclotide, a synthetic guanylyl cyclase C (GC-C) agonist, and the prostone analog, Lubiprostone, are approved to manage chronic idiopathic constipation and constipation-predominant irritable bowel syndrome. Lubiprostone also protects intestinal mucosal barrier function in ischemia. GC-C signaling regulates local fluid balance and other components of intestinal mucosal homeostasis including epithelial barrier function. The aim of this study was to compare if select dosing regimens differentially affect linaclotide and lubiprostone modulation of ion transport and barrier properties of normal human colonic mucosa. Normal sigmoid colon biopsies from healthy subjects were mounted in Ussing chambers. Tissues were treated with linaclotide, lubiprostone, or vehicle to determine effects on short-circuit current (I_{sc}). Subsequent I_{sc} responses to the cAMP agonist, forskolin, and the calcium agonist, carbachol, were also measured to assess if either drug caused desensitization. Barrier properties were assessed by measuring transepithelial electrical resistance. I_{sc} responses to linaclotide and lubiprostone were significantly higher than vehicle control when administered bilaterally or to the mucosal side only. Single versus cumulative concentrations of linaclotide showed differences in efficacy while cumulative but not single dosing caused desensitization to forskolin. Lubiprostone reduced forskolin responses under all conditions. Linaclotide and lubiprostone exerted a positive effect on TER that was dependent on the dosing regimen. Linaclotide and lubiprostone increase ion transport responses across normal human colon but linaclotide displays increased sensitivity to the dosing regimen used. These findings may have implications for dosing protocols of these agents in patients with constipation.

Abbreviations

CFTR, cystic fibrosis transmembrane conductance regulator; cGMP, cyclic guanosine monophosphate; CIC, chronic idiopathic constipation; ClC-2, chloride channel type 2; CLCA, calcium-activated chloride channels; EMA, European Medicines Agency; FDA, Food and Drug Administration; GC-C, guanylyl cyclase C; IBS-C, constipation-predominant irritable bowel syndrome; IBS, irritable bowel syndrome; IEC, intestinal epithelial cell; I_{sc}, short-circuit current; LPS, lipopolysaccharide; PD, potential difference; PKG, protein kinase G; TER, transepithelial electrical resistance; UC, ulcerative colitis.

Introduction

In recent years, a number of agents that act by promoting epithelial chloride secretion have been approved for the alleviation of chronic constipation. Linaclotide (Forest Laboratories, Inc.; Ironwood Pharmaceuticals Inc.) has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of irritable bowel syndrome (IBS) patients with constipation (IBS-C) and adults with chronic idiopathic constipation (CIC). It has also been approved by the European Medicines Agency (EMA) for the treatment of moderate to severe IBS-C in adults (McWilliams et al. 2012; Blackshaw and Brierley 2013). Linaclotide is a potent agonist of the guanylyl cyclase C (GC-C) receptor, which is located on the luminal surface of intestinal epithelial cells (ICE) throughout the gut mucosa (Li and Goy 1993). This first-in-class synthetic GC-C agonist is composed of a 14 amino acid peptide that is converted in vivo by carboxypeptidase A into a 13 amino acid active form metabolite, MM-419447 (Busby et al. 2013). Linaclotide strongly binds to the GC-C receptor in a pH-independent manner and results in increased conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP) via GC-C catalysis (Busby et al. 2013). Elevated cGMP activates protein kinase A (PKA) and protein kinase G (PKG) in a cGMP-dependent manner (Field et al. 1978; Giannella and Drake 1979). Activated PKA and PKG II result in secretion of chloride and bicarbonate ions into the luminal space by inducing phosphorylation and opening of the cystic fibrosis transmembrane conductance regulator (CFTR) (Vaandrager et al. 1997, 2000). By this effect, linaclotide can increase intestinal electrolyte and fluid secretion and then accelerate lumenal passage, thus relieving constipation.

GC-C signaling plays a critical role in intestinal function through its involvement in regulating the control of local fluid balance, electrolyte homeostasis and maintaining the protective mucus layer (Lorenz et al. 2003; Vaandrager et al. 2005). Additionally, it is considered that GC-C signaling is a fundamental promoter of intestinal mucosa integrity and barrier function through crypt renewal dynamics, cell differentiation, and metabolism (Lucas et al. 2000; Pitari et al. 2007). GC-C signaling protects IEC integrity by localization of tight junction proteins in the apical membrane, which promotes tight junction assembly and reduced IEC permeability (Lucas et al. 2000; Han et al. 2011). Hence, induction of GC-C activity by pharmacologic agonists may have value in preventing further damage, or promoting mucosal barrier restitution in patients with disturbed barrier function, including ulcerative colitis (UC) for which a next generation GC-C agonist is currently being tested (www.synergypharma.com) (Lin et al. 2012). Linaclotide acts locally in the GI tract with minimal systemic exposure, resulting in low oral bioavailability and thus a low risk of systemic adverse effects (Layer and Stanghellini 2014).

Lubiprostone (Sucampo Pharmaceuticals Inc., Bethesda, MD, U.S.A.) has been used to treat constipation in patients with IBS-C and CIC since 2006 (Ginzburg and Ambizas 2008; Barish et al. 2010; Schey and Rao 2011). Lubiprostone is an analog of endogenous prostones that act as functional fatty acids physiologically generated in the human body. Lubiprostone induces efflux of anions such as chloride by activating the chloride channel type 2 (ClC-2) in IEC although evidence indicates a possible role for the CFTR chloride transporter in the overall response to lubiprostone (Cuppoletti et al. 2004a, 2014; Bao et al. 2008; Bijvelds et al. 2009; Ao et al. 2011). The stimulation of chloride secretion promotes the passage of water into the luminal space and facilitates the passage of stool thus significantly improving symptoms associated with CIC and IBS-C. Moreover, lubiprostone has been shown to exert a protective effect on the intestinal mucosal barrier function through ClC-2 activation. Lubiprostone stimulated rapid repair of intestinal barrier function in ischemic-injured porcine ileum (Moeser et al. 2007). Activation of ClC-2 resulted in co-localization of ClC-2 with tight junction proteins such as occludin in the region of the apical tight junction (Gyomorey et al. 2000; Moeser et al. 2004, 2008). More recently, it has been shown that ClC-2 modulates tight junction barrier function via intracellular trafficking of occludin (Nighot and Blikslager 2012).

It is important to note, however, that even though both lubiprostone and linaclotide are capable of promoting intestinal secretion and alleviating constipation, they do not act via identical mechanisms nor do they appear to have uniform outcomes on other parameters of intestinal epithelial function that contribute to the overall transporting capacity of the intestine. Specifically, in one recent study using isolated ischemia-damaged pig jejunum, linaclotide failed to effectively repair or protect epithelial barrier function and IEC homeostasis after exposure to cell stressors, in contrast to lubiprostone (Cuppoletti et al. 2012). In addition, there is controversy as to the localization of the proposed molecular target of lubiprostone, the ClC-2 chloride channel. In spite of lubiprostone's ability to promote overall apical chloride secretion by epithelial cells, several immunohistochemical studies indicate that in intestinal tissues across a range of species ClC-2 is localized to the basolateral not the apical surface of epithelial cells (Catalan et al. 2004; Pena-Munzenmayer et al. 2005). Moreover, although lubiprostone and linaclotide are both administered to patients by the oral route, lubiprostone is capable of

stimulating short-circuit current (I_{sc}) when administered to the serosal surface of intestinal tissues ex vivo (Moeser et al. 2007; Johanson et al. 2008a,b; Johnston et al. 2009; Chey et al. 2012). Therefore, the aim of this study was to determine if multiple dosing regimens differentially affect linaclotide and lubiprostone modulation of ion transport and barrier properties of normal human colonic mucosa.

Materials and Methods

Human subjects

Study subjects were enrolled among patients referred for colonoscopy for general evaluation (anemia of unknown origin, previous diverticulitis, polyp surveillance, etc.) at Thornton Hospital, University of California San Diego Health Care System, La Jolla, United States. Six biopsies from sigmoid colon in which the colonic mucosa was macroscopically normal were obtained from 18 subjects (10 men; mean age 57 $\pm$ 5 years and eight women; mean age; 62 $\pm$ 3 years) by a gastroenterologist (M. J. D.). Studies were performed according to the guidelines of the Declaration of Helsinki. Approval was granted by the Human Research Protections Program, University of California San Diego, and written informed consent was obtained from all study subjects.

Biopsy collection

Colonic biopsies from sigmoid colon were obtained with a large capacity forceps (Olympus, Tokyo, Japan) and placed on gel foam inserts (Ethicon US LLC, Cincinnati, OH) (mucosal side facing up) by a Gastroenterologist (M. J. D). Biopsied tissues were immediately placed into cold, preoxygenated Ringer's solution (pH 7.4) with the following composition (in mmol/L): 140 Na^+, 5.2 K^+, 1.2 Ca^{2+}, 0.8 Mg^{2+}, 119.8 Cl^-, 25 HCO_3^-, 2.4 $H_2PO_4^-$, and 10 glucose. Tissues were transported to the laboratory within 30 min of collection. A paired biopsy was placed in formalin for subsequent immunohistochemical staining.

Immunohistochemical staining

Immunostaining was performed on 4 mm thick, formalin-fixed, paraffin-embedded tissue sections mounted on positively charged X-tra slides (Surgipath, Richmond, IL). Paraffin sections were deparaffinized in xylene, rehydrated, and washed in H_2O. Tissues were stained with hematoxylin and eosin (H&E) to confirm that tissues were noninflamed. Images were taken using an Olympus IX71 microscope.

Electrophysiological studies of human colon

Mucosal biopsies were mounted on specially designed Ussing chamber inserts with a window area of 0.031 cm^2 (Physiologic Instruments, San Diego, CA). Tissues were bathed bilaterally in 5 mL oxygenated Ringer's solution (composition as above) at 37°C. The tissues were short-circuited by an automated multichannel voltage/current clamp (VCC MC8) and the (I_{sc}), expressed in μA, across the tissues was monitored at intervals as an indication of net active ion transport. Tissues were allowed to equilibrate for a 20 min period, at which point baseline potential difference (PD) expressed in mV, short I_{sc}, and tissue conductance (G) were measured prior to administration of any reagents (Hemlin et al. 1988; Clarke 2009).

Test compound dosing procedures

Electrical conductance was determined by application of a 5 mV pulse prior to addition of compounds to confirm tissue viability. Three different treatment protocols were utilized. In study 1, biopsies were collected from each subject; one biopsy was treated with dimethyl sulfoxide (DMSO) (0.045%) as a negative control, while remaining biopsies were treated with increasing concentrations (0.01, 0.1, and 1.0 μmol/L) of linaclotide (prepared in H_2O; provided by Ferring Research Institute Inc., San Diego, CA) or lubiprostone (purchased from TLC PharmaChem, Vaughan, Ontario, Canada) on both the mucosal and serosal surfaces added to individual tissue preparations. After 30 min, tissues were treated with forskolin (20 μmol/L; Sigma-Aldrich, St. Louis, MO) applied to the mucosal and serosal side. After ~5 min (at the peak of the forskolin response plateau phase), tissues were treated with the Ca^{2+}-dependent agonist Carbachol (300 μmol/L; Sigma-Aldrich) to the serosal side of chamber. Concentrations based on maximally induced responses observed in Ussing chamber studies of ex vivo intestine (McCole et al. 2005). This acted as not only a reference point for linaclotide and lubiprostone efficacy but also as a test for tissue viability. Tissues that failed to respond to both forskolin and carbachol were excluded from the data analysis in all studies. In study 2, linaclotide and lubiprostone were added to only the mucosal side of the chamber in a cumulative dosage regimen (from 10^{-10} to 10^{-4} mol/L). After addition of each concentration of compound, the I_{sc} was recorded for 10 min so that responses to later additions were not compromised by changes in tissue integrity. Forskolin and carbachol were added as previously described in study 1. In study 3, individual tissues were treated with a single concentration of linaclotide (1.0 or 10 μmol/L) or lubiprostone (1.0 or 10 μmol/L) to the mucosal side of the

chamber only. Concentrations of linaclotide and lubiprostone were selected based on clinical dosing ranges and ranges used in experimental studies of these agents (Johanson et al. 2008b; Chey et al. 2012; Cuppoletti et al. 2012). Data were recorded and analyzed using Labchart Pro 7 software (AD Instruments, Colorado Springs, CO).

Data analysis

I_{sc} responses (ΔI_{sc}) to linaclotide and lubiprostone were calculated by subtracting the baseline I_{sc} from peak I_{sc}. ΔI_{sc} responses to forskolin and carbachol were calculated in the same manner. Transepithelial electrical resistance (TER) was calculated from the conductance and I_{sc} based on Ohm's law ($R = V/I$). Percent change in TER was also calculated from basal and post treatment TER.

Statistics

Data are presented as mean ± standard error of mean (SEM). Comparisons between groups were performed by using analysis of variance followed by Newman–Student–Keuls post test or unpaired Student's *t*-test where appropriate, using GraphPad prism software (version 5; GraphPad Software, La Jolla, CA). A *P*-value of <0.05 was considered statistically significant.

Results

Bilateral administration of linaclotide and lubiprostone increased I_{sc} responses across human sigmoid colon

As lubiprostone is capable of modulating I_{sc} when administered to mucosal or serosal surfaces of ex vivo porcine intestine, we first determined the effect of lubiprostone or linaclotide on ion transport across human sigmoid colon following administration to both the mucosal and serosal surfaces was examined and compared with control (DMSO vehicle only) (Moeser et al. 2007). H&E staining was used to confirm that tissues were noninflamed and mucosal biopsies did not contain any submucosal structures (Fig. 1A). I_{sc} responses were recorded as the change in short-circuit current (ΔI_{sc}). There were no differences in baseline I_{sc} (Fig. 1B) or baseline TER (Fig. 1C) between the different groups prior to treatment. Linaclotide treatment increased I_{sc} in a concentration-dependent manner (Fig. 2A). In particular, 0.1 and 1.0 μmol/L concentrations of linaclotide showed a significant increase in I_{sc} compared with control. The ion transport response to lubiprostone (ΔI_{sc}) was also concentration-dependent as lubiprostone at 1.0 μmol/L showed a significant increase in I_{sc} compared with control (Fig. 2A). Additionally, 1.0 μmol/L of linaclotide showed a significantly greater ΔI_{sc} than the equivalent concentration of lubiprostone indicating greater efficacy of linaclotide in stimulating I_{sc} across human colon under these experimental conditions.

Lubiprostone, but not linaclotide, desensitizes human colonic mucosa to subsequent cAMP-dependent but not calcium-dependent ion transport responses

Forskolin has been widely used to induce cAMP-mediated I_{sc} in the intestinal mucosa of various species, in addition to anion secretion across intestinal epithelial monolayers (Clarke et al. 1992; Mall et al. 1998). Forskolin acts in a receptor-independent manner to activate the enzyme adenylyl cyclase and increase intracellular levels of cAMP (Metzger and Lindner 1981). To investigate whether cAMP-mediated I_{sc} was altered by linaclotide or lubiprostone pretreatment, forskolin (20 μmol/L) was administered bilaterally after the mucosal biopsies had been exposed to linaclotide or lubiprostone for 30 min. Although forskolin-stimulated I_{sc} responses in linaclotide-treated groups and lubiprostone-treated groups, lubiprostone pretreatment caused a significant reduction in the I_{sc} response to forskolin in a concentration-dependent manner compared with control (Fig. 2B).

Carbachol, also known as carbamylcholine, is a more stable analog of the neurotransmitter acetylcholine. Carbachol is a well-established secretagogue that induces anion secretion across ICE (Barrett and Keely 2000). Carbachol triggers Cl^- secretion in colonocytes by activation of Ca^{2+}-dependent pathways through activation of muscarinic M3 receptors (Barrett and Keely 2000). To investigate whether the responsiveness to carbachol was altered by linaclotide or lubiprostone pretreatment, carbachol (300 μmol/L) was administered to the serosal side of each chamber after the forskolin-induced peak response had reached a plateau (~5 min). Pretreatment with linaclotide or lubiprostone had no significant impact on the capacity of carbachol to stimulate I_{sc} thus indicating that neither of these agents impaired calcium-dependent I_{sc} responses across human colon (Fig. 2C).

A cumulative concentration response regimen revealed similar responsiveness to mucosal administration of linaclotide on cAMP-dependent transport

Multiple drug dosing regimens are used to test not just pharmacokinetic properties but also to determine if there are issues with tolerance and efficacy of an agent when administered in a single concentration versus a cumulative concentration regimen (Schechter 1997). With this in

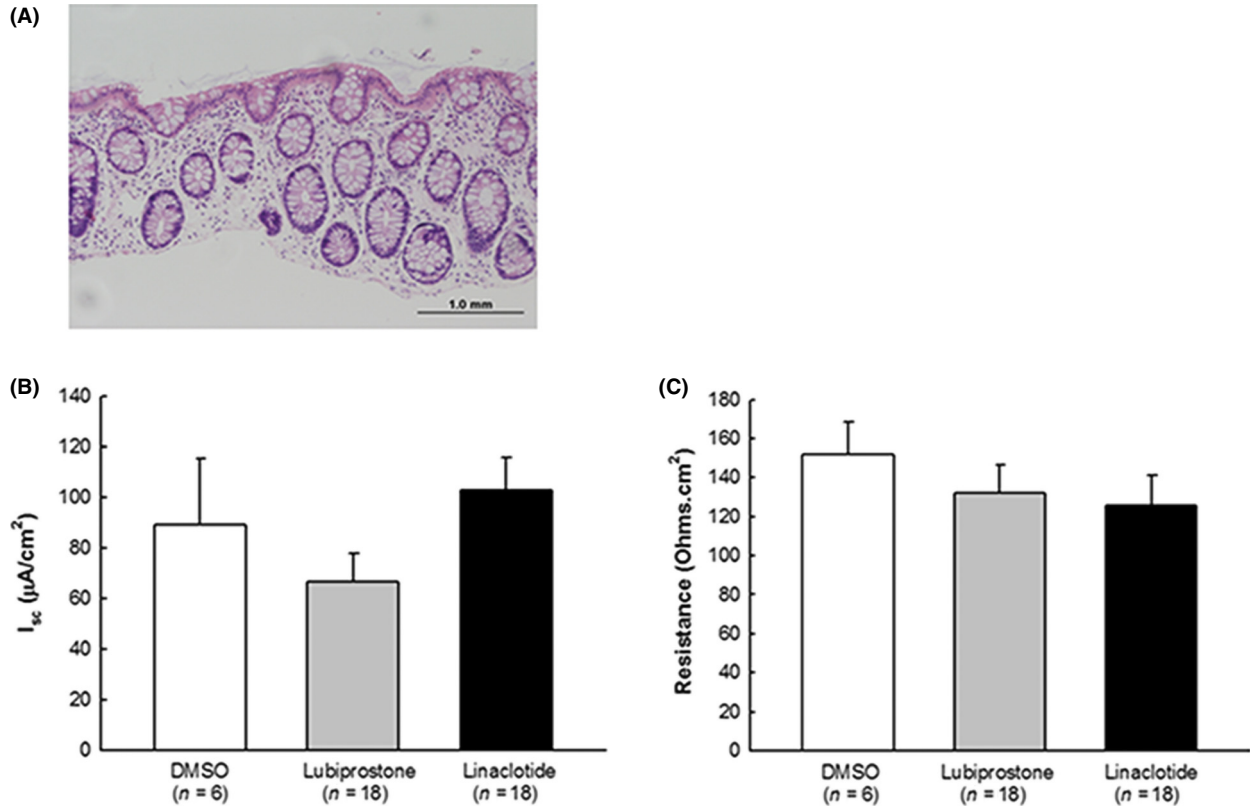

Figure 1. Basal electrophysiological properties of human colonic mucosal biopsies mounted in Ussing chambers. (A) Formalin-fixed mucosal biopsy from the sigmoid colon of a normal subject stained with hematoxylin and eosin. Adjacent biopsies were used for electrophysiological studies. (B) The baseline short-circuit current (I_{sc}), expressed in $\mu A/cm^2$, of tissues in individual drug treatment groups prior to addition of pharmacologic agents. (C) Baseline transepithelial resistance (TER) expressed in Ohms.cm^2 prior to addition of pharmacologic agents. All data were plotted as mean ± SEM. There was no difference in baseline I_{sc} and TER between the different groups (B and C). TER, transepithelial electrical resistance.

mind, we performed a cumulative concentration response to linaclotide and lubiprostone to complement the single-concentration studies performed in Figure 2. In addition, we examined the efficacy of each agent only when administered mucosally as the oral route is the preferred route of administration for both of these agents. Increasing concentrations of each agent were administered to individual biopsies at 10-min intervals from 10^{-10} mol/L concentration to 10^{-4} mol/L concentration. Both lubiprostone and linaclotide increased ΔI_{sc} in a concentration-dependent manner compared with DMSO (Fig. 3A). At the 1.0 and 10 μmol/L concentrations, both lubiprostone and linaclotide showed significantly higher I_{sc} responses than DMSO control. Forskolin increased I_{sc} in control, linaclotide-treated, and lubiprostone-treated tissues. However, in contrast to data using a single-concentration administration, (c.f. Fig. 2A), linaclotide significantly inhibited the subsequent I_{sc} response to forskolin (Fig. 3B). As with single-concentration administration, lubiprostone pretreatment also inhibited forskolin-stimulated I_{sc} (Fig. 3B). Carbachol responses were not significantly affected by linaclotide or lubiprostone pretreatment (Fig. 3C). Given the reported effects of lubiprostone in improving TER, a measure of epithelial barrier function, in intestinal tissues we also assessed if either agent had an effect on TER of mucosal biopsies (Cuppoletti et al. 2012). Interestingly, DMSO, the vehicle control, caused a drop in TER over time but this was mitigated in lubiprostone-treated tissues, whereas linaclotide significantly preserved TER compared with DMSO (Fig. 3D).

Effect of single concentration, mucosal side only administration of lubiprostone and linaclotide on ion transport and mucosal resistance of colonic biopsies

Clinically, linaclotide and lubiprostone are both administered orally with linaclotide administered once per day, whereas lubiprostone is administered twice daily (Johanson et al. 2008a,b; Johnston et al. 2009; Chey et al. 2012).

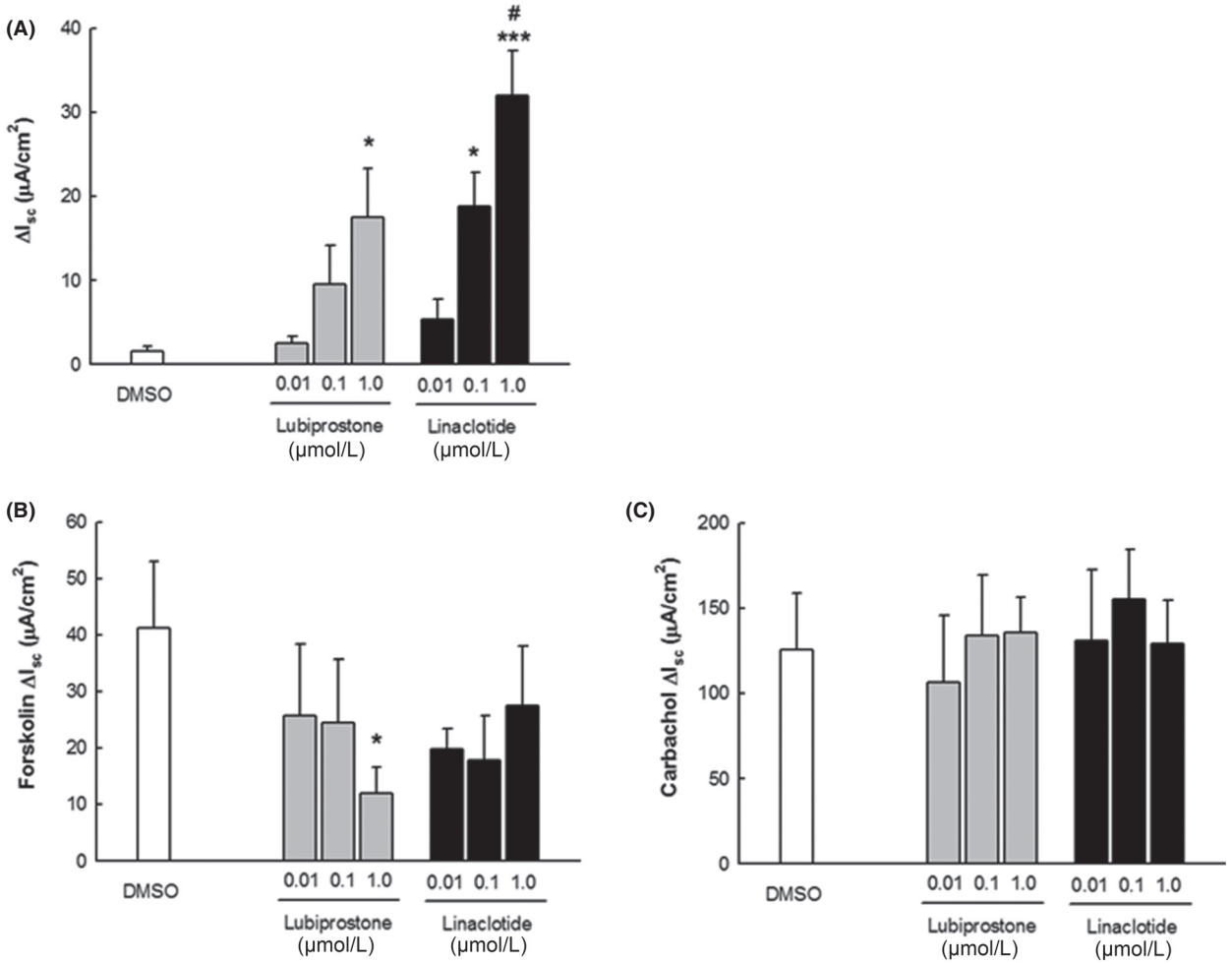

Figure 2. Single-concentration administration of linaclotide and lubiprostone increased I_{sc} across human colon in a concentration-dependent manner. (A) Changes in short-circuit current (I_{sc}) to bilateral administration of linaclotide or lubiprostone (0.01, 0.1, 1.0 μmol/L; $n = 6$) were measured. Each concentration was added to an individual human colon biopsy mounted in an Ussing chamber. (B) ΔI_{sc} responses to forskolin (20 μmol/L; bilateral) and (C) carbachol (300 μmol/L; serosal) were also determined subsequent to lubiprostone or linaclotide treatments. All data were plotted as mean ± SEM. *$P < 0.05$, ***$P < 0.01$ versus DMSO; #$P < 0.05$ versus lubiprostone 1.0 μmol/L; $n = 6$.

Therefore, and with consideration of the results of the cumulative concentration study (c.f. Fig. 3), 1.0 and 10 μmol/L concentrations of both compounds were selected to investigate the effect of single-concentration administration to the mucosal surface only of human colonic biopsies. I_{sc} responses to both concentrations of lubiprostone and linaclotide were significantly higher than control (Fig. 4A). Subsequent treatment with forskolin increased I_{sc} in all groups, however, only pretreatment with lubiprostone at the 10 μmol/L concentration significantly attenuated the forskolin-stimulated I_{sc} response compared with control ($P < 0.05$; Fig. 4B). Interestingly, I_{sc} responses to carbachol were greater in tissues mucosally pretreated with lubiprostone and linaclotide compared with control but this increase did not reach statistical significance (Fig. 4C). With respect to their effects on TER, mucosal lubiprostone and linaclotide at both 1.0 and 10 μmol/L concentration preserved TER, from baseline recording of TER through to the end of the experiment, in contrast to the decrease induced by DMSO control (Fig. 4D).

Discussion and Conclusion

The mechanism of action of lubiprostone enhancement of Cl^- secretion has attracted a lot of research interest given the clinical efficacy of this drug in alleviating constipation. Initial studies by Cuppoletti et al. (2004a, b) reported that lubiprostone activates ClC-2 to increase Cl^- secretion in T_{84} colonic epithelial cells (Cuppoletti et al. 2004a). Follow-up studies also indicated that lubiprostone mainly targets ClC-2 to induce Cl^- secretion, whereas Fei et al.

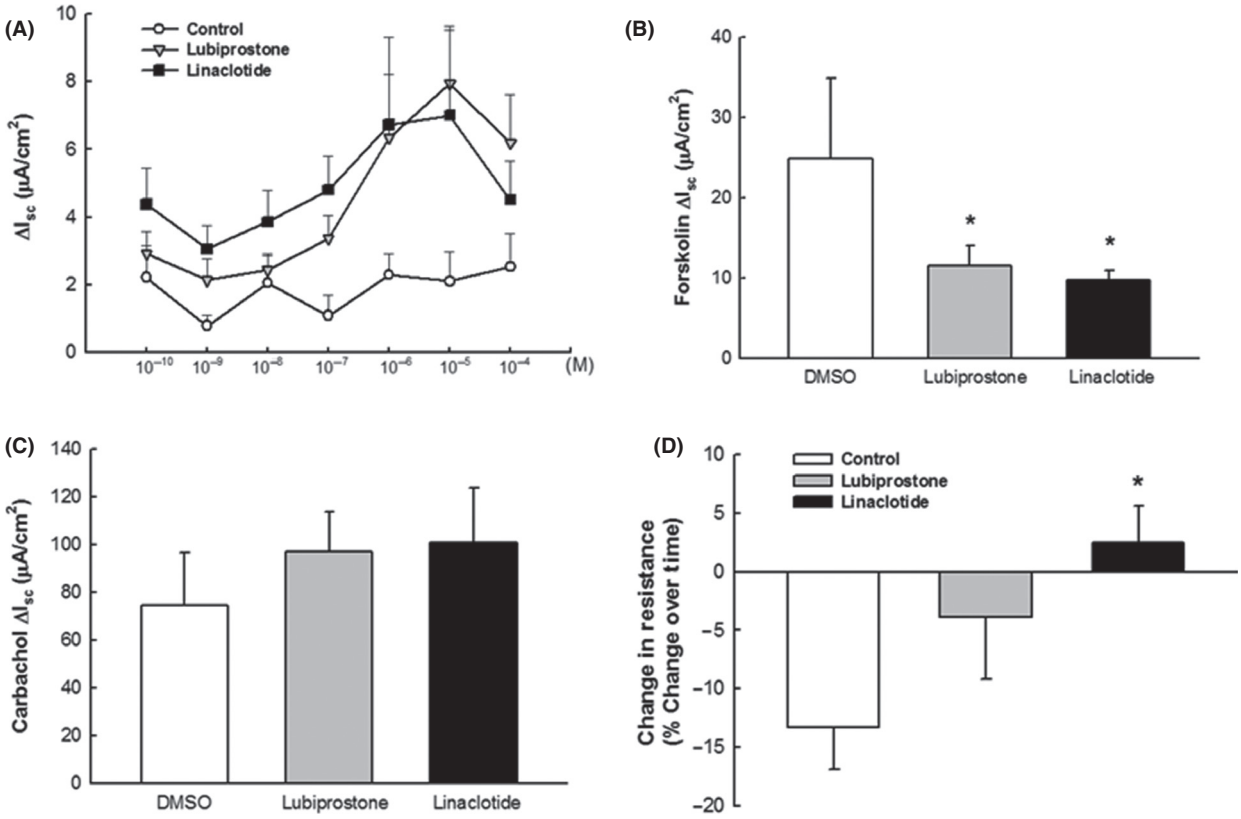

Figure 3. A cumulative concentration response regimen reveals similar I_{sc} responsiveness to mucosal administration of linaclotide. Duplicate tissues from individual subjects were used for drug treatments and the mean for each individual subject was used for statistical analysis (each $n = 1$ subject). (A) At the 0.1, 1.0 and 10 μmol/L concentrations, both lubiprostone and linaclotide induced higher I_{sc} responses than DMSO ($n = 5$). (B) In particular, pretreatment with either lubiprostone or linaclotide (cumulative concentration of 10^{-4} mol/L) inhibited subsequent I_{sc} responses to forskolin ($P < 0.05$; $n = 5$). (C) Pretreatment with lubiprostone or linaclotide (cumulative concentration of 100 μmol/L) did not affect carbachol-stimulated I_{sc}. (D) The change in TER over the full duration of the experiment (120 min) was assessed. Treatment with linaclotide significantly preserved TER versus control; (*$P < 0.05$, vs. DMSO; $n = 5$). TER, transepithelial electrical resistance.

(2009) reported that lubiprostone acts through a channel other than the CFTR transporter in guinea pig ileum (Fei et al. 2009). However, other groups have generated evidence of a role for CFTR activity in the response to lubiprostone. These reports identified that lubiprostone induces Cl^- secretion through CFTR and cAMP signaling in T_{84} cells, whereas responses to lubiprostone are diminished in tissues from CFTR knockout mice and in intestinal biopsies from pediatric cystic fibrosis patients expressing the ΔF508 CFTR mutation that reduces CFTR trafficking to the epithelial apical membrane (Bijvelds et al. 2009; Ao et al. 2011). Forskolin is an inducer of CFTR-mediated anion secretion in intestinal epithelium and acts by increasing adenylyl-cyclase-driven production of intracellular cAMP, although it can also stimulate a low level of K^+ secretion (Cuthbert et al. 1999). In our study, the effect of forskolin on I_{sc} was largely suppressed by lubiprostone pretreatment. This finding mirrors previous studies indicating that lubiprosotone and forskolin both activate Cl^- secretion through cAMP, and that responses to forskolin are densitized by prior treatment with lubiprostone (MacVinish et al. 2007; Bijvelds et al. 2009; Ao et al. 2011). Indeed, lubiprostone appears to exert part of its effect via prostanoid receptors as the transport response to lubiprostone in T_{84} cells, as well as smooth muscle contraction in mouse intestine, was sensitive to inhibition of the cAMP-coupled EP4 receptor, as well as EP1 receptors in smooth muscle (Bassil et al. 2008; Bijvelds et al. 2009). Interestingly, this effect of 1 μmol/L lubiprostone on subsequent forskolin-stimulated I_{sc} appeared to be specific for bilateral preadministration (1 μmol/L; c.f. Fig. 2B) as mucosal administration of a single concentration of 1 μmol/L lubiprostone did not affect subsequent forskolin responses (c.f. Fig. 4B). A cumulative dosing regimen which culminated in a final concentration of 10^{-4} mol/L lubiprostone administered to the mucosal surface only, also inhibited subsequent I_{sc} responses to forskolin (c.f. Fig. 3B). These findings may

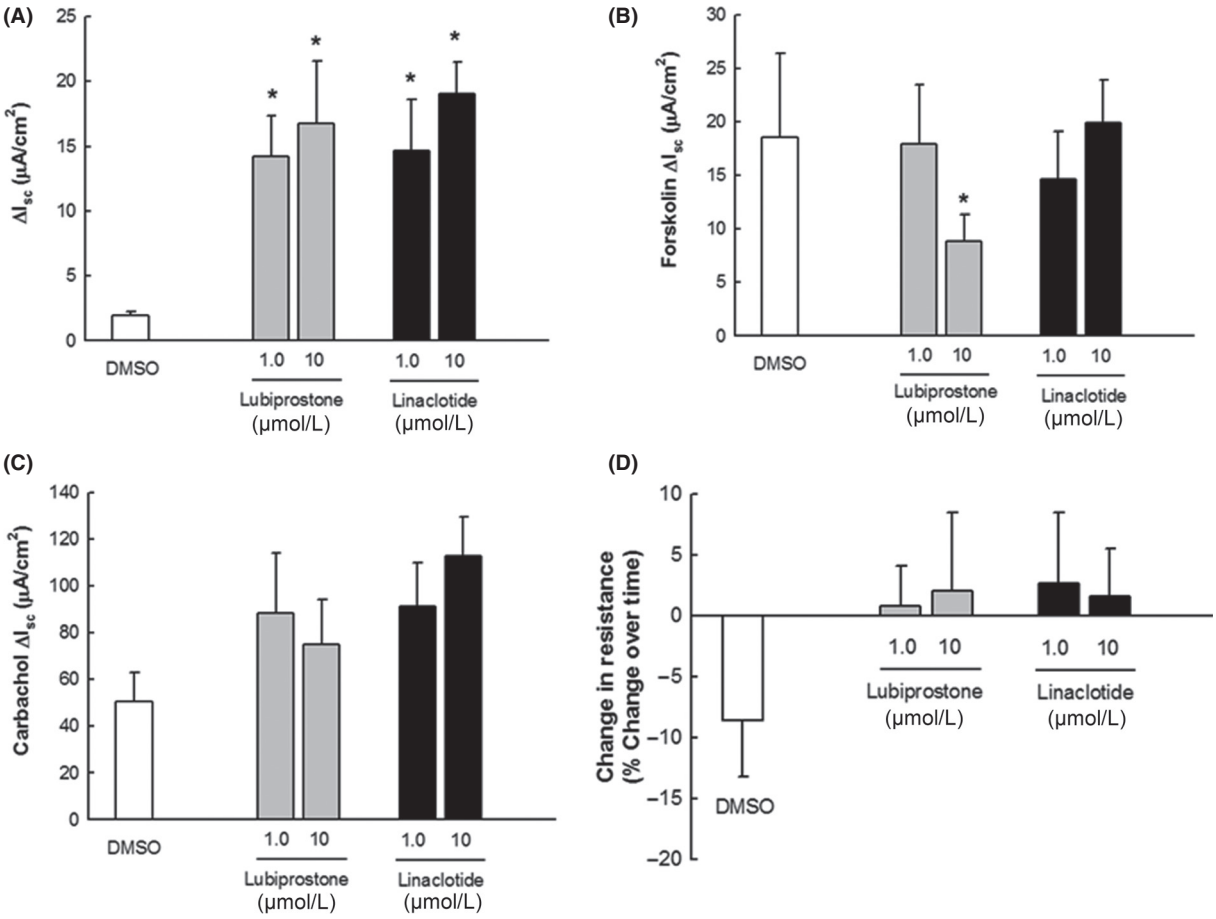

Figure 4. Single-concentration mucosal administration of lubiprostone but not linaclotide inhibits subsequent cAMP-dependent I_{sc} responses. (A) Single-concentration mucosal administration of lubiprostone and linaclotide [1.0, 10 μmol/L] induced significantly higher I_{sc} responses than DMSO vehicle ($P < 0.05$; $n = 7$). (B) Pretreatment with lubiprostone (10 μmol/L) significantly decreased the I_{sc} response to forskolin (*$P < 0.05$, vs. DMSO; $n = 7$). (C) Pretreatment with lubiprostone or linaclotide did not affect carbachol-stimulated I_{sc}. (D) Linaclotide or lubiprostone pretreatment preserved TER over the duration of the experiment (30 min) compared with DMSO vehicle control. TER, transepithelial electrical resistance.

have implications for dosing strategies in vivo and desensitization to endogenous cAMP-dependent stimuli of fluid secretion.

In contrast to the inhibitory effect of lubiprostone on subsequent responses to the cAMP agonist, forskolin, responses to the calcium-dependent agonist, carbachol did not appear to be affected by lubiprostone. It is not surprising that lubiprostone did not inhibit carbachol-driven fluid secretion given that carbachol acts through calcium-dependent opening of basolateral potassium channels and subsequent opening of apical calcium-activated chloride channels (CLCA) (Barrett and Keely 2000). However, given that carbachol stimulation of Cl^- secretion on a background of elevated cAMP leads to a synergistic increase in the I_{sc} response to carbachol, it is somewhat surprising that responses to carbachol were not significantly greater in lubiprostone-treated tissues than DMSO controls (Dharmsathaphorn and Pandol 1986). Similar to lubiprostone, linaclotide also increased I_{sc} compared to DMSO in a concentration-dependent manner. Moreover, when administered to both the mucosal and serosal surfaces of the colonic biopsy, a single concentration of 1 μmol/L linaclotide showed a significantly higher ΔI_{sc} than the same concentration of lubiprostone (c.f. Fig. 2A). This indicates increased responsiveness to linaclotide. However, this increased response to linaclotide was not apparent following cumulative (c.f. Fig. 3A) administration where responses to both agents were lower than with single-concentration administration, possibly due to partial densensitization or differential recruitment of signaling pathways. The increased responsiveness to linaclotide was also absent following single-concentration administration to the mucosal side only (c.f. Fig. 4A). Therefore, mucosal administration of linaclotide and lubiprostone appear to

have equal efficacy in stimulating I_{sc} across human colonic mucosa. However, it is worth noting that the inhibition of subsequent I_{sc} responses to forskolin by lubiprostone was observed at 10 μmol/L following mucosal exposure versus 1 μmol/L when added bilaterally (c.f. Fig. 2B).

Although the mechanism by which lubiprostone suppresses forskolin-stimulated I_{sc} responses is likely quite complex and context-dependent, in addition to possible crosstalk between forskolin signals and lubiprostone signaling downstream of EP4 receptor signaling pathways that may impinge upon I_{sc} responses to forskolin, forskolin itself has been shown to activate recombinant hClC-2 (Cuppoletti et al. 2014). Moreover, human (but not rat or mouse) ClC-2 is also activated by forskolin-IBMX through a PKA pathway thus adding an extra layer of complexity to our understanding of these events (Cuppoletti et al. 2004b, 2013, 2014). What effect linaclotide administered to the serosal surface has is unclear as functional assays with GC-C stimuli indicate receptor localization on the mucosal surface, whereas guanylin secretion also occurs on the mucosal (apical) surface of intestinal epithelium (Martin et al. 1999). In addition, expression of GC-C in human colon has been reported to be restricted to surface as opposed to crypt epithelial cells and thus should be maximally activated by mucosal addition of linaclotide (Swenson et al. 1996). There was an additional consequence of the different dosing regimens on linaclotide regulation of I_{sc} in human colon. It was striking that following a cumulative dosing on the mucosal surface only, did linaclotide exert a significant inhibitory effect on subsequent cAMP-dependent responses to forskolin (c.f. Fig. 3B). This effect was identical to that of lubiprostone pretreatment. One possible explanation for this may be recruitment of additional PKA regulated pathways to modify CFTR activity in response to a subsequent cAMP-PKA agonist such as forskolin. Additionally, it has been reported that GC-C activation can give rise to secondary effects on cAMP generation (Field et al. 1978; Chao et al. 1994). Thus, the possibility that a cumulative versus single concentration of linaclotide may reduce forskolin-induced cAMP levels in isolated human mucosa cannot be ruled out.

As mentioned previously, lubiprostone but not linaclotide demonstrated a protective and reparative effect on the epithelial barrier under conditions of stress (Cuppoletti et al. 2012). To determine if these agents modified barrier properties in normal human colon, we calculated the change in TER (ΔTER) over the course of the cumulative concentration administration of lubiprostone or linaclotide (120 min). Surprisingly, linaclotide but not lubiprostone significantly preserved TER in contrast to tissues treated with vehicle control (c.f. Fig. 3D). Although we did not assess whether linaclotide affects the molecular composition of tight junctions, our functional data suggest that linaclotide may exert some degree of protection on mucosal integrity in the absence of pharmacological stressors to prevent declining barrier function ex vivo. Our finding is worthy of more detailed evaluation with respect to the effects of linaclotide on barrier function as loss of GC-C signaling has been shown to lead to intestinal barrier defects in a GC-C knockout mouse model following challenge with bacterial lipopolysaccharide (LPS) (Han et al. 2011).

To our knowledge, this is the first ex vivo study using human intestinal mucosa to perform a side-by-side comparison of electrolyte transport responses to linaclotide and lubiprostone. In summary, we have shown that linaclotide and lubiprostone both increase I_{sc} responses in isolated human colonic mucosa. The side-by-side comparisons of multiple dosing regimens suggest a close comparability of each compound with respect to induction of electrolyte transport across human colonic mucosa. However, discreet differences in the effects of linaclotide in particular are apparent depending on the dosing regimen used and whether the drug is administered bilaterally, or solely to the mucosal surface. This may have implications for dosing strategies in patients with constipation and desensitization to pro-secretory agents, as well as patients with compromised barrier function that permits access of luminally administered agents to the serosal surface of intestinal epithelium.

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Author Contributions

S. B. K. (acquisition of electrophysiological data; analysis, and drafting of the manuscript); R. R. M., H. M. P. (acquisition of data; immunohistochemistry; review of manuscript); M. J. D. (acquisition of mucosal biopsies and critical revision of the manuscript for important intellectual content and editing); D. F. M. (study design, obtained funding for the study, data analysis, critical revision of the manuscript for important intellectual content, and editing). All authors approved the final version of the manuscript.

Disclosures

D. F. M. has served as a consultant to Ferring Research Institute Inc. The remaining authors have no conflicts of

interest to declare in relation to the topics and content discussed in this article.

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