MRP4 Modulation of the Guanylate Cyclase-C/cGMP Pathway: Effects on Linaclotide-Induced Electrolyte Secretion and cGMP Efflux#

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**Running title: MRP4 modulates GC-C dependent electrolyte and cGMP secretion**

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Abstract

Multidrug resistance protein 4 (MRP4) mediates the efflux of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) and acts as an important regulator of these secondary messengers, thereby affecting signaling events mediated by cGMP and cAMP. Immunofluorescence staining showed high MRP4 expression localized predominantly to the apical membrane of rat colonic epithelium. In vitro studies were performed using rat colonic mucosal layer mounted in an Ussing chamber. Linaclotide activation of the guanylate cyclase-C (GC-C)/cGMP pathway induced a concentration-dependent increase in transepithelial ion current (Isc) across rat colonic mucosa (EC50: 9.2 nM). Pretreatment of colonic mucosa with the specific MRP4 inhibitor MK571 potentiated linaclotide-induced electrolyte secretion and augmented linaclotide-stimulated intracellular cGMP accumulation. Notably, pretreatment with the phosphodiesterase 5 inhibitor sildenafil increased basal Isc, but had no amplifying effect on linaclotide-induced Isc. MRP4 inhibition selectively affected the activation, but not the deactivation phase of linaclotide. In contrast, incubation with a GC-C/Fc chimera binding to linaclotide abrogated linaclotide-induced Isc, returning to baseline. Further, linaclotide activation of GC-C induced cGMP secretion from the apical and basolateral membranes of colonic epithelium. MRP4 inhibition blocked cGMP efflux from the apical, but not the basolateral membrane. These data reveal a novel, previously unrecognized mechanism that functionally couples GC-C-induced luminal electrolyte transport and cGMP secretion to spatially restricted, compartmentalized regulation by MRP4 at the apical membrane of intestinal epithelium. These findings have important implications for gastrointestinal disorders with symptoms associated with dysregulated fluid homeostasis, such as irritable bowel syndrome with constipation, chronic idiopathic constipation and secretory diarrhea.
Introduction

Maintenance of intestinal homeostasis is closely linked to secretory mechanisms controlling fluid and electrolyte secretion, regulated by coordinated actions of a complex network of transporters and ion channels located in the apical and basolateral membranes of intestinal epithelial cells (IEC). The movement of Cl ions across the apical membrane of IEC generates an electrogenic ion current that drives transepithelial sodium flux. This process is further associated with inhibition of sodium/hydrogen exchanger 3 (NHE3)-mediated sodium reabsorption, thereby establishing an osmotic gradient for concomitant movement of water into the luminal compartment.

The main conductive pathway for Cl ions across the apical membrane of IEC is the anion-selective cystic fibrosis transmembrane conductance regulator (CFTR) channel. Functional activity of this ion channel, which forms membrane-associated, macromolecular complexes including transporters and signaling molecules, is partly regulated by protein kinase A following CFTR coupling to adenylate cyclase, resulting in elevated local concentrations of the second messenger cyclic adenosine 3’,5’-monophosphate (cAMP) (Wang et al., 2000; Huang et al., 2001; Li and Naren, 2005). Multidrug resistance protein 4 (MRP4) is an ubiquitously expressed member of the ATP-binding cassette transporter superfamily, which mediates energy-dependent, unidirectional efflux of prostaglandins and the cyclic nucleotides cAMP and cyclic guanosine 3’,5’-monophosphate (cGMP) (Dean et al., 2001; Wielinga et al., 2003; Sager, 2004; Ritter et al., 2005). The physical and functional coupling of MRP4 with CFTR has been reported, directly implicating MRP4 in spatially restricted and tightly modulated compartmentalization of cAMP-signaling, and regulation of CFTR-induced ion secretion (Li et al., 2007). Altered ratios of CFTR to MRP4 due to changes in expression and/or subcellular localization, resulting in enhanced or
attenuated CFTR-mediated Cl⁻ transport, have been implicated in the pathology of irritable bowel syndrome with constipation (IBS-C) and secretory diarrhea (Li et al., 2007), and decreased expression of MRP4 in IBS-C patients has recently been reported (Harrington et al., 2014).

Guanulate cyclase C (GC-C), a type I transmembrane receptor with intrinsic guanylate cyclase activity, is expressed predominantly on the luminal surface of intestinal epithelial cells along the gastrointestinal tract, is a key regulator of intestinal homeostasis and bowel function (Schulz et al., 1990; Carrithers et al., 1996; Castro et al., 2013; Silos-Santiago et al., 2013). GC-C is activated by the natural hormones guanylin and uroguanylin, and heat-stable enterotoxin (ST) peptides, members of the guanylin family of cGMP regulating peptides that bind GC-C and activate the intracellular guanylate cyclase domain of the receptor (Currie et al., 1992; Hamra et al., 1993; Kita et al., 1994; Forte 1999, 2004). Stimulation of GC-C by these peptides leads to significantly increased intracellular concentrations of cGMP, the sole second messenger generated by GC-C, which is involved in a broad range of physiological processes including activation of cGMP-dependent protein kinase II (PKG-II) (Schlossmann et al., 2005; Pfeiffer et al., 1996). PKG-II phosphorylation regulates the activity of CFTR, a well-established pathway involved in the regulation of intestinal fluid homeostasis (Seidler et al., 1997; Vaandrager et al., 1998). More recently, activation of the GC-C/cGMP pathway has also been associated with the modulation of gastrointestinal sensory signaling as a mechanism potentially underlying the analgesic effects of GC-C agonism in models of visceral pain, through an active transport of cGMP into the submucosa following GC-C activation (Eutamene et al., 2010; Castro et al., 2013; Silos-Santiago et al., 2013). It is not currently known whether MRP4 modulates GC-C mediated cGMP signaling pathways and its potential clinical implications.
Linaclotide is an orally administered, minimally absorbed, potent and selective agonist of GC-C that has been approved in the United States and European Union for the treatment of adult patients with irritable bowel syndrome with constipation (IBS-C) and in the United States for chronic idiopathic constipation (CIC) (Lembo et al., 2011; Chey et al., 2012; Rao et al., 2012). This 14-amino acid peptide is a member of the guanylin family of cGMP-regulating peptides that includes the natural hormones guanylin and uroguanylin (Bryant et al., 2010; Busby et al., 2010). In the studies described here, we have investigated the pharmacological effects of MRP4 inhibition on linaclotide-induced transepithelial ion currents and apical/basolateral cGMP efflux in vitro in rat colon mucosa and propose a novel mechanism that functionally couples compartmentalized linaclotide-induced electrolyte secretion and apical cGMP efflux to spatially restricted regulation by MRP4, following linaclotide activation of the GC-C/cGMP pathway.
Materials and Methods

Animals. Female Sprague-Dawley rats (8-9 weeks of age, average body weight approximately 200 grams) were obtained from Charles River Laboratories (Wilmington, MA) and Harlan Laboratories (Dublin, VA) and were housed in an environmentally controlled room with access to food and water ad libitum. All animal studies were approved either by the Ironwood Pharmaceuticals Institutional Animal Care and Use Committee or by the University of Kansas Animal Care and Use Committee.

Reagents. The 14-amino acid peptide linaclotide (CCEYCCNPACTGCY) (Bryant et al., 2010; Busby et al., 2010), synthesized by solid phase synthesis, was obtained from Polypeptide Laboratories (Torrance, CA). MK571, isobutylmethylxanthine (IBMX) and sildenafil were obtained from R&D Systems (Minneapolis, MN).

Transepithelial Short-Circuit Current (Isc) in Rat Colonic Mucosa. Proximal colons were collected from Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA; n=6-10) and seromuscular layers were removed by blunt dissection. Colonic tissue was mounted on slides with the 0.5 cm² aperture. Krebs Ringer Bicarbonate solution (KRB, 3 ml) containing (in mM) 115 NaCl, 15 NaHCO₃, 2.4 K₂HPO₄, 1.2 CaCl₂, 1.2 MgCl₂, and 0.4 KH₂PO₄ at pH 7.4 was added to the apical and basolateral chambers of the Ussing chamber. The apical and basolateral chambers also contained mannitol (10 mM) and glucose (10 mM), respectively. The temperature in both chambers was maintained constant at 37°C, and KRB was oxygenated throughout the experiment. Measurements of transepithelial short-circuit current (Isc) were performed using an automatic voltage clamp (Model VCC MC8; Physiologic Instruments, San Diego, CA). Linaclotide, MK571, or sildenafil was added to rat colonic mucosal preparations at indicated
time points for specific periods of time shown for each assay. For all studies, changes in the short circuit currents ($\Delta$Isc) were calculated using the equation $\Delta$Isc=−(|Isc|−|Isc^{\text{min}}|) and GraphPad Prizm software. Where |Isc| represents the absolute value of Isc recorded at a given time, and |Isc^{\text{min}}| represents a minimal absolute value of Isc recorded at baseline.

**MRP4 Immunofluorescence.** Rat colonic mucosa (Sprague-Dawley rats, Charles River Laboratories, Wilmington, MA) was dissected, embedded in Tissue-Tek® O.C.T. Compound (VWR # 4583, Radnor, PA), and frozen on a dry ice block. Colon mucosa sections (10 $\mu$m thickness) were cut on a cryostat and mounted on Fisher brand Superfrost Plus microscope slides (Fisher Scientific #12-550-15, Waltham, MA). Tissue sections were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed with 1x PBS and blocked for 1.5 hours with PBS buffer containing 5% donkey serum and 0.5% Triton X-100 at room temperature. A rabbit anti-MRP4 antibody (gift from Dr. A.P. Naren, University of Cleveland, Ohio) was applied to each section and incubated overnight at 4°C. The Alexa Fluor® 568 donkey anti-rabbit IgG secondary antibody (Life Technologies# A10042, Carlsbad, CA) was used for the detection of MRP4. Negative control staining was performed as described for MRP4 immunofluorescence, with the exception that the primary anti-MRP4 antibody was omitted from the procedure. Hoechst 33342 (Life Technologies #H3570, Carlsbad, CA) was used to stain the cell nuclei. Prolonged anti-fade mounting media (Life Technologies, Grand Island, NY) was used to cover the tissue sections. Fluorescent image was taken by Nikon microscope (Video Instruments, Avon, MA).

**Cyclic GMP Accumulation in Rat Colon Epithelium.** Isolated colon mucosa tissues (Sprague-Dawley rats, Charles River Laboratories, Wilmington, MA) were incubated in 24-well tissue culture plates with KRB (1 ml) containing either vehicle (0.1% DMSO in KRB), MK571
(20 µM) or the phosphodiesterase inhibitor IBMX (100 µM) for 20 minutes at 37°C on an orbital shaker. Twenty minutes later, linaclotide (6, 1000 nM) was added to vehicle and MK571-treated tissues, and the plates were incubated for another 20 minutes. Following treatment, mucosal tissues were collected and snap frozen in liquid nitrogen. For cGMP measurements, mucosal tissues were cryohomogenized using a Geno Grinder (SPEX SamplePrep, Metuchen, NJ). Colon mucosa homogenates were resuspended in 0.2 ml of cold passive lysis buffer (Promega, Madison, WI) containing IBMX (1 mM) and incubated on ice for 30 minutes, followed by centrifugation (30 minutes, 4°C). Supernatants were collected and protein concentrations determined using the Bradford assay. Cyclic GMP was acetylated and quantified using the cGMP Biotrak Enzyme Immunoassay System (GE Healthcare, Little Chalfont, UK).

**Cyclic GMP Efflux from Rat Colonic Mucosa.** To measure linaclotide-induced cGMP secretion, rat colonic mucosa (Sprague-Dawley rats, Charles River Laboratories, Wilmington, MA) was dissected and mounted in the Ussing chamber as described above. Vehicle (water, 4.6 µl) was added to the luminal chamber for a 60-minute equilibration period, followed by addition of linaclotide (1 µM). To determine the effect of MRP4 inhibition on cGMP secretion, rat colon mucosa was treated with the selective MRP4 inhibitor MK571 (20 µM) added to the apical and basolateral chambers at the 30-minute time point. Aliquots (0.12 ml) from the apical and basolateral chambers were collected in 15-minute intervals to measure cGMP secretion. The volume of KRB solution in both chambers was kept constant throughout the experiment by adding fresh buffer (0.12 ml) to each chamber at the same time aliquots were collected for cGMP measurements. Extracellular cGMP concentrations were determined using the cGMP Biotrak Enzyme Immunoassay System, using the cGMP acetylation protocol (GE Healthcare, Little Chalfont, UK).
Measurement of In Vivo cGMP Secretion into the Submucosal Layer by Microdialysis. Female Sprague-Dawley rats (Harlan Laboratories, Dublin, VA; n=10) were anesthetized (2.54% isoflurane), and middle line laparotomy was performed to expose the proximal colon. Feces were removed by injection of saline (4.5 ml) into the lumen. An isolated segment of proximal colon (colonic loop) was created by two firm ligations 2 cm apart from each other. A linear microdialysis probe consisting of a 5 mm polyacrylonitrile membrane connected on both sides to a polyimide tubing (15 cm) probe was implanted in the colon submucosa layer. Saline was perfused through the microdialysis probe at a rate of 1 µl/minute and fractions of dialysate were collected during 5 minute intervals. The microdialysis probe was equilibrated for 25 minutes and fractions were collected to determine the basal concentrations of cGMP concentrations in the submucosa (Woo and Lunte, 2008). Vehicle (D-glucose: 10 µg in H2O) was injected into the lumen of the colon loop and the collection of dialysate fractions was continued for 30 minutes. Thereafter, linaclotide (0.5, 1.7, and 5 µg in 0.2 ml of vehicle) was injected into the lumen of the colon loop and dialysate fractions were collected for another 30 minutes. The concentration of cGMP in collected dialysate fractions was determined by liquid chromatography-mass spectrometry (LC-MS/MS). The extraction efficiency of cGMP was determined for each probe following the experiment.

Statistical Analysis. Statistical analyses were performed using the Student’s paired or unpaired two-tailed t test, and two-way analysis of variance (ANOVA). Differences between mean values were determined by Bonferroni correction post hoc for multiple t tests. All data are expressed as the mean ± standard error of the mean (SEM). A P value <0.05 is considered statistically significant.
Results

**Linaclotide Induces Transepithelial Ion Currents in the Rat Colon.** The effects of linaclotide on transepithelial ion currents (Isc) as an indicator of net ion transport across intestinal mucosa, were measured in in vitro Ussing chamber assays using mucosa isolated from rat proximal colon. Following a 15-minute baseline period, addition of linaclotide (30 nM) to the apical chamber induced a strong Isc across the colonic mucosa that rapidly reached its maximum and remained stable in the presence of linaclotide (Fig. 1). To determine whether the generation of this transepithelial ion current is mediated through activation of GC-C and further, is dependent on continuous linaclotide stimulation of GC-C, a bivalent human GC-C/Fc fusion protein capable of binding linaclotide was employed. Preliminary in vitro molecular and functional studies had confirmed expression in HEK293 cells of a glycosylated GC-C/Fc fusion protein in the predicted molecular weight range (~125 kDa for the reduced chimera). This chimera had an estimated purity of 85% following protein-A sepharose affinity chromatography and bound antibodies specific to the extracellular portion of human GC-C and IgG1 (Supplemental Fig. 1A,B). Furthermore, the GC-C/Fc fusion protein concentration-dependently inhibited linaclotide-induced cGMP secretion from human colon carcinoma T84 cells (Supplemental Fig. 2). Addition of this GC-C/Fc fusion protein to the apical chamber resulted in a fast decrease of the linaclotide-stimulated Isc and return to baseline within 15 minutes (Fig. 1). The effective concentration of linaclotide which produced 50% of the maximal activity (EC$_{50}$) increasing transepithelial ion currents in the rat colonic mucosa was 9.2 nM, using a linaclotide concentration-response curve ranging from 1 nM to 1000 nM (Fig. 2 A,B).

**MRP4 Inhibition Amplifies Linaclotide-induced Transepithelial Ion Currents.**

MRP4 mRNA expression has previously been detected in rat mucosal tissue extruded from each
region of the intestine, ranging from the duodenum to the rectum (Silos-Santiago et al., 2013). We further investigated the cellular localization of MRP4 expressed in rat colonic mucosa by immunohistochemistry and found MRP4-specific staining predominantly associated with the apical membrane, indicating high expression of this cGMP transporter on the apical membrane of rat colonocytes, with very minor staining associated with the basolateral membrane also detectable, a pattern similar to that described recently in human and mouse colonic mucosa (Harrington et al., 2014), and previously reported for human colonic T84 and HT29-CL19A epithelial cells (Li et al., 2007) (Fig. 3). We further assessed the effects of MK571-mediated inhibition of MRP4 on linaclotide-induced transepithelial ion currents (Reid et al., 2003). Linaclotide effects were measured at concentrations below the calculated EC$_{50}$ (6 nM) and at 1000 nM, a concentration of linaclotide that elicits a maximum increase in transepithelial ion currents. Colonic mucosa samples were pretreated with MK571 (20 µM) added to the apical and basolateral chambers, prior to sequential addition of 6 nM and 1000 nM linaclotide for 20-minute incubation periods. During the 20-minute pretreatment period, MK571 elicited no effect on the Isc in rat colon mucosa, compared to vehicle-treated tissue (Fig. 4A). In the presence either of 6 nM or 1000 nM linaclotide, MK571 augmented linaclotide-induced transepithelial ion currents (Fig. 4A), and significantly ($P<0.05$) increased the maximal Isc response at both peptide concentrations (Fig. 4B).

**MRP4 Inhibition Differentially Affects Activation/Deactivation Kinetics of Linaclotide-induced Transepithelial Ion Currents.** We next investigated the effects of MK571 (20 µM) and the phosphodiesterase-5 inhibitor sildenafil (100 nM) on the activation/deactivation kinetics of linaclotide-induced transepithelial ion currents. Pretreatment of colonic mucosa for 20 minutes with sildenafil, but not MK571, significantly increased the Isc. Addition of linaclotide (6
nM) for a 20-minute period to the apical chamber induced a marked increase in transepithelial ion current (Fig. 5A), which was further potentiated in MK571-pretreated mucosa (Fig. 5B), but elicited no changes in transepithelial ion currents in sildenafil-pretreated mucosa (Fig. 5C). Apical GC-C/Fc fusion protein (60 nM) rapidly abrogated the linaclotide-induced increase in transepithelial ion currents, returning to baseline within 20 minutes. Similar deactivation kinetics of linaclotide-induced transepithelial ion currents were observed in colonic mucosa pretreated either with MK571 or sildenafil, indicating that continuous GC-C stimulation with linaclotide is required to generate increased Isc (Fig. 5A,B,C).

**MRP4 Inhibition Increases Linaclotide-stimulated cGMP Accumulation in Rat Colon Tissue.** In vitro linaclotide-stimulated cGMP accumulation in human colon carcinoma T84 cells has previously been reported (Busby et al., 2010). Here, we further investigated the effect of MK571-mediated inhibition of the efflux pump MRP4 on cGMP accumulation in rat colon mucosa. Following in vitro incubation of rat colon mucosa with MK571 (20 µM) added to both the apical and basolateral chambers, no changes in tissue cGMP levels were observed compared to vehicle-treated colon mucosa, while treatment with the phosphodiesterase inhibitor IBMX (100 µM) elicited a significant increase ($P<0.01$) in cGMP levels (Fig. 6). Linaclotide (6, 1000 nM) added to the apical chamber elicited a marked increase in cGMP levels in the colon mucosa, pharmacological effects consistent with activation of GC-C expressed on the apical surface of IEC. Preincubation of linaclotide-treated colon mucosa with MK571 (20 µM) further increased tissue cGMP levels ($P<0.01$, 1000 nM linaclotide), indicative of a role of MRP4 in this process (Fig. 6).

**MRP4 Inhibition Decreases Linaclotide-stimulated Apical cGMP Efflux, but has no Effect on Basolateral cGMP Efflux.** Previous studies using surgically ligated intestinal loops
have demonstrated in vivo linaclotide-stimulated secretion of cGMP into the luminal compartment (Bryant et al., 2010; Busby et al., 2010). Similarly, linaclotide stimulated in vitro cyclic nucleotide efflux pump-dependent basolateral release of cGMP from polarized Caco-2 cells that was concentration-dependently inhibited by the cGMP transporter inhibitor, probenecid (Castro et al., 2013). Here, we further investigated the effect of MK571-mediated MRP4 inhibition in vitro on linaclotide-stimulated cGMP secretion from the apical and basolateral sides of rat colonic epithelium. No effects on either apical or basolateral cGMP release were observed during a 60-minute preincubation period with vehicle or MK571 (20 μM) (Fig. 7A,B). After 60 minutes, linaclotide (1 μM) was added to the apical chamber, and aliquots were collected every 15 minutes for another 60 minutes to measure cGMP release from the apical and basolateral sides of the colonic mucosa. Linaclotide stimulated apical cGMP efflux was markedly decreased by MK571 (Fig. 7A), while basolateral efflux was not affected by MK571 (Fig. 7B). Kinetics of cGMP efflux indicated an early phase spike in cGMP release from the apical side, with slower but continuous release in the later phase, while cGMP efflux from the basolateral side was characterized by a slow, continuous pattern. Simultaneous recording of transepithelial ion currents throughout the experiment confirmed that linaclotide-induced Isc decreased following MK571 pretreatment of the colonic mucosa (data not shown).

**Linaclotide Induces In Vivo cGMP Efflux into the Colonic Submucosal Layers.**

Linaclotide-induced cGMP transporter-dependent extracellular release of cGMP from basolateral membranes of IEC is believed to mediate inhibition of colonic nociceptors located in the submucosa, resulting in decreased visceral pain (Castro et al., 2013, Silos-Santiago et al., 2013). However, in vivo release of cGMP from colonic epithelial cells into the colonic submucosal layer has not been studied. We have developed an in vivo colonic microdialysis assay consisting
of a microdialysis probe implanted between the epithelium and submucosal layer of the rat ascending colon (Woo and Lunte, 2008). Here we measured for the first time in vivo linaclotide-induced cGMP efflux into the colonic submucosal layer. Control experiments confirmed that linaclotide administration at doses of 0.5, 1.7 and 5 μg into the colonic loop significantly ($P<0.001$) increased fluid secretion (data not shown). Following administration of vehicle (D-glucose: 10 μg in H2O) into the colonic loop, dialysate fractions were collected for 30 minutes to measure baseline concentrations of submucosal cGMP. Administration of linaclotide (0.5, 1.7, 5 μg) induced a dose-dependent (83%, 90%, 182%, respectively), significant ($P<0.001$) increase in submucosal cGMP levels after 30 minutes (Fig. 8A); the corresponding time course of linaclotide-stimulated cGMP efflux, measured in 5-minute intervals is presented in figure 8B. Linaclotide-induced increases in submucosal cGMP levels were time-dependent, with the highest dose reaching maximal effect within 12 minutes of linaclotide administration (data not shown).
Discussion

We provide evidence for a direct role of MRP4 as a key modulator of linaclotide pharmacology in the rat colon: potentiation of electrolyte secretion, augmentation of tissue cGMP accumulation, and inhibition of cGMP efflux selectively from the apical membrane of polarized IEC. These data reveal a novel, previously unrecognized mechanism that functionally links MRP4 to intestinal electrolyte and cGMP secretion following linaclotide activation of the GC-C/cGMP pathway.

The role of the endogenous hormones guanylin and uroguanylin as intestinal secretagogues is firmly established, and the intracellular GC-C/cGMP pathway is thus considered the principal regulator of intestinal fluid homeostasis (Joo et al., 1998; Donowitz et al., 2005). Maintenance of fluid homeostasis is achieved through vectorial transport of electrolytes and water from secretory IEC, controlled by the coordinated activity of a network of transporters and ion channels. The CFTR anion channel, located in the apical membrane of IEC, is one of the key membrane proteins regulating overall fluid movement (Anderson et al., 1991; Bear et al., 1992). Under steady-state conditions, CFTR is located predominantly in subapical vesicular compartments; however, under conditions of linaclotide-induced cGMP production that leads to electrolyte and fluid secretion, the total number of CFTR channels in the apical membrane is rapidly up-regulated by exocytic trafficking while simultaneously, the sodium/hydrogen exchanger-3 (NHE3) is transiently down-regulated by internalization and endocytosis, preventing electroneutral sodium reabsorption (Jakab et al., 2011; Kravtsov et al., 2013; Ameen et al., 2014). In addition, regulation of the CFTR channel activity is also mediated through activation of this epithelial channel that couples to adenylate cyclase (AC) and raises cellular cAMP (Huang et al., 2001; Li et al., 2007). In the gut, adenosine-stimulated CFTR-
mediated chloride currents are potentiated by MRP4 inhibition, and this potentiation is directly
coupled to attenuated cAMP efflux through the apical cAMP transporter, MRP4. Indeed, CFTR
forms macromolecular signaling complexes also containing MRP4 and PDZK1 that have been
shown to have important implications in vivo, such as mice lacking MRP4 expression are more
prone to CFTR-mediated secretory diarrhea (Li et al., 2007).

Using an in vitro rat colonic mucosa model, we have shown that linaclotide stimulation at
low concentrations (1-10 nM; EC$_{50}$: 9.2 nM) induced a marked increase in electrolyte secretion
measured by increased Isc, accumulation of intracellular cGMP in colonic epithelium and cGMP
secretion from both the apical and basolateral membranes of colonic epithelium. Notably,
preincubation with the selective MRP4 inhibitor MK571 potentiated linaclotide-induced
electrolyte secretion, augmented intracellular cGMP accumulation and selectively decreased
cGMP secretion from the apical membrane, without impacting basolateral cGMP secretion.
Together, these data provide evidence that GC-C induced transcellular electrolyte transport and
cGMP secretion are functionally coupled to spatially restricted, compartmentalized regulation by
MRP4 at the apical membrane of colonic epithelium. This model is further supported by
immunohistochemistry data revealing MRP4 expression predominantly at the apical membrane
and the apparent clustering of MRP4 locally in close proximity to the site of cGMP production.
Decreased MRP4-mediated cGMP efflux may result in cGMP accumulation locally in close
proximity to CFTR, thus leading to increased Isc. Highly compartmentalized regulation of CFTR
functional activity in a MRP4-dependent manner has previously been reported in response to
local elevation of the second messenger cAMP, supporting the concept that the specific location
of physically and/or functionally coupled multiprotein signaling complexes in distinctive
subcellular microdomains increases their signaling efficacy at their local target sites (Li et al.,
2007). In contrast, the formation of a macromolecular signaling complex at the apical membrane that physically links GC-C to MRP4 to provide an efflux path for cGMP via MRP4 in response to locally elevated cGMP levels in close proximity to CFTR has not yet been reported. However, several findings support the hypothesis that a GC-C interactome is formed in the subapical space of IEC. Firstly, our finding that pretreatment of colonic mucosa with the PDE5 inhibitor sildenafil increased basal Isc, but had no amplifying effect on linaclotide-induced ion currents suggests an initial global increase in cellular cGMP levels due to inhibition of the cytoplasmic enzyme PDE5, while MK571-mediated MRP4 inhibition results in compartmentalized elevation of cGMP locally at the apical membrane, thereby increasing the amplitude and duration of the linaclotide-induced Isc response. Furthermore, MRP4 inhibition only affected the activation phase of linaclotide-induced Isc, but had no effect on the deactivation phase, an effect elicited by the human GC-C/Fc chimera, suggesting that linaclotide-induced increases in Isc are dependent on linaclotide activation of the GC-C/cGMP pathway. Secondly, CFTR phosphorylation and activation by membrane-associated PKGII following activation of the GC-C/cGMP pathway is well documented. Thirdly, the isolation of the GC-C interacting protein IKEPP (intestinal and kidney-enriched PDZ protein), an adaptor protein closely related to PDZ1 found to be enriched at the apical membrane of human IEC has been described, but its role remains incompletely defined (Scott et al., 2002).

When we investigated the effect of MK571-mediated MRP4 inhibition on linaclotide-stimulated extracellular transport of cGMP from rat colonic epithelium, we found selective inhibition of cGMP secretion from the apical membrane consistent with the subcellular localization of MRP4, but no effect on basolateral cGMP secretion suggesting the involvement of other cyclic nucleotide efflux pumps such as MRP5 in this process. While the involvement of
an intracellular GC-C/cGMP pathway in the regulation of intestinal fluid homeostasis is firmly
established, recent studies in rodent models of visceral pain have uncovered a novel role of
extracellular cGMP transported out of IEC into the submucosal space by cyclic nucleotide efflux
pumps following local activation of the GC-C/cGMP pathway (reviewed in: Hannig et al., 2014).
Evidence strongly supports a model in which increased extracellular cGMP levels modulate
intestinal nociceptor function resulting in peripheral analgesia, emphasizing the importance of
MRP-mediated extracellular cGMP transport as a source of cGMP for paracrine actions (Ritter et
al., 2005; Castro et al., 2013; Silos-Santiago et al., 2013). Importantly, two pivotal phase 3 trials
in linaclotide-treated IBS-C patients support translation of these findings in the clinic, validating
the approach of therapeutically targeting the GC-C/cGMP pathway in this disorder. Orally
administered linaclotide at a dose of 290 μg, once-daily, significantly improved abdominal
symptoms (abdominal pain, discomfort, bloating, fullness) compared with placebo. Moreover,
post hoc analysis of IBS-C patient subpopulations with severe abdominal symptoms at baseline
further confirmed significant improvements of all abdominal symptoms in these patients, as well
as significant improvements in global measures and in the IBS-related quality-of-life score
(Layer and Stanghellini, 2014; McCormack, 2014; Rao et al., 2014).

Our studies using a novel microdialysis model in rats demonstrated in vivo cGMP transport into
the submucosal layers following local linaclotide-stimulated activation of GC-C on the luminal
surface of colonic epithelium, further consistent with this model. MRP4 localization primarily at
the apical membrane of IEC suggests that cyclic nucleotide efflux pumps other than MRP4 are
mediating this process; elevated levels of MRP5 expression have been reported in different
segments of the rat colon, and predominantly basolateral expression of MRP5 in human colonic
mucosal biopsies, implying a prominent role for this cGMP efflux pump (Silos-Santiago et al.,
2013; Harrington et al., 2014). Notably, cGMP production following activation of GC-C occurs locally in close proximity to the apical membrane, but the mechanism(s) potentially involved in the translocation of cGMP to the basolateral cell membrane to drive extracellular transport, such as encapsulation into transport vesicles and/or passive diffusion, is currently not known.

Importantly, in recto-sigmoid mucosal biopsies of IBS-C patients, but not in IBS patients with mixed (IBS-M) bowel habits (constipation and diarrhea) MRP4 expression was found significantly down-regulated, compared to healthy subjects, while no changes in expression of the hormones guanylin and uroguanylin, GC-C and MRP5 were observed in either group. However, immunohistochemistry revealed a spatially different pattern of MRP expression: MRP4 located at the apical and MRP5 at the basolateral membrane of IEC, respectively (Harrington et al., 2014). Together, these data show that distinct alterations are evident in the GC-C/cGMP pathway between different IBS subtypes that may contribute to the pathophysiology of IBS, which could further help explain some aspects of the symptom complex associated with IBS, such as differential stool frequency. Further consistent with this notion, Mrp4-deficient mice are more prone to CFTR-mediated secretory diarrhea than their wild-type littermates (Li et al., 2007). On the molecular level, changes in MRP4 expression levels in IBS-C patients not only highlight the need to understand which proteins form the postulated GC-C interactome in IEC, but importantly, this knowledge could improve our understanding how specific changes in the composition of the GC-C interactome may contribute to disease.

In summary, these studies have demonstrated the presence of a previously unrecognized mechanism that functionally couples the secretory GC-C/cGMP pathway to spatially restricted compartmentalized modulation by the cyclic nucleotide efflux pump MRP4 at the apical membrane of the colonic epithelium. Translation of this concept into the clinic may have
important implications for patients suffering from gastrointestinal disorders such as IBS-C, CIC and secretory diarrhea, and MRP4 could evolve as a novel predictive biomarker in the clinic that may help stratify patients who would benefit most from GC-C agonist therapy.
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Authorship Contributions

Participated in research design: Ge, Lunte, Silos-Santiago, Tchernychev, Thomas, Tobin

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Wrote or contributed to the writing of the manuscript: Bryant, Currie, Ge, Fretzen, Hannig, Kurtz, Silos-Santiago, Tchernychev
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Footnotes

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Reprint requests

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Figure legends

**Fig. 1.** Linaclotide induces transepithelial anion transport (Isc) in rat colonic mucosa. Rat proximal colon tissue (female Sprague-Dawley rats), with the seromuscular layers removed by blunt dissection, was mounted on slides with the 0.5 cm² aperture in the Ussing chamber. Following a 15-minute equilibration period, linaclotide (30 nM) was added to the apical bath of the Ussing chamber. At the 30-minute time point, a GC-C/Fc fusion protein (300 nM) capable of binding linaclotide was added to the apical chamber. Linaclotide-induced changes in Isc were measured using an automatic voltage clamp and expressed as $\Delta$Isc ($\mu$A/cm²). Time points of addition of linaclotide and GC-C/Fc fusion protein are marked by arrows. Data is presented as the mean ± SEM ($n$=8).

**Fig. 2.** Linaclotide-induced changes in transepithelial anion transport (Isc) in rat colonic mucosa are concentration-dependent. Rat proximal colon tissue was dissected and mounted in the Ussing chamber as described in figure 1. (A) Traces of linaclotide-induced changes in Isc, added sequentially in half-log increasing concentrations to the apical chamber every 20 minutes, ranging from 1 nM – 1000 nM. (B) The effective concentration of linaclotide which produced 50% of the maximal activity (EC$_{50}$) increasing transepithelial ion currents in the rat colonic mucosa was calculated with 9.2 nM. Linaclotide-induced changes in Isc were measured using an automatic voltage clamp and expressed as $\Delta$Isc ($\mu$A/cm²). Data is presented as the mean ± SEM ($n$=6).

**Fig. 3.** Localization of MRP4 in rat colonic epithelium by immunofluorescence microscopy. (A) MRP-4 specific antibody stain, (B) negative control (primary antibody omitted). Epithelial cell nuclei were counterstained using the cell-permeant DNA stain Hoechst 33342. Magnification: 40X, the scale bar represents 50 $\mu$m.
Fig. 4. MRP4 inhibition amplifies linaclotide-induced transepithelial ion currents. Rat proximal colon tissue was dissected and mounted in the Ussing chamber as described in figure 1. (A) Following a 20-minute equilibration period, a sub-EC$_{50}$ concentration of linaclotide (6 nM) was added to the apical chamber for 20 minutes, followed by addition of a concentration of linaclotide inducing a maximum increase in Isc (1000 nM) for another 20-minute period. In a parallel experiment, colonic mucosa was pretreated with MK571 (20 µM) for 20 minutes prior to addition of linaclotide (6 nM, 1000 nM) at the indicated time points. Time points of addition MK571 and linaclotide are marked by arrows. Linaclotide-induced changes in Isc were measured using an automatic voltage clamp and expressed as $\Delta$Isc (µA/cm$^2$). (B) The effect of MK571 potentiation on linaclotide-induced changes in Isc, compared to vehicle is expressed as maximum response (%) for each test article. Data is presented as the mean ± SEM ($n=6$). *$P<0.05$ versus vehicle.

Fig. 5. Effect of MRP4 and phosphodiesterase 5 inhibition on activation/deactivation kinetics of linaclotide-induced transepithelial ion currents (Isc). Following an equilibration period, rat proximal colon tissue was dissected and mounted in the Ussing chamber as described in figure 1. (A) Rat colonic mucosa was pretreated either with vehicle (0.1% DMSO in KRB), (B) MK571 (20 µM), or (C) sildenafil (100 nM) for 20 minutes, followed by incubation with linaclotide (6 nM) for 20 minutes. After 40 minutes, a GC-C/Fc fusion protein (60 nM) capable of binding linaclotide was added to the apical chamber for another 20 minutes. Time points of addition of vehicle, MK571, and GC-C/Fc fusion protein are marked by arrows. Linaclotide-induced changes in Isc were measured using an automatic voltage clamp and expressed as $\Delta$Isc (µA/cm$^2$). Data is presented as the mean ± SEM ($n=6$).
Fig. 6. Effect of MRP4 inhibition on linaclotide-induced cGMP accumulation in rat colonic mucosa. Rat proximal colon tissues were incubated for 20 minutes in 24-well tissue culture plates with KRB (1 ml) containing either vehicle (0.1% DMSO in KRB), MK571 (20 µM), or IBMX (100 µM) to determine mucosal cGMP levels under unstimulated condition (control). Mucosa pretreated with MK571 was stimulated with linaclotide (6 nM, 1000 nM) for another 20 minutes, and mucosal cGMP levels (pmol/mg protein) were determined by ELISA. Data is presented as the mean ± SEM (n=6). *P<0.05; **P<0.01 versus vehicle.

Fig. 7. Effect of MRP4 inhibition on linaclotide-induced cGMP efflux from rat colonic mucosa. Rat proximal colon tissue was dissected and mounted in the Ussing chamber as described in figure 1. Tissues were incubated for 60 minutes in the presence either of vehicle (0.1% DMSO in KRB) or MK571 (20 µM), added to both the apical and basolateral chambers. At the indicated time point (60 minutes), linaclotide (1 µM) was added to the apical chamber for another 60-minute incubation period. Aliquots (120 µl) were collected in 15-minute intervals from the apical (A) and basolateral (B) chambers and cGMP concentrations (fmol/well) determined by ELISA. Data is presented as the mean ± SEM (n=6).

Fig. 8. Linaclotide induces in vivo cGMP efflux into rat colonic submucosal layer. (A) A linear microdialysis probe was implanted between the colon epithelium and submucosal layer of a loop generated in the ascending colon of anesthetized female Sprague-Dawley rats. Saline was perfused at a rate of 1 µl/minute and fractions of dialysate were collected during 5 minute intervals. The microdialysis probe was equilibrated for 25 minutes and fractions were collected to determine cGMP baseline concentrations in the serosa. Vehicle (D-glucose (10 µg/ml) in H2O) was injected into the lumen of the colon loop and the collection of dialysate fractions was continued for 30 minutes. Thereafter, linaclotide (0.5, 1.7, 5 µg in 0.2 ml of vehicle) was injected.
into the lumen of the colon loop and dialysate fractions were collected for another 30 minutes. Data is presented as the mean ± SEM (n=10). ***P<0.001 versus vehicle. (B) Time course of linaclotide-stimulated cGMP efflux, measured in 5-minute intervals. Time points of addition of vehicle and linaclotide are marked by arrows. Each animal serves as its own control versus basal and vehicle cGMP levels. Dose groups: 1 = 0.5 µg, 2 = 1.7 µg, 3 = 5.0 µg. The concentration of cGMP in the dialysate fractions was determined by liquid chromatography-mass spectrometry (LC-MS/MS). Data is expressed as fold increase over average baseline.
Figure 1
Figure 2
Figure 3
Figure 4

A

MK571  Linaclootide (6 nM)  Linaclootide (1000 nM)

[Graph showing time course of ΔI_{sc} (μA/cm²) over time (min) with data points for Vehicle and MK571 treatments.]

B

Max response (%)

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* indicates statistically significant difference.

Figure 4
Figure 6
Figure 7
Figure 8