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Novel mechanisms of neuronal and hormonal regulation of large intestinal electrolyte transport

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Novel mechanisms of neuronal and hormonal regulation of large intestinal electrolyte transport

Andrew J. Nickerson

Dissertation submitted to the College of Medicine at West Virginia University

In partial fulfillment of the requirements for the degree of Doctor of Philosophy in Cellular and Integrative Physiology

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ABSTRACT:

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Andrew J. Nickerson

The mammalian colon plays a vital role in maintaining overall electrolyte and fluid homeostasis in the body, and it possesses the ability to either secrete or absorb water depending upon the needs of the organism. Secretion or absorption of electrolyte ions, such as Na^+ , Cl^- and K^+ , drives the movement of water across the colonic epithelium – the inner gut lining where all transport takes place. Aberrant absorptive and/or secretory activity within the colonic epithelium is central to the pathogenesis of many gastrointestinal diseases, such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), constipation and infectious diarrhea. As such, therapeutic control of colonic electrolyte transport is critical to the management of these conditions. Two of its most important regulators – the enteric nervous system (ENS) and the corticosteroid hormones – provide key avenues by which colonic epithelial transport activity can be modulated or corrected, in the case of pharmacotherapeutic intervention. The work described in this dissertation characterizes two separate, novel aspects of regulatory control that may be the targets of future therapeutic drug development. First is the discovery and characterization of voltage gated K^+ ($\text{K}_{\text{V}}7$) channels in the ENS as governors of ENS activity and resultant epithelial transport function. Second is the study of the epithelial N^+ , K^+ , 2Cl^- (NKCC1) co-transporter and its regulation by the corticosteroid hormone, aldosterone, in controlling epithelial K^+ and Cl^- transport. The findings from this work set the stage for development of targeted therapies aimed at these two mechanisms, which may lead to improved management of intestinal pathologies such as IBS, IBD or other diarrheal conditions.

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CHAPTER 1: LITERATURE REVIEW

Part 1: electrolyte transport in the gastrointestinal tract.

1.1 A brief introduction

Large organisms, including humans and other mammals, face the distinct biological challenge of supplying vital nutrients, water and oxygen to cells and tissues buried deep beneath a largely impenetrable body exterior. Simple diffusion of essential materials directly from our environment is not a viable option, as it is for our single-celled relatives. To address such a challenge, evolution has produced a complex arrangement of transport and exchange systems to allow for the safe and regulated uptake of these precious resources. A critical component to this process, the gastrointestinal (GI) tract – also known as the *alimentary canal* – is comprised of several distinct, yet inter-connected tissues whose primary function is the facilitation of water, nutrient and electrolyte (i.e., ion) transport into the body, and the simultaneous removal of waste materials and non-absorbed dietary substances (1,2).

A wide variety of cell types are located within the alimentary canal, each of which is required to execute its many functions. Among these are: epithelial cells, which form the inner gut lining and carry out the transport of nutrients and electrolytes (3); layers of smooth muscle, which mix and propel material to aid in digestion and motility (4); vascular networks, carrying newly-absorbed water and nutrients off to their destinations within the body (5); neuronal networks that regulate epithelial transport, smooth muscle contraction and blood flow, while also communicating with central and peripheral nervous systems (6); and immune cells that constantly surveil the environment for potentially harmful materials or organisms (7). Beyond this, within the

large intestine resides an entire community of microbes (often referred to as the *microbiome*) that outnumber the host's own cells 10:1 (8), and are essential for proper G.I. function (9,10).

The focus of the studies within our laboratory has been to characterize and understand the mechanisms underlying the transport of electrolytes within the large intestine, which is just one of the many aspects of GI function. Appropriate absorption and/or secretion of osmotically active ions (a.k.a. “electrolytes”), such as sodium (Na^+), chloride (Cl^-) or potassium (K^+), is paramount to maintaining fluid balance (11,12). Coordinated transport of these ions creates the driving force for the movement of water into or out of the body, across the intestinal barrier. Consequently, perturbations in electrolyte transport can lead to severe, and even life-threatening conditions (13). A review of the currently accepted principles of intestinal electrolyte transport will provide a useful context in which to present the findings from our recent studies, beginning with a discussion of the basic principles of ion transport within the gut.

For water to be transported from the lumen of the gut to the inside of the body, it must travel down an osmotic gradient. Water molecules cannot be transported directly, but will move across a (semi)permeable barrier to whichever compartment has a higher solute concentration (14). This phenomenon is referred to as *osmosis* and serves as the basis for all biological fluid transport (15). Therefore, absorption or secretion of solutes is the mechanism by which epithelial cells carry out their transport duties. In the intestine, this is mainly accomplished either by actively transporting Na^+ ions from inside the lumen across the epithelium and into the interstitial space, or by actively secreting Cl^- into the lumen (1). Depending upon the region of the GI tract, a variety of other solutes may travel with Na^+ or Cl^- and contribute to the generation of an osmotic gradient which favors water absorption or secretion (16). The details of the major absorptive and secretory

pathways will be described in later sections. However, an understanding of the overall structure of intestinal epithelium is required to discern the function of its various transport mechanisms.

1.2 Basic structure and function of the intestinal epithelium.

The epithelium itself – the main constituent of the *mucosa* – has a particular spatial arrangement and cellular architecture to allow for vectorial (i.e., directional) ion transport. Simple columnar epithelial cells are situated adjacent to one another with their apical membranes facing the gut lumen and their basolateral membranes facing the interstitial space, or *lamina propria*. Histologically, the intestinal mucosa can generally be compartmentalized into absorptive surface and secretory crypt regions (17) (Fig. 1). At the base of each crypt are LRG5⁺ stem cells which give rise to the other epithelial cell types along the crypt/surface axis (18). The cell populations inhabiting these two regions develop along a continuum as they traverse the crypt structure. Cells along the crypt/surface axis have separate physiological roles and biomolecular characteristics, which will be described later. With respect to an individual epithelial cell, the apical and basolateral membranes are also functionally distinct from one another (Fig. 2). These membranes are segregated, in part, by the presence of tight junction proteins that form a decisive boundary between the two domains (apical vs. basolateral regions) (19). Tight junctions also contribute to the formation of a restrictive extracellular barrier separating the lumen from the interstitial space. Forming a tight seal near the apical poles of adjacent epithelial cells prevents indiscriminate leaking of water, solutes or even pathogenic material (20) – any of which would undermine the absorptive process altogether.

Tight junctional complexes are formed by a number of different proteins, including the *claudin* and *occludin* gene families. These proteins extend through the plasma membrane and

articulate with their counterpart proteins originating from adjacent cells, fortifying the epithelial monolayer and allowing very few substances to pass through (21). The pathway between epithelial cells and around tight junctions is termed the “paracellular” pathway and is quite restrictive in allowing the passage of most solutes - including ions. As a result, many epithelial ion transport pathways are described as “trans-cellular,” passing through the intracellular compartment of the epithelial cell (Fig. 2). Trans-cellular solute absorption requires the coordinated actions of specialized transport proteins - ion channels or transporters - that are deliberately situated within the apical and/or basolateral membranes of absorptive epithelial cells. Specialized water channels, called aquaporins, are also required to allow the passage of water across the epithelial barrier (22), as tight junctions impede its movement through the para-cellular pathway. Aquaporin channel proteins are a heterogenous population with multiple gene family members (AQP1-12) differentially expressed in apical and basolateral membranes of many tissues. In the intestine AQP3,4 and 8 are the predominant isoforms located on in intestinal epithelial cells (23,24) and trafficking to and from the plasma membrane is an important component in control the rate of water transport.

In the small intestine, absorptive epithelial cells reside in the numerous pillar-like structures called *villi*, while in the colon they are configured in flat surface regions that interface with the lumen (Fig. 1). In both cases, the absorptive domains (i.e. villus or surface epithelium) are interspersed with crypts Lieberkühn, where a separate population of secretory cells can be found. Fluid and electrolytes are transported both into and out of the gut lumen throughout the GI tract, as is necessary for certain digestive functions and hydration of the mucus layer that covers the epithelium (11). However, the main function of the colon is to desiccate fecal material by removing most of the remaining water content, as absorption of all dietary nutrients (amino acids,

carbohydrates, fats and most minerals) is completed in the small intestine. In the colon, desiccation is accomplished by absorption of Na^+ via two predominant mechanisms: Cl^- -dependent, electroneutral or Cl^- -independent electrogenic absorption. Both of these processes occur primarily in absorptive surface colonocytes.

1.2.1 Electroneutral NaCl absorption is mediated by coupled Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers

To understand a discussion of the mechanistic details of electrolyte transport, it is important to become at least somewhat familiar with one of the most common and useful experimental techniques in studying epithelial transport: *Ussing chamber electrophysiology*. In brief, when polarized tissues, such as the intestinal mucosa, are mounted in a chamber apparatus with separate mucosal (apical) and serosal (basolateral) baths and are *voltage-clamped*, this allows for the continuous measurement of an electrical parameter referred to as “short-circuit current”, or I_{SC} (25). I_{SC} is defined as the amount of applied current necessary to maintain (i.e., clamp) the trans-epithelial voltage (V_{TE}) to a potential of 0 mV, thus “short circuiting” the system. If left alone after mounting, intestinal mucosae will otherwise spontaneously develop this V_{TE} as charged ions are moved across the tissue, resulting in a lumen-negative potential. This is typically on the order of -15 to -30 mV in the colon of most mammalian species. Measuring I_{SC} provides a means of quantitating the total net electrogenic transport across a tissue, while at the same time eliminating the electromotive force imparted by the negative V_{TE} , if it were allowed to persist. Directional isotopic fluxes performed under these conditions therefore indicate the total *active* transport for a particular ion. Pharmacological treatments and/or bath solution ion substitution experiments have provided a great deal of information regarding the identity and properties of many colonic epithelial transport proteins.

Early studies by Binder and colleagues using radioisotope ($^{22}\text{Na}^+$ and $^{36}\text{Cl}^-$) flux experiments performed in rat colon under voltage-clamped conditions led to the identification of electroneutral and electrogenic Na^+ absorptive mechanisms (26,27). A large component of Na^+ absorption was found to be dependent on the presence of Cl^- in the apical tissue bath. In addition, net Na^+ and Cl^- fluxes were found to be greater than the I_{SC} generated by the tissues during these experiments, indicating that much of the active Na^+ absorption was electroneutral (27). Foster et al first identified the presence of a Na^+/H^+ exchange process in the apical membranes of rat colonocytes that brings a Na^+ ion into the cell across the apical membrane in exchange for a proton (28). Later, functional coupling to a $\text{Cl}^-/\text{HCO}_3^-$ exchange system via a pH-dependent mechanism was discovered (29). Intracellular pH (pH_i) rises as protons are transferred out of the cell in exchange for Na^+ . Outward HCO_3^- efflux occurs to offset this pH_i change, and Cl^- is brought into the cell in return. This parallel Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange system can also be found in the apical membranes of the small intestine (30,31) and proximal tubule of the kidney (32). It accounts for the majority of whole-body NaCl and water absorption among mammals.

In the colon, apical Na^+/H^+ exchange is primarily attributable to the activity of Na^+/H^+ exchanger isoform 3 (NHE-3; *Slc9a3*), with some involvement of NHE-2 (*Slc9a2*) (33) (Fig. 3A). Apical $\text{Cl}^-/\text{HCO}_3^-$ exchange is mediated by a gene called “down-regulated in adenoma” (DRA; *Slc26a3*) (34,35) (Fig. 3A), and possibly putative anion transporter 1 (PAT1; *Slc26a6*), although its activity appears to be restricted to the small intestine (36). Cl^-/OH^- exchange may also be coupled to Na^+/H^+ exchange via a pH-dependent, HCO_3^- -independent mechanism (37,38). However, the identity of a Cl^-/OH^- exchanger in the colon is yet undetermined. Genetic disruption of *Slc26a3* (DRA) produces severe diarrhea in mice (34). In humans, several diarrheal diseases, such as congenital sodium or congenital chloride diarrhea, are the result of mutations in neutral

Na^+/H^+ (NHE-3) or $\text{Cl}^-/\text{HCO}_3^-$ (DRA) exchangers, respectively (39,40). These conditions are very rare and often present with a severe disease phenotype.

Coupled Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers mediate Na^+ and Cl^- uptake at the apical membranes of epithelial cells (41). However, the driving force generating the electrochemical gradient that allows for sustained Na^+ entry (and Cl^- entry that is coupled to it) is established by the continuous activity of the basolateral Na^+/K^+ ATPase (a.k.a. the Na^+/K^+ pump), which functions to extrude 3 Na^+ across the basolateral membrane in exchange for 2 K^+ (11) (Fig. 3A). Na^+ extrusion via Na^+/K^+ ATPase occurs continuously and is sensitive to cardiac glycosides, such as ouabain (42). Though costly to operate, this enzyme serves the dual purpose of maintaining Na^+ at low intracellular levels and providing a pathway across the basolateral membrane for Na^+ to be taken up into the blood. $\text{Cl}^-/\text{HCO}_3^-$ exchange occurs secondarily to Na^+/H^+ exchange and both are ultimately dependent on Na^+/K^+ ATPase activity. As intracellular Cl^- ions accumulate, basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchange via anion exchanger 2 (*Slc4a2*) (43), basolateral K^+/Cl^- co-transporter (KCC1; *Slc12a4*) (44) and parallel intermediate conductance K^+ (IK; *Kcnn4*) channel and CLC-2 (*clcn2*) Cl^- channel proteins (45) can all facilitate Cl^- efflux into the interstitial space, completing the NaCl absorption process (Fig. 3A).

1.2.2 Electroneutral NaCl absorption is also coupled to short-chain fatty acid absorption

Short-chain fatty acids (SCFAs) are produced via the metabolism of non-absorbed dietary carbohydrates by commensal bacteria in the colon (46). Acetate, propionate and butyrate are the predominant SCFA species generated, and their total concentration within the gut lumen can be upwards of 100 mM (47). Among these species, butyrate has received the most investigative attention because it not only provides a major fuel source for colonocytes (46), but also heavily

influences gene expression by acting as a histone deacetylase (HDAC) inhibitor (48,49). The absorptive pathways of Na^+ and butyrate are unquestionably linked. Butyrate significantly enhances electroneutral NaCl uptake and butyrate uptake is dependent on Na^+ as well (50,51). Still, the exact pattern for butyrate-dependent NaCl absorption has not been fully elucidated at this point in time.

Monocarboxylate transporters (MCTs) are widely accepted as the major butyrate cellular intake mechanism in a variety of cell types (52). These transporters are dependent on either an inward H^+ gradient (MCT-1; *Slc16a1*) or inward Na^+ (sodium) gradient (SMCT-1; *Slc5a8*). Both proteins have been localized to the colonic epithelium via immunolabeling studies (53,54). However, the involvement of additional butyrate transport pathways is likely. Functional evidence points to Na^+ -dependent butyrate requiring an outward-directed HCO_3^- gradient (55), and is not necessarily related directly to pH_i , as would be expected for H^+ -coupled transport via MCT-1. The involvement of SMCT-1 in butyrate uptake is also brought into question because its K_M value for butyrate is very high – nearly 50mM (52). Studies using isolated apical membrane vesicles suggest that Cl^- /butyrate and HCO_3^- /butyrate exchangers are both present on the apical membranes of surface epithelial cells, and operate in parallel to each other (56). However, the molecular identity of these putative anion antiporters has not been elucidated as of yet.

Operation of Cl^- /butyrate or HCO_3^- /butyrate transport systems is functionally coupled to the Na^+/H^+ exchangers described in the previous section. NHE-2/NHE-3 dependent Na^+ absorption is augmented in the presence of butyrate, even without HCO_3^- present in the apical bath, but to a greater extent when both anions are present (51,57). An outward-directed HCO_3^- gradient likely drives a HCO_3^- /butyrate exchanger to bring butyrate into the cell across the apical membrane. The resultant fall in pH_i triggers activation of apical NHE-2/NHE-3. Finally, intracellular butyrate is

exchanged for Cl^- across the apical membrane, completing the cycle. While apical butyrate- and HCO_3^- -dependent neutral Na^+ absorptive mechanisms are interconnected, they each can function independently as well. In addition, NHE-2 and NHE-3 were shown to differentially regulate butyrate-dependent Na^+ absorption in healthy versus inflamed colon (58), with NHE-2 mediated, butyrate-dependent Na^+ absorption predominating under inflammatory conditions.

1.2.3 Electrogenic Na^+ absorption is mediated by the epithelial Na^+ channel; ENaC.

Neutral NaCl absorption occurs throughout most of the small and large intestine. In some species, including rabbit, mouse and human, the distal segment of the colon is also characterized by an *electrogenic* Na^+ absorptive pathway that is not dependent on the presence of luminal Cl^- (1,11). This is mediated by an apical membrane epithelial Na^+ channel (ENaC) (Fig. 3B), first described functionally by Foster et. al. (27), who observed that net $^{22}\text{Na}^+$ absorption was roughly equivalent to the I_{SC} (i.e., was entirely electrogenic) measured across colonic epithelial sheets under certain conditions. The channel carrying this current is now known to be encoded by the *Scnn* gene family. ENaC channels are comprised of three highly homologous subunits (ENaC α, β, γ ; *Scnna*, *Scnnb*, *Scnng*) which are transcriptionally and post-translationally regulated by a variety of factors (59) (more later).

Apical ENaC-mediated Na^+ influx is also driven by basolateral Na^+/K^+ ATPase activity (Fig. 3B), which produces an electrochemical gradient favoring Na^+ entry from the lumen, similar to what has been described for electroneutral absorption. Unlike NHE-3-mediated absorption however, opening of ENaC channels generates an electrical current because no other charged solutes are transported in exchange. ENaC is very sensitive to inhibition by the diuretic, amiloride ($\text{IC}_{50} \sim 100 \text{ nM}$) (59). Application of $10 \text{ }\mu\text{M}$ amiloride dramatically inhibits both I_{SC} and $^{22}\text{Na}^+$

absorption in colonic tissues with high ENaC activity (60). NHE-3 and NHE-2 are also inhibited by amiloride but are much less sensitive, with IC_{50} values in the hundred μM range. Both NHE-3 and ENaC-mediated Na^+ absorption take place primarily in surface epithelial cells of the colon, although there is some evidence of neutral Na^+ absorption within the crypt regions under certain experimental conditions (61).

Both electroneutral and electrogenic Na^+ absorptive processes are present in the colon under normal physiological conditions. In many species, including humans, mice and rats, ENaC-mediated absorption becomes more prominent in the distal regions of the colon and rectum (1). NHE-2/3 mediated electroneutral absorption is the primary mechanism within the proximal segment of the colon (62), as well as in the small intestine (33). The gradual transition from electroneutral to electrogenic Na^+ absorption from proximal to distal regions bears resemblance to Na^+ transport within the kidney as well, as neutral Na^+ absorption predominates tubular transport until urine reaches the collecting duct (32). Many diseases related to mutations in ENaC, such as Liddle syndrome and pseudo-hyperaldosteronism, present with disturbances in renal function (63). In the colon, ENaC plays a role mostly in the final desiccation of fecal material, aiding in the final removal of its water content in preparation for defecation.

1.2.4 Cl^- secretion is electrogenic and occurs within the crypt

The colon also possesses the ability transport fluid *into* the lumen by secreting Cl^- across the apical membranes of crypt epithelial cells (64). Cl^- is the electrolyte generally associated with fluid secretion, whereas Na^+ is associated with fluid absorption. This is because the electrochemical driving forces heavily favor Na^+ entry into the epithelial cell as a result of continuous Na^+/K^+ ATPase activity maintaining very low Na^+ levels inside the cell. Efflux of Na^+

against its electrochemical gradient toward the lumen is nearly impossible from a biophysical standpoint. Intracellular Cl^- concentrations are not as rigorously maintained because apical and basolateral membranes are relatively permeable to the ion. Consequently, Cl^- can readily move into or out of the epithelial cell in response to the activities of other transport proteins.

The cystic fibrosis transmembrane conductance regulator (CFTR; *Cftr*) is an apical membrane-localized Cl^- channel protein found in cells within the crypt region (65,66) (Fig. 3C). The channel protein was first identified as the defective gene product associated with cystic fibrosis (CF) (67), an inherited condition characterized by inadequate hydration of airway epithelia that leads to impaired mucociliary clearance and persistent, life-threatening bacterial infections. Patients with cystic fibrosis have a small disruption in the gene (usually a deletion of phenylalanine 508; Δf508) that results in non-functional CFTR channels because of inappropriate membrane trafficking (68). Normally, opening of CFTR channels in the colon is triggered by cyclic AMP (cAMP)-dependent phosphorylation in response to any number of stimuli, including stimulation by enteric nervous system inputs or the presence of pathogen-derived substances, such as cholera toxin (69).

CFTR mediates most of, if not all luminal Cl^- secretion in the colon, as CF mice fail to generate any Cl^- secretory response to physiological agonists (70). CFTR contributes significantly to the basal and/or stimulated I_{SC} observed in most GI tissues because Cl^- efflux through the channel is also electrogenic. There is some controversy regarding the participation of other apical membrane Cl^- channels, including the Ca^{2+} -activated TMEM16A (*Ano1*) (71) (Fig. 3C), and conflicting results have been produced using pharmacological and transgenic animal models (72,73). In the absence of CFTR activity via genetic knockout (73) or drug inhibition (74), basal and agonist (e.g., cAMP)-stimulated Cl^- secretion is essentially absent. Many newborn CF patients

are also affected by a condition known as *meconium ileus*, where non-functional CFTR channels in the GI tract results in poor hydration of the gut lumen and subsequent buildup of fecal material (75).

Basolateral $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC1; *Slc12a1*) supports CFTR-dependent Cl^- secretion in the crypts of the large intestine (11,76) (Fig. 3C). NKCC1 brings these ions across the basolateral membrane in this stoichiometry (1:1:2) by harnessing the inward Na^+ gradient established by the Na^+/K^+ ATPase. To allow sustained Cl^- efflux across the apical membrane, Na^+ and K^+ are immediately recycled back across the basolateral membrane through the Na^+/K^+ ATPase and K^+ channels, respectively (77). The latter play a particularly critical role by maintaining the electromotive force for Cl^- efflux via depolarization as K^+ ions exit the cell. Intermediate conductance (IK; *Kcnn4*) (78) and voltage-gated subfamily Q member 1 ($\text{K}_{\text{v}}7.1$; *Kcnq1*) (79) K^+ channels have been shown to support CFTR-mediated efflux, as drug inhibition or genetic ablation of either channel substantially reduces the capacity for Cl^- secretion. Expression of CFTR, NKCC1, IK and $\text{K}_{\text{v}}7.1$ is localized primarily to cells within the crypt region, making this the principal location for fluid secretion in the colon (Fig. 3C).

Closely related to the secretion of Cl^- is electrogenic HCO_3^- secretion, which most frequently is mediated by canonical Cl^- channels, including CFTR (80). HCO_3^- permeability through the CFTR channel is ~25% that of Cl^- (80) and is thus a major carrier of anionic current in the epithelium. CFTR-mediated HCO_3^- secretion is critical for proper release and functionality of Mucin proteins secreted by Goblet cells (81), which help to form a protective mucous lining on the surface of the epithelium. While it remains unclear whether Goblet cells express CFTR, another Ca^{2+} -activated anion channel Bestrophin-2 (BEST-2; *Best2*), is highly expressed specifically in Goblet cells and is critical for HCO_3^- secretion in the colon (82). HCO_3^- secretion mediated by

BEST-2 may also be necessary for protection of the epithelium from luminal microbes, as *Best2*^{-/-} mice display increased sensitivity to DSS-induced colitis (82).

1.2.5 K⁺ is both actively absorbed and secreted in the colon

Na⁺ and Cl⁻ are transported in the colon for the purpose of generating osmotic forces to drive fluid absorption and/or secretion. On the other hand, vectorial colonic K⁺ transport plays a role in overall K⁺ homeostasis within the body (83,84). Blood K⁺ levels are very tightly regulated because even slight alterations in extracellular K⁺ can be fatal. The colon serves as a complement to the kidney in terms of taking in or eliminating excess K⁺ from the luminal contents in an effort to maintain blood K⁺ levels (85,86). Depending on the needs of the organism, the colon has the capacity to either actively absorb or secrete K⁺ (83). This is in sharp contrast to K⁺ transport in the small intestine, which is believed to occur only passively due to the mass absorption of water itself in a process called *solvent drag* (87). It should be noted that the basolateral K⁺ recycling necessary for Cl⁻ secretion is not considered as vectorial transport because K⁺ ions are continuously moved back and forth across the basolateral membrane and do not enter or exit the body (i.e., transport is not unidirectional).

Active K⁺ absorption is mediated by apical membrane H⁺/K⁺ ATPases (*ATP12a1*) (Fig. 3B) that can be either sensitive or insensitive to the cardiac glycoside, ouabain (88). These H⁺/K⁺ ATPases share about 60% homology with the basolateral Na⁺/K⁺ ATPase and all three transporters are inhibited by the P-type ATPase inhibitor, Na⁺-orthovanadate (89). H⁺/K⁺ ATPases are expressed by surface epithelial cells only in the distal colon, as determined by uptake studies using isolated apical membrane vesicles (88). K⁺ efflux across the basolateral membrane is facilitated by IK channels functioning in parallel to CLC-2 Cl⁻ channels (45), or by the K⁺/Cl⁻ co-transporter

(KCC) (44). Active K^+ absorption in the colon is therefore an electroneutral process. Besides providing a pathway for K^+ uptake into the body, H^+/K^+ ATPase activity may support electrogenic (ENaC-mediated) Na^+ absorption by supporting K^+ recycling at the apical membrane to help sustain the electrochemical driving force necessary for continuous Na^+ entry (90). Evidence supporting this model is limited, however. A more apparent role for the colonic H^+/K^+ ATPase in regulating whole-body K^+ balance is evidenced by the finding that deletion of functional colon-specific H^+/K^+ ATPase in mice resulted in an increased sensitivity to dietary K^+ deprivation (91).

While absorption is electroneutral, colonic K^+ secretion is electrogenic and is mediated by apical membrane large conductance (BK; *Kcnma1*) (92,93), with possible involvement of intermediate conductance (IK) K^+ channels (94) (Fig. 3B). Uptake of K^+ to support apical efflux is mediated by basolateral Na^+/K^+ ATPase and NKCC1 activity (86). There is evidence supporting both crypt (95) and surface (96,97) localization of electrogenic K^+ secretion systems, and species variation likely exists as well (98,99). Active K^+ secretion is present throughout the entire large intestine, whereas absorption occurs only in the distal segment (83). Colonic K^+ secretion provides an alternate outlet for K^+ , in addition to the kidney. This pathway is important for preventing life-threatening hyperkalemia in patients with end-stage renal disease (100,101). Excessive K^+ secretion has also been implicated in ulcerative colitis (UC) (96) and colonic pseudo-obstruction (102). Both of these conditions are associated with increased stool K^+ content which is likely the result of enhanced BK-mediated K^+ secretion.

Part 2: Physiological regulation of colonic electrolyte transport

The process of digestion requires a great deal of fluid secretion from various sources into the small intestine for proper enzymatic and digestive function. These secretions originate from

the small intestine itself, as well as salivary, pancreatic and biliary secretions carrying essential factors like HCO_3^- , enzymes and bile (3). Nutrients are broken down and absorbed along with Na^+ , Cl^- , and K^+ as the food products (by this point, referred to as *chyme*) travel along the length of the small intestine. The majority of all water presented to the GI tract is also absorbed in the small intestine under normal conditions. Over the course of a normal day for the average adult, the small intestine typically absorbs between 6 and 7L of fluid, with about 2L remaining, which enters the colon. Another 1.8 - 1.9L is absorbed by the colon in the process of desiccation (103). However, the colon has a large overhead capacity for water absorption (up to 5L), as well as secretion (e.g., in the case of enterobacterial infection). The colon is therefore a very dynamic absorptive tissue. Regulation of colonic electrolyte and water transport is of critical importance in maintaining fluid balance. This is achieved by a host of different factors, including neuronal and hormonal influences.

2.1 - Regulation of colonic ion transport by the enteric nervous system.

The enteric nervous system (ENS) is a vast network of neurons and glia located entirely within the GI tissue. More than 100 million neurons constitute the ENS, which can operate independent of inputs from other central or peripheral neural networks (6,104), although significant crosstalk does occur *in vivo*. The ENS is macroscopically organized into two main plexuses: the myenteric (Auerbach's) plexus – located between inner and outer smooth muscle layers, and the submucosal (Meissner's) plexus – located beneath the lamina propria and muscularis mucosae in the submucosal space. The myenteric and submucosal plexuses are generally considered to control smooth muscle contraction/motility and epithelial secretion/absorption, respectively (105).

Sensory afferents also permeate the GI tissue to gather information about luminal contents, pH of the epithelial environment, gut wall distension, presence of foreign substances and other stimuli (106,107). This information is carried via ascending pathways, often to both CNS and ENS divisions, which in turn modulate epithelial transport functions (108). There is a staggering complexity in the anatomical and functional articulations between intrinsic ENS neurons (located entirely within the GI tissue, e.g., myenteric/submucosal plexuses) and extrinsic neurons, whose cell bodies reside outside of the GI tissue (e.g., spinal afferents, sympathetic and parasympathetic fibers) (104).

2.1.1 - Major neurotransmitter systems mediating neuronal-epithelial communication

Within the ENS exist many subpopulations of neurons that directly control smooth muscle or epithelial activity, collectively known as “secretomotor” neurons (109). These are connected to sensory neurons, interneurons and glia to form an expansive network capable transducing sensory input directly from the GI tissue and generating a secretory and/or motor response to a stimulus (110). Secretomotor neurons of the submucosal plexus are the primary effector cells of this reflex circuit with control over epithelial transport (111). These neurons, which typically release pro-secretory neurotransmitters (a.k.a., secretagogues) to the epithelium, are often localized to submucosal ganglia with projections in close contact to the mucosa. Further innervation is also provided by divisions of the Vagus nerve, a major component to the parasympathetic system (112). More than 30 different common neurotransmitters have been identified in ENS neurons, including (but not limited to) acetylcholine, vasoactive intestinal peptide (VIP), and serotonin (5-hydroxytryptamine; 5-HT) (6,113). Acetylcholine and VIP are the most abundant ENS-derived neurotransmitters in the large intestine that heavily influence epithelial secretions (113,114). Serotonin plays an important role in the conveyance of sensory information retrograde from the

epithelium to primary sensory neurons, as well as in peristalsis – the coordinated contractions that propel luminal contents onward through the intestine – which frequently occurs together with epithelial secretions (115). Each of these transmitter systems will be discussed below.

Acetylcholine

Acetylcholine (ACh) is among the most abundant signaling molecules in the body and is vitally important for both neuro-neuronal and neuro-epithelial communication in the gut (110,116). Ganglionic transmission (i.e., neuro-neuronal communication) in most peripheral neural pathways is mediated by ACh. The ENS is no exception to this, as nicotinic ACh receptors (nAChRs) are widely expressed throughout (117,118). Inhibition of nicotinic transmission has a profound effect on secretomotor ENS function, highlighting the importance of cholinergic pathways in the gut (119). Activation of neuron-localized nAChRs triggers the propagation of action potentials through descending pathways until the effector secretomotor neurons are activated. When ACh is delivered to the colonic epithelium via cholinergic secretomotor neurons, it primarily binds to and activates M₃ muscarinic receptors, which are expressed ubiquitously on the basolateral membranes of colonocytes along the entire crypt axis (120,121). M₁ muscarinic receptors are also expressed on epithelial cell basolateral membranes (122), as well as post-synaptic membranes in ENS neurons (123), although their involvement in epithelial signaling is less than that of the M₃ receptor subtype.

M₃ receptors in the colonic epithelium are coupled to G_{αq} proteins that trigger a rapid increase in cytosolic Ca²⁺ when activated, through release of Ca²⁺ from ER stores that is mediated by inositol triphosphate (IP₃) (121). This robust, but transient Ca²⁺ event leads to a number of outcomes related to ion transport. Opening of Ca²⁺-activated K⁺ channels on the crypt cell

basolateral membrane creates an immediate electromotive force favoring apical Cl^- efflux through CFTR and other Cl^- channels, which may also be activated directly by Ca^{2+} (124,125). At the same time, NKCC1 is immediately inserted into the basolateral membrane from sub-membrane vesicles in response to M_3 receptor activation to allow for enhanced Cl^- uptake (126). Ca^{2+} /calmodulin-activated PKC has also been shown to regulate basolateral K^+ channel activation via direct phosphorylation (127), while indirect Ca^{2+} -dependent activation of PKA leads to phosphorylation of NKCC1 (126,128), supporting maximal Cl^- efflux in this context. Further, Goblet cells that secrete mucus and HCO_3^- are also sensitive to cholinergic input (129,130) and when stimulated, release large amounts of HCO_3^- via CFTR or possibly Bestrophin channels (82).

Muscarinic receptors are also found in the surface epithelial cells as well. Cholinergic input in this region of the tissue leads to the rapid internalization of NHE-3 from the apical membrane (131). This process is dependent on phospholipase C (PLC; activated by muscarinic stimulation) and involves extensive rearrangement of the large NHE-3 macromolecular complex, as well as cytoskeletal elements (132,133). Termination of Na^+ uptake prevents the concurrent uptake of Cl^- through apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers (e.g., DRA) because the activity of these transporters is coupled by intracellular pH_i (134). Inhibition of absorptive processes in surface cells, along with stimulation of anion secretion within the crypt, combine to produce a powerful secretory response to cholinergic input to the epithelium.

Vasoactive Intestinal Peptide (VIP)

The other predominant secretory neurotransmitter in the large intestine of most mammalian species is VIP (113,116). VIP is a 28-residue amino acid peptide produced by secretomotor neurons in the ENS, but is also found in other populations within the ENS (inhibitory motor

neurons, interneurons), as well as the CNS (135). In the colon, VPAC1 receptors (which bind VIP) are expressed by crypt epithelial cells and located on the basolateral membrane (136). These receptors are coupled to G_{s} and their activation leads to production of cAMP by adenylate cyclase, the target of G_{s} proteins. cAMP-dependent protein kinase A (PKA) activation leads to the phosphorylation of apical membrane CFTR Cl^- channels (137,138) and cAMP-activated K^+ ($\text{K}_{\text{v}}7.1$) channels on the basolateral membrane (139) to support sustained Cl^- and HCO_3^- secretion. Patients with diarrheal disorders, such as IBS-D, have been reported to have higher VIP concentrations within the gut (140,141). Conversely, genetic ablation of VIP completely disrupts intestinal transit and basal secretory function in mice (142), mimicking Hirschsprung's Disease – a congenital condition where newborns lack ENS innervation to the colon and rectum.

Post-synaptic VIP-ergic neurons also innervate submucosal blood vessels, as their nomenclature implies (143). The vasodilatory response can support secretion by supplying fluid to the epithelium, which will follow the osmotic gradient into the gut lumen if active anion secretion persists (144). This effect is likely mediated by VPAC1 receptors in the endothelium, although current supporting data have only been provided for extra-intestinal vessels (145). VPAC2 receptors are expressed in the smooth muscle itself (146), where release of VIP triggers muscle relaxation, contributing to the hyperemic response. Furthermore, VIP is found in inhibitory myenteric neurons where it plays a role in suppressing motility (147,148). The combined effects of local VIP release culminate in the formation of watery stool, similar to that observed with cholinergic stimulation.

Often, cAMP and Ca^{2+} signaling (e.g., from M3R and VPAC1 receptors) work synergistically to produce a maximal secretory response (149,150,151). There is significant

overlap in these two signaling pathways and neither can proceed in true isolation. For example, some adenylyate cyclases (which generate cAMP and lead to CFTR activation) are activated by the Ca^{2+} -binding protein, calmodulin (152), which is a down-stream mediator of muscarinic signaling. Conversely, the inositol triphosphate receptor (IP_3R) – an integral component of Ca^{2+} release from endoplasmic reticulum (ER) stores – is sensitized by cAMP (153), and Ca^{2+} transients in epithelia can be elicited by treatment with VIP (154). Even so-called Ca^{2+} -activated Cl^- channels (CaCCs), now identified as the TMEM16 gene family, have been reported to be dependent on the function of cAMP-regulated CFTR channels (73,155), although these two pathways were once viewed as entirely independent. Aberrant cholinergic (Ca^{2+} -mediated) and VIP-ergic (cAMP-mediated) signaling have both been associated with ENS-driven pathologies, such as irritable bowel syndrome (IBS) (13,156,157).

Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) was first identified in the 1940s (158), but its influence over intestinal motility was documented even earlier, before the molecule itself was known (159). Although widely understood to be an important CNS neurotransmitter implicated in the control of mood, emotion and sleep, the vast majority (>95%) of all serotonin is housed within the gut (160). The name, “serotonin,” itself is related to its vasoconstrictive properties, which results from platelets (the major carrier of serotonin in the blood) acquiring gut-derived serotonin as they pass through the GI circulation (161). The major source of serotonin production is the enterochromaffin cells (EC cells), a type of endocrine cell in the epithelium. Despite their scarcity, the overwhelming majority of serotonin production in the body is confined to this niche cell type. EC cells are electrically excitable and respond to mechanical distension by releasing serotonin through degranulation across their basolateral membranes (162,163,164), where the “post-

synaptic” intrinsic primary afferent terminals of myenteric neurons (as well as others) reside. This initiates a multi-step, reflexive secretomotor response necessary to secrete fluid and propel luminal contents forward (164). Cholinergic and VIP-ergic submucosal neurons are the end-effectors in the secretory reflex circuit (164,165), while myenteric motor neurons simultaneously induce smooth muscle contraction (166). The ability of the gut to carry out this coordinated secretomotor reflex, independently of central innervation, is largely dependent on this phenomenon of serotonergic “synaptic” transmission from EC cells to the intrinsic afferents.

5-HT₃ receptors on intrinsic afferent terminals in close proximity to EC cells (162,167), as well as on post-synaptic terminals of efferent motor neurons (168), have the greatest involvement of the 5-HT receptor subtypes. Alosetron, a 5-HT₃R antagonist, was a commonly used treatment for IBS-D because it interferes with aberrant serotonergic transmission from EC cells to afferent terminals, thus preventing unwanted peristalsis (i.e., frequent bowel movements) (169,170). On the other hand, 5-HT receptor agonists, such as Tegaserod, can be effective in alleviating constipation, as they promote motility (171). Both of these drugs have declined in use however, due to deleterious side effects (169). Serotonin transporter (SERT) is expressed in epithelial cells and facilitates its uptake from the synaptic space (172), just as in the CNS. Selective serotonin reuptake inhibitors (SSRIs), a class of anti-depressant drugs (e.g., fluvoxamine), are consequently associated with a host of GI-related side effects (173).

Experiments using transgenic mice have revealed that serotonergic transmission is important primarily for motility (174). Deletion of tryptophan hydroxylase, the rate-limiting enzyme required for serotonin synthesis, in either EC cells (175) or ENS neurons (176) impairs peristalsis. Interestingly, while EC cell production of serotonin dwarfs that of ENS neurons themselves, it is the production of serotonin specifically within myenteric neurons that seems to

be the most important for normal motive function (177). With respect to secretion, serotonin released from ENS neurons is not known to specifically act upon epithelial cells in the crypt or surface regions. Thus, it is not generally considered to be a classical “secretagogue.” However, enterocytes in the crypt do express 5-HT₂ receptors that can be activated by the paracrine release of serotonin from nearby EC cells (168).

2.1.2 Neuro-immune communication

Many populations of immune cells reside within the GI tissue and are critical for host defense, as the lumen of the gut – and in particular, the colon – is home to innumerable microorganisms. Collectively, intestinal immune cells are referred to as gut-associated lymphoid tissue (GALT), and constitute over 75% of the body’s total immune system (178). One of the important GALT components are *Peyer’s patches*, concentrated foci of lymphocytes that form nodules just beneath the mucosa throughout the GI tract (179). These allow for a robust adaptive immune response to pathogenic substances, as T- and B-lymphocytes are very tightly packed in these structures, awaiting the signal to mobilize into action. Innervation of Peyer’s patches by peptidergic (e.g., VIP and substance P) submucosal neurons has been known for some time (180). However, the extent to which the ENS interacts with resident immune populations – including not only lymphocytes in the Peyer’s patches, but also resident macrophages and monocytes, M cells, circulating leukocytes, and others – is only recently beginning to emerge in its true scale.

Communication is now known to proceed both ways: neuron to immune effector cell and *vice versa*. For example, most circulating leukocytes express receptors for common neurotransmitters, such as acetylcholine and norepinephrine (originating from sympathetic nerve fibers) (181). Cholinergic signaling from the ENS, as well as the parasympathetic system, to these

immune populations has a powerful immunosuppressive effect systemically and in the gut as well (7). Intestinal macrophages have also been shown to be heavily influenced by physical contact with myenteric or submucosal neurons, which differentially regulate their development into specific M2 or M1 phenotypes, respectively, through release of neurochemicals (182). As another example, Mast cells in the *lamina propria* are activated by certain neuropeptides (e.g., Substance P), and they subsequently degranulate to release large quantities of histamine (183). Mast cell number and degree of neurogenic activation are increased in patients with IBS-D (184). Histamine receptors are expressed in both enterocytes (185) – where their activation triggers Cl⁻ secretion, and sensory afferents (186) – where they contribute to nociception and sensitization to other stimuli. Similarly, Substance P induces the production of pro-inflammatory cytokines (e.g., TNF- α , IL-6) from monocytes in the *lamina propria* (187), contributing to epithelial secretion as well.

Cytokines can also, in turn, potentiate neuronal firing and induce secretion and/or motility. This is believed to be central in the pathogenesis of many GI disorders like inflammatory bowel disease (IBD) and enterobacterial/enteroviral infection. Inflammation-induced changes to ENS activity can take the form of increased excitability. For example, IL-6 is dramatically upregulated in IBD and IBS patients, and potentiates the effects of other secretagogues in the submucosal plexus, contributing to enhanced secretory activity (188). In fact, application of plasma from IBD patients increased firing in rat submucosal neurons, but this was attenuated by neutralization of IL-6 and/or corticotrophin releasing factor (CRF) (189). In addition, anatomical changes to the ENS are associated with an inflammatory state as well. IL-1 β and TNF- α have can activate neurite outgrowth via the NF- κ B signaling pathway (190). Changes in ganglion density have also been associated with IBD as well (191).

In summary, the ENS is a multifaceted and highly complex governing entity that controls both motility and secretion in the colon. Modulation of epithelial ion transport, specifically, by the ENS is multidimensional as well, as interplay between environmental, immunological and endocrine factors each have their own bearing on ENS-driven secretion (7). Many structural and functional aspects of ENS circuitry have been brought to light in recent years. Further exploration of the precise mechanisms of interaction between the ENS and effector cells in the epithelium, smooth muscle, and lymph tissue will give us insight into this relatively new platform from which to approach the treatment of diseases like IBS and IBD.

2.2 – Endocrine Regulation of Colonic Electrolyte Transport

Modulation of colonic ion transport by the ENS takes place on a short time scale. ENS-driven secretion across the epithelium occurs rapidly in response to various stimuli (discussed above). In addition, input from the ENS to the epithelium is generally *reductive* in terms of its influence on basal absorptive processes. That is to say that in the absence of input from the ENS, Na^+ and Cl^- absorption is allowed to proceed unabated, and thus the release of ENS-derived neurotransmitters inhibits basal Na^+/Cl^- absorption and induces Cl^- and/or HCO_3^- secretion simultaneously (Fig. 4). Most of these short-term alterations in ion transport are the result of transient changes in epithelial signaling pathways, such as receptor-induced increases in intracellular Ca^{2+} or cAMP. The activities of transporters and channel proteins can be rapidly adjusted in this context via changes in trafficking or phosphorylation. This allows for very dynamic, fine-tuned control over absorption and secretion.

However, long-term adjustments to epithelial transport are often mediated at a transcriptional level through endocrine mechanisms that either: 1) directly increase the production

of transporters and channel proteins, or 2) indirectly regulate gene products that control the expression or trafficking of key transporter systems (192,193). Furthermore, endocrine signals are the only external influences that can *increase* basal epithelial absorption, as neural and immune factors almost always induce a secretory response (103). For example, dehydration and pregnancy are both physiological conditions that require a sustained increase in colonic (and renal) Na^+ absorption to maintain fluid volume in the body over relatively long time periods (194,195). Endocrine regulation of epithelial transport is a crucial element in these situations because Na^+ absorptive processes must be maintained above baseline levels over time. Hormonal factors bring about long-term changes necessary to maintain fluid and electrolyte homeostasis. Cortisol, aldosterone, somatostatin and the sex hormones estradiol and progesterone all play an important role in this process, as do many other endocrine signals.

2.2.1 – Corticosteroid regulation of colonic ion transport.

Corticosteroid hormones are produced in the adrenal cortex and have diverse biological functions. Among these are the regulation of metabolism and blood sugar levels, maintenance of fluid balance, adaptation to stress and suppression of inflammation (196). Two subsets of corticosteroid hormones – glucocorticoids and mineralocorticoids – are particularly important in regulating electrolyte transport in the intestine (192), as well as in the kidney (197,198). Both of these are synthesized from cholesterol, although the activity of the enzymes required for glucocorticoid and mineralocorticoid synthesis are differentially expressed by specific cells in the adrenal cortex (199). Glucocorticoids are produced in the *zona fasciculata* via the 11β -hydroxylase enzyme, which converts the cholesterol-derived precursors into the active cortisol (200) (or corticosterone, in rodents). Exclusively expressed in the *zona glomerulosa* is the aldosterone

synthase enzyme, which converts corticosterone into aldosterone. The activity of these various enzymes is also regulated by different biological signals as well.

Production of glucocorticoid hormones from the *zona fasciculata* cells of the adrenal cortex is mainly triggered by binding of circulating adrenal corticotrophic hormone (ACTH) to its receptor on the plasma membrane (201). Upstream of this, ACTH produced by the anterior pituitary in response to corticotropin-releasing hormone (CRH) sent from the hypothalamus. This pathway is a critical component to adaptation to stress by increasing circulating blood glucose levels and conferring behavioral and emotional changes in the CNS (202). The acute effects of a robust stress response generally inhibit absorption and associated blood flow (203), in lieu of diverting resources to brain and muscle to meet immediate demands. Under normal conditions however, basal glucocorticoid production is required for proper absorption, as described below.

Mineralocorticoid production is mainly triggered by the renin-angiotensin system in response to a reduction in circulating volume (i.e., hemorrhage or dehydration) via angiotensin-II receptors (204), or directly by an increase in extracellular K^+ (hyperkalemia) in *zona glomerulosa* cells (205). Glucocorticoids therefore regulate basal colonic transport in a normal, homeostatic manner, while mineralocorticoids become increasingly important in restoring fluid and electrolyte balance in response to significant perturbations to the system. While there is significant overlap in the end effects of GR and MR signaling with respect to colonic ion transport (206), some key differences to exist in terms of both their site of action and the specific transport pathways which the two hormones regulate.

The primary mechanism of action for corticosteroids (and any other steroid hormone) is different from that of the neurotransmitters, paracrine factors and cytokines described earlier.

Glucocorticoid and mineralocorticoid receptors (GR and MR, respectively) are located within the cytosol of epithelial cells (207), as opposed to the plasma membrane receptors for neurotransmitters and cytokines. These hormones typically exert their effects through translocation to the nucleus upon binding to their hormone ligand, and they subsequently initiate the transcription of specific gene products (207). Some non-genomic actions have also been described as well (see below). Although synthetic corticosteroids are widely used in the clinic (208), including in the treatment of inflammatory bowel disease (209), the main biologically active, endogenous glucocorticoid and mineralocorticoid hormones are *cortisol* and *aldosterone*, respectively. Cortisol and aldosterone are similar in structure and closely related in the steroid synthesis pathways (199). However, cortisol has more widespread effects in the body, as almost every somatic tissue expresses the glucocorticoid receptor (210). On the other hand, mineralocorticoid receptors are present mainly within salt-transporting epithelia, including the distal colon. Circulating levels of cortisol are typically much higher than aldosterone – on the order of 100-fold or more.

The distal nephron of the kidney, along with the distal colon constitute the main aldosterone-sensitive tissues, and both constitutively express MR (211). However, glucocorticoids can also bind to MR with similar affinity compared to GR. Because of this, and also because glucocorticoids are so much more abundant in the circulation, an enzyme called 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD 2) is required for aldosterone sensitivity and is specifically expressed in these tissues (211,212). 11 β -HSD 2 works by converting cortisol into the inactive *cortisone*, thus enabling the less abundant aldosterone to bind to MR and exert its effects. In this way, small changes in circulating aldosterone levels can have a substantial impact on MR-regulated distal colonic electrolyte transport. Mutations in 11 β -HSD 2 that render the enzyme non-

functional cause a form of juvenile hypertension called the “syndrome of apparent mineralocorticoid excess,” due to increased occupancy of MR by cortisol, which cannot be eliminated in aldosterone-sensitive tissues (213). Excessive salt and water absorption ensues, causing hypervolemic hypertension.

Electroneutral NaCl absorption is stimulated by corticosteroid signaling in the proximal colon

In humans and rodents, infusion of corticosteroid hormones including cortisol (or corticosterone in rodents) and aldosterone, as well as synthetic compounds, transcriptionally up-regulates the expression of electroneutral transporters in the proximal colon to allow for increased capacity for Na^+ and Cl^- absorption (206, 214, 215). In particular, NHE-3 protein abundance is enhanced in animals infused with mineralocorticoids over a period of hours to days (216). Low dose glucocorticoids produce a similar effect in the small intestine (217). Mineralocorticoid response elements (MREs) and glucocorticoid response elements (GREs) are found in the promoter region of NHE-3, where steroid-bound receptors can dock and initiate transcription (218,219). As it turns out, corticosteroids were used in the treatment of IBD initially for their anti-inflammatory effects (220), resulting from suppression of pro-inflammatory genes which are regulated by GREs in T-cells. Later, after subsequent investigation into the mechanism of action, it was discovered that intestinal NaCl absorption was also dramatically impacted by corticosteroid use, contributing to symptom alleviation as stool water content is reduced (221).

Basal circulating glucocorticoid levels are necessary for development of salt-transporting epithelia, including in the intestine. In healthy adults, basal electroneutral NaCl (NHE-3 dependent) absorption in the small and large intestine is also driven by constitutive glucocorticoid production (222). Adrenalectomized animals, which are unable to synthesize corticosteroids, have

severely impaired absorptive capacity (223,224). Replenishment of exogenous glucocorticoids is sufficient to restore normal absorptive function in adrenalectomized animals (225). Although mineralocorticoid (aldosterone) production is also eliminated in these animals, evidence suggests that mineralocorticoid activity is not essential for electroneutral absorption in the proximal colon, nor the small intestine (225). On the other hand, electrogenic (ENaC-mediated) absorption in the distal colon is critically dependent on mineralocorticoid signaling.

The rate of Cl^- absorption in the proximal colon also increases in response to corticosteroids, though the net flux of Na^+ remains greater than that of Cl^- (62,215). In addition, expression of electroneutral $\text{Cl}^-/\text{HCO}_3^-$ exchangers is not increased in parallel to NHE-3 (226), suggesting the enhanced Cl^- absorption occurs secondarily to NHE activity. Efflux of Cl^- across the apical membrane, or inability of Cl^- to transit the basolateral membrane as readily as Na^+ may be responsible for this discrepancy in the rates of flux for the two ion species. There may also be some involvement of apical $\text{Cl}^-/\text{butyrate}$ or $\text{HCO}_3^-/\text{butyrate}$ exchangers as well, although this possibility has not been explored. It is not known whether SCFA transporters are transcriptionally regulated by MR or GR signaling. Rates of Na^+ and Cl^- uptake are elevated by both butyrate and corticosteroids, independently. However, the effect of corticosteroids on butyrate uptake (which is now known to be closely tied to Na^+ uptake) has not been studied. If Na^+ uptake is more tightly coupled to butyrate uptake (as opposed to Cl^-) under conditions of high MR/GR activity, this may explain the difference in corticosteroid-induced Na^+ versus Cl^- absorption in the proximal colon. Interestingly, butyrate has transcriptional effects as well because it acts as a histone deacetylase inhibitor (49). In fact, there is some evidence to suggest that butyrate is necessary to confer aldosterone sensitivity in the colon (227,228), raising the possibility of a powerful combined effect of the two.

Na^+/K^+ ATPase activity drives the overall absorptive process, as described in earlier sections. Na^+/K^+ ATPase activity in the colon is also regulated by corticosteroids (86,229), although there is some evidence that aldosterone controls Na^+/K^+ ATPase expression indirectly through an increase in transporter density in the basolateral membrane rather than through direct transcriptional control (85,230). This may be partially attributable to transcriptional regulation of non-transporter/channel gene targets of MR signaling. Among the most well-described of these are the serum- and glucocorticoid-regulated kinase 1 (SGK-1) (231,232) and corticosteroid hormone inducing factor (CHIF) (233,234), both of which are necessary for corticosteroid hormones to produce their full effects in transporting epithelial tissues, including the colon.

Electrogenic Na^+ absorption is stimulated and electroneutral NaCl absorption is inhibited by mineralocorticoid signaling in the distal colon.

As mentioned before, ENaC facilitates electrogenic Na^+ absorption and is found only in the distal segment of the colon. ENaC is comprised of three highly homologous subunits (α , β and γ), two of which (β and γ) are under direct transcriptional control by aldosterone (235,236,237,238). ENaC degradation via the proteasome pathway is also inhibited by aldosterone through activation of SGK-1 (239). Apical ENaC-mediated Na^+ absorption is driven by basolateral Na^+/K^+ ATPase activity, just as electroneutral absorption is in the proximal colon. In animals infused with aldosterone, or in animals with diet-induced secondary hyperaldosteronism, nearly all Na^+ absorption is inhibited by low-dose amiloride and is therefore dependent on ENaC (235,240). At the same time, NHE-3 expression is inhibited by aldosterone (241). Effects on $\text{Cl}^-/\text{HCO}_3^-$ or other anion exchangers are not currently known. However, given that anion exchangers are driven by Na^+/H^+ exchange, and that $^{36}\text{Cl}^-$ fluxes are reduced by aldosterone (27), the

functionality of anion exchangers in the distal colon is down-regulated by MR signaling, regardless of any potential effects on protein expression directly. Importantly, aldosterone-induced inhibition of electroneutral NaCl absorption, as well as stimulation of electrogenic Na⁺ absorption, is prevented by treatment with the MR antagonist, spironolactone (242,243). As such, MR-regulated Na⁺ absorption is entirely electrogenic and electroneutral NaCl absorption completely suppressed.

Mineralocorticoid, but not glucocorticoid signaling induces K⁺ secretion in the proximal and distal colon.

The production of aldosterone is typically associated with the renin-angiotensin system, which is activated after a loss in blood volume (244). In this context, aldosterone serves to enhance Na⁺ and water absorption to minimize fluid loss and restore balance. The other major trigger for aldosterone production is a rise in extracellular K⁺ [K⁺]_o, known as hyperkalemia. Even minute changes in [K⁺]_o can be life-threatening because of the depolarizing effect on important electrically-excitable cell populations (i.e., neurons in the hypothalamus and brain stem). *Zona glomerulosa* cells in the adrenal cortex are depolarized by an increase in [K⁺]_o, directly leading to Ca²⁺ influx that triggers a signaling cascade resulting in aldosterone synthesis (245). This mechanism bypasses the renin-angiotensin system, which also ultimately cause zona glomerulosa cell depolarization indirectly through binding of angiotensin II to receptors and downstream inhibition of plasma membrane K⁺ channels (245).

Colonic K⁺ transport is mediated by apical membrane large conductance K⁺ (BK) (97,246) and possibly intermediate conductance K⁺ (IK) (94) channels, and aldosterone induces the expression of BK and IK (94) channels in the colon. Under normal conditions, there is a small amount of K⁺ secretion in the proximal colon, but K⁺ is absorbed in the distal colon by apical

membrane H^+/K^+ ATPases (83, 88). In the context of elevated MR signaling, net K^+ secretion is enhanced in the proximal colon and induced in the distal colon, primarily through an iberiotoxin (i.e., BK channel-dependent) mechanism (92,95,246). Colonic K^+ secretion that is regulated by aldosterone is an imperative outlet mechanism in patients with end-stage renal disease (ESRD) (100, 101), who cannot adequately eliminate excessive K^+ through the kidney. Thorough characterization of the supporting elements of sustained K^+ secretion in response to aldosterone, such as accessory BK channel subunits (β/γ) and basolateral K^+ loaders (i.e., Na^+/K^+ ATPase versus NKCC1) is not yet complete.

Non-genomic effects of aldosterone on colonic ion transport.

Steroid hormones are now recognized to exert rapid effects on cellular activity which are not dependent on transcription. Often these effects are in line with transcriptional outcomes but require much less time to develop. This holds true for modulation of colonic epithelial transport by aldosterone as well (247), which produces a rapid induction of Na^+ absorptive and K^+ secretory process dependent one or more of the protein kinase C (PKC) isoforms (248, 249, 250). At the same time, aldosterone also inhibits basolateral IK channel currents necessary for supporting Cl^- secretion in the crypts (251). Characterization of the non-genomic actions of aldosterone is an emerging and rapidly evolving field. Many aspects of rapid corticosteroid effects have yet to be explored.

2.2.2 – Regulation of colonic ion transport by somatostatin.

Somatostatin (SS) is a 14- or 28-amino acid peptide hormone produced in the pancreas, hypothalamus, or in the intestinal mucosa (252). The effects of SS are generally considered to be inhibitory, preventing the release of growth hormone from the pituitary and glucagon from the

pancreas. The GI tract is no exception, where treatment with exogenous SS inhibits basal and agonist-induced Cl^- secretion (253,254). The somatostatin receptor is coupled to $\text{G}_{\alpha i}$, which inhibits the production of cAMP upon receptor binding. The inhibitory effects of SS with respect to epithelial secretion are thought to be dependent on the prevention of cAMP-dependent $[\text{Ca}^{2+}]_i$ increase, thus attenuating the stimulatory effect of other secretagogues (255,256). BK-mediated K^+ secretion is also attenuated by SS in colonic epithelial cells (257). SS is one of the only known non-steroid agents that *enhances* basal absorption. Somatostatin analogs (SSAs) are used clinically for the treatment of a variety of endocrine disorders and conditions related to neo-endocrine tumors (258). However, certain GI-related conditions, such as refractory diarrhea and some post-surgical syndromes can be relieved by treatment with SSAs (258).

2.2.3 – Regulation of colonic ion transport by sex steroids.

Recent attention to sex as a biological variable has led to many discoveries related to sex differences across all disciplines of biomedical research. Epithelial transport in many tissues, including the colon, is governed by sex steroids in addition to the corticosteroids discussed previously (259). Sex steroid hormones usually exert their effects in the same way – by binding to intracellular receptors and causing their translocation to the nucleus to initiate the transcription of gene products. Evidence suggests that some sex steroids, especially 17- β estradiol (E_2), modulate epithelial growth (260) and tight junction barrier development (261), and may even have anti-tumorigenic properties (262) that are all mediated by transcriptional mechanisms. However, many of the documented effects on epithelial ion *transport* seem to occur through non-genomic effects (263,264,265). There is some speculation that the steroid molecule itself can interact directly with target proteins to produce these effects (266). Recently, a membrane-bound estrogen receptor (ER)

variant was also identified that probably mediates some non-transcriptional events via modulation of kinase-dependent signaling cascades (267).

Administration of exogenous E₂ (estradiol) to isolated colonic mucosa inhibits both basal and cAMP-stimulated Cl⁻ secretion through acute internalization of the basolateral K_v7.1 channel (268), whose activity is critical for sustained Cl⁻ efflux (see section 1.2.4). This was found to be dependent on the activity of PKC, similar to studies involving the corticosteroids, while inhibition of ER signaling via Tamoxifen did not interfere with the response. E₂ also dampened the secretory response to *V. cholera* and *E. coli*-derived enterotoxins *in vitro* through a similar mechanism (269). There is only very limited evidence to suggest a similar effect of progesterone, with a very mild effect seen in cultured epithelial cells with respect to cAMP-induced secretion (270). Meanwhile, the predominant male sex steroid, testosterone, has not been shown to produce any alterations to colonic electrolyte transport. Although E₂ clearly influences epithelial growth and secretion via genomic and non-genomic mechanisms, respectively, the exact biological importance of these effects is unknown.

Part 3: Specific Aims.

As reviewed above, the regulation of ion transport in the intestine is a complex, but essential element of overall health. Many pathological conditions are either 1) the direct result of, 2) exacerbated by, or 3) ameliorated by changes to electrolyte transport in the colon. The focus of our recent studies has been centered around two areas in which the current understanding of colonic electrolyte transport regulation is lacking. The first involves identifying and characterizing a novel target, voltage-gated K_v7 channels, in the neurogenic control of epithelial transport. The

other is related to deepening our knowledge of the changes in epithelial transport mediated by mineralocorticoids (aldosterone), including investigation into K^+ and Cl^- secretory pathways.

3.1 – Specific Aim 1A: Determine the effect of neuronal K_v7 (KCNQ) channel activation on epithelial Cl^- secretion. The enteric nervous system is known to regulate epithelial ion transport mainly by providing stimulatory (pro-secretion) input to epithelial cells. The *KCNQ* family of voltage-gated (K_v7) K^+ channels are a neuron-localized group of channels that hyperpolarize the plasma membrane of neurons and reduce excitability. K_v7 channel activators have been used clinically to treat other conditions related to excessive neuronal firing (i.e., epilepsy or chronic pain), but the effects on ENS-driven secretion are not known. Using I_{SC} recording techniques, pharmacological approaches and immunofluorescence microscopy, we tested the hypothesis that activation of ENS-localized K_v7 channels suppresses epithelial Cl^- secretion.

3.2 – Specific Aim 1B: Determine the effect of neuronal K_v7 (KCNQ) channel activation on epithelial Na^+ absorption. Stimulatory ENS input to the colonic epithelium is characterized by the additive effects of Cl^- secretion and simultaneous inhibition of Na^+ absorption. This often produces a robust secretory response to physiological or pathological stimuli. Electroneutral Na^+ absorption is known to be inhibited by various secretagogues originating from secretomotor ENS neurons. I_{SC} and $^{22}Na^+$ radioisotope flux experiments were performed to assess the effect of neuronal K_v7 channel activation on epithelial Na^+ transport, with the prediction that reduced ENS input via a K_v7 channel-activating drug would enhance basal Na^+ absorption in parallel to any suppressive effects on Cl^- secretion.

3.3 – Specific Aim 2A: Investigate the role of the basolateral co-transporter, NKCC1, in supporting aldosterone-induced K^+ secretion in the distal colon. Although stimulation of K^+ secretion in the distal colon by aldosterone has been well-described, the role of NKCC1 in supporting K^+ secretion is not fully understood – in particular, transcriptional regulation of NKCC1 by aldosterone has not been established in the colon. Furthermore, it is not known whether changes in NKCC1 expression also influence Cl^- secretion in parallel to K^+ secretion. Electrophysiological and molecular biological techniques were used to test the hypothesis that aldosterone transcriptionally up-regulates NKCC1 in the distal colon, specifically to support K^+ secretion without concurrent enhancement of Cl^- secretion.

3.4 – Specific Aim 2B: Determine whether aldosterone regulates the expression of critical components to electrogenic K^+/Cl^- secretion in the proximal colon. This work expands upon the existing literature which demonstrates that net basal K^+ and Cl^- transport is differentially regulated by aldosterone in the proximal versus distal colon. Evidence of transcriptional regulation has not been provided, nor has a thorough assessment of agonist-stimulated changes in Cl^- transport. We hypothesized that aldosterone reduces the capacity for Cl^- secretion, likely via transcriptional effects on Cl^- channel proteins and/or other components to the canonical secretory pathway. At the same time, K^+ secretion is known to be enhanced by aldosterone; thus, we predict the pathway mediating this is similar to the distal colon (i.e., upregulation of apical membrane BK and/or IK channels). Short-circuit current (I_{SC}) electrophysiology and molecular biological approaches were used to assess the effect of aldosterone on basal and agonist (Ca^{2+} /cAMP)-stimulated Cl^- and K^+ secretion and expression of critical molecular components (i.e., CFTR, NKCC1, BK and IK channels).

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A

B

Figure 1 – Histological structure of the colonic epithelium. Mouse colonic epithelium stained with hematoxylin and eosin and sectioned at 5 μm . Images captured at 20x magnification; scale bars = 50 μm . *A*: Full-thickness colon tissue depicted, with surface and crypt epithelial regions shown, as well as underlying submucosa and smooth muscle containing blood vessels and ganglia. B.V.= blood vessel; A.M. = apical membrane; B.M. = basolateral membrane; L.P. = lamina propria.

Figure 2 – Basic structure and function of colonic epithelial cells. Individual epithelial cells of the colon (colonocytes) are connected to adjacent cells via tight junction (shown in blue) to form a tight barrier which lines the gut. Net absorption of electrolyte ions, such as Na^+ and Cl^- , through the trans-cellular compartment and into the interstitial space allows for water absorption to occur through osmosis (left panel). Conversely, secretion of electrolytes into the luminal space drives water secretion (right panel). Electrolyte movement in either direction requires transport (either active or passive) across the apical and basolateral cell membranes, shown here in red and green, respectively. Water transport also occurs largely through the trans-cellular compartment. The colon is capable of both absorption and secretion, depending upon the needs of the organism.

A**Electroneutral NaCl absorption (proximal/mid-distal colon)***Surface epithelial cell* **B****Electrogenic Na^+ absorption and K^+ secretion (distal colon)***Surface epithelial cell* **C****Electrogenic Cl^- and K^+ secretion (proximal and distal colon)***Crypt cell*

Figure 3 – Summary of the major electrolyte transport systems within the colonic epithelium.

A: Electroneutral NaCl absorption occurs in surface epithelial cells of the proximal and mid-distal colon. Na^+/H^+ exchanger isoform 3 (NHE-3) and the “down-regulated in adenoma” $\text{Cl}^-/\text{HCO}_3^-$ exchanger (DRA) mediate electroneutral Na^+ and Cl^- absorption, respectively, which are coupled via intracellular pH. Basolateral Na^+/K^+ ATPase activity drives this process by continuously extruding Na^+ across the basolateral membrane. Intermediate conductance K^+ (IK) and Cl^- channel 2 (CLC-2) channels primarily facilitate electroneutral K^+ and Cl^- uptake across the basolateral membrane. *B:* Electrogenic Na^+ absorption occurs via the epithelial Na^+ channel (ENaC) in surface epithelial cells of the distal colon. Apical membrane H^+/K^+ ATPase and large conductance K^+ (BK) channels mediate active K^+ absorption and secretion, respectively. Basolateral transporter/channel functions are similar to panel *A*. *C:* Electrogenic Cl^- and K^+ secretion occurs within the crypt region of the colonic epithelium in both proximal and distal regions of the tissue. Apical membrane BK channels and cystic fibrosis transmembrane conductance regulator (CFTR) and transmembrane protein 16A (TMEM16A) likely mediate efflux of K^+ and Cl^- , respectively. Basolateral Na^+/K^+ ATPase activity drives K^+ and Cl^- uptake via the NKCC1 co-transporter, which ultimately supports active secretion. Basolateral IK or voltage gated $\text{K}_v7.1$ channels participate in K^+ recycling in conjunction with NKCC1 in order to maintain the electrical driving force necessary for sustained Cl^- efflux.

Figure 4 – Enteric nervous system (ENS) input generally inhibits Na^+ absorption and stimulates Cl^- secretion in the colonic epithelium. Release of ENS neurotransmitters (e.g., acetylcholine; Ach, or vasoactive intestinal peptide; VIP) stimulates basolateral receptors (e.g., M_3R and VPAC1 , respectively) on colonic epithelial cells. These receptors are typically linked to $\text{G}\alpha_q$ (M_3R) or $\text{G}\alpha_s$ (VPAC1), which induce Ca^{2+} - and/or cAMP-dependent signaling events, respectively, leading to inhibition of Na^+ absorption and stimulation of Cl^- secretion. This outcome is largely the result of trafficking of channels and transporters to (e.g., NKCC1) and from (e.g., NHE-3) the plasma membrane to regulate their activities. In addition, protein kinase A or C (PKA/PKC)-induced phosphorylation of channels, transporters or regulatory complex proteins is often a critical regulatory component. The end result of these events is typically decreased NaCl absorption, which leads to decreased water absorption and increased stool volume (i.e. diarrhea).

CHAPTER 2

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Running title: KCNQ (K_v7) activation in ENS neurons inhibits epithelial Cl⁻ secretion

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ABSTRACT

K_V7 (KCNQ) K⁺ channels are expressed in many neuronal populations, and play an important role in regulating membrane potential by generating a hyper-polarizing K⁺ current and decreasing cell excitability. However, the role of K_V7 channels in the neural regulation of intestinal epithelial Cl⁻ secretion is not known. Cl⁻ secretion in mouse distal colon was measured as a function of short circuit current (I_{SC}), while pharmacological approaches were used to test the hypothesis that activation of K_V7 channels in enteric neurons would inhibit epithelial Cl⁻ secretion. Flupirtine, a non-selective K_V7 activator, inhibited basal Cl⁻ secretion in mouse distal colon and abolished or attenuated the effects of drugs that target various components of enteric neurotransmission, including tetrodotoxin (Na_V channel blocker), Veratridine (Na_V channel activator), Nicotine (nicotinic acetylcholine receptor agonist) and Hexamethonium (nicotinic antagonist). In contrast, flupirtine did not block the response to epithelium-targeted agents VIP (endogenous VPAC receptor ligand) or carbachol (non-selective cholinergic agonist). Flupirtine inhibited Cl⁻ secretion in both full-thickness and seromuscular-stripped distal colon (containing the submucosa, but not myenteric plexus), but generated no response in epithelial T84 cell monolayers. K_V7.2 and K_V7.3 channel proteins were detected by immunofluorescence in whole-mount preparations of the submucosa from mouse distal colon. ICA 110381 (K_V7.2/7.3 specific activator) inhibited Cl⁻ secretion comparably to flupirtine. We conclude that K_V7 channel activators inhibit neurally-driven Cl⁻ secretion in the colonic epithelium and may therefore have therapeutic benefit in treating pathologies associated with hyper-excitable enteric nervous system, such as irritable bowel syndrome with diarrhea (IBS-D).

KEY WORDS: Colonic epithelium . K_V7 K⁺ channels . Enteric nervous system (ENS). Irritable bowel syndrome with diarrhea (IBS-D) . Flupirtine. Ussing Chamber .

INTRODUCTION

Irritable bowel syndrome with diarrhea (IBS-D) is one of the most common gastrointestinal (GI) disorders (43). Symptoms such as frequent and watery bowel movements are often related to hyperactivity of the enteric nervous system (ENS) (11,20,53). Pharmacological agents that inhibit hyperactivity of secretomotor neurons of the ENS have been shown to increase colonic transit time and reduce stool water content in IBS-D (24,30,36). Although ENS-targeted drug therapies, such as eluxadoline (opioid receptor agonist) or alosetron (5-HT₃ receptor antagonist) alleviate IBS-D symptoms, they are also commonly associated with adverse side effects such as drowsiness, blurred vision and constipation (12). Development of novel therapeutic strategies therefore remains an important and active area of research.

The KCNQ (K_v7) gene family is a group of small conductance, voltage-gated K⁺ channels, which open at characteristically negative membrane potentials (4,10,46,54). It has been shown that some members of K_v7 family (K_v7.2, K_v7.3 and K_v7.5) can assemble to form heterotetramers that carry the “M” current, a stabilizing K⁺ current that is inhibited by muscarinic receptor stimulation in neurons (51,59). In addition, targeted KCNQ gene disruptions have been shown to cause seizures and hyperalgia in experimental models, while K_v7 channel activators that inhibit neural excitability have been used in the treatment of epilepsy (8,22,50,60). K_v7 channels have thus been implicated as important regulators of resting membrane potential in neurons.

K_v7 channels have recently been localized in neurons of the ENS along the human GI tract, as well as afferent fibers innervating the distal colon in both human and mouse (49). It was demonstrated that neuronal K_v7.3 activation inhibited both mechanical- and chemical-stimulated sensory response, providing evidence that K_v7 channels could be a target in the treatment of GI pain. However, the effects of K_v7 channel activation with respect to epithelial electrolyte transport

are not known. Because neural stimulation of epithelial Cl^- secretion through apical Cl^- channels (e.g. CFTR) can provide a driving force for water secretion in the GI tract (i.e. diarrhea) (17,34), there is potential therapeutic value in understanding the role of K_v7 channels in this process.

We hypothesized that K_v7 channel activation would dampen stimulatory ENS input to the intestinal epithelium, thus inhibiting epithelial Cl^- secretion. To test this hypothesis, we examined the effect of the K_v7 activator, flupirtine, on Cl^- secretion in mouse distal colon. Following the initial observation that flupirtine inhibited basal short-circuit current (I_{sc} ; i.e. Cl^- secretion), pharmacological approaches were used to examine whether flupirtine acted on the ENS or the epithelium directly, and immunofluorescence microscopy was used to confirm the expression of K_v7 subunits in enteric neurons. The results presented in this study provide evidence that inhibition of epithelial Cl^- secretion by flupirtine is likely due to activation of ENS-localized K_v7 channels.

MATERIALS AND METHODS

Animals

Male BALB/c mice (8 weeks old, Charles River, NY) were housed in groups of 2-4, exposed to 12-hour light/dark cycles and provided normal chow and water ad libitum. Female mice were excluded from this study for the purpose of controlling for unwanted variability in Cl^- secretion resulting from female sex hormone activity (2,14). Animals were anesthetized using 5% isoflurane (Patterson Veterinary, SC) balanced with oxygen, and the whole colon was removed by gently cutting away mesenteric attachments. Animals were then euthanized via cervical dislocation. Tissues harvested from each mouse were randomly assigned to treatment groups. All experimental protocols utilized in this study were approved by the West Virginia University Institutional Animal Care and Use Committee.

Ussing chamber experiments

Mouse colonic tissue:

Whole colon was removed from anesthetized mice and flushed gently with ice-cold phosphate-buffered saline (PBS) to remove fecal contents. The colon was then incubated for 5-10 minutes in ice-cold PBS containing 5 μ M indomethacin to inhibit prostaglandin production during tissue manipulation, before being pinned near the caecum in a sylgard plate. A longitudinal incision was made along the mesenteric border and the tissue was pinned mucosal side down. After removing the attached mesentery, segments of mid-distal colon (2-5 cm proximal to the rectum) were then excised and mounted on plastic sliders with an oval aperture of 0.04 cm² for use in an EasyMount Ussing-style chamber (Physiological Instruments, San Diego, CA). Up to 4 tissues were taken from each colon. For experiments using seromuscular-stripped tissue, a lateral incision was made into the serosal side of the tissue and the musculature was gently peeled back using ultra-fine forceps under a dissecting microscope. Removal of smooth musculature and the myenteric plexus contained within was performed to examine the effects of flupirtine on submucosal neurons, which were left intact in these preparations. Selected segments of unstripped (full-thickness) and seromuscular-stripped colon were set aside for histological assessment, as described below.

Mounted tissues were bathed bilaterally in 5 mL of modified Kreb's solution containing, in mM: 115 NaCl, 25 NaHCO₃, 1.6 K₂HPO₄, 0.4 KH₂PO₄, 2.8 KCl, 1.2 CaCl₂, 1.2 MgCl₂ and 10 glucose (pH 7.4). Tissues were monitored and allowed to equilibrate under open-circuit conditions for a period of 15-20 minutes until trans-epithelial potential (V_{TE}) had stabilized, while being maintained at 37°C and continuously gassed with 5% CO₂, 95% O₂. Short-circuit current (I_{SC}) recordings were performed as described previously (52). In brief, all mounted tissues were voltage-

clamped at 0 mV using a multichannel voltage clamp (VCC MC8; Physiologic Instruments, San Diego, CA) and I_{SC} was measured continuously by computer-operated software (Acquire & Analyze; Physiologic Instruments). A brief (200 ms) bipolar 5 mV pulse was applied every 5 seconds to monitor trans-epithelial resistance. Under these conditions, positive I_{SC} values reflect anion (Cl^-) secretion. I_{SC} recordings were allowed to reach a steady-state for at least 5 minutes prior to the addition of any drugs. Up to four adjacent tissues were obtained from each animal. When four tissues were taken from the same animal, the average intra-animal basal I_{SC} range was 56.6 and the average standard deviation was $\pm 25.4 \mu A/cm^2$ (n=10).

It should be noted that while electrogenic Na^+ absorption via epithelial Na^+ channel (ENaC) can also generate a positive I_{SC} in the colon of some species under certain conditions, normal mouse colon exhibits very minimal ENaC activity and this is restricted to only the most distal segment of the tissue (32). For all experiments in this study, tissues were taken from between 2 and 5 cm proximal to the rectum (mid-distal colon). Thus, we make the assumption that positive I_{SC} values under our experimental conditions reflect *predominantly* Cl^- secretion. This approach was chosen to eliminate any potential confounding effects of using an ENaC inhibitor, such as amiloride.

T84 cells

T84 cells (a human colonic adenocarcinoma cell line, ATCC, Manassas, VA) were grown and maintained in DMEM/F12 media (ThermoFisher, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Hampton, NH) and 1% penicillin-streptomycin (Mediatech, Manassas, VA) at 37°C in 5% CO_2 incubator. After passaging, cells were seeded at near confluence onto 1.12 cm^2 Snapwell inserts that contain permeable support (Corning, Corning, NY). Upon reaching confluence, the cells were maintained in monolayers for 18 days to ensure

complete development of tight junctions and trans-epithelial potential (V_{TE}). Fresh culture media was replaced every other day until utilization in Ussing chamber studies. On the day of experiments, the inserts containing T84 monolayers were removed and gently washed with warm PBS. The monolayers were then mounted in the Ussing chamber and bathed on both sides with Ringers' solution. I_{SC} measurements were performed as described above. Monolayers were allowed to equilibrate and come to a steady-state (~15 minutes) prior to the application of any drugs.

Histology

Full-thickness and seromuscular-stripped segments of mid-distal colon were fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature and embedded in paraffin. The colons were then sectioned (5 μ m), mounted on glass slides and stained with hematoxylin and eosin for visualization of tissue structures. Images were captured with an Olympus VS210 slide scanner (Tokyo, Japan) using a 20x objective.

Immunofluorescence studies

To obtain a whole-mount preparation of the submucosal plexus, the distal colon was first opened along the mesenteric border and pinned mucosal side down. Similar to the tissue preparation for experiments using seromuscular-stripped colon, a lateral incision was made into the serosal side and the musculature was peeled away using forceps. The tissue was then placed mucosal side facing upward and the epithelium was removed by scraping with a glass slide. The remaining tissue was then fixed for 48 hours at 4°C in 4% paraformaldehyde in PBS. After permeabilizing with 1% Triton-X 100 (Fisher Scientific, Hampton, NH) plus 5% goat serum (Fisher Scientific, Hampton, NH) in PBS for 30 minutes at room temperature, tissues were incubated at 4°C overnight in blocking solution (5% goat serum, 0.01% Triton-X in PBS)

containing the following primary antibodies: mouse anti-microtubule-associated protein 2 (MAP2; Millipore #MAB3418; 1:200 dilution) plus either rabbit anti-K_v7.2 (Alomone #APC-050; 1:100 dilution) or rabbit anti-K_v7.3 (Alomone #APC-051; 1:100 dilution). All of these commercially-available antibodies have been used in immunofluorescence microscopy experiments to detect their respective proteins in mouse tissues (39,56,61). The following day, tissues were washed 5 times for 5 minutes each with PBS + 0.01% Triton-X 100 (PBST) before incubating in blocking solution containing AlexaFluor 568 goat anti-mouse (Thermo #A11031; 1:2,000 dilution) and AlexaFluor 488 goat anti-rabbit (Thermo #A11008; 1:2,000 dilution) for 30 minutes at room temperature to allow detection of MAP2 (anti-mouse) and K_v7.2 or K_v7.3 (anti-rabbit) within the same tissue preparation. After washing again 5 times in PBST for 5 minutes each, the tissues were placed on charged glass slides and mounted in Slow-Fade Diamond anti-fade mountant with DAPI (Thermofisher Scientific, Waltham, MA). Images were captured with a Zeiss 710 confocal microscope (Carl Zeiss, Oberkochen, Germany) using a 20x objective.

Data analysis

Flupirtine concentration-response analysis

Flupirtine was prepared as 1, 10 and 100 mM stock solutions and diluted to reach a final working concentration range of 1 nM to 250 μ M in the Ussing chamber bath. Concentrations were increased sequentially in the chamber bath, accounting for the residual drug concentration from the previous drug application. Tissues were allowed to reach a steady-state prior to the application of the next administered concentration. A separate group of tissues ($n = 4$) were given an equivalent volume of DMSO (vehicle) at each time point of flupirtine administration, to account for time/vehicle-dependent reduction in I_{SC} when calculating % inhibition values. The total volume of DMSO added during the experiment was 13 μ L (0.26% of bath volume). The average change in

I_{SC} after each DMSO “dose” was subtracted from the response at each corresponding concentration of flupirtine. This was done to avoid arbitrary pairing between vehicle- and flupirtine-treated tissues. % inhibition was calculated using the following equation:

$$\% \text{ inhibition of basal } I_{SC} = (\Delta I_{SC} \text{FLU}_{[X]} - \Delta I_{SC} \text{DMSO}) / \text{basal } I_{SC} \cdot 100$$

$\Delta I_{SC} \text{FLU}_{[X]}$ and $\Delta I_{SC} \text{DMSO}$ refer to the difference between basal I_{SC} and residual I_{SC} after flupirtine or DMSO application, respectively, for each concentration (X). % inhibition values were fitted to a curve using non-linear regression (log_[drug] vs response; variable slope) in GraphPad Prism 8.0 software with the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC50} - X) \cdot \text{HillSlope}))}$.

Statistical analysis

All presented data represent mean ± standard error (SEM). *n* values ranged from 4-5, depending on the experiment and are given in each of the figure legends. Statistical comparison between two treatment groups was done with an unpaired Student’s t-test, with Bonferroni correction for multiple t-tests, using GraphPad Prism 8.0 software (San Diego, CA). *p* < 0.05 was used for the determination of statistical significance (*).

Chemicals and stock solutions

Indomethacin and carbamoylcholine (carbachol; CCH) were purchased from Sigma Aldrich (St. Louis, MO). Veratridine (VER) was purchased from Alomone Labs (Jerusalem, Israel). Flupirtine (FLU), Tetrodotoxin (TTX), Vasoactive Intestinal polypeptide (VIP), Nicotine (NICO), Hexamethonium (HEX) and ICA 110381 were purchased from Tocris Pharmaceuticals (Minneapolis, MN). All drugs were prepared as 1000x stocks and diluted to their working concentrations in the Ussing chamber bath, with the exception of FLU for concentration response experiments (described above). TTX, CCH, VIP, NICO and HEX were dissolved in water.

Indomethacin was dissolved in ethanol. VER, FLU and ICA 110381 were dissolved in DMSO. When added to the Ussing chamber bath, the DMSO concentration was increased by 0.1% after each drug administration (5 μ L of DMSO into 5 mL bath).

RESULTS

Flupirtine, a K_v7 channel activator, inhibits basal Cl⁻ secretion in mouse distal colon.

Initial experiments were conducted in order to assess the effect of a non-selective K_v7 activator, flupirtine (FLU), on basal I_{SC} in ENS-intact mouse distal colon. A concentration of 100 μ M was chosen based on the previous studies involving *ex vivo* GI tissues (1,28). Application of 100 μ M flupirtine to the serosal bath inhibited I_{SC} significantly, compared to vehicle (DMSO) control (FLU vs DMSO: $\Delta I_{SC} = -87.5 \pm 10.4$ vs -14.8 ± 4.2 μ A/cm²; $p < 0.01$) (Fig. 1A-C). Inhibition of I_{SC} was not the result of impaired tissue integrity, as trans-epithelial conductance was also reduced after flupirtine administration ($\Delta G_t = -0.6 \pm 0.1$ mS/cm²). A residual steady-state I_{SC} of 57.0 ± 11.9 μ A/cm² persisted post-treatment, corresponding to Cl⁻ secretion that was not inhibited by flupirtine.

The concentration-dependence of I_{SC} inhibition in response to sequentially increasing concentrations of flupirtine in the basolateral bath was determined next (Fig. 1D). An apparent half-maximal inhibitory concentration (IC₅₀) for flupirtine was calculated to be 5.8 μ M by fitting a non-linear regression to normalized data (% inhibition of basal I_{SC}) obtained from 4 separate animals. In the experiments to follow, flupirtine was administered at 100 μ M, as this concentration produced a consistent maximal inhibition of I_{SC}.

Flupirtine and tetrodotoxin produce similar inhibitory effect on basal Cl⁻ secretion in mouse distal colon.

If the inhibitory effect of flupirtine on I_{SC} is attributable to its hyperpolarizing effects on ENS neurons via activation of K_v7 channels, then subsequent addition of an alternative ENS-targeted inhibitor following pre-treatment with flupirtine should produce minimal or no further effect. Tetrodotoxin (TTX; a Na_v channel blocker) is routinely used for the purpose of diminishing ENS-stimulated secretion in intestinal preparations (13). Because Na_v channels produce the depolarizing current necessary to generate an action potential, inhibition by tetrodotoxin renders the cell unable to propagate an action potential and thus, neurotransmitter cannot be released (6). In contrast, flupirtine induces a hyperpolarizing K^+ current, inhibiting action potential generation through the mechanism that is distinct from that of tetrodotoxin.

Flupirtine (100 μ M) and tetrodotoxin (1 μ M) both inhibited basal I_{SC} in mouse colon when administered to serosal bath (Fig. 2 A-B). G_t was also reduced by tetrodotoxin ($G_t = -0.4 \pm 0.1$ mS/cm²). Following treatment with either flupirtine or tetrodotoxin, subsequent addition of the other drug produced only minimal further inhibition. Flupirtine-mediated I_{SC} inhibition was significantly attenuated by pre-treatment with tetrodotoxin (FLU vs TTX/FLU: $\Delta I_{SC} = -75.8 \pm 13.3$ vs -9.3 ± 2.9 μ A/cm²; $p < 0.01$) (Fig. 2C). Conversely, tetrodotoxin-mediated I_{SC} inhibition was significantly attenuated by pre-treatment with flupirtine (TTX vs FLU/TTX: $\Delta I_{SC} = -65.3 \pm 17.9$ vs -3.2 ± 0.7 μ A/cm²; $p < 0.01$) (Fig. 2D). Residual I_{SC} values following either flupirtine or tetrodotoxin were not significantly different ($I_{SC} = 48.3 \pm 7.4$ vs 57.3 ± 4.7 μ A/cm², respectively; $p = 0.29$) and did not appear to be dependent on the size of the ΔI_{SC} for either flupirtine or tetrodotoxin. These findings indicate that flupirtine-induced inhibition of I_{SC} likely occurs via a tetrodotoxin-sensitive pathway.

Flupirtine modulates the stimulatory response to veratridine in mouse distal colon.

Following the observation that flupirtine completely negated tetrodotoxin-induced I_{SC} inhibition, the effect of flupirtine on the I_{SC} response to Na_V channel activation was determined next. Veratridine (VER) is a selective Na_V channel opener that induces action potential firing in neurons and triggers subsequent stimulation of the colonic epithelium, generating a secretory response (21). If flupirtine hyperpolarizes ENS neuronal membrane potential via K_V7 channel activation, then the secretory response to depolarization by veratridine may be diminished or delayed in the presence of flupirtine.

Addition of veratridine to the serosal bath induced a rapid and transient increase in I_{SC} (Fig. 3A), consistent with previous reports (21). Tissues pre-treated with flupirtine had an altered I_{SC} response to veratridine (Fig. 3B), although the magnitude of veratridine-induced secretion was not significantly reduced in flupirtine-treated tissues (VER vs FLU/VER: $\Delta I_{SC} = 66.2 \pm 11.4$ vs $41.8 \pm 8.0 \mu A/cm^2$; $p = 0.085$) (Fig. 3C). However, the time necessary to reach half-maximal activation of I_{SC} was dramatically increased in flupirtine-treated compared to untreated tissues (with vs without FLU: 4.1 ± 0.7 vs 1.3 ± 0.2 minutes; $p < 0.01$) (Fig. 3D). The observed delay in the response to veratridine may be attributable to flupirtine-induced hyper-polarizing K_V7 current opposing the depolarization induced by Na_V channel opening, resulting in a longer time period necessary to reach full stimulatory effect. This observation further suggests that flupirtine acts upon cell types which express Na_V channels (i.e. neurons).

Nicotine- and hexamethonium-induced I_{SC} responses are attenuated by flupirtine in mouse distal colon.

Nicotinic acetylcholine receptors (nAChRs) are found within the ENS, where they contribute to ganglionic transmission and resultant stimulation of epithelial secretion (19,23). As they are primarily expressed by neurons, pharmacological targeting of nAChRs provides another avenue for assessing the effect of flupirtine on stimulatory or inhibitory events that are largely (if not entirely) dependent on the ENS. Experiments were therefore conducted to determine if flupirtine interferes with the response to either activation or inhibition of nAChRs.

Serosal nicotine (NICO; nAChR agonist) application induced a robust and transient increase in I_{SC} , which was completely abolished by pre-treatment with flupirtine (NICO vs FLU/NICO: $\Delta I_{SC} = 93.0 \pm 18.9$ vs $0.3 \pm 1.1 \mu A/cm^2$; $p < 0.01$) (Figs. 4A - 4C). The response to nicotine in the presence of flupirtine was not significantly different from zero ($p = 0.82$). Hexamethonium (HEX; nAChR antagonist) administration partially reduced basal I_{SC} , while its inhibitory effect was negated by pre-treatment with flupirtine (HEX vs FLU/HEX: $\Delta I_{SC} = -51.2 \pm 6.1$ vs $-2.3 \pm 0.6 \mu A/cm^2$; $p < 0.01$) (Figs. 5A - 5C). Hexamethonium reduced G_t ($\Delta G_t = -0.5 \pm 0.1 mS/cm^2$) and pre-treatment with hexamethonium also significantly reduced I_{SC} inhibition in response to flupirtine (FLU vs HEX/FLU: $\Delta I_{SC} = -82.6 \pm 7.0$ vs $-52.0 \pm 4.3 \mu A/cm^2$; $p < 0.01$) (Fig. 5D), suggesting that at least a portion of the basal I_{SC} inhibited by flupirtine is mediated by nicotinic transmission. The remaining hexamethonium-insensitive, flupirtine-sensitive I_{SC} may be attributable to basal neurogenic secretion that is not dependent on nicotinic ganglionic transmission (e.g. serotonin- or ATP-dependent mechanisms). Nonetheless, as nAChRs have a well-established role in enteric neuronal communication, flupirtine interference with both nAChR stimulation and inhibition lends further support to flupirtine having a site of action within the ENS.

Epithelium-targeted secretagogues, VIP and carbachol, stimulate Cl⁻ secretion in the presence and absence of flupirtine in mouse distal colon.

If flupirtine inhibits stimulatory neurotransmitter release by hyperpolarizing ENS neurons, then application of exogenous epithelium-targeted secretagogues should still elicit a response in flupirtine-treated tissues. Experiments were thus performed utilizing secretagogues which target the epithelium directly by acting on basolateral receptors for endogenous neurotransmitters: vasoactive intestinal polypeptide (VIP), the endogenous ligand of VPAC receptors and carbachol (CCH), a non-selective nicotinic and muscarinic acetylcholine receptor (mAChR) agonist. VIP and carbachol were selected because VIP-ergic and cholinergic neurons were recently demonstrated to be two of the major populations of secretomotor neurons present in mouse colon (16). VIP and carbachol also stimulate Cl⁻ secretion via cAMP- and Ca²⁺-dependent pathways, respectively, representing the two primary pathways involved in epithelial Cl⁻ secretion.

As expected, VIP and carbachol both elicited a robust secretory response (Fig. 6 A,D). Pre-treatment with flupirtine did not significantly inhibit the response to VIP (VIP vs. FLU/VIP: $\Delta I_{SC} = 88.2 \pm 21.9$ vs 61.1 ± 17.0 $\mu A/cm^2$; $p = 0.36$) (Fig. 6 B-C). Flupirtine pre-treatment did partially, but significantly inhibit the response to carbachol (CCH vs FLU/CCH: $\Delta I_{SC} = 79.2 \pm 9.6$ vs 44.0 ± 3.3 $\mu A/cm^2$; $p < 0.01$) (Fig. 6 E-F), but did not abolish the response altogether, as was the case for nicotine (Fig. 4). This may be due to carbachol acting both on ENS-localized nAChRs and epithelial/neuronal mAChRs, whereas nicotine acts solely upon nAChRs. It should be mentioned that the use of a selective muscarinic receptor agonist would provide no significant advantage over a general cholinergic agonist (such as carbachol), as muscarinic receptors are expressed by ENS neurons as well as on the epithelium, making it impossible to completely distinguish between the two sites of action. Ultimately, the response to either VIP or carbachol in the presence of flupirtine

further suggests that flupirtine inhibits I_{SC} via an ENS-dependent mechanism, and not through effects on the epithelium directly.

Flupirtine does not inhibit Cl^- secretion in epithelial T84 monolayers.

T84 monolayers have been used as a model to study the colonic epithelium for several decades (57,58). The T84 cell line was originally isolated from a lung metastasis of colorectal adenocarcinoma, and consists of a homogenous population of secretory epithelium (15). Therefore, any drug acting specifically on ENS neurons should not elicit a response in T84 cells. When flupirtine was added to the serosal (basolateral) bath of T84 monolayers mounted in the Ussing chamber, no measurable I_{SC} response was produced (Fig. 7A). Forskolin (FSK) was used to stimulate I_{SC} (via adenylate cyclase activation; cAMP-induced Cl^- secretion) to confirm that the monolayers were properly developed and capable of generating a secretory response. Application of basolateral flupirtine following forskolin stimulation produced only a minor change in I_{SC} ($-0.8 \pm 0.2 \mu A/cm^2$) (Fig. 7B), although statistically greater than zero ($p < 0.01$). The response to forskolin stimulation was not significantly altered by pre-treatment with flupirtine (FSK vs FLU/FSK: $\Delta I_{SC} = 39.3 \pm 4.2$ vs $32.4 \pm 1.9 \mu A/cm^2$; $p = 0.18$) (Fig. 7C). The absence of an effect on either basal or forskolin-stimulated I_{SC} when applied to T84 cells provides additional functional support for flupirtine having a non-epithelial site of action.

Flupirtine inhibits basal and stimulated Cl^- secretion in longitudinal and circular smooth muscle stripped mouse distal colon.

Neurons in the submucosal plexus of the ENS are generally associated with epithelial secretion, while neurons of the myenteric plexus are considered to regulate motility (20). To

determine if basal I_{SC} inhibition by flupirtine was attributable to effects on the submucosal plexus specifically, flupirtine was administered to colonic tissues stripped of longitudinal and circular smooth muscle, and thus lacking the myenteric plexus. Representative images of H&E-stained unstripped (i.e. full-thickness) (Fig. 8A-B) and seromuscular-stripped (Fig. 8C-D) mouse distal colon are shown to illustrate the depth of seromuscular stripping used in these experiments. It should be noted that while these representative images depict sectioned tissues that have been folded over, tissues used for Ussing chamber experiments were flattened when mounted on the chamber sliders. Representative recordings demonstrating flupirtine-induced I_{SC} inhibition are shown for unstripped (Fig. 8E) and seromuscular-stripped tissues (Fig. 8F).

Basal I_{SC} inhibition by flupirtine was not different between unstripped and stripped colon in terms of magnitude (stripped vs unstripped: $\Delta I_{SC} = -70.4 \pm 13.4$ vs $-73.6 \pm 7.4 \mu A/cm^2$; $p = 0.802$) (Fig. 8G), or the time necessary to reach half-maximal inhibition (stripped vs unstripped: 3.5 ± 0.6 vs 2.5 ± 0.4 minutes; $p = 0.14$) (Fig. 8H). Residual I_{SC} after flupirtine treatment was also not significantly different between full-thickness and seromuscular-stripped tissues (stripped vs unstripped: $I_{SC} = 73.3 \pm 11.0$ vs $61.7 \pm 9.8 \mu A/cm^2$; $p = 0.37$).

In addition, the secretory response to nicotine was abolished by flupirtine pre-treatment in stripped tissues (NICO vs FLU/NICO: $\Delta I_{SC} = 72.1 \pm 21.9$ vs $0.8 \pm 2.3 \mu A/cm^2$; $p < 0.05$) (Fig. 9 A,C,E), similar to the previous experiments using intact tissues (Fig. 4). Hexamethonium added to the serosal bath also inhibited basal I_{SC} in stripped tissues, but this effect was attenuated by pre-treatment with flupirtine as well (HEX vs FLU/HEX: $\Delta I_{SC} = -51.4 \pm 11.4$ vs $-11.9 \pm 4.7 \mu A/cm^2$; $p < 0.05$) (Fig. 9 B,D,F). Additionally, flupirtine inhibited a portion of residual I_{SC} in tissues treated with hexamethonium ($\Delta I_{SC} = -44.1 \pm 15.1 \mu A/cm^2$) and this ΔI_{SC} value was significantly greater than 0.0 ($p = 0.04$). This suggests that flupirtine-mediated basal I_{SC} inhibition is not dependent on

the presence of the myenteric plexus. It is therefore plausible that K_V7 channels within the submucosal plexus mediated the inhibition of basal I_{SC} induced by flupirtine in the experiments described above.

$K_V7.2$ and $K_V7.3$ channel subtypes are expressed in the submucosal plexus of mouse distal colon.

Immunofluorescent labelling of $K_V7.2$ and $K_V7.3$ within the submucosa was performed, as these two channel subtypes together produce the “M” current and are widely known to regulate membrane potential in neurons. Tissues were labelled for either $K_V7.2$ or $K_V7.3$ in conjunction with microtubule-associated protein 2 (MAP2; a neuron-specific marker). Figure 10 illustrates that cells were co-labelled with $K_V7.2$ and MAP2 (Fig. 10A) or $K_V7.3$ and MAP2 (Fig. 10B), indicating that these two K_V7 subtypes are localized within the submucosal ENS neurons in mouse distal colon. $K_V7.2$ or $K_V7.3$ was detected in the majority of MAP2-positive cells. $K_V7.2$ and $K_V7.3$ were also detected in cells not labelled with MAP2, possibly corresponding to glial or smooth muscle cells. Further investigation of non-neuronal expression of $K_V7.2$ and $K_V7.3$ was not pursued in this study, but may be of interest for future work.

ICA 110381 inhibits basal Cl^- secretion in mouse colon.

Immunofluorescence studies indicated the presence of $K_V7.2$ and $K_V7.3$ proteins in submucosal neurons. Co-localization of $K_V7.2$ and $K_V7.3$ could not be determined by our immunofluorescence experiments because both primary antibodies were raised in rabbit, precluding the use of multiple fluorophore-conjugated secondary antibodies. However, the $K_V7.2/3$ heterotetramer is known to play a vital role in the function of many neuronal cell populations in central and peripheral nervous systems. ICA 110381, a selective activator for

K_V7.2/3 heterotetramers (9) was used to determine if activation of K_V7.2/3 channels would inhibit basal I_{SC} comparably to non-selective K_V7 activation by FLU. The reported EC₅₀ values for ICA110381 activation for K_V7.1 and K_V7.2/3 activation are 15 and 0.38 mM, respectively (3), indicating the drug's specificity for K_V7.2/3 heterotetramers. Addition of ICA 110381 to the serosal bath inhibited basal I_{SC} (Fig. 11A). Figure 10B shows a representative response to flupirtine for comparison. Residual I_{SC} following ICA 110381 administration was $42.8 \pm 9.2 \mu\text{A}/\text{cm}^2$. The magnitude of FLU-mediated I_{SC} inhibition appears to be slightly greater compared to ICA 110381, although this difference was not statistically significant (FLU vs ICA: $\Delta I_{SC} = -86.6 \pm 10.4$ vs. $-60.0 \pm 9.4 \mu\text{A}/\text{cm}^2$; $p = 0.09$) (Fig. 11C). Inhibition of basal I_{SC} by ICA 110381 indicates that K_V7.2/3 heterotetramers may be expressed in ENS neurons, where their activation decreases epithelial Cl⁻ secretion.

DISCUSSION

This study demonstrates that K_V7 channel activation in submucosal enteric neurons inhibits basal Cl⁻ secretion in mouse distal colon. This conclusion is supported by the following observations: 1) in Ussing chamber studies using unstripped mouse colon, flupirtine (FLU) inhibited basal I_{SC} (Fig. 1). Flupirtine also abolished or attenuated the effects of ENS-targeted drugs (tetrodotoxin, veratridine, nicotine, hexamethonium) (Figs. 2-5). However, flupirtine did not abolish the effects of epithelium-targeted drugs, VIP or carbachol (Fig. 6). 2) Flupirtine had no effect on basal or stimulated I_{SC} the epithelial T84 cell line, which lacks an ENS (Fig. 7), but inhibited basal I_{SC} in seromuscular-stripped mouse colon, which lacks a myenteric plexus but contains submucosal neurons (Fig. 8). 3) K_V7.2 and K_V7.3 channel subunits were detected in

submucosal neurons (Fig. 9), while the selective $K_v7.2/3$ activator, ICA 110381, also inhibited basal I_{SC} in mouse distal colon (Fig. 10).

Addition of flupirtine to the serosal bath inhibited basal I_{SC} (i.e., Cl^- secretion) in unstripped (full-thickness) mouse distal colon (Fig. 1A-C). The residual I_{SC} after flupirtine treatment likely corresponds to Cl^- secretion generated by the epithelium itself as part of its basal transport function. The identities of the epithelial channels and transporters which facilitate the observed Cl^- secretion (basal or residual) were not investigated here, although existing literature suggests that the basolateral co-transporter, NKCC1, and apical CFTR channel are likely responsible (32). An IC_{50} for flupirtine was calculated to be $\sim 5.8 \mu M$ (Fig. 1D). This value falls between the those reported for flupirtine in patch clamp experiments using rat hippocampal neurons ($0.6 \mu M$) (25) and in motor activity experiments using human *taenia coli* ($29.9 \mu M$) (1). Indeed, IC_{50} and EC_{50} values for drugs are generally higher in Ussing-style chamber experiments compared to other methods, owing to drug diffusion barriers, precipitation by HCO_3^- etc. (13), and the IC_{50} value of $5.8 \mu M$ calculated here lies within the expected range.

The ability of flupirtine to completely attenuate the inhibitory effect of tetrodotoxin (Fig. 2) and significantly delay the stimulatory response to veratridine (Fig. 3) indicates that flupirtine mediates its effects through cell types which express Na_v channels. Although the response to veratridine was not diminished significantly in terms of magnitude, a substantial delay in onset of secretion was observed. Interestingly, veratridine-induced I_{SC} levels did not return to baseline in the presence of flupirtine, as they did in untreated tissues. The reason for this finding is unclear but may be the result of flupirtine (hyperpolarizing K_v7 currents) preventing a tachyphylactic response to veratridine within ENS neurons, or to the subsequently-released neurotransmitters at the level of the epithelium. However, this interpretation is purely speculative.

Another major finding of this study is that nicotine and hexamethonium produced no response in flupirtine-treated tissues (Figs. 4,5 and 9), further supporting the working model of an ENS site of action for flupirtine, as nicotinic receptor (nAChR) stimulation is a generally associated with neuron-to-neuron communication. There have been reports of nAChR subtype expression in colonic epithelial cells, but their function may be related to Na^+/K^+ ATPase regulation (metabotropic) and not membrane de-polarization (ionotropic), as epithelial cells are not excitable in the classical sense (40). However, it is well-established that nAChR's play an important role in neurotransmission in the ENS (19,23). Thus, the interference of nicotinic transmission by flupirtine further supports an ENS site of action.

Vasoactive intestinal polypeptide (VIP) and carbachol both stimulate the colonic epithelium directly through VPAC1 and M_3R receptors, respectively, which are expressed on the basolateral membrane of crypt cells (5,27), and thus are not dependent on ENS neurons to mediate their effects. As predicted, both agents generated a response in the presence and absence of flupirtine (Fig. 6). Pre-treatment with flupirtine did not reduce the response to VIP, but did partially reduce the response to carbachol. This finding is still consistent with the working hypothesis because carbachol stimulates both nAChRs (neuronal) and mAChRs (neuronal and epithelial). The decrease in carbachol-stimulated I_{SC} in flupirtine-treated tissues is expected because nAChR activation by carbachol had presumably been negated by the hyperpolarizing effect of flupirtine, while stimulation of epithelial M_3R remains intact. Enteric neurons also express VPAC receptors and VIP signaling is important for neuron-glia communication as well (17). However, under these conditions, any inter-neuronal or glial component to VIP-stimulated Cl^- secretion appears to be unaffected by pre-treatment with flupirtine.

Previous reports have identified K_V7 channel proteins in the myenteric plexus, as well as within GI smooth muscle (28,49). Other K_V7 channel activators (e.g. retigabine) have been shown to reduce colonic motility and smooth muscle contraction – functions generally associated with the myenteric plexus and the smooth muscle itself, which also expresses K_V7 channels (28). Here we find evidence of K_V7 channel subunits, K_V7.2 and K_V7.3, in submucosal neurons (Fig. 10). Additionally, a selective K_V7.2/3 activator, ICA 110381, also inhibited I_{SC} in mouse distal colon (Fig. 11). K_V7.2/3 channels are known to regulate the function of other neuronal cell types (51). Our findings suggest they may modulate ENS-driven Cl⁻ secretion in the colon as well. This, coupled with previous reports showing efficacy of K_V7 channel activators in attenuating GI-localized pain (49), as well as reducing smooth muscle contractile activity (28), make K_V7 channels an attractive target for drug development. Future studies should be directed at characterizing the expression of specific K_V7 proteins and their homo/heterotetrameric assembly within enteric neurons, as well as identifying whether flupirtine/ICA-sensitive channels are confined to a particular neuronal subpopulation.

Another member of the K_V7 family, K_V7.1, has been well-characterized in terms of colonic epithelial cell function. K_V7.1 is expressed on the basolateral membrane of crypt epithelial cells, where its activation supports sustained apical Cl⁻ secretion by providing an electrochemical driving force for apical Cl⁻ exit (31,42). While flupirtine is a “non-selective” K_V7 activator, and may therefore act upon epithelium-localized K_V7.1, in theory this would *increase* Cl⁻ secretion by creating an electrical gradient favoring Cl⁻ efflux. In this study, the opposite effect was observed, suggesting that flupirtine either 1) does not stimulate Cl⁻ secretion by activating basolateral K_V7.1, or 2) any stimulatory effect is overshadowed by the inhibitory effects on ENS-driven secretion as described above. Moreover, flupirtine had no effect on baseline Cl⁻ secretion and did not enhance

or alter the effect of forskolin stimulation in T84 cells (Fig. 7). T84 cells therefore may not express flupirtine-sensitive K_V7 channels, or their activation does not alter basal or forskolin-stimulated secretion under these experimental conditions.

Interestingly, the anti-convulsant, retigabine – a structural analogue of flupirtine – was found to have no effect on K_V7.1 channels in cardiac cells, and preferentially activates K_V7.2 – K_V7.5 (44). Unfortunately, there is little data available regarding the sensitivity of each K_V7 family subunit (K_V7.1 – K_V7.5) to flupirtine, particularly in peripheral neural networks, such as the ENS. However, it is possible that K_V7.1 is less sensitive or insensitive to flupirtine as well, given the similar structural and pharmacological profiles of the two drugs (44,33). K_V7.1 is also known to associate with a regulatory β -subunit, KCNE3, in intestinal crypt cells (7,37). This association may also have some bearing on the apparent insensitivity of epithelial K_V7.1 to flupirtine in the experiments described here.

Flupirtine is considered to be a non-steroidal, non-opioid analgesic (35). Investigation into its analgesic properties has revealed several possible mechanisms and sites of action besides direct activation of K_V7 channels. These possible effects include indirect antagonism of *N*-methyl-*D*-aspartate (NMDA) receptors through voltage-dependent Mg²⁺ block resulting from K_V7-mediated hyperpolarizing current (29), as well as direct activation of G-protein-activated inward rectifying K⁺ (GIRK) channels (26) and γ -aminobutyric acid (GABA) receptors (35). As the concentration of flupirtine used in the present study was relatively high (100 μ M), the possible involvement of alternative mechanisms, such as those listed above, cannot be excluded. Nonetheless, if flupirtine has a suppressive effect on ENS neuronal excitability which is partially attributable to one or more of these alternative mechanisms, the end result of reduced stimulatory input to the epithelium would be effectively equivalent in terms of measured secretory activity within the epithelium. A

detailed pharmacanalysis of these “off-target” effects, specifically in ENS neurons, should be conducted in future studies to determine the exact nature of the flupirtine’s apparent suppressive effects on neurogenic secretion.

Previous work has demonstrated that several gastrointestinal disorders, including IBS-D, result in an overall increase in the excitability of ENS neurons (24,38,41,48). However, current clinical options are limited for these pathologies, with many observed adverse effects (18,47). Flupirtine had been available for nearly three decades in several European countries for the treatment of chronic pain, as well as neurological disorders such as epilepsy, before being recently removed from the market due to issues of hepatotoxicity (44,35,45). Additionally, flupirtine has been associated with a wide variety of generalized GI-related side effects, including constipation, diarrhea, abdominal pain and nausea (55). To our knowledge, there has not been an assessment to determine whether patients with pre-existing GI disorders (such as IBS) reported any of these side effects differentially (i.e. whether they were more or less likely to experience constipation or diarrhea, etc.). However, given that flupirtine is generally well-tolerated, the use of K_v7 channel activators (whether flupirtine, ICA-110381 or a novel related compound) may prove to be beneficial in treating patients that have been unresponsive to traditional treatments for IBS-D, and these possibilities should be explored in the future.

In summary, our results demonstrate that activation of K_v7 channels significantly inhibits epithelial Cl⁻ secretion in mouse distal colon. This effect is likely mediated through K_v7 channels located within enteric neurons of the submucosal plexus, which decrease neuronal excitability and prevent stimulatory input to the epithelium. This discovery may have clinical significance with respect to future development of therapeutic agents to reduce ENS hyper-excitability in gastrointestinal pathologies, such as IBS-D.

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FIGURES AND LEGENDS

Figure 1. Flupirtine inhibits basal I_{SC} in mouse distal colon in a concentration-dependent manner in mouse distal colon. Representative I_{SC} recording of unstripped mouse distal colon treated with 100 μM flupirtine (FLU) (A) or vehicle (DMSO) (B) in the serosal bath. C: Group data showing the change in I_{SC} (ΔI_{SC}) in response to FLU or DMSO treatment. D: FLU concentration-response curve fitted using non-linear regression. Half-maximal inhibitory concentration (IC_{50}) for FLU was calculated to be 5.8 μM . As mentioned above, correction was applied for time and for vehicle administration for subsequent concentrations of flupirtine, based upon I_{SC} recordings from adjacent tissues ($n=4$) treated only with DMSO at each corresponding time/concentration point. Those values, subtracted from the $DI_{SC(FLU)}$ from 0.1, 1, 5, 10, 50, 100

and 250 μM flupirtine concentrations, were 5.0, 16.3, 22.3, 31.5, 45.3, 47.5 and 50.5 $\mu\text{A}/\text{cm}^2$, respectively. These adjusted values were used for fitting of the concentration/response curve. Additionally, the “top” and “bottom” parameters of the non-linear fit were calculated to be 44.6 ± 2.3 and 6.4 ± 3.1 $\mu\text{A}/\text{cm}^2$, respectively. The “bottom” parameter value (6.4 ± 3.1 $\mu\text{A}/\text{cm}^2$) was not significantly different from 0.0 ($p = 0.11$). The “Hill slope” parameter was calculated as 1.18 ± 0.4 , and this was not significantly different from 1.0 ($p = 0.60$). Data are presented as mean $\pm$ SEM from 4 separate animals. $*p < 0.05$.

Figure 2. Flupirtine and tetrodotoxin inhibit basal I_{SC} in a similar and non-additive manner in mouse distal colon. *A*: Representative I_{SC} recording of unstripped mouse distal colon treated with 100 μM flupirtine (FLU), followed by 1 μM tetrodotoxin (TTX) in the serosal bath. *B*:

Representative I_{SC} recording of unstripped mouse distal colon treated with 1 μ M TTX, followed by 100 μ M FLU in the serosal bath. *C*: Comparison of the change in I_{SC} induced by 100 μ M FLU ($\Delta I_{SC(FLU)}$) in the presence and absence of 1 μ M TTX. *D*: Comparison of the change in I_{SC} induced by 1 μ M TTX ($\Delta I_{SC(TTX)}$) in the presence and absence of 100 μ M FLU. Data are presented as mean $\pm$ SEM from 5 different animals. * $p < 0.05$.

Figure 3. Flupirtine delays the response to veratridine in mouse distal colon. *A*: Representative I_{SC} recording of unstripped mouse distal colon treated with 10 μ M veratridine (VER) in the serosal bath. *B*: Representative I_{SC} recording of unstripped mouse distal colon treated with 10 μ M VER in the presence of 100 μ M flupirtine (FLU). *C*: Comparison of the change in I_{SC} induced by 10 μ M VER ($\Delta I_{SC(VER)}$) in the presence (+) and absence (-) of 100 μ M FLU. *D*: Comparison of the time to half-maximal activation following VER administration in the presence (+) and absence

(-) of 100 μ M FLU. Data are presented as mean $\pm$ SEM from 5 different animals. * $p < 0.05$.

Figure 4. Flupirtine abolishes the response to nicotine in mouse distal colon. *A*: Representative I_{SC} recordings of unstripped mouse distal colon treated with 100 μ M nicotine (NICO) in the serosal bath. *B*: Representative I_{SC} recording of unstripped mouse distal colon treated with 100 μ M NICO in the presence of 100 μ M flupirtine (FLU). *C*: Comparison of the change in I_{SC} induced by 100 μ M NICO ($\Delta I_{SC(NICO)}$) in the presence (+) and absence (-) of 100 μ M FLU. Data are presented as mean $\pm$ SEM from 5 different animals. * $p < 0.05$.

Figure 5. Flupirtine abolishes the response to hexamethonium in mouse distal colon. *A:* Representative I_{SC} recordings of unstripped mouse distal colon treated with 100 μ M hexamethonium (HEX), followed by 100 μ M flupirtine (FLU) in the serosal bath. *B:* Representative I_{SC} recordings of unstripped mouse distal colon treated with 100 μ M HEX in the presence of 100 μ M FLU. *C:* Comparison of the change in I_{SC} induced by 100 μ M HEX ($\Delta I_{SC(HEX)}$) in the presence (+) and absence (-) of 100 μ M FLU. *G:* Comparison of the change in I_{SC} induced by 100 μ M FLU ($\Delta I_{SC(FLU)}$) in the presence (+) and absence (-) of 100 μ M HEX. Data are presented as mean $\pm$ SEM from 5 different animals. * $p < 0.05$.

Figure 6. Flupirtine does not abolish the response to VIP or carbachol in mouse distal colon.

A: Representative I_{SC} recordings of unstripped mouse distal colon treated with 100 nM vasoactive intestinal polypeptide (VIP) in the serosal bath. *B:* Representative I_{SC} recordings of full-thickness mouse distal colon pre-treated with 100 μM flupirtine (FLU), followed by subsequent addition of 100 nM VIP to the serosal bath. *C:* Comparison of the change in I_{SC} induced by 100 nM VIP ($\Delta I_{SC(VIP)}$) in the presence (+) and absence (-) of 100 μM FLU. *D:* Representative I_{SC} recordings of unstripped mouse distal colon treated with 100 μM carbachol (CCH) in the serosal bath. *E:* Representative I_{SC} recordings of unstripped mouse distal colon pre-treated with 100 μM FLU, followed by subsequent addition of 100 μM CCH to the serosal bath. *F:* Comparison of the change in I_{SC} induced by 100 μM CCH ($\Delta I_{SC(CCH)}$) in the presence (+) and absence (-) of 100 μM FLU. Data are presented as mean $\pm$ SEM from 5 different animals. * $p < 0.05$.

Figure 7. Flupirtine does not inhibit Cl^- secretion in T84 Monolayers. *A*: Representative I_{SC} recording from T84 monolayers that were treated with basolateral 100 μM flupirtine (FLU), followed by 10 μM forskolin (FSK). *B*: Representative I_{SC} recording from T84 monolayers that were treated with 10 μM FSK, followed by 100 μM FLU. *C*: Comparison of the change in I_{SC} induced by 10 μM FSK ($\Delta I_{SC(FSK)}$) in the presence (+) and absence (-) of 100 μM FLU. Data are presented as mean $\pm$ SEM from 4 different experiments. $*p < 0.05$.

Figure 8. Flupirtine inhibits basal Cl⁻ secretion in both full-thickness and seromuscular-stripped mouse distal colon. Hematoxylin and eosin stained mouse distal colonic tissue in unstripped (*A-B*) and seromuscular-stripped (*C-D*) preparations. Brackets in panels A and B mark the muscularis propria in unstripped tissues which is absent in seromuscular-stripped preparations. Brackets in panel B show the outer longitudinal and inner circular smooth muscle layers, with arrowheads indicating the presence of myenteric ganglia between these two layers. Scale bars = 50 μ m. Representative I_{SC} recording of unstripped (*E*) and seromuscular-stripped (*F*) mouse distal colon treated with 100 μ M flupirtine (FLU) in the serosal bath. *G*: Comparison of the change in I_{SC} induced by 100 μ M FLU (Δ I_{SC(FLU)}) in unstripped and seromuscular-stripped mouse distal colon. *H*: Comparison of the time necessary to reach half-maximal inhibition following basolateral addition of 100 μ M FLU in unstripped and seromuscular-stripped mouse distal colon. Data are presented as mean $\pm$ SEM from 5 different animals. **p* < 0.05.

Figure 9. Flupirtine inhibits the response to nicotine and hexamethonium in seromuscular-stripped mouse distal colon. Representative I_{SC} recording of stripped mouse distal colon treated with 100 μM nicotine (NICO) (A) and 200 μM hexamethonium (HEX) (B) in the serosal bath. Representative I_{SC} recordings of stripped mouse distal colon pre-treated with 100 μM flupirtine (FLU), followed by subsequent addition of 100 μM NICO (C) or 200 μM HEX (D) to the serosal

bath. Comparison of the change in I_{SC} induced by 100 μ M NICO or 200 μ M HEX in the presence (+) and absence (-) of 100 μ M FLU. Data are presented as mean $\pm$ SEM from 5 different animals. $*p < 0.05$.

Figure 10. K_V7.2 and K_V7.3 channel subtypes are expressed in the submucosal plexus of mouse distal colon. Immunofluorescent labelling of whole-mount preparation of mouse distal colon submucosa, captured at 20x magnification. *Row A:* Whole-mount submucosa labelled for K_V7.2 (green), microtubule-associated protein 2 (MAP2; red), and nuclei (DAPI; blue), as well as a merge of the red, green and blue channels. *Row B:* Whole-mount submucosa labelled for K_V7.3 (green), microtubule-associated protein 2 (MAP2; red), and nuclei (DAPI; blue), as well as a merge of the red, green and blue channels. Scale bars = 20 μ m.

Figure 11. ICA 110381, a specific activator of $K_v7.2/3$, inhibits basal Cl^- secretion in mouse distal colon. *A*: Representative I_{SC} recording of unstripped mouse distal colon treated with 100 μM ICA 110381 (ICA) in the serosal bath. *B*: Representative I_{SC} recording of unstripped mouse distal colon treated with 100 μM flupirtine (FLU) in the serosal bath. *C*: Comparison of the change in I_{SC} (ΔI_{SC}) induced by 100 μM ICA and 100 μM flupirtine (FLU). Data are presented as mean $\pm$ SEM from 5 different animals.

CHAPTER 3

TITLE: Flupirtine enhances NHE-3 mediated Na⁺ absorption in rat colon via an ENS-dependent mechanism

RUNNING TITLE: Flupirtine enhances colonic Na⁺ absorption

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ABSTRACT

Recent studies in our lab have shown that the K_v7 channel activator, flupirtine, inhibits colonic epithelial Cl^- secretion through effects on submucosal neurons of the enteric nervous system (ENS). We hypothesized that flupirtine would also stimulate Na^+ absorption as a result of reduced secretory ENS input to the epithelium. To test this hypothesis, unidirectional $^{22}Na^+$ fluxes were measured under voltage-clamped conditions. Pharmacological approaches using an Ussing-style recording chamber, combined immunofluorescence microscopy techniques were used to determine the effect of flupirtine on active Na^+ transport in the rat colon. Flupirtine stimulated electroneutral Na^+ absorption in partially seromuscular stripped colonic tissues, while simultaneously inhibiting short circuit current (I_{SC} ; i.e., Cl^- secretion). Both of these effects were attenuated by pre-treatment with the ENS inhibitor, tetrodotoxin. The NHE-3-selective inhibitor, S3226, significantly inhibited flupirtine-stimulated Na^+ absorption whereas the NHE-2-selective inhibitor HOE-694 did not. NHE-3 localization near the apical membranes of surface epithelial cells was also more apparent in flupirtine-treated colon versus control. Flupirtine did not alter epithelial Na^+ channel (ENaC)-mediated Na^+ absorption in distal colonic tissues obtained from hyperaldosteronaemic rats and had no effect in the normal ileum, but did stimulate Na^+ absorption in the proximal colon. Finally, the parallel effects of flupirtine on I_{SC} (Cl^- secretion) and Na^+ absorption were significantly correlated with each other. Together, these data indicate that flupirtine stimulates NHE-3-dependent Na^+ absorption, likely as a result of reduced stimulatory input to the colonic epithelium by submucosal ENS neurons.

NEW AND NOTEWORTHY: We present a novel mechanism regarding regulation of epithelial ion transport by enteric neurons. Activation of neuronal K_v7 K^+ channels markedly stimulates Na^+ absorption and inhibits Cl^- secretion across the colonic epithelium. This may be useful in developing new treatments for diarrheal disorders, such as irritable bowel syndrome with diarrhea (IBS-D).

KEY WORDS: enteric nervous system; Ussing chamber; IBS-D; KCNQ.

INTRODUCTION

The epithelium of the colon plays a critical role in maintaining overall electrolyte and fluid homeostasis (1,2,3,4). Although the total volume of absorbed water is typically less than in the small intestine, the colon possesses a hugely dynamic range in absorptive capacity and readily adapts its electrolyte transport activity to fit the needs of the organism (5). Having tremendous influence over this activity is the enteric nervous system (ENS), one of the most important and powerful regulators of epithelial ion transport (6,7,8,9). Na^+ , Cl^- and water are actively absorbed under basal conditions, but the release of pro-secretory neurotransmitters from ENS neurons can induce a robust efflux of Cl^- and HCO_3^- from the crypt cells (10,11), driving water into the gut lumen through osmosis. At the same time, ENS-driven secretion is augmented by the concurrent inhibition of basal absorptive processes, such as Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange in surface epithelial cells (12,13).

Recent work in our lab has revealed that pharmacological activation of voltage-gated $\text{K}_\text{V}7$ (KCNQ) K^+ channels in the ENS inhibits neurogenic Cl^- secretion significantly within the colon (14). This is likely a result of suppressed activity of secretomotor neurons, as $\text{K}_\text{V}7$ channels mediate a hyperpolarizing K^+ current that reduces excitability and action potential firing (15,16,17). The importance of these channels has been highlighted within many central and peripheral circuits (18,19,20), and $\text{K}_\text{V}7$ channel activators have been used clinically to treat chronic pain and epilepsy – both of which are related to neuronal hyper-excitability (16,21,22). However, the role of $\text{K}_\text{V}7$ channels in the ENS with respect to epithelial transport remains incompletely understood.

Many common gastrointestinal (GI) disorders, such as irritable bowel syndrome with diarrhea (IBS-D), are related to a hyperexcitable ENS (6,11,23,24). IBS-D, while not usually life-

threatening, presents a massive economic burden to the healthcare systems in the U.S. and abroad (25). Many current treatment options that target ENS activity are effective in relieving symptoms but are commonly associated with adverse side effects (26,27,28). Thus, development of improved therapeutic strategies for IBS-D management is of great clinical importance. Gaining a deeper understanding of epithelial transport modulation by K_V7 channels in the ENS may provide new avenues for treatment, as the use of K_V7 activators has not yet been explored in this context.

In light of the recent evidence concerning the effects on neurogenic Cl⁻ secretion (14), we predicted that activation of K_V7 channels in the ENS would also enhance Na⁺ absorption simultaneously, as a result of diminished stimulatory ENS input. Na⁺ absorption – in particular, the activity of the predominant electroneutral Na⁺/H⁺ exchanger isoform 3 (NHE-3) – is known to be active at rest and inhibited by various ENS-derived neurotransmitters (29). We therefore tested the hypothesis that the K_V7 channel activator, flupirtine, stimulates active Na⁺ absorption in the colon. Unidirectional ²²Na⁺ fluxes and short-circuit current (I_{SC}) were measured under voltage-clamped conditions in an Ussing chamber system. Our findings suggest that active Cl⁻ secretion is inhibited, while NHE-3-mediated Na⁺ absorption is enhanced by activation of K_V7 channels within the ENS.

METHODS

Animals

Non-fasting male Sprague-Dawley rats (150-200g) were housed 2 per cage in climate-controlled rooms under 12-hour light/dark cycles and given free access to standard chow and water. One group of rats was given a substitute Na⁺-depleted diet for 7 consecutive days to induce secondary hyperaldosteronism, as has been described previously (30,31). Rats given Na⁺-depleted

diet were also allowed free access to water. Dietary Na^+ depletion was used to study the effect of ENS K_v7 activation specifically on *electrogenic* versus *electroneutral* epithelial Na^+ absorption, as electroneutral (primarily NHE-3-mediated) absorption is down-regulated while electrogenic (epithelial Na^+ channel; ENaC-mediated) absorption is induced in hyperaldosteronaemic rats.³² On the day of experiments, all rats were anesthetized with 5% isoflurane balanced with oxygen and maintained under a deep plane of anesthesia with 2-3% isoflurane delivered via nose cone. The colon was removed by cutting away mesenteric attachments and excising the tissue from caecum to rectum. Colons were immediately flushed with ice-cold modified Krebs solution containing the following, in mM: 140 Na^+ , 5.2 K^+ , 1.2 Ca^{2+} , 1.2 Mg^{2+} , 119.8 Cl^- , 25 HCO_3^- , 2.4 HPO_4^{2-} , 0.4 H_2PO_4^- , and 10 glucose. Krebs solution was bubbled with 5% CO_2 balanced with oxygen and the was adjusted to pH 7.41. After removal of the colon, rats were euthanized by cutting the diaphragm and the colon was used for down-stream Ussing chamber, immunofluorescence, or Western blotting experiments. For some experiments, the ileum was also removed and flushed in the same manner. All experimental procedures were approved by West Virginia University Animal Care and Use Committee.

Previous work in our lab regarding the effects of K_v7 activation in the ENS has been conducted using mice. In the present study, rats were chosen as the primary model system for several reasons: 1) to establish that the effect(s) of K_v7 activation on colonic epithelial transport are not unique to the mouse, 2) to allow for more substantiated interpretation of the findings regarding the modulation of Na^+ transport, as much of the previous work in field of colonic Na^+ transport has been conducted in rats (23,33,34,35,36), and 3) to minimize the number of required animals, as the larger surface area of the rat colon permits the use of multiple tissue segments per animal for unidirectional flux experiments. In addition, only male rats were used in this study

because many aspects of colonic ion transport are markedly different in females versus males, likely owing to modulation by sex steroids (37,38,39). Future studies will be aimed at exploring any sex differences in the response to flupirtine or other ENS-modulating drugs.

Ussing chamber experiments

After flushing, the colon was opened longitudinally along the mesenteric border and placed mucosal side down in a dissecting dish, submerged in ice-cold bubbled Krebs solution. The serosa, longitudinal and circular smooth muscle layers were gently stripped away first by making a lateral incision at the junction between proximal and distal segments (i.e., at the distal end of the proximal striations). Muscle and serosal layers were then peeled back using a glass slide and fine forceps to obtain a mucosa/muscularis mucosae/submucosa preparation. Tissues prepared in this way contain functional submucosal neurons, but lack the myenteric plexus altogether. Colonic segments were then mounted on 1.1cm² sliders with a circular aperture for use in an Ussing-style recording chamber (Physiologic Instruments, San Diego, CA). Mounted tissues were bathed bilaterally in 5mL of cold Krebs buffer, bubbled with 5% CO₂ balanced with oxygen and gradually warmed to 37° C. For experiments using proximal colon, the tissue was prepared the same way as described here using segments of proximal colon characterized by striation patterns. For experiments using ileum, tissues were partially stripped and mounted on 0.3 cm² oval aperture sliders. This difference in slider aperture was corrected for in the electrical recordings and Na⁺ flux calculations described below.

Chambers were equipped with a pair voltage-sensing AgCl pellet electrodes and current-injecting AgCl wire electrodes connected to the chamber bath via agar-salt bridge (3.5% agar in 3M KCl) embedded in the electrode tips. After mounting, tissues were allowed ~15 minutes to

equilibrate and be brought up to temperature under open circuit conditions, during which time trans-epithelial potential (V_{TE}) was monitored. Tissues were then voltage-clamped to 0 mV using a multi-channel voltage clamp/amplifier (VCC MC8; Physiologic Instruments) and short-circuit current (I_{SC}) was continuously monitored by automated computer software (Acquire & Analyze; Physiologic Instruments). 5 mV, 200 ms bipolar pulses were applied at 30 sec intervals to monitor trans-epithelial conductance (G_{TE}). By convention, positive I_{SC} values reflect net anion (Cl^-/HCO_3^-) secretion and/or cation (Na^+) absorption; negative I_{SC} values reflect net anion absorption or cation (K^+) secretion.

Unidirectional $^{22}Na^+$ fluxes were performed by first assigning tissue pairs with G_{TE} values within 15% of each other. 2 μCi of $^{22}Na^+$ was then added to either the serosal (basolateral) or mucosal (apical) chamber bath and allowed 25 minutes to reach a steady-state rate of flux. 250 μL samples were taken in duplicate from the opposite (“cold”) chamber bath at the beginning and end of each 30-minute flux period and replaced with 500 μL of fresh buffer. Drugs were added as 1000x stock solutions, administered in 5 μL volumes to the 5mL chamber bath immediately following sample collection. Drug concentration and placement details are given in the figure legends. At the end of each experiment, 50 μL samples were taken from the “hot” chamber bath (where $^{22}Na^+$ tracer was added) to obtain a standard value used in the calculation of Na^+ flux rate. Samples were then mixed with liquid scintillation cocktail (Sigma Aldrich, #03999-5L) and counted using a Tri-carb 4910TR liquid scintillation counter (Perkin Elmer, Waltham MA). Unidirectional $^{22}Na^+$ flux rates were calculated from the measured counts per minute (cpm) values using the following equation:

$$Na^+ \text{ flux } (J) (\mu Eq/cm^2 \cdot hr) = (5 \times B - [A \times 0.9]) / ([S/140] \times 1.1 \times 0.5)$$

“A” and “B” represent cpm/mL values measured from the beginning and end of a flux period, respectively. 5 is the total volume (in mL) of the chamber bath and 0.9 is dilution factor accounting for removal of 500 μ L of bath volume upon sample collection. “S” represents the cpm/ μ Eq (in 1 mL) measured from the standard obtained from the “hot” bath of the chamber. 1.1 corresponds to the slider aperture (in cm^2) and 0.5 is the time (in hours) for each flux period (40). Net Na^+ fluxes were calculated by subtracting the serosal to mucosal ($S-M$) from the mucosal to serosal ($M-S$) fluxes from paired tissues for a given flux period.

Immunofluorescence microscopy

To identify the presence of flupirtine-sensitive $\text{K}_{\text{v}}7$ channels in submucosal neurons, segments of mid-distal colon were dissected under a microscope to obtain a submucosal preparation, as described previously (14). First, the colon was opened longitudinally and pinned mucosal side down on a dissecting stage. A shallow lateral incision was made near the end of the striations of the proximal colon to transect the longitudinal and circular smooth muscle layers, which were then peeled away in a distal direction until completely removed. The tissue was then inverted and pinned mucosal side up. Another lateral incision was made near the rectum, just deep enough to isolate the mucosa/muscularis mucosae from the underlying submucosal layer. The mucosal/muscularis mucosae was removed by carefully creating a plane of separation using ultra-fine forceps, and then gently rubbing in a proximal direction with the index finger. The remaining submucosal layer was immediately fixed on 4% paraformaldehyde (PFA) dissolved in phosphate buffered saline (PBS) overnight at 4° C.

The following day, the tissue was permeabilized with PBS + 1% Triton-X + 5% goat serum for 1 hour at room temperature and blocked for an additional 30 minutes in PBS + 0.01% Triton-

X (PBST) + 5% goat serum. The tissue was then incubated in PBST + 5% goat serum containing the primary antibodies: mouse anti-microtubule associated protein 2 (MAP-2; neuronal marker) (Millipore #MAB3418; 1:200 dilution) (41) and rabbit anti-KCNQ2 (flupirtine-sensitive K_v7 channel subtype) (Thermo #PA1-929; 1:100 dilution) (20) overnight at 4° C. The next day, the tissue was washed 5 times for 5 minutes each in PBST before incubation in PBST + 5% goat serum containing AlexaFluor 488 goat anti-rabbit (Thermo #A11008; 1:2,000 dilution) and AlexaFluor 568 goat-anti-mouse (Thermo #A11031; 1:2,000 dilution) for 30 minutes at room temperature, after which it was again washed 5 times for 5 minutes each in PBST. The tissue was then rinsed briefly in water and placed flat on a charged glass microscope slide. SlowFade Diamond mountant with DAPI (Thermo #36971) was used to mount a coverslip on the slide, which was then sealed with clear fingernail polish. Images were captured using a Zeiss 710 confocal microscope under 20x magnification (Carl Zeiss, Oberkochen, Germany).

For immunofluorescence experiments to visualize NHE-3 localization in the epithelium, intact (unopened) colons were stripped of longitudinal and circular smooth muscle and incubated in bubbled Krebs solution with or without flupirtine for 10 minutes at 37° C. The tissues were then immediately fixed in pre-warmed 4% PFA in PBS for 15 minutes. After washing 3 times in cold PBS, tissues were flash frozen in isopentane cooled with liquid nitrogen and embedded in tissue freezing medium (Scigen #4586, Gardena, CA). 10 µm sections were cut with a cryostat and mounted on charged glass slides. Sections were permeabilized in PBS + 0.1% Triton-X + 5% goat serum for 20 minutes at room temperature, washed briefly in PBST and blocked in PBST + 5% goat serum for an additional 30 minutes. Next, sections were incubated in PBST+ 5% goat serum containing NHE-3-specific primary antibody (Abcam #95299; 1:100 dilution) (39) for 1 hour at 37° C in a humidifying chamber. The sections were subsequently washed 5 times or 5 minutes

each and incubated in PBST+ 5% goat serum containing AlexaFluor 488 goat anti-rabbit (Thermo #A11008; 1:2,000 dilution) and TRITC-conjugated phalloidin (filamentous actin labelling) (Thermo #R415; 1:1,000 dilution) for 30 minutes at room temperature. Finally, sections were washed again 5 times for 5 minutes each and mounted using SlowFade Diamond mountant with DAPI (Thermo #36971). Slides were sealed with clear fingernail polish and imaged using a Zeiss 710 confocal microscope under 20x magnification (Carl Zeiss, Oberkochen, Germany).

Western blotting

Colons from normal or dietary Na⁺-depleted rats were flushed with ice-cold Krebs solution to remove fecal contents. A clamp was then placed on the distal end of the colon, near the rectum, and the colons were filled with ice-cold epithelial isolation solution containing the following, in mM: 30 NaCl, 8 HEPES-Tris (pH 7.5), 5 ethylenediaminetetraacetic acid (EDTA), 1 phenylmethylsulphonyl fluoride (PMSF), and 0.5 dithiothreitol (DTT). After filling, the colons were secured on the proximal end with a small ligature and incubated in this same solution on ice for 30 minutes. Then the colons were emptied, opened longitudinally along the mesenteric border and scraped with a glass slide to remove the mucosa, which was flash frozen in liquid nitrogen and stored at -80° C until use.

Proteins were extracted from the tissues by homogenizing in ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer and briefly sonicating to disrupt membranes. Samples were centrifuged for 20 minutes at 12,000 x g and the supernatants were collected and mixed with 4x Laemmli buffer. The samples were then heated to 90° C for 5 minutes and immediately chilled on ice before adding 2-mercaptoethanol (5% w/v). Proteins were resolved on 8% polyacrylamide gels and transferred to PVDF membranes, which were subsequently blocked

in 3% bovine serum albumin (BSA) in tris-buffered saline plus 0.1% Tween-20 (TBST) for 1 hour at room temperature. Membranes were then incubated overnight in TBST plus 3% BSA solution containing rabbit anti-NHE-3 (Abcam #95299; 1:500 dilution) or horseradish peroxidase (HRP)-conjugated mouse anti- β -actin (SCBT #SC-47778; 1:1,000 dilution) primary antibody. The following day, membranes were washed in TBST 5 times for 5 minutes each. Membranes that were probed for NHE-3 were then incubated in HRP-conjugated goat anti-rabbit secondary antibody (Thermo #31462) for 1 hour at room temperature and subsequently washed an additional 5 times for 5 minutes each. Immune complexes were detected via chemiluminescence using West Dura extended duration substrate (Thermo #34075) and imaged on a G-box. Protein band intensity was quantified using FIJI (Image J) software.

Drugs and chemicals

Flupirtine, vasoactive intestinal polypeptide (VIP) and tetrodotoxin were purchased from Tocris (Minneapolis, MN). Veratridine was purchased from Alomone labs (Jerusalem, IL). Amiloride and S3226 were purchased from Sigma Aldrich (St. Louis, MO). HOE694 was a generous gift from Dr. Selvi Krishnan. All other chemicals and salts were purchased either from Sigma Aldrich or Fisher Scientific (Waltham, MA).

Data analysis

I_{SC} and G_{TE} values were averaged from each tissue over the final 5 minutes for a given flux period when used for comparison between treatment groups or values obtained from consecutive flux periods unless otherwise noted. Duplicate cpm values were averaged for use in the calculation of unidirectional $^{22}\text{Na}^+$ flux for a given 30-minute period. Evaluation of statistical significance was determined using GraphPad Prism 9 software, via unpaired Student's t-test for comparison

between two treatment groups/flux periods, or via one-way analysis of variance (ANOVA) with Tukey's *post-hoc* when comparing more than two treatment groups/flux periods. Statistical test details are given in the figure legends. For correlation analysis of change in I_{SC} (ΔI_{SC}) versus change in M-S Na^+ (ΔJ_{M-S}) flux, Pearson's r coefficient was determined using product-moment correlation analysis in GraphPad Prism 9 software. Statistical significance was defined as $p < 0.05$ for all analyses.

RESULTS

K_v7 channel activator, flupirtine, attenuates neurogenic secretion in rat distal colon.

Previously studies in our lab have shown that electrogenic Cl^- secretion (measured as I_{SC}) was dramatically inhibited in mouse colon by the application of flupirtine to the serosal side of the tissue. Pre-treatment with flupirtine also blunted or abolished the effects of neural-targeted secretory agents, veratridine (Na_v channel opener) and nicotine (nAChR agonist), while sparing the effects of the epithelium-targeted secretagogues VIP (VPAC receptor agonist) and carbachol (non-selective cholinergic agonist). Figure 1 demonstrates that a similar pattern is observed in the rat colon. Segments of mid-distal colon pre-treated with flupirtine (100 μM) exhibited markedly reduced basal I_{SC} compared to control (untreated vs. FLU: 18.6 ± 1.2 vs. $121.2 \pm 11.6 \mu A/cm^2$; $p < 0.01$) (Fig. 1A-B). Flupirtine-treated tissues responded to serosal VIP (untreated vs. FLU: $\Delta I_{SC(VIP)} = 84.1 \pm 16.0$ vs. $94.1 \pm 15.8 \mu A/cm^2$; $p = 0.67$) (Fig. 1D), but displayed a significantly diminished response to serosal veratridine (untreated vs. FLU: $\Delta I_{SC(VER)} = 69.5 \pm 11.5$ vs. $24.9 \pm 4.0 \mu A/cm^2$; $p < 0.01$) (Fig. 1C). Labelling of $K_v7.2$ in MAP-2⁺ cells of rat submucosal ganglia (Fig. 1E) confirmed the presence of flupirtine-sensitive channels in these tissues, consistent with those observed in mouse. These preliminary studies confirm that the effects of flupirtine are similar

between mouse and rat with respect to neurogenic secretion and support the use of the rat colon as a valid model for studying the effects flupirtine on Na^+ transport in the experiments to follow.

Flupirtine enhances Na^+ absorption in rat distal colon in an ENS-dependent manner.

After establishing that the effects of flupirtine are consistent between mouse and rat regarding neurogenic Cl^- secretion, $^{22}\text{Na}^+$ flux experiments were performed next to determine whether epithelial Na^+ transport was also altered by flupirtine. For initial experiments, colonic tissues were allowed to equilibrate for 15 minutes with or without flupirtine in the serosal bath, prior to the addition of isotope. After isotope equilibration (25 minutes), unidirectional and net Na^+ fluxes were calculated for the subsequent 30-minute flux period as described above. Na^+ fluxes, as well as I_{SC} and G_{TE} data are listed in *Table 1*. Under these conditions, flupirtine enhanced net Na^+ absorption by ~75% (untreated vs. FLU: 6.6 ± 1.2 vs $11.7 \pm 1.2 \mu\text{Eq}/\text{cm}^2\cdot\text{hr}$; $p<0.05$) via a significant increase in the mucosal-to-serosal (M-S) flux ($p<0.05$), as well as a slight decrease in serosal-to-mucosal (S-M), which was not statistically significant ($p=0.148$). At the same time, basal I_{SC} and G_{TE} were both reduced in flupirtine-treated tissues ($p<0.01$ for both).

Next, experiments were designed to determine whether the enhancement of Na^+ absorption by flupirtine was mediated through effects on the ENS, as is the case for the inhibition of epithelial Cl^- secretion. To test this, Na^+ fluxes were measured in sequential 30-minute periods, before and after application of flupirtine, using tissues that were either untreated (control) or pre-treated with $1 \mu\text{M}$ tetrodotoxin (Na_v channel blocker) to abolish ENS activity (12,42). Flupirtine-sensitive I_{SC} was much greater in the absence versus in the presence of tetrodotoxin (control vs. TTX: $\Delta I_{\text{SC(FLU)}} = -92.8 \pm 11.4$ vs. $-9.5 \pm 2.6 \mu\text{A}/\text{cm}^2$; $p<0.01$) (Fig. 2C), as was flupirtine-sensitive G_{TE} (control vs. TTX: $\Delta G_{\text{TE(FLU)}} = -2.9 \pm 0.5$ vs. $-1.0 \pm 0.1 \text{ mS}/\text{cm}^2$; $p<0.01$) (Fig. 2D). These results mirror

those described in mouse. Na^+ absorption was acutely enhanced by flupirtine in control tissues (basal vs. FLU: 2.4 ± 1.0 vs 7.8 ± 0.8 $\mu\text{Eq}/\text{cm}^2\cdot\text{hr}$; $p<0.05$) via an increase in M-S flux ($p<0.01$) and a slight decrease in S-M flux ($p<0.05$). However, pre-treatment with tetrodotoxin negated this effect (basal vs. FLU: 10.4 ± 1.6 vs 9.9 ± 0.4 $\mu\text{Eq}/\text{cm}^2\cdot\text{hr}$; $p=0.55$). Flupirtine also reduced I_{SC} and G_{TE} significantly in both control and tetrodotoxin-treated tissues ($p<0.01$ for all), although the change from baseline was greater in control tissues, as basal I_{SC} and G_{TE} values were higher in the absence of ENS blockade via tetrodotoxin. *Table 2* provides the details of Na^+ fluxes, I_{SC} and G_{TE} data from these experiments.

Figure 3A summarizes the net Na^+ absorption measured before and after flupirtine treatment in the presence and absence of tetrodotoxin. When the net flux values were compared to each other via one-way ANOVA with Tukey's *post-hoc*, a significant increase was again observed only in control tissues (pre- vs. post-flupirtine; $p<0.01$), but not tetrodotoxin-treated tissues (pre- vs. post flupirtine; $p>0.97$). In addition, net Na^+ absorption was significantly greater in both tetrodotoxin- and tetrodotoxin/flupirtine-treated tissues compared to the basal flux for control ($p<0.01$ for both). However, there was not a significant difference in Na^+ flux between control tissues post-flupirtine and tetrodotoxin-treated tissue either before or after flupirtine application ($p=0.11$ and 0.23 , respectively).

In these experiments, $^{22}\text{Na}^+$ fluxes were measured in *sequential* 30-minute periods, before and after administration of flupirtine. This differs from the experiments summarized in *Table 1*, where fluxes were *time-matched* and measured either in the presence or absence of flupirtine. The additional equilibration time at the beginning of those experiments may explain the relatively higher rates of net Na^+ flux, as more time had passed before the addition of isotope. For additional comparison, time-matched Na^+ fluxes were compiled from both sets of experiments (*Tables 1 and*

2) and are depicted in Figure 3B. When compared via one-way ANOVA with Tukey's *post-hoc*, Na^+ absorption was again significantly greater in flupirtine-treated tissues ($p<0.05$), while no difference was observed between treatment with flupirtine and tetrodotoxin ($p=0.799$). Although not statistically significant ($p=0.076$), Na^+ absorption was also greater in tetrodotoxin-treated colon compared to control. These studies demonstrate that the effects of flupirtine on Na^+ absorption likely occur via a tetrodotoxin-sensitive mechanism (i.e., by acting on ENS neurons), consistent with the hypothesis that inhibition of neurogenic input via activation of neuronal K_v7 channels both dampens epithelial secretion enhances Na^+ absorption.

Flupirtine enhances Na^+ absorption that is primarily mediated by NHE-3.

Increased Na^+ absorption without an accompanying increase in I_{SC} indicates that flupirtine enhanced Na^+ flux through an electroneutral absorptive pathway. Na^+/H^+ exchanger isoform 3 (NHE-3) is the predominant electroneutral Na^+ transporter within the intestinal mucosa (29). Importantly, NHE-3 is also known to be regulated by intracellular signaling events occurring in direct response to ENS stimulation, such as an increase in cytosolic Ca^{2+} or cAMP following cholinergic or VIP-ergic input (12). However, Na^+/H^+ exchanger isoform 2 (NHE-2) activity has also been reported to play an important role in electroneutral Na^+ absorption under certain conditions as well (43,44,45). Studies were therefore initiated to evaluate the relative contributions of NHE-2 and NHE-3 transporters in flupirtine-stimulated Na^+ absorption.

Na^+ fluxes were measured in the presence of flupirtine alone (control) or in the presence of 50 μM HOE694 (NHE-2 selective inhibitor) (45), 10 μM S3226 (NHE-3 selective inhibitor) (46) or 1 mM amiloride (non-selective inhibitor of electroneutral/electrogenic Na^+ absorption) (47) in the mucosal bath. As illustrated in Figure 4, mucosal HOE694 had no discernable effect (FLU

vs. HOE694: 10.5 ± 0.4 vs 10.0 ± 0.5 $\mu\text{Eq}/\text{cm}^2\cdot\text{hr}$; $p=0.55$). ($p=0.98$). However, net Na^+ absorption was substantially inhibited by both S3226 (FLU vs. S3226: 10.5 ± 0.4 vs 4.2 ± 0.3 $\mu\text{Eq}/\text{cm}^2\cdot\text{hr}$; $p<0.01$) and amiloride (FLU vs. AMIL: 10.5 ± 0.4 vs 1.9 ± 0.5 $\mu\text{Eq}/\text{cm}^2\cdot\text{hr}$; $p<0.01$), owing to a large decrease in M-S flux (details given in *Table 3*). Amiloride inhibited Na^+ absorption to a greater extent than S3226 amiloride (S3226 vs. AMIL: 4.2 ± 0.3 vs 1.9 ± 0.5 $\mu\text{Eq}/\text{cm}^2\cdot\text{hr}$; $p<0.01$), also a result of reduced M-S flux (see *Table 3*), indicating that a concentration of 10 μM S3226 may not be sufficient to maximally inhibit NHE-3 under these conditions. Nonetheless, S3226 abolished the majority (~70%) of amiloride-sensitive Na^+ absorption in the presence of flupirtine, suggesting critical involvement of NHE-3 activity in this process. These observations fit nicely with the current understanding of NHE-3 regulation by the ENS, as the apparent suppression of ENS activity via K_v7 activation triggers a considerable increase in NHE-3-dependent absorption.

Extensive characterization of acute NHE-3 regulation in the intestine has revealed a primary mechanism involving rapid changes in membrane localization through trafficking between the apical membrane and an intracellular vesicular pool (48). Stimulation of various basolateral receptors, such as M_3R via acetylcholine, triggers the internalization of NHE-3, thus inhibiting Na^+ uptake across the apical membrane. If activation of ENS-localized K_v7 channels via flupirtine causes reduced stimulatory neurogenic input that is associated with enhanced NHE-3-dependent Na^+ absorption (Figs 1-4), then NHE-3 trafficking is likely to play a role in this process. Qualitatively, seromuscular-stripped rat distal colon that was treated with flupirtine appeared to have more NHE-3 protein localized near the apical membrane (labelled with phalloidin) compared with control (Figure 5). The NHE-3-specific labelling was more diffuse beneath the apical surface in untreated control tissue, while appearing more focused around the apical membrane in flupirtine-treated tissue. While quantitative analysis was not performed here,

the apparent change in NHE-3 localization in response to flupirtine-mediated suppression of ENS activity is congruent with the results from our Ussing chamber experiments, as well as with the currently accepted model of acute NHE-3 regulation in colonic epithelia.

Flupirtine does not enhance ENaC-mediated Na⁺ absorption in hyperaldosteronaemic rat distal colon.

Both electroneutral and (NHE-2/3-mediated) and electrogenic (ENaC-mediated) Na⁺ absorption are present in the colon of most mammalian species, but there is considerable variability in the functional importance, as well as the anatomical distribution of these pathways (43). Along with this, second messenger systems responding to ENS-derived neurotransmitters, such as Ca²⁺ (M₃R activation) or cAMP (VPAC1 activation), differentially regulate NHE-3 and ENaC as well. For example, cAMP is known rapidly inhibit NHE-3 activity, whereas ENaC has been shown to be activated by elevated cAMP (49,50) and its expression may be governed by Ca²⁺ events (51). Beyond this, there has been little work done to elucidate the regulation of ENaC by ENS neurotransmission, specifically, within the colon.

The data presented thus far support the notion that activation of K_v7 channels in the ENS augments NHE-3-mediated absorption. However, the potential effects of K_v7 activation on ENaC-mediated electrogenic absorption remain undetermined. Na⁺ absorption in the normal rat distal colon is almost exclusively electroneutral with very little involvement of ENaC-mediated electrogenic Na⁺ absorption, except for the most distal segment of the colon and rectum (4). To address this problem, the effect of flupirtine on Na⁺ flux was measured in distal colon of dietary Na⁺-depleted rats, which exhibit secondary hyperaldosteronism (30). One of the hallmark

characteristics of this model is the induction of electrogenic (ENaC-mediated) Na^+ absorption and the concurrent down-regulation of NHE-3, as well as NHE-2 (43).

Basal I_{SC} is typically much higher (2- to 4-fold) in the distal colon of hyperaldosteronaemic (a.k.a. “aldo”) rats compared to normal controls, which is attributable mostly to ENaC activity. In “aldo” distal colon, addition of flupirtine to the serosal bath caused a reduction in I_{SC} (basal vs. FLU: 297.8 ± 7.2 vs. $193.5 \pm 11.8 \mu\text{A}/\text{cm}^2$; $p < 0.01$) and G_{TE} (basal vs. FLU: 11.8 ± 0.7 vs. $7.4 \pm 0.3 \text{ mS}/\text{cm}^2$; $p < 0.01$) (Fig. 5A-B). However, this inhibition appears qualitatively to be more gradual in onset when compared the I_{SC} inhibition observed in normal rats. I_{SC} declined steadily over the 30-minute flux period in “aldo” tissues, whereas I_{SC} in controls reached a new baseline within a few minutes (see Fig. 2). Net Na^+ flux was unaffected by flupirtine in “aldo” distal colon (basal vs. FLU: 11.4 ± 0.7 vs $10.6 \pm 0.8 \mu\text{Eq}/\text{cm}^2 \cdot \text{hr}$; $p = 0.66$) (Fig. 5C), despite the concurrent reduction in I_{SC} and G_{TE} , suggesting a portion of the basal I_{SC} was not mediated by electrogenic Na^+ transport (e.g., $\text{Cl}^-/\text{HCO}_3^-$ secretion). However, subsequent application of $10 \mu\text{M}$ amiloride (ENaC-selective at this concentration) (52) dramatically inhibited I_{SC} (FLU vs. AMIL: 193.5 ± 11.8 vs. $-27.9 \pm 2.5 \mu\text{A}/\text{cm}^2$; $p < 0.01$) and G_{TE} (FLU vs. AMIL: 7.4 ± 0.3 vs. $5.0 \pm 0.2 \text{ mS}/\text{cm}^2$; $p < 0.01$) (Fig. 5A-B), as well as Na^+ absorption (FLU vs. AMIL: 10.6 ± 0.8 vs $2.0 \pm 0.3 \mu\text{Eq}/\text{cm}^2 \cdot \text{hr}$; $p < 0.01$) (Fig. 5C), due to a substantial reduction in M-S flux ($p < 0.01$).

The profound effects of low-dose amiloride on I_{SC} , G_{TE} and Na^+ flux indicate the prominent activity of ENaC in the distal colon of these animals. In addition, Western blot analysis using distal colonic mucosae from normal and “aldo” rats confirmed that NHE-3 expression was significantly down-regulated (<25% of control), as shown in the representative blot image (Fig. 5D) and densitometric quantification (Fig. 5E). Based on these observations, activation of K_v7 channels in

the ENS may thus only enhance electroneutral NHE-3-mediated absorption in rat colon, as ENaC-mediated absorption is already quite high at baseline, and not altered by treatment with flupirtine.

Effects of flupirtine on Cl⁻ secretion and Na⁺ absorption are specific to the colon.

The results described above suggest that enhanced Na⁺ absorption in response to K_V7 activation was mediated only by electroneutral, NHE-3-mediated transport. Indeed, NHE-3 is the major Na⁺ absorptive mechanism along the length of the small and large intestine. The compositional make-up of submucosal ENS neuronal networks is widely varied between anatomical regions, however. Decades of investigation into the neurochemical coding of GI innervation has helped to characterize the various ENS secretomotor subpopulations. The spatial and functional configuration of the major neuronal subtypes (e.g., cholinergic, VIP-ergic, nitrergic) has been fairly well-defined (8). Still undetermined is whether segmental differences exist regarding the response to flupirtine on either Cl⁻ secretion (I_{SC}) or Na⁺ absorption, as the specific distribution pattern of K_V7 channel subtypes has not been described in these networks to date. Thus, the effects of K_V7 activation on Cl⁻ secretion and Na⁺ absorption were assessed in both the proximal colon and the ileum to determine whether the response was region-specific.

Flupirtine inhibited I_{SC} (basal vs. FLU: 70.7 ± 14.2 vs. 25.8 ± 2.2 μA/cm²; *p*<0.01) and G_{TE} (basal vs. FLU: 9.7 ± 0.5 vs. 7.6 ± 0.4 mS/cm²; *p*<0.01) (Fig. 6A,C), while stimulating Na⁺ absorption (basal vs. FLU: 3.1 ± 0.8 vs 5.7 ± 0.6 μEq/cm²·hr; *p*<0.05) (Fig. 6E) in rat proximal colon, suggesting the presence of flupirtine-sensitive K_V7 channels in this region. Flupirtine-stimulated Na⁺ absorption was also inhibited by mucosal S3226 (FLU vs. S3226: 5.7 ± 0.6 vs 2.8 ± 0.2 μEq/cm²·hr; *p*<0.01) (not illustrated), suggesting that NHE-3 is the primary mediator in the proximal colon as well. In the ileum, however, flupirtine did not inhibit I_{SC} (basal vs. FLU: 96.2 ±

6.0 vs. $97.9 \pm 6.4 \mu\text{A}/\text{cm}^2$; $p=0.84$), slightly *increased* G_{TE} (basal vs. FLU: 23.9 ± 0.9 vs. $26.7 \pm 0.8 \text{ mS}/\text{cm}^2$; $p<0.05$) (Fig. 6B,D) and did not alter Na^+ absorption (basal vs. FLU: 4.9 ± 0.6 vs $5.7 \pm 0.7 \mu\text{Eq}/\text{cm}^2\cdot\text{hr}$; $p=0.41$) (Fig. 6F). Together, these data indicate the effects of K_v7 channel activation in ENS neurons may be specific to the colon, at least in rats.

Stimulation of Na^+ absorption and inhibition of Cl^- secretion are significantly correlated in flupirtine treated rat distal colon.

Release of ENS-derived neurotransmitters to the intestinal epithelium has effects on both secretion and absorption within cells of the crypt and surface regions, respectively (7). By inducing secretion and inhibiting absorption together in a coordinated manner, ENS activity can produce a flushing secretory response that is often coupled to enhanced motility for the purpose of removing potentially harmful substances from the bowel. However, this response is often inappropriate and can become pathological, as in IBS-D (11,24). Our data suggest that activation of K_v7 channels in the ENS both inhibits electrogenic secretion and stimulates electroneutral absorption in the colon. To assess whether these two outcomes were correlated, the change in M-S Na^+ flux was plotted against the change in I_{SC} recorded from the same tissue. Tissues from all experiments in this project were used for analysis that incorporated normal distal or proximal colon, where fluxes were measured before and after flupirtine administration ($n=20$). Simple linear regression and correlation analysis revealed a significant relationship ($p<0.0001$; $r^2=0.6051$) between the magnitude of I_{SC} inhibition and the magnitude in M-S flux enhancement (Figure 8). Such a strong correlation between these effects is presumably the result of varying degrees of basal ENS activity being inhibited by K_v7 activation, leading to a proportional (but opposite) response with respect to Cl^- secretion and Na^+ absorption because both processes are governed by ENS input.

DISCUSSION

Here we provide evidence that a K_v7 channel activator, flupirtine, both suppresses neurogenic Cl^- secretion and enhances NHE-3-dependent Na^+ absorption simultaneously in the rat colon. In support of this claim are the following observations: 1) Flupirtine-sensitive $K_v7.2$ channels were detected by immunofluorescence in submucosal ganglia, and ENS-driven Cl^- secretion (via veratridine) was suppressed by flupirtine, while the response to epithelium stimulation directly (via VIP) was unaffected. 2) Flupirtine inhibited I_{SC} and G_{TE} , and increased both M-S and Net Na^+ fluxes across rat colonic tissues, but this effect was not observed in the presence of ENS blockade via tetrodotoxin. 3) S3226 and amiloride, but not HOE694, significantly inhibited M-S and Net Na^+ fluxes in the presence of flupirtine, while NHE-3 localization near the apical membranes of surface epithelial cells was apparently enhanced in flupirtine-treated colon compared to control. 4) Flupirtine did not alter Na^+ fluxes in “aldo” rat distal colon, which has significantly reduced NHE-3 expression/activity. 5) Flupirtine inhibited I_{SC} and G_{TE} , and increased both M-S and Net Na^+ fluxes in the proximal colon, but not in the ileum, suggesting a colon-specific effect.

Under normal conditions, the primary function of the colon is to desiccate fecal material via active absorption of Na^+ , Cl^- and water. Efferent signals from the ENS can halt these absorptive processes and induce a secretory response to either normal (e.g., mechanical distension) (53,54) or pathological stimuli (e.g., infection or stress) (55,56). Often, epithelial secretion occurs in conjunction with increased motility of colonic smooth muscle in order to propagate material onward toward its eventual elimination (57). Secretomotor neurons of the ENS are thus frequently targeted in the treatment of diarrheal GI disorders, such as IBS-D. Alosetron (serotonin receptor antagonist) and loperamide (opioid receptor agonist) are two of the most commonly prescribed

IBS-D treatments, both of which dampen ENS activity (58,59). However, these drugs and others that modulate these ENS activity are associated with adverse side effects, such as nausea, abdominal pain, constipation and dependence (26). Novel pharmacotherapies that dampen ENS-driven symptoms may therefore potentially have great therapeutic potential. The results from this study demonstrate that K_v7 channel activators may be of use in this context, as both inhibition of Cl^- secretion and stimulation of Na^+ absorption favor water absorption and would likely combat diarrhea. Furthermore, if the effects observed here are truly specific to the colon (see Fig. 7), potential off-target effects of interfering with ENS function in the small intestine (i.e., related to normal digestive function) may possibly be avoided as well. *In vivo* studies will be required for a more complete assessment of efficacy.

The clinical use of K_v7 channel activators, including flupirtine, has historically been centered around the treatment of chronic pain and epilepsy (21). These drugs are effective analgesics and anticonvulsants because of their suppressive effects on neuronal excitability. Opening of K_v7 channels in the neuronal plasma membrane produces a hyperpolarizing K^+ current that stabilizes membrane potential and thus dampens the response to incoming stimuli (60). However, the exact mechanism of action in terms of their analgesic effects is not completely clear, and it may involve interactions with γ -amino butyric acid (GABA) receptors, G protein-coupled inward rectifier K^+ (GIRK) channels, or possibly voltage-dependent Mg^{2+} block of NMDA receptors (61,62,63). Whether direct or indirect, here we show for the first time that the suppressive effects of K_v7 channel activators can be seen in the context of the ENS and its modulation of multiple ion transport pathways in the colon. Somewhat related to this, Peiris et. al. demonstrated that activation of K_v7 channels in sensory afferents with retigabine – a structural analog of flupirtine – blunted the nociceptive response to noxious stimuli in mouse and human colon (20).

The potential benefits of targeting neuronal K_v7 channels in the treatment of GI disorders such as IBS-D may therefore be multifaceted. Generalized GI-related side effects (i.e., nausea, abdominal discomfort) are associated with flupirtine use in patients treated for chronic pain. However, to our knowledge, there has not been an analysis to determine whether patients with pre-existing GI conditions, such as IBS-D, experienced these side effects differentially. A thorough review of the clinical data surrounding the use of flupirtine in patients would be useful in this respect.

One of the major findings from this study is that specifically NHE-3-mediated Na⁺ absorption is enhanced by K_v7 activation. Modulation of NHE-3 activity by secretagogues originating from ENS neurons has been thoroughly described (29). Cholinergic and VIP-ergic neurons constitute the two primary secretomotor classes within the submucosal plexus of the colon (8,64). Both of the second messenger systems induced by the activation of their target receptors – Ca²⁺ and cAMP, respectively – inhibit NHE-3 activity by triggering its rapid internalization from the apical membranes of surface colonocytes. Our observations that NHE-3 surface localization is enhanced by treatment with flupirtine, and that NHE-3-dependent Na⁺ absorption is increased by flupirtine to a degree which is correlated to the inhibition of Cl⁻ secretion, fits well into the existing model of Na⁺ and Cl⁻ transport modulation by ENS input. In spite of this, the experimental data presented here offer no definitive insight regarding the intracellular events underlying the observed effects on Na⁺ or Cl⁻ transport. Further studies will be required to assess the involvement of cholinergic or VIP-ergic (or other) pathways, as well as to resolve the details of NHE-3 trafficking and/or other possible means of regulation in response to flupirtine.

Flupirtine is generally considered to be non-selective for K_v7 channels. One member of this family, K_v7.1, has a well-defined role in the colonic epithelium itself (65). K_v7.1 is expressed in crypt cells and localizes to the basolateral membrane to support apical Cl⁻ secretion by

maintaining the electrochemical driving force for sustained Cl^- efflux (66). In this study, as well as recent studies from our lab, there was no indication that flupirtine stimulates Cl^- secretion by opening basolateral $\text{K}_v7.1$ channels, despite the drug being “non-selective” for the family subtypes. Importantly, another non-selective K_v7 channel activator, retigabine, does not act on the $\text{K}_v7.1$ subtype *in vitro* (16). While analysis of the effects of flupirtine, specifically, on $\text{K}_v7.1$ is lacking, there have been reports that the channel may also be insensitive to flupirtine (21). Indeed, flupirtine and retigabine are very closely related in both structure and function. We have also observed no effect from flupirtine on basal or stimulated Cl^- secretion in T84 cells, which express $\text{K}_v7.1$ channels. $\text{K}_v7.1$ does exhibit significant homology to other K_v7 family members (67). The reason for the apparent insensitivity to these non-selective activators are unknown but may be related to the association of $\text{K}_v7.1$ with an auxiliary minK (*KCNE*) subunit in the colonic epithelium (68,69), or may be related to secondary effects on GABA or NMDA receptors, as mentioned above. Nevertheless, flupirtine clearly modulates epithelial transport in an ENS-dependent manner, as evidenced by the attenuating effect of pre-treatment with tetrodotoxin on the response to flupirtine, as well as the similarity in I_{SC} , G_{TE} and Na^+ absorption properties among tissues treated with either of the two agents. The mechanistic details underlying K_v7 activation of neuronal versus epithelial subtypes, as well as possible “off-target” inhibitory effects within ENS neurons, remain to be elucidated but should be the subject of future studies.

CONCLUSIONS

In summary, these data show that the K_v7 channel activator, flupirtine, simultaneously inhibits Cl^- secretion and stimulates NHE-3-dependent Na^+ absorption in the rat colon. Further, these effects are likely mediated by an ENS-dependent mechanism. Both of inhibition of Cl^-

secretion and stimulation of Na^+ absorption may have significant bearing on water absorption in the colon. Therefore, the use of $\text{Kv}7$ -activating drugs for the treatment of ENS-associated diarrheal conditions – such as IBS-D – should be explored in the future.

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TABLES

²² Na ⁺ Fluxes							
Condition	<i>n</i>	J_{M-S} (μEq/cm ² ·hr)	J_{S-M} (μEq/cm ² ·hr)	J_{Net} (μEq/cm ² ·hr)	<i>n</i>	I_{SC} (μA/cm ²)	G_{TE} (mS/cm ²)
Untreated	6	11.2 ± 0.6	4.2 ± 0.7	6.6 ± 1.2	12	73.7 ± 9.4	6.6 ± 0.4
Flupirtine	6	14.3 ± 0.9	3.0 ± 0.4	11.7 ± 1.2	12	19.1 ± 2.5	4.7 ± 0.4
<i>p</i>		0.0220*	0.1480	0.0117*		<0.0001*	<0.0031*

Table 1 – Effect of flupirtine on unidirectional ²²Na⁺ fluxes in rat distal colon. Unidirectional ²²Na⁺ fluxes were measured across seromuscular-stripped rat distal colon under voltage-clamped conditions in the presence and absence of 100 μM serosal flupirtine. Mucosal-to-serosal (M-S) and serosal-to-mucosal (S-M) fluxes were measured over a 30-minute period. Net fluxes were calculated by subtracting S-M from M-S fluxes using tissues pairs matched by conductance, as described in *Methods*. Flux values are given as mean ± SEM from 6 tissue pairs. I_{SC} and G_{TE} values represent the averages recorded over the 30-minute flux period and are given as mean ± SEM from 12 tissues (6 tissues each for M-S and S-M fluxes). *p* values are given for each treatment group/parameter. * indicates significance compared to control (untreated), as determined by unpaired Student's t-test.

²² Na ⁺ Fluxes								
	Flux Period	<i>n</i>	J_{M-S}	J_{S-M}	J_{Net}	<i>n</i>	I_{SC}	G_{TE}
			(μEq/cm ² ·hr)	(μEq/cm ² ·hr)	(μEq/cm ² ·hr)		(μA/cm ²)	(mS/cm ²)
<i>Control</i>	Basal	6	6.1 ± 0.9	3.7 ± 0.2	2.4 ± 1.0	12	109.2 ± 11.3	7.4 ± 0.5
	Flupirtine	6	10.7 ± 0.7	2.9 ± 0.2	7.8 ± 0.8	12	15.8 ± 1.4	4.5 ± 0.3
	<i>p</i>		0.0022*	0.0365*	0.0017*		<0.0001*	<0.0001*
<i>TTX-treated</i>	Basal	5	14.0 ± 0.8	3.6 ± 0.3	10.4 ± 1.6	10	24.4 ± 3.0	5.7 ± 0.2
	Flupirtine	5	13.8 ± 0.6	3.9 ± 0.3	9.9 ± 0.4	10	14.9 ± 0.8	4.7 ± 0.2
	<i>p</i>		0.8437	0.5640	0.5541		<0.0001*	<0.0031*

Table 2 – Effect of tetrodotoxin on flupirtine-stimulated ²²Na⁺ fluxes in rat distal colon.

Unidirectional ²²Na⁺ fluxes were measured across seromuscular-stripped rat distal colon under voltage-clamped conditions before (Basal) and after the addition of 100 μM flupirtine (Flupirtine) to the serosal chamber bath in the absence (*Control*) and presence of tetrodotoxin (*TTX-treated*). Flux values are given mean ± SEM from 6 tissue pairs from control and 5 tissue pairs from the tetrodotoxin-treated group. I_{SC} and G_{TE} values represent the averages recorded over final 5 minutes of each 30-minute flux period and are given as mean ± SEM from 12 control and 10 tetrodotoxin-treated tissues. *p* values are given for each treatment group/parameter. * indicates significance when compared to the basal flux period, as determined by unpaired Student's t-test.

²²Na⁺ Fluxes

Condition	<i>n</i>	J _{M-S} (μEq/cm ² ·hr)	J _{S-M} (μEq/cm ² ·hr)	J _{Net} (μEq/cm ² ·hr)	<i>n</i>	I _{SC} (μA/cm ²)	G _{TE} (mS/cm ²)
Flupirtine	5	14.1 ± 0.3	3.2 ± 0.2	10.5 ± 0.4	10	19.6 ± 2.0	5.3 ± 0.5
HOE694	5	13.0 ± 0.7	3.0 ± 0.2	10.0 ± 0.5	10	17.7 ± 1.8	5.6 ± 0.5
S3226	5	6.5 ± 0.4 ^{*,#}	2.2 ± 0.2 ^{*,#}	4.2 ± 0.3 ^{*,#}	10	12.8 ± 0.8 [*]	4.0 ± 0.1 ^{*,#}
Amiloride	5	4.3 ± 0.5 ^{*,#,\u00a3}	2.4 ± 0.1 [*]	1.9 ± 0.5 ^{*,#,\u00a3}	10	13.3 ± 0.9 [*]	4.5 ± 0.1

Table 3 – Effect of HOE694, S3226 and amiloride on unidirectional ²²Na⁺ fluxes in flupirtine-treated rat distal colon. Unidirectional ²²Na⁺ fluxes were measured across seromuscular-stripped rat distal colon under voltage-clamped conditions in the presence of 100 μM serosal flupirtine alone, or flupirtine plus 50 μM mucosal HOE694, 10 μM mucosal S3226 or 1 mM mucosal amiloride. Flux values are given as mean ± SEM from 5 tissue pairs for all treatment groups. I_{SC} and G_{TE} values were averaged from 10 tissues (5 tissue pairs) over the 30-minute flux period for each treatment group and are given as mean ± SEM. ^{*} *p*<0.01 versus flupirtine alone, [#] *p*<0.05 versus flupirtine plus HOE694, ^{\u00a3} *p*<0.05 versus flupirtine plus S3226, as determined by one-way ANOVA with Tukey's *post-hoc*.

²² Na ⁺ Fluxes							
Condition	<i>n</i>	J _{M-S} (μEq/cm ² ·hr)	J _{S-M} (μEq/cm ² ·hr)	J _{Net} (μEq/cm ² ·hr)	<i>n</i>	I _{SC} (μA/cm ²)	G _{TE} (mS/cm ²)
Basal	5	13.9 ± 0.7	2.4 ± 0.2	11.4 ± 0.7	10	297.8 ± 7.2	11.8 ± 0.7
Flupirtine	5	12.3 ± 0.8	1.6 ± 0.2 [*]	10.7 ± 0.8	10	195.5 ± 11.8 [*]	7.4 ± 0.3 [*]
Amiloride	5	3.7 ± 0.3 ^{*,#}	1.7 ± 0.1 [*]	2.0 ± 0.3 ^{*,#}	10	-27.9 ± 2.5 ^{*,#}	5.0 ± 0.2 ^{*,#}

Table 4 – Effect of flupirtine and amiloride on unidirectional ²²Na⁺ fluxes in “aldo” rat distal colon. Unidirectional ²²Na⁺ fluxes were measured across seromuscular-stripped “aldo” rat distal colon under voltage-clamped conditions for three sequential flux periods: under basal conditions, in the presence of 100 μM serosal flupirtine, and flupirtine plus 10 μM mucosal amiloride. Flux values are given as mean ± SEM from 5 tissue pairs for all treatment groups. I_{SC} and G_{TE} values were averaged from the 10 tissues (5 tissue pairs) over the final 5 minutes of each 30-minute flux period for each treatment group and are also given as mean ± SEM. ^{*} *p*<0.01 versus basal flux period, [#] *p*<0.05 versus flupirtine, as determined by one-way ANOVA with Tukey’s *post-hoc*.

FIGURES AND LEGENDS

Figure 1 – Flupirtine inhibits neurogenic Cl⁻ secretion in rat distal colon. *A-B*: Short-circuit current (I_{SC}) recordings from control (open circles) and 100 μ M flupirtine-treated (closed circles) rat distal colon in response to 10 μ M veratridine (VER) (*A*) or 100 nM vasoactive intestinal polypeptide (VIP) (*B*). All drugs were added to the serosal chamber bath. *C*: Group data showing the change in I_{SC} following veratridine administration in control and flupirtine-treated tissues. *D*: Group data showing the change in I_{SC} following VIP administration in control and flupirtine-treated tissues. Lines and error bars represent mean $\pm$ SEM. $n=5$ tissues obtained from at least 3 separate animals for all groups; * $p<0.05$, as determined by unpaired Student's t-test. *E*: Immunofluorescent labeling of flupirtine-sensitive $K_V7.2$ channels (green) in $MAP2^+$ cells (red) from a submucosal ganglion of rat distal colon. Nuclei are labeled with DAPI (blue). Images were captured at 20x magnification. Scale bar = 20 microns.

Figure 2 – Flupirtine-induced I_{SC} and G_{TE} inhibition is attenuated by tetrodotoxin. *A-B:* I_{SC} and G_{TE} recordings from control (open circles) and 1 μ M tetrodotoxin (*TTX*)-treated (closed circles) seromuscular-stripped rat distal colon in response to 100 μ M flupirtine (*FLU*). All drugs were added to the serosal chamber bath. *C:* Group data showing the change in I_{SC} following flupirtine administration in control and tetrodotoxin-treated tissues. *D:* Group data showing the change in G_{TE} following flupirtine administration in control and tetrodotoxin-treated tissues. Lines and error bars represent mean $\pm$ SEM. $n=12$ for control and $n=10$ for tetrodotoxin-treated tissues, obtained from 6 and 5 separate animals, respectively; ** $p<0.01$, as determined by unpaired Student's t-test. $^{22}\text{Na}^+$ fluxes were also performed during these experiments and are summarized in *Table 2*.

A Sequential flux periods (from Table 2)

B Time-matched flux periods (from Tables 1 and 2)

Figure 3 – Flupirtine enhances Na^+ absorption via a tetrodotoxin-sensitive mechanism. *A*: Net Na^+ fluxes before (open circles/squares) and after (closed circles/squares) addition of 100 μM serosal flupirtine in control and 1 μM tetrodotoxin (*TTX*)-treated seromuscular-stripped rat distal colon. Values in panel *A* are derived from *Table 2*. *B*: Net Na^+ fluxes in time-matched tissues that were untreated (*Control*, open triangles), treated with 100 μM serosal flupirtine (*FLU*; closed triangles) or treated with 1 μM serosal tetrodotoxin (*TTX*; open squares). Values in panel *B* are derived from *Tables 1 and 2*. Lines and error bars represent mean $\pm$ SEM from 6 (control) or 5 (tetrodotoxin-treated) tissue pairs obtained from 6 and 5 separate animals, respectively. * $p < 0.05$, ** $p < 0.01$, as determined by one-way ANOVA with Tukey's *post-hoc*.

Figure 4 - Flupirtine-stimulated Na^+ absorption is primarily mediated by Na^+/H^+ exchanger isoform 3 (NHE-3). Net Na^+ fluxes in the presence of 100 μM serosal flupirtine alone (open circles), or flupirtine plus 50 μM mucosal HOE694 (NHE-2-selective inhibitor; closed circles), 10

μ M mucosal S3226 (NHE-3-selective inhibitor; light gray squares) or 1 mM mucosal amiloride (AMIL, non-selective NHE/ENaC inhibitor; dark gray triangles). Lines and error bars represent mean $\pm$ SEM from 5 tissue pairs, obtained from 5 separate animals, for all groups. ** $p < 0.01$, as determined by one-way ANOVA with Tukey's *post-hoc*.

Figure 5 – Effect of flupirtine on surface localization of NHE-3 in rat distal colon.

Seromuscular-stripped rat distal colon, incubated for 10 minutes in the absence (control, row *A*) or presence of flupirtine (FLU, row *B*), were labeled for NHE-3 (green) and filamentous actin (phalloidin, red). Nuclei are labeled with DAPI (blue). Zoom of control (*C*) and flupirtine-treated (*D*) colon is also shown below. Arrows indicate the presence of sub-apical NHE-3 proteins, which are more prominent in control versus flupirtine-treated rat distal colon. Images were captured at 20x magnification. Scale bar = 25 microns.

Figure 6 - Flupirtine does not enhance ENaC-mediated Na⁺ absorption in “aldo” rat distal colon. *A-B*: I_{SC} (*A*) and G_{TE} (*B*) recordings from seromuscular-stripped hyperaldosteronaemic (“aldo”) rat distal colon during basal, 100 μM serosal flupirtine (FLU) and 10 μM apical amiloride (AMIL) flux periods. *C*: Net ²²Na⁺ fluxes, measured during the sequential 30-minute periods shown in *A-B*. *D*: Representative Western blot showing NHE-3 protein expression in control and “aldo” rat distal colonic epithelium. Protein ladder is shown in the extreme left lane and corresponding molecular weights are given to the left of the blot images. *E*: Densitometric quantification of two blots similar to the one shown in panel *D* (*n*=4 separate animals, for both control and “aldo” groups). NHE-3 band intensity was normalized to actin as a loading control and represented as relative abundance in reference to control samples. Lines and error bars represent mean ± SEM. ** *p*<0.01, as determined by one-way ANOVA with Tukey’s *post-hoc* (*C*) or unpaired Student’s *t*-test (*E*).

Figure 7 – Flupirtine inhibits Cl⁻ secretion and stimulates Na⁺ absorption in normal rat proximal colon, but not ileum. *A-B:* I_{sc} recordings from seromuscular-stripped rat proximal colon (*A*) and ileum (*B*) before and after addition of 100 μM flupirtine (FLU) to the serosal chamber bath. *C-D:* G_{TE} recordings from seromuscular-stripped rat proximal colon (*C*) and ileum (*D*) before and after addition of 100 μM flupirtine (FLU) to the serosal chamber bath. *E-F:* Net

$^{22}\text{Na}^+$ fluxes measured in seromuscular-stripped rat proximal colon (E) and ileum (F), corresponding to the sequential 30-minute flux periods before (*Basal*) and after addition of flupirtine (*FLU*) as illustrated in panels A-D. Lines and error bars represent mean $\pm$ SEM from 5 tissue pairs obtained from 5 separate animals for proximal colon, and 3 separate animals for ileum.

* $p < 0.05$, as determined by unpaired Student's t-test.

Figure 8 – Stimulation of Na⁺ absorption is significantly correlated to the inhibition of Cl⁻ secretion in rat colonic tissues treated with flupirtine. The absolute value of the change in I_{sc} (inhibition of Cl⁻ secretion) was plotted against change in M-S $^{22}\text{Na}^+$ flux (stimulation of Na⁺ absorption) from rat colonic tissues that were treated with flupirtine. Every tissue used in this study was included that was assigned as “M-S”, where unidirectional $^{22}\text{Na}^+$ fluxes were measured before and after addition of 100 μM flupirtine to the serosal chamber bath ($n=20$). Simple linear

regression analysis was performed using GraphPad Prism 9.0 software, and the fit is shown as a solid black line. $p < 0.0001$ indicates the line of best fit has a slope significantly greater than zero.

CHAPTER 4

TITLE: Aldosterone up-regulates basolateral $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter-1 to support enhanced large conductance K^+ channel-mediated K^+ secretion in rat distal colon.

SHORT TITLE: Regulation of NKCC1 by aldosterone in colon

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ABBREVIATIONS: I_{SC} – short circuit current, G_{TE} – transepithelial conductance, IbTX – iberiotoxin; BK – large conductance K^+ channel, IK – intermediate conductance K^+ channel, Bumet – bumetanide; ESRD - end stage renal disease, ENaC – epithelial Na^+ channel.

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ABSTRACT

BACKGROUND AND AIMS: Na^+ - K^+ - 2Cl^- cotransporter-1 (NKCC1) facilitates basolateral K^+ and Cl^- uptake, supporting their efflux across the mucosal membranes of colonic epithelial cells. NKCC1 activity has also been shown to be critical for electrogenic K^+ secretion induced by aldosterone, a hormone which is known to stimulate the expression of large conductance K^+ (BK) channels in mucosal membranes. This study was aimed to 1) identify whether aldosterone enhances NKCC1 expression specifically to support BK-mediated K^+ secretion and 2) to determine whether increased NKCC1 supports electrogenic Cl^- secretion in parallel to K^+ secretion. **METHODS:** Dietary Na^+ depletion was used to induce secondary hyperaldosteronism in rats, or aldosterone was administered *ex vivo* to rat distal colonic mucosae. NKCC1-dependent electrogenic K^+ or Cl^- secretion was measured as a function of short circuit current (I_{SC}). qRT-PCR, western blot and immunofluorescence analyses were performed using standard techniques. **RESULTS:** Aldosterone enhanced NKCC1 and $\text{BK}\alpha$ expression and electrogenic K^+ secretion in the distal colon, which was inhibited by either serosal bumetanide (NKCC1 inhibitor) or mucosal iberiotoxin (IbTX; BK channel blocker), but not TRAM-34 (IK channel blocker). Expression of NKCC1 and $\text{BK}\alpha$ proteins was enhanced in crypt cells of hyper-aldosterone rats. However, neither NKCC1-dependent Cl^- secretion nor CFTR (apical Cl^- channel) expression was enhanced by aldosterone. **CONCLUSIONS:** Aldosterone enhances NKCC1 to support BK-mediated K^+ secretion independently of Cl^- secretion in the distal colon. The regulation of NKCC1 expression/ K^+ secretion by aldosterone may be a therapeutic target in treating gastrointestinal disorders associated with alterations in colonic K^+ transport, such as colonic pseudo-obstruction, and hyperkalemia associated with end stage renal disease.

Key words: short circuit current, pseudo-obstruction, hyperkalemia, end stage renal disease.

INTRODUCTION

The mammalian colon plays a critical role in maintaining body K^+ homeostasis, as it exhibits both active K^+ absorptive and secretory processes^{1,2}. In the distal colon, H^+, K^+ -ATPases mediate electroneutral active K^+ absorption across mucosal membranes^{3,4,5,6}, while both K^+ - Cl^- cotransport (via KCC1) and the parallel function of intermediate conductance K^+ (IK) channels and Cl^- (CLC2) channels have been shown to mediate K^+ exit across the basolateral membranes in an “electroneutral” manner.^{7,8} Conversely, active K^+ secretion in the distal colon is “electrogenic” and mediated primarily by large conductance K^+ (BK) channels in the mucosal membranes.^{1,9,10} The Na^+, K^+ -ATPase and $Na^+-K^+-2Cl^-$ cotransporter (NKCC1) both contribute to K^+ uptake across the basolateral membrane, and both transporters are required for supporting electrogenic K^+ secretion in colon.^{5,11} However, NKCC1 is particularly important to this process, because although its activity is dependent on the Na^+ gradient established by the Na^+, K^+ -ATPase, inhibition of NKCC1 alone is sufficient to disrupt K^+ secretion, while leaving Na^+, K^+ -ATPase activity intact.¹¹

Alterations in colonic K^+ transport can have a profound impact on disease pathogenicity, either harmful or beneficial. For example, excessive colonic K^+ secretion is associated with various diarrheal diseases, either secondarily as a result of large stool volume losses, as in *V. cholera* infection¹², or as a primary driver of watery stool production, as has been suggested in colonic pseudo-obstruction (Ogilvie’s syndrome)^{13,14} or ulcerative colitis.^{15,16} On the other hand, increased colonic K^+ secretion is often a critical adaptation in end stage renal disease (ESRD), providing a secondary outlet for K^+ from the body, and preventing or delaying life-threatening hyperkalemia.^{17,18} As such, furthering our current understanding of colonic K^+ transport and its regulation is of high clinical value.

Aldosterone, a mineralocorticoid hormone, is a major physiological regulator of electrolyte transporters (including K^+ transporters) in the kidney and colon.^{11,19,20} In addition to its more prominent role in the regulation of Na^+ absorption,^{21,22} aldosterone is also known to induce active K^+ secretion by enhancing BK channel expression in the distal colon.^{15,23,24} Aldosterone-induced K^+ secretion was shown to be dependent on NKCC1 function¹¹ and there is evidence to suggest that aldosterone regulates NKCC1 expression, although a direct functional connection between aldosterone-stimulated NKCC1 and BK channels has not yet been established.^{10,25,26} Beyond this, it is unclear whether aldosterone-stimulated NKCC1 activity also supports an enhanced capacity for Cl^- secretion. Cl^- absorption, which is electroneutral and coupled to Na^+ absorption in the distal colon, is reduced by aldosterone.²⁷ However, aldosterone's influence on active (i.e. stimulated) Cl^- secretion – a process dependent on NKCC1 activity – is unknown.

This study was therefore designed to test the hypothesis that aldosterone directly upregulates NKCC1 in the distal colon, specifically to facilitate active BK-mediated K^+ secretion, without concurrently enhancing Cl^- secretion in the distal colon. Using the model of secondary hyperaldosteronism induced by dietary Na^+ depletion in rats, or using exogenous aldosterone applied directly to *ex vivo* tissues, we provide novel evidence that aldosterone increases the expression of NKCC1 along the crypt/surface axis in the distal colon, and that coordinated NKCC1 and BK channel activity is a key feature of aldosterone-induced colonic K^+ secretion. Additional studies confirmed that enhanced NKCC1 expression and activity is specific to the K^+ transport pathway, as basal and cAMP-stimulated anion (Cl^-) secretion were not altered by aldosterone. Regulation of NKCC1 by aldosterone may therefore be relevant to developing treatment strategies targeting pathologies associated with colonic K^+ handling, or others involving aberrant NKCC1 function.

MATERIALS AND METHODS

Animals: Male and female Sprague-Dawley rats (200 - 250g) were either purchased from Charles River laboratories or bred inhouse (generously provided by Dr. Timothy Nurkiewicz, Department of Physiology and Pharmacology, West Virginia University, Morgantown, WV). All procedures used in this study were approved by WVU's animal care and use committee. Rats were randomly assigned to one of two groups receiving either standard chow or Na⁺-deficient diet (MP biomedical #0296023210) for six day to induce secondary hyperaldosteronism.²⁸ All rats were given diet and water *ad libitum*. While anesthetized under 5% isoflurane, whole colon was removed and washed with ice cold Ringer's solution containing (in mM): 140 Na⁺, 119.8 Cl⁻, 25 HCO₃⁻, 5.2 K⁺, 1.2 Ca²⁺, 1.2 Mg²⁺, 2.4 HPO₄²⁻, 0.4 H₂PO₄⁻, and 10 glucose (pH 7.4) before being used for mucosal scraping or electrophysiological studies. Euthanasia was then immediately performed by cutting the diaphragm and blood was collected via cardiac puncture.

Aldosterone assay: Serum aldosterone level was determined using an ELISA kit (Invitrogen #EIAALD). Aldosterone concentration was calculated by fitting sample absorbance values to a standard curve using non-linear regression in GraphPad Prism 8.0 software, as indicated in the kit's instructions.

Mucosal scraping: Distal colon sacs were made by filling with ice cold buffer containing (in mM): 15.4 NaCl, 8 HEPES/Tris (pH 7.5), 5 ethylenediaminetetraacetic acid (EDTA), 0.5 dithiothreitol (DTT), and 0.5 phenylmethylsulphonyl fluoride (PMSF) and incubated in the same solution on ice for 45 minutes. The drained sacs were opened along the mesenteric border. The distal mucosal (epithelial) layer was scraped using glass slides and immediately frozen in liquid nitrogen until use. Scrapped mucosae were used for RNA isolation and protein extraction.

RNA isolation and *qRT-PCR analysis*: Total RNA was isolated from frozen colonic mucosa (50 mg) using Trizol method and quantitated with NanoDrop spectrometer (Thermo Sci. ND2000C). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using One-Step kit (NEB #E3005) per the manufacturer's protocol. In brief, custom gene-specific primers were designed using the published DNA sequence and synthesized by Thermo Scientific, and are listed below in Table 1.

<i>Gene Target and Accession number</i>	<i>Primer Sequence</i>	<i>Amplicon length</i>
<i>NKCC1 (Slc12a2)</i> <i>NM_001270617.1</i>	F: 5'- TGGCAAGACTTCAACTCAGC - 3' R: 5'- GGTATCAACAAGGTCAAACCTCC - 3'	177
<i>ENaCγ (Scnn1g)</i> <i>NM_017046.2</i>	F: 5'- ATGATACCTCTGACTGCGCC - 3' R: 5'- AAAAGCGTGAAGTTCCTGGC - 3'	199
<i>BKα (Kcnma1)</i> <i>NM_001393699.1</i>	F: 5'- ATGTCTACAGTGGGTTACGG - 3' R: 5'- TGGGTGGTAGTTCTTTATGG - 3'	464
<i>NaKα (ATP1a1)</i> <i>NM_012504.1</i>	F: 5'- CATCAACGCAGAGGATGTCG - 3' R: 5'- AGTGAGGAGTTATCCACCTTGC - 3'	117
<i>CFTR (Cftr)</i> <i>NM_031506.1</i>	F: 5'- TTCTTCAGCTGGACCACACC - 3' R: 5'- TGGAAGCTTGTTCCCTGTCC - 3'	106
<i>β-actin (Actb)</i> <i>NM_031144.3</i>	F: 5'- AGATCAAGATCATTGCTCCTCC - 3' R: 5'- AGTAACAGTCCGCCTAGAAGC - 3'	165

Table 1: Primer sequences used in qRT-PCR analysis of RNA extracts from rat distal colon.

qRT-PCR reactions performed on a 96-well plate were read using a CFX96 real time PCR machine (BioRad). Differential mRNA abundance was determined using the delta-delta C_t method, with β -actin as a reference housekeeping gene.

Western blot analysis: Proteins were extracted from frozen colonic mucosa (50 mg) by homogenizing in lysis buffer (150 mM NaCl, 50 mM Tris base, 0.5% sodium deoxycholate, 0.1% SDS, 1.0 % Triton-X100) plus protease inhibitor cocktail (Thermo A32965), followed by a brief sonication (Fisher Sonic Dismembrator Model 100). Following centrifugation, the supernatant was mixed with 4x Laemmli buffer (Life Technologies NP0007) and heated to 90°C for 5 minutes. Samples were then chilled on ice before adding β -mercaptoethanol (5% w/v), and the aliquoted samples were flash frozen and stored at -80°C. Proteins (~20 μ g) were resolved on 8% Tris-glycine polyacrylamide gels and transferred on to PVDF membranes. Membranes were incubated in blocking buffer (3% BSA in tris-buffered saline plus 0.1% Tween-20 (TBS-T) for 1 hour at room temperature, then incubated overnight with either rabbit anti-NKCC1 (Cell Signaling Technologies 14581, 1:1000)²⁹ or horseradish peroxidase-conjugated rabbit anti-actin (Santa Cruz Biotechnologies SC-4778, 1:1000) at 4°C. The membranes were then washed in TBST (5 minutes x5). Blots incubated with anti-NKCC1 were incubated with secondary antibody solution containing goat anti-rabbit (Thermo 31462, 1:20,000) for 1 hour at room temperature and washed again in TBST (5 min x 5). Immune complexes were detected using enhanced chemiluminescence (West Dura, Thermo 34075). Images were captured using a G:BOX (Syngene) and band intensities were quantified by densitometry using Image J software and normalized to β -actin as a loading control.

Immunofluorescence studies: Distal colon segments were fixed in 4% paraformaldehyde in PBS overnight at 4°C, washed in PBS, and flash frozen in isopentane cooled with liquid nitrogen before embedding in tissue freezing medium (Thermo 1437365) and storing at -80°C. Sections (8 μ m) were cut and mounted on charged glass slides. Antigen retrieval was then performed by heating to 70° C in 10 mM citrate (pH 6.0) for 40 minutes using a microwave on low power. After

cooling, sections were permeabilized and blocked in 5% goat serum and 0.1% Tween-20 in PBS (PBST) for 30 minutes, then incubated in blocking solution containing rabbit anti-NKCC1 (1:500) and mouse anti-BK α (Millipore MABN70, 1:100)³⁰ antibodies overnight at 4°C. The following day, sections were washed in PBST (5 minutes x 5) and incubated for 1 hour at room temperature in blocking solution containing Alexa Fluor 488 goat anti-rabbit (Thermo A11008) and Alexa Fluor 568 goat anti-mouse (Thermo A11031). After washing again in PBST, coverslips were mounted with SlowFade Diamond mountant containing DAPI (Thermo S36973) and sealed with clear nail polish. Images were captured with a Zeiss 710 confocal microscope (Carl Zeiss, NY).

Ussing chamber studies: Distal colon segments placed in ice cold Ringer's solution containing indomethacin (5 μ M; to inhibit prostaglandin production)³¹ were opened along the mesenteric border and the mucosal layers were separated, as described previously⁸. Briefly, the colons were placed in a dissecting dish and a shallow lateral incision was made into the mucosal layer just proximal to the most distal Peyer's patch (~2 cm from the rectum). The distal mucosal layer (2-6 cm proximal to the rectum) was then separated from the underlying smooth muscle layers. Mucosae mounted on EasyMount sliders (1.1 cm² aperture) placed Ussing Style chambers (Physiologic Instruments, San Diego CA) were bathed bilaterally with Ringer's solution, bubbled with 5% CO₂ balanced with oxygen, and warmed to 37°C. Tetrodotoxin (1 μ M) was added to the serosal bath to inhibit residual neuronal activity³¹. Tissues were allowed to equilibrate until trans-epithelial potential (V_{TE}) had stabilized (~20 minutes). Then, tissues were voltage clamped and short-circuit current (I_{SC}) was measured by computer-operated software (Physiologic Instruments, San Diego CA). For all data presented here, positive I_{SC} reflect anion secretion (serosal to mucosal direction) and/or cation absorption (mucosal to serosal direction). Trans-epithelial conductance (G_{TE}) was monitored by generating a bipolar 5 mV pulse at 5 second intervals. All tissues were

allowed to reach steady-state I_{SC} (i.e., plateau) for at least 10 minutes prior to the addition of any drugs.

RESULTS

Dietary Na^+ depletion induces secondary hyperaldosteronism in a sex-dependent manner.

Dietary Na^+ depletion is commonly used to generate experimental hyperaldosteronism.^{27,32,33} In the past, the effects of experimental hyperaldosteronism on kidney and colon electrolyte transport have been mostly characterized in male, but not in female animals. However, in accordance with current NIH guidelines regarding biological variability related to sex, we examined the effect of dietary Na^+ depletion on plasma aldosterone levels in both male and female rats. Although the serum aldosterone levels were similar in both normal male and female (244.9 ± 29.9 vs 245.9 ± 61.7 pg/mL), dietary Na^+ depletion induced a 14.2-fold increase in serum aldosterone level in male rats (normal vs Na^+ -depleted: 244.9 ± 29.9 vs. $3,485.0 \pm 949.1$ pg/mL), but only a 3.4-fold increase in female rats (normal vs Na^+ -depleted: 245.9 ± 61.7 vs. 827.5 ± 57.3 pg/mL) (Supp. Fig. 1A).

Because dietary Na^+ depletion-induced hyperaldosteronism is known to induce epithelial Na^+ channel (ENaC)-mediated Na^+ absorption^{21,22}, we also examined whether the differentially-increased aldosterone levels were reflected in ENaC activity in male and female rat distal colon. Similar to the difference observed regarding aldosterone generation, Na^+ depletion induced ENaC activity proportionately lower (~4-fold) in dietary Na^+ -depleted female compared to male rat distal colon, as measured by 100 μ M amiloride-sensitive short circuit current (I_{SC} ; ENaC-mediated Na^+ absorption) (Na^+ -depleted male vs female: $\Delta I_{SC(AMIL)} = -388.3 \pm 28.3$ vs -101.2 ± 31.6 μ A/cm²) (Supp. Fig. 1B). A concentration of 100 μ M amiloride was selected based on similar published

experiments.^{23,24} To assess whether a sex-specific effect of dietary Na⁺ depletion had occurred, a Two-way analysis of variance (ANOVA) with Tukey's *post-hoc* was performed. Analysis showed significantly elevated serum aldosterone level in dietary Na⁺-depleted male ($p < 0.01$), but not in female ($p = 0.82$) rats; and significantly induced ENaC activity in dietary Na⁺-depleted male ($p < 0.001$), but not female ($p = 0.06$) rat distal colon.

Although serum aldosterone and ENaC activity were appreciably high in dietary Na⁺-depleted female rats, and thus the dietary effects are certainly not negligible, the response was not as robust as in the males and therefore requires separate independent study to identify the mechanistic nature of lower aldosterone generation level in Na⁺-depleted female rats. However, as the specific goal of this investigation is to evaluate the regulation of NKCC1 and BK-mediated K⁺ secretion by aldosterone, all subsequent experiments were designed to incorporate only male rats to avoid confounding effects resulting from sex differences, as well as to maintain consistency with existing literature regarding aldosterone-regulated K⁺ transport.

Dietary Na⁺ depletion stimulates electrogenic Na⁺ absorption and K⁺ secretion in rat distal colon.

Further initial studies were performed to confirm that dietary Na⁺ depletion induces ENaC-mediated electrogenic Na⁺ absorption and BK channel-mediated electrogenic K⁺ secretion in rat distal colon, as has been previously reported.^{24,34} As shown in Supplemental Fig. 2A, the large, positive basal I_{SC} measured in Na⁺-depleted rat distal colon indicates the presence of ENaC activity (normal vs dietary Na⁺-depleted: basal I_{SC} = 28.3 ± 3.2 vs 353.8 ± 8.9 $\mu\text{A}/\text{cm}^2$; $p < 0.001$), while inhibition by mucosal amiloride further confirmed that ENaC activity was the source of this basal I_{SC} in dietary-Na⁺ depleted rat distal colon (normal vs dietary Na⁺-depleted: I_{SC} post-amiloride = 17.9 ± 0.9 vs -40.0 ± 3.3 $\mu\text{A}/\text{cm}^2$; $p < 0.001$). Negative I_{SC} in the presence of amiloride indicates electrogenic K⁺ secretion (Fig. 2A). Lower basal I_{SC} that was less sensitive to amiloride, as well

as a positive residual I_{SC} after amiloride application, suggested that normal distal colon does not exhibit a significant amount of electrogenic Na^+ absorption and K^+ secretion, respectively. The positive I_{SC} in normal rat distal colon likely represents anion (Cl^-/HCO_3^-) secretion (Supp. Fig. 2A). A parallel increase in ENaCγ subunit (12.5-fold), as well as BKα subunit (2.3-fold) specific mRNA abundance indicated that ENaC and BK channel expression were induced in dietary Na^+ -depleted rat distal colon (Supp. Fig. 2B), consistent with the observed functional increase in both electrogenic Na^+ absorption and electrogenic K^+ secretion in distal colon from normal versus Na^+ -depleted rats. These data validate the model of secondary hyperaldosteronism by Na^+ deprivation used in the experiments to follow.

Dietary Na^+ depletion stimulates NKCC1 expression in rat distal colon.

qRT-PCR analyses were performed to determine whether, in parallel to BKα mRNA, the abundance of basolateral K^+ transporters, Na^+,K^+ -ATPase and NKCC1 were also increased in dietary Na^+ -depleted rat distal colon. NKCC1 (2.2-fold; $p < 0.01$), but not NaKα (catalytic subunit of Na^+,K^+ -ATPase) specific mRNA abundance was significantly increased (Fig. 1A). Previous studies have also found that Na^+,K^+ -ATPase subunits are not transcriptionally regulated by aldosterone, but rather an increase in activity is achieved via an increase in transporter density within the basolateral membrane^{35,36}. A single product of predicted size (177 bp) confirmed the specific amplification of NKCC1 mRNA in the qRT-PCR reactions (Fig. 1B). Western blot analysis also showed increased NKCC1 protein expression in dietary Na^+ -depleted compared to normal rat distal colon (2.3-fold; $p < 0.01$) (Figs. 1C and 1D).

Dietary Na^+ depletion induces bumetanide-sensitive K^+ secretion in rat distal colon.

Experiments were next performed to establish that basolateral K^+ uptake via NKCC1 supports electrogenic K^+ secretion in Na^+ -depleted rat distal colon. The contribution of NKCC1-supported K^+ secretion was measured as a function of bumetanide-sensitive I_{SC} in an Ussing-style recording chamber. Bumetanide (NKCC1-specific inhibitor; 200 μM) -sensitive I_{SC} was measured in the presence of mucosal amiloride (100 μM), to eliminate interference by highly electrogenic ENaC activity. A concentration of 200 μM bumetanide was selected based on previous literature.^{11,24} In the presence of amiloride, positive and negative I_{SC} values were measured in normal and dietary Na^+ -depleted rat distal colon, respectively (normal vs dietary Na^+ -depleted: $I_{SC} = 16.8 \pm 0.7$ vs -39.9 ± 9.8 $\mu A/cm^2$; $p < 0.01$) (Figs. 2A and 2C). Application of bumetanide to the serosal bath induced a reversal from negative to positive I_{SC} in Na^+ -depleted rat distal colon (basal vs bumetanide: $I_{SC} = -39.9 \pm 4.4$ vs 7.1 ± 1.7 $\mu A/cm^2$; $p < 0.01$), while having no significant effect in normal distal colon (basal vs bumetanide: $I_{SC} = 16.8 \pm 0.7$ vs 13.0 ± 0.7 $\mu A/cm^2$; $p = 0.74$) (Fig. 2A and 2C). The average change in I_{SC} induced by bumetanide was significantly larger in Na^+ -depleted vs normal rat distal colon (normal vs Na^+ -depleted: $\Delta I_{SC(BUMET)} = -4.6 \pm 0.8$ vs 47.0 ± 3.1 $\mu A/cm^2$; $p < 0.01$) (Fig. 2E).

Serosal application of bumetanide also significantly inhibited G_{TE} in dietary Na^+ -depleted (basal vs bumetanide: $G_{TE} = 6.2 \pm 0.5$ vs. 4.2 ± 0.4 mS/cm^2 ; $p < 0.01$), but not normal (basal vs bumetanide: 4.6 ± 0.2 vs. 4.3 ± 0.2 mS/cm^2 ; $p = 0.26$) rat distal colon (Fig. 2B and 2D), suggesting the positive change in I_{SC} induced by bumetanide was due to a *reduction* in cation (K^+) secretion rather than an increase in anion (Cl^-/HCO_3^-) secretion or cation (Na^+) absorption. The average change in G_{TE} induced by bumetanide was also significantly greater in Na^+ -depleted vs normal rat distal colon (normal vs Na^+ -depleted: $\Delta G_{TE(BUMET)} = -0.01 \pm 0.04$ vs -2.0 ± 0.2 mS/cm^2 ; $p < 0.01$) (Fig. 2F). These observations suggest that NKCC1 functions as a major basolateral K^+ loader

during electrogenic K^+ secretion in dietary Na^+ -depleted rat distal colon, while having little effect on basal secretion in normal distal colon.

Ex vivo aldosterone induces bumetanide-sensitive K^+ secretion and NKCC1 expression in normal rat distal colon.

To establish that aldosterone, but not the dietary Na^+ depletion *per se*, is critical for induced electrogenic K^+ secretion and enhanced NKCC1/BK α expression, the effect of aldosterone applied *ex vivo* was examined in normal rat distal colon mucosae mounted in the recording chamber. Similar to dietary Na^+ depletion, *ex vivo* aldosterone (10 nM, based upon measured serum aldosterone levels) over an 8-hour incubation period induced both ENaC activity (i.e., increased amiloride-sensitive I_{SC}), as well as electrogenic K^+ secretion (i.e., negative I_{SC}) in presence of amiloride, which were not observed in control tissues or tissues treated with aldosterone plus 10 μ M spironolactone, a mineralocorticoid antagonist (Fig. 3A-B). It should be noted that colonic mucosae remain viable for many hours under these conditions, as previously demonstrated.³⁷ Serosal addition of bumetanide completely inhibited the aldosterone-induced electrogenic K^+ secretion (basal vs bumetanide: $I_{SC} = -29.9 \pm 1.3$ vs 8.6 ± 1.9 μ A/cm²; $p < 0.01$). Bumetanide-sensitive I_{SC} was significantly greater in aldosterone-treated versus control tissues (control vs. aldosterone-treated: $\Delta I_{SC(BUMET)} = -5.7 \pm 1.4$ vs. 30.9 ± 1.3 μ A/cm²; $p < 0.001$) and aldosterone alone compared to aldosterone plus spironolactone-treated tissues (aldosterone vs. aldosterone + spironolactone: $\Delta I_{SC(BUMET)} = -4.0 \pm 1.1$ vs. 30.9 ± 1.3 μ A/cm²; $p < 0.001$) (Fig. 3C). Bumetanide-sensitive- G_{TE} followed the same pattern (control vs. aldosterone-treated: $\Delta G_{TE(BUMET)} = -0.2 \pm 0.02$ vs. -1.0 ± 0.04 mS/cm²; $p < 0.001$); (aldosterone vs. aldosterone + spironolactone : $\Delta G_{TE(BUMET)} = -0.2 \pm 0.01$ vs. -1.0 ± 0.04 mS/cm²; $p < 0.001$) (Fig. 3D). qRT-PCR analyses performed on total

RNA isolated from the same tissues revealed that aldosterone treatment significantly increased the NKCC1 (1.8-fold; $p < 0.05$) and BK α (5.9-fold; $p < 0.01$) specific mRNA abundance compared to control or aldosterone plus spironolactone-treated tissues (Fig. 3E). Interestingly, BK α mRNA abundance was still increased in aldosterone plus spironolactone-treated tissues compared to control (2.7-fold; $p < 0.05$), suggesting either that 10 μ M spironolactone is insufficient to completely inhibit BK α induction by aldosterone, or that aldosterone-induced BK α expression may be partially mediated by a spironolactone-insensitive mechanism. Nonetheless, these data indicate that the increased serum aldosterone, but not the dietary Na⁺ depletion alone, was likely responsible for the enhanced NKCC1 and BK α expression and electrogenic K⁺ secretion in rat distal colon observed in earlier experiments.

Bumetanide-sensitive electrogenic K⁺ secretion is mediated by mucosal BK channels in dietary Na⁺-depleted rat distal colon.

We have previously found increased mucosal BK and IK channel activities in dietary Na⁺-depleted rat distal colon.³⁷ Thus, to identify whether BK and/or IK channels mediate bumetanide-sensitive K⁺ exit across mucosal membranes, the effect of mucosal K⁺ channel blockers Ba²⁺ (a nonspecific K⁺ channel blocker), Iberitoxin (IbTX; a selective BK channel blocker) and Tram-34 (a selective IK channel blocker) were examined. K⁺ channel blocker concentrations were selected based on similar experiments described in the literature.^{9,38} Mucosal Ba²⁺ (3mM) inhibited basal K⁺ secretion ($\Delta I_{SC(Ba^{2+})} = 19.8 \pm 3.1 \mu A/cm^2$) (Fig. 4 A,D) and significantly attenuated the effect of bumetanide in Na⁺-depleted rat distal colon (no inhibitor vs. Ba²⁺: $\Delta I_{SC(BUMET)} = 47.0 \pm 3.1$ vs. $19.3 \pm 2.5 \mu A/cm^2$; $p < 0.001$) (Fig. 4F). Mucosal IbTX also inhibited basal K⁺ secretion ($\Delta I_{SC(IbTX)} = 33.8 \pm 4.0 \mu A/cm^2$) (Fig. 4 B,D) and attenuated the effect of serosal bumetanide as well (no

inhibitor vs. IbTX: $\Delta I_{SC(BUMET)} = 47.0 \pm 3.1$ vs. $15.9 \pm 1.8 \mu A/cm^2$; $p < 0.001$) (Fig. 4F), although a much longer time period was required to achieve inhibition with IbTX, as is expected.²³ Addition of mucosal Tram-34 (250 nM) produced no appreciable change in basal K^+ secretion ($\Delta I_{SC(Tram-34)} = -1.7 \pm 1.8 \mu A/cm^2$) (Fig. 4 C,D) and did not alter the response to serosal bumetanide (no inhibitor vs. Tram-34: $\Delta I_{SC(BUMET)} = 47.0 \pm 3.1$ vs. $44.5 \pm 4.2 \mu A/cm^2$; $p > 0.98$) (Fig. 4F). Together, these data suggest that NKCC1-dependent K^+ secretion in Na^+ -depleted rat distal colon is likely mediated by mucosal membrane BK, but not IK channels.

Addition of Ba^{2+} (non-selective K^+ channel blocker) produced a similar effect to IbTX with respect to inhibition of basal K^+ secretion (Fig. 4C), although IbTX-induced inhibition was significantly greater than that of Ba^{2+} ($\Delta I_{SC(IbTX)}$ vs. $\Delta I_{SC(Ba^{2+})} = 33.8 \pm 4.0$ vs. $19.8 \pm 3.1 \mu A/cm^2$; $p < 0.05$) (Fig. 4D). However, residual I_{SC} following IbTX or Ba^{2+} administration was not different (IbTX vs. Ba^{2+} : $I_{SC(RES)} = -8.8 \pm 2.4$ vs. $-12.9 \pm 2.5 \mu A/cm^2$; $p = 0.61$) (Fig. 4E), suggesting this difference may have resulted from variability in the initial I_{SC} values. In the continued presence of mucosal Ba^{2+} , IbTX or Tram-34, serosal bumetanide completely inhibited the electrogenic K^+ secretion and unmasked the apparent presence of a small degree of anion secretion (i.e. positive I_{SC}) (Fig. 4A-C), similar to Figures 3 and 4. It is also to be noted that only a negligible amount of IbTX-sensitive K^+ secretion was present in normal compared to dietary Na^+ -depleted rat distal colon (normal vs dietary Na^+ -depleted: $\Delta I_{SC(IbTX)} = 3.1 \pm 1.2$ vs. $33.8 \pm 4.0 \mu A/cm^2$; $p < 0.001$), supporting the earlier finding that $BK\alpha$ subunits expression is upregulated by dietary Na^+ depletion (Supp. Fig. 2).

Unexpectedly, serosal bumetanide appeared to inhibit residual K^+ secretion even in the presence of the non-selective K^+ channel blocker, Ba^{2+} . Incomplete inhibition of apical K^+ channels may have resulted from the effective (free) Ba^{2+} concentration being reduced by mucins

or carbonate anions present in the apical bath, or from tissue architecture impeding the diffusion of drugs to the target cell membranes. Nonetheless, the inhibitory effect of bumetanide on basal K^+ secretion was substantially diminished by IbTX and Ba^{2+} (60-70%), suggesting that coordinated uptake via NKCC1 at the basolateral membrane and exit via BK channels at the apical membrane are both essential for basal secretion of K^+ induced by aldosterone in rat distal colon.

Dietary Na^+ depletion enhances NKCC1 and $BK\alpha$ channel protein expression together in rat distal colonic crypts.

Inhibition of basal electrogenic K^+ secretion by bumetanide and/or IbTX suggested that basolateral NKCC1 and mucosal BK channels may be coordinately regulated by aldosterone in dietary Na^+ -depleted rats. Thus, immunofluorescence studies were performed to identify whether NKCC1 and $BK\alpha$ channel specific proteins are localized in the basolateral and mucosal membranes of the same cell(s) in distal colon sections. As shown in Fig. 5, NKCC1-like proteins in normal distal colon were localized predominantly in the lateral membranes of lower crypt epithelia, with minimal labelling observed in surface cells (Fig. 5 A,D,I,J). $BK\alpha$ -like proteins were diffusely localized in surface epithelial cells, with some apical membrane localization apparent within the crypt epithelium (Fig. 5 B,D,I,J). In contrast, NKCC1-like proteins are localized in the lateral membranes of both upper- and lower-crypt epithelia, as well as surface cells in Na^+ depleted rat distal colon (Fig. 5 E,H,K,L). In addition, $BK\alpha$ -like proteins were concentrated on the mucosal membranes of both upper- and lower-crypt epithelia of dietary Na^+ -depleted rat distal colon (Fig. 5B), while remaining diffuse in surface epithelial cells. $BK\alpha$, but not NKCC1 proteins were also localized in smooth muscle cells, as well as other unidentified cell types in the lamina propria of both normal and dietary Na^+ -depleted rat distal colon (Fig. 5 B,F).

Merged images clearly indicate that NKCC1 and BK α -channel proteins are localized in basolateral and mucosal membranes, respectively, in epithelial cells of the crypts of normal and dietary Na⁺-depleted rat distal colon (Fig. 5 D,H,J,L). Qualitatively, it appears that both NKCC1 and BK-channel protein expression were substantially increased in dietary Na⁺-depleted rat distal colon, consistent with our qRT-PCR and Western blot data (Fig. 1). These observations further support the working hypothesis that the expression of basolateral NKCC1 and mucosal BK α channel proteins are coordinately regulated to produce the electrogenic K⁺ secretion induced by aldosterone in the distal colon, and further suggest that the crypt region may be the primary location of aldosterone-induced K⁺ secretion that is mediated by NKCC1 and BK channels.

Enhanced NKCC1 expression does not support enhanced Cl⁻ secretion in dietary Na⁺ depleted rat distal colon.

Studies were next designed to determine whether in addition to functioning as a basolateral K⁺ loader, increased NKCC1 expression would also function as Cl⁻ loader supporting active Cl⁻ secretion in dietary Na⁺-depleted rat distal colon, as active Cl⁻ secretion is known to be dependent on basolateral NKCC1 activity.³⁹ ³⁶Cl⁻ flux experiments have previously demonstrated that both absorption and secretion of Cl⁻ are reduced in Na⁺-depleted rat distal colon²⁷, indicating that NKCC1 up-regulation may be unrelated to basal Cl⁻ secretion. Our I_{SC} data support this, as in the presence of mucosal K⁺ channel blockers Ba²⁺ or IbTX, I_{SC} remained negative (Fig. 4). However, aldosterone's regulation of stimulated Cl⁻ secretion in the distal colon – and its dependence on NKCC1 – is not known. Thus, the effect of forskolin (FSK; an adenylyl cyclase activator that stimulated cAMP-dependent Cl⁻ secretion) was assessed in normal and dietary Na⁺-depleted rat distal colon.

When added to the serosal bath, FSK induced a robust increase in I_{SC} (i.e., Cl^- secretion) in both normal and dietary Na^+ -depleted rat distal colon (normal vs dietary Na^+ -depleted: $\Delta I_{SC(FSK)} = 123.6 \pm 15.1$ vs. $92.4 \pm 22.8 \mu A/cm^2$; $p = 0.28$) (Fig. 6A,C). The increased I_{SC} was accompanied by increased G_{TE} (normal vs dietary Na^+ -depleted: $\Delta G_{TE(FSK)} = 4.2 \pm 0.6$ vs. $6.2 \pm 1.6 mS/cm^2$; $p = 0.30$) (Fig. 6B,D). Importantly, cAMP is also known to stimulate electrogenic K^+ secretion independently of Cl^- secretion in normal distal colon⁴⁰, which may ostensibly “mask” the FSK-stimulated Cl^- secretion under these conditions. However, when Na^+ -depleted rat distal colon was pre-treated for 60 minutes with mucosal IbTX, neither $\Delta I_{SC(FSK)}$ ($77.7 \pm 11.4 \mu A/cm^2$) nor $\Delta G_{TE(FSK)}$ ($4.0 \pm 0.6 mS/cm^2$) were significantly different from either normal or untreated Na^+ -depleted tissues ($p > 0.15$ for both) (Fig. 6A-D). Congruent with the finding that stimulated Cl^- secretion is not enhanced by aldosterone, abundance of cystic fibrosis transmembrane conductance regulator (CFTR; cAMP-activated Cl^- channel) mRNA was also found to be unaffected by Na^+ depletion (Fig. 6E). Thus, it is likely that the increased NKCC1 expression induced by aldosterone primarily functions to support enhanced electrogenic K^+ secretion, without a concurrent increase in basal and/or stimulated Cl^- secretion.

DISCUSSION

This study provides novel evidence that aldosterone increases the expression of both NKCC1 and BK channels in a coordinated manner to facilitate electrogenic K^+ , but not Cl^- secretion in rat distal colon. This conclusion is supported by the following observations: 1) both the activity (bumetanide-sensitive I_{SC}) and expression of NKCC1 (mRNA and protein) is enhanced by aldosterone, via dietary Na^+ depletion or by aldosterone application *ex vivo*, 2) K^+ secretion induced by aldosterone is mediated by basolateral NKCC1 and mucosal BK channels working in

tandem, as indicated by the attenuating effect of mucosal IbTX on bumetanide-sensitive I_{SC} , as well as the apparent robust co-expression of $BK\alpha$ and NKCC1 in crypt cells of Na^+ -depleted rats, and 3) aldosterone-induced upregulation of NKCC1 does not also facilitate an increase in basal or FSK-stimulated Cl^- secretion, suggesting its regulation by aldosterone is specific to the K^+ secretory pathway. Figure 8 summarizes these observations and proposes a basic cellular model of the regulation of K^+ secretion by aldosterone in the rat distal colon.

Stimulation of K^+ secretion is a fundamental effect of aldosterone signaling in the colonic epithelium and occurs independently of its parallel effects on electrogenic Na^+ absorption.²⁴ While the effects of aldosterone on apical K^+ channels have been described in detail, here we provide evidence that expression of the basolateral NKCC1 co-transporter is also under the control of aldosterone in the distal colon, which had not been demonstrated previously in rat. Further, we have shown that augmented NKCC1 activity contributes to the vectorial transport of K^+ , but not Cl^- , across the colonic epithelium, suggesting a critical role for NKCC1 specifically in aldosterone-regulated K^+ transport.

Short-term aldosterone treatment has been recently shown to regulate NKCC1 in several epithelial and non-epithelial cell types (e.g. cochlear and smooth muscle) *in vitro*,^{25,41–43} indicating the phenomenon is not specific to intestinal epithelia. However, the role of NKCC1 in vectorial BK -mediated K^+ secretion had not been investigated. Moreover, the reported mechanism of NKCC1 up-regulation was suggested to be that aldosterone prevents its degradation via the proteasomal pathway, not via transcription upregulation,^{25,41} in contrast to our findings. This discrepancy may be due to inherent differences in the response to aldosterone between in native tissue and immortalized cell lines, or because of different environment factors associated with cell culture versus within native tissue. For example, short chain fatty acids produced by the

microbiota, as well as neuronal input are both known to have a substantial impact on cellular homeostasis and electrolyte transport *in vivo*,^{44–48} but these factors are typically absent under normal cell culture conditions. Whether aldosterone also regulates the proteasomal degradation of NKCC1 in native tissue remains to be determined but is entirely possible.

A somewhat controversial issue regarding colonic K^+ transport is whether crypt or surface epithelial regions (or both) participate in luminal K^+ secretion,^{10,34,38,49} and there may be considerable variation among species. Sorensen et. al. identified the presence of BK channels and BK-mediated K^+ secretion to be restricted to the crypt region in mice, and also found NKCC1 mRNA transcript to be elevated ~ 2-fold in the crypts of mice fed a high K^+ diet, another dietary model of hyperaldosteronism.¹⁰ Although obtained from rats and not mice, our I_{SC} and immunofluorescence data are in agreement with these findings, as IbTX significantly attenuated the inhibition of K^+ secretion by bumetanide, while strong labelling of both NKCC1 and $BK\alpha$ was apparent only in the crypts of Na^+ -depleted rat distal colonic sections. Jakob et. al. also demonstrated that NKCC1 expression is confined primarily to the crypt region throughout the rat GI tract.⁵⁰ It is therefore likely that NKCC1/ $BK\alpha$ -mediated K^+ secretion, a process enhanced by aldosterone, occurs mainly in the crypt region of the distal colonic epithelium. However, aldosterone-induced K^+ secretion in surface epithelial cells has also been suggested to occur as a consequence of increased ENaC activity, favoring apical efflux of K^+ through one or more voltage-gated K^+ channels in response to the depolarization brought on by Na^+ influx.⁵¹ Thus, luminal K^+ secretion may occur in both surface and crypt regions simultaneously. Here, NKCC1 labelling was detected in surface epithelial cells of Na^+ -depleted animals, but clear membrane localization of BK channels was not apparent. Unfortunately, while NKCC1/BK-mediated K^+ secretion was clearly enhanced by aldosterone, the precise localization of this transport pathway within the tissue

– whether solely in the crypt or the surface (or both) – cannot be determined with certainty based upon these experiments.

It should be noted that the degree of hyperaldosteronism induced by dietary Na^+ depletion was dependent on the sex of the animals. As described above, serum aldosterone was somewhat elevated in Na^+ -depleted females versus females fed a normal diet, but the effect of diet was much smaller in females compared to males. It is possible that sex hormone activity in some way modulates the response to aldosterone production or confers some resistance to the impact of Na^+ depletion itself. Vivas et. al., using an acute model of Na^+ depletion by restricting dietary Na^+ and administering furosemide (a loop diuretic), found that male mice were more sensitive to the effects of Na^+ depletion in terms of resultant Na^+ appetite, as well as other cardiovascular and neuronal outcomes.⁵² However, serum aldosterone was not measured in these studies. While little work has been done to date, the mechanism(s) underlying the sex-specific effects of Na^+ depletion and accompanying hyperaldosteronism warrant further investigation, as this important aspect of mineralocorticoid function remains incompletely understood.

Alterations in colonic K^+ transport play an important role in various pathologies.^{1,5,53} For example, active colonic K^+ secretion via mucosal BK channels becomes imperative in patients with ESRD, providing an alternative outlet for K^+ and thus preventing life-threatening hyperkalemia.^{17,18} On the other hand, excessive K^+ secretion is a hallmark of colonic pseudo-obstruction, contributing to diarrhea and hypokalemia in severe cases,^{13,14} with at least one documented case of a patient who was successfully treated with spironolactone, a mineralocorticoid antagonist, to reduce fecal K^+ excretion and hypokalemia associated with diarrhea.¹⁴ Another report demonstrated that spironolactone reduced deleterious fecal K^+ losses and significantly increased stool $\text{Na}^+:\text{K}^+$ ratio in cholera patients, although overall stool rate was

not affected.¹² Accordingly, there is clinical value in furthering our understanding of mineralocorticoid regulation of colonic K^+ transport, as development of new interventions may prove useful in treating conditions like those described above.

In conclusion, here we provide evidence that aldosterone stimulates the expression of basolateral NKCC1, supporting luminal K^+ , but not Cl^- secretion in rat distal colon. Establishing that NKCC1 is an aldosterone-regulated component to the K^+ secretory pathway in the colonic epithelium may aid in the development of novel therapeutic strategies centered around the regulation of K^+ transport, such as colonic pseudo-obstruction or ESRD.

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FIGURES AND LEGENDS

Figure 1. Dietary Na^+ depletion induces NKCC1 expression in rat distal colon. (A) NKCC1 and Na^+ , K^+ -ATPase ($\text{NaK}\alpha$) mRNA abundance measured by qRT-PCR using total-RNA isolated from normal and dietary- Na^+ depleted rat distal colon. (B) qRT-PCR-amplified NKCC1 transcript products were resolved on 1.5% agarose gel show a single product of expected size (177 bp) in total-RNA isolated from both normal and dietary Na^+ -depleted rat distal colon. DNA ladder and corresponding fragment sizes are indicated on the left. (C) Representative Western blot of NKCC1 and β -actin (internal control) protein expression in mucosal homogenates from normal (N) and dietary Na^+ -depleted (D) rat distal colon. Protein ladder is shown in the extreme left lane, and corresponding molecular weights in kDa are indicated. (D) Densitometry quantitation of NKCC1

protein normalized to β -actin. NKCC1 band intensity was measured and divided by the β -actin band intensity for each sample using FIJI (ImageJ) software. Data were then transformed so that control NKCC1/ β -actin values were set equal to 1. Lines and error bars represent means $\pm$ SEM, $*p < 0.01$, as determined by Student's unpaired t-test.

Figure 2. Inhibition of NKCC1 significantly inhibits electrogenic K^+ secretion in dietary Na^+ -depleted, but not in normal rat distal colon. I_{SC} (A) and G_{TE} (B) measurements from normal

(open circles) and dietary Na⁺-depleted (closed circles) distal colonic mucosae in the presence of 100 μ M amiloride in the mucosal bath. Tissues were treated with 200 μ M bumetanide (BUMET) in the serosal bath where indicated. (C-D) Group data showing I_{SC} (C) and G_{TE} (D) from normal and aaldo tissues are before and after application of bumetanide, in the presence of mucosal amiloride. Average I_{SC} and G_{TE} values over the course of 1 minute immediately prior to bumetanide addition (*basal*), and 1 minute at the end of the recording period (*BUMET*) were calculated for each tissue. (E-F) Change in (Δ) I_{SC} (E) and G_{TE} (F) induced by bumetanide in normal and dietary Na⁺-depleted rat distal colon in the presence of mucosal amiloride. Δ I_{SC} and Δ G_{TE} were calculated for each tissue by subtracting the basal value from the value at the end of the recording period, after BUMET addition. Lines and error bars represent means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ as determined by one-way ANOVA with Tukey's post-hoc (panels C-D) or Student's unpaired t-test (panels E-F).

A **B** **C** **D** **E**

Figure 3. *Ex vivo* aldosterone induces ENaC activity and bumetanide-sensitive K⁺ secretion and increases NKCC1 and BK α mRNA abundance in normal rat distal colon. I_{SC} (A) and transepithelial resistance (G_{TE}; B) were continuously monitored in normal rat distal colonic mucosae mounted under voltage clamp conditions in the presence and absence of 10 nM aldosterone, or 10 nM aldosterone plus 10 μ M spironolactone. Following an 8-hour incubation period, I_{SC} and G_{TE} were progressively increased (evidence for induced ENaC activity) in aldosterone-treated tissues (closed circles), while no change was observed in control (open circles) or aldosterone plus spironolactone-treated tissues (grey triangles). I_{SC} and G_{TE} data over the 8-hour incubation period are not shown here, so as to prevent the depicted data from being distorted by the lengthy time scale. I_{SC} recordings depicted here were begun at 8 hours post-treatment. All tissues were treated with mucosal amiloride (AMIL; 100 μ M), followed by serosal bumetanide (BUMET; 200 μ M) where indicated. (C-D) Group data showing the bumetanide-sensitive change in I_{SC} (Δ I_{SC}; C) and G_{TE} (Δ G_{TE}; D) in control, aldosterone-, and aldosterone plus spironolactone-treated tissues. Average I_{SC} and G_{TE} values over the course of 1 minute immediately prior to bumetanide addition, and 1 minute at the end of the recording period were calculated for each tissue. Δ I_{SC} and Δ G_{TE} were calculated by subtracting the basal value from the value at the end of the recording period, after bumetanide application. (E) NKCC1 and BK α mRNA transcript abundance measured by qRT-PCR analysis using RNA extracted from control, aldosterone- and aldosterone plus spironolactone-treated mucosae used in the above experiments. Lines and error bars represent means $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$, as determined by one-way ANOVA with Tukey's post-hoc.

Figure 4. Mucosal K^+ channel blockers inhibit basal and bumetanide-sensitive electrogenic K^+ secretion in dietary Na^+ -depleted rat distal colon. I_{SC} recordings from Na^+ -depleted rat distal colonic mucosae treated with mucosal Ba^{2+} (3 mM) (A), Iberitoxin (IbTX; 250 nM) (B),

or TRAM-34 (250 nM) (C) in the presence of 100 μ M mucosal amiloride. Tissues were subsequently treated with serosal bumetanide (BUMET; 200 μ M). (D-E) Group data showing change in I_{SC} (ΔI_{SC} ; D) in the presence of Ba^{2+} , IbTX or TRAM-34, as well as the residual I_{SC} ($I_{SC(RES)}$; E) after the application of each drug. Average I_{SC} values over the course of 1 minute immediately prior to Ba^{2+} , IbTX or TRAM-34 application, 1 minute immediately prior to the addition of bumetanide, and 1 minute at the end of the recording period were calculated for each tissue. (F) Change in I_{SC} induced by bumetanide ($\Delta I_{SC(BUMET)}$) in the presence IbTX, Tram-34 or Ba^{2+} . ΔI_{SC} values were calculated by subtracting residual I_{SC} after Ba^{2+} , IbTX or TRAM-34 application, from the final I_{SC} measured after bumetanide application. Data points with no inhibitor (-) (far left) are re-plotted from Fig. 4E for comparison. Lines and error bars represent means $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$, as determined by one-way ANOVA with Tukey's post-hoc.

Figure 5. Immunofluorescence localization of NKCC1 and BK α channel specific proteins in normal and dietary Na⁺-depleted rat distal colon. Normal (A-D) and dietary Na⁺-depleted (E-H) rat distal colon sections were labelled with both anti-NKCC1 (green) and anti-BK α (red) antibodies, while nuclei were labelled with DAPI (blue). Merged images are shown in panels D and H. All images were captured at 20x magnification, scale bar = 50 μ m. Magnified surface cell regions are shown for normal (I) and dietary Na⁺-depleted (K) rat distal colon images, as indicated by the white boxes in panels D and H. Magnified crypt regions are also shown for normal (J) and dietary Na⁺-depleted (L) rat distal colon. Arrows indicate basolateral membrane labelling of NKCC1 in the lower crypt region of normal (A,J), and the whole crypt as well as surface cells in Na⁺-depleted rat distal colon sections (E,K,L). Brackets in panels A and E illustrate that NKCC1

labelling appears mostly restricted to the lower crypt in normal, while extending upward to the surface region in Na^+ -depleted rat distal colon. Arrowheads indicate the apparent mucosal membrane-localized BK channels in the crypts of both normal (B,J) and Na^+ -depleted (F,L) rat distal colon sections, while BK labelling in the surface regions was diffuse in both normal and Na^+ -depleted colon sections.

Figure 6. Enhanced NKCC1 expression does not support increased capacity for stimulated Cl^- secretion. I_{SC} (A) and G_{TE} (B) measurements from normal (open circles) and dietary Na^+ depleted (closed circles) rat distal colon, as well as dietary Na^+ -depleted rat distal colon pre-treated with mucosal IbTX (250 nM) (grey triangles) in the presence of mucosal 100 μM amiloride. Tissues were treated with 10 μM forskolin (FSK) in the serosal bath where indicated. (C-D) Group data showing change in (Δ) I_{SC} (C) and G_{TE} (D) induced by FSK in each group. Average I_{SC} and G_{TE} values over the course of 1 minute immediately prior to FSK addition, and 1 minute at the end of the recording period were calculated for each tissue. ΔI_{SC} and ΔG_{TE} were calculated by subtracting the basal value from the value at the end of the recording period, after FSK addition. Lines and error bars represent means $\pm$ SEM.

Figure 7. Cellular model of aldosterone-regulated electrogenic K^+ secretion in the distal colon. Normal distal colon secretes both K^+ and Cl^- across the apical membrane. Na^+ , K^+ ATPase (~) activity drives overall transport by establishing the Na^+ gradient that allows basolateral uptake

of K^+ and Cl^- via NKCC1. Apical membrane secretion of K^+ and Cl^- is supported by basolateral exit of Cl^- and K^+ ions, respectively, to maintain the necessary electrochemical gradient. Multiple basolateral K^+ and Cl^- channels may be involved in this process, including intermediate conductance (IK) and voltage-gated ($K_v7.1$) K^+ channels, as well as CLC-2 Cl^- channels. Aldosterone stimulates electrogenic K^+ secretion by increasing basolateral Na^+ , K^+ ATPase activity (represented by enlarged arrows) and increasing the expression and activity of BK channels and NKCC1 co-transporter (represented by enlarged image objects and arrows, respectively). Electrogenic K^+ secretion is inhibited by either IbTX (apical membrane BK channels) or bumetanide (basolateral NKCC1). To support the aldosterone-induced sustained K^+ secretion at the apical membrane, basolateral Cl^- exit also must presumably be enhanced, although the identity of the channel(s) involved remains undetermined. Despite the increase in NKCC1 expression and activity, electrogenic Cl^- secretion is *not* enhanced by aldosterone, suggesting that the regulation of NKCC1 by aldosterone is specific to the K^+ transport pathway.

Supplemental Figure 1. Dietary Na⁺ depletion induces secondary hyperaldosteronism differentially in male versus female rats. (A) Aldosterone concentrations measured in serum collected from male and female rats fed normal diet (open circles) or Na⁺- deficient diet (closed circles) for six consecutive days. (B) Change in short-circuit current (ΔI_{SC}) induced by mucosal amiloride (100 μ M) in distal colonic mucosae isolated from male and female rats fed rats fed normal or Na⁺- deficient diet, measured in an Ussing-style recording chamber. * $p < 0.05$ as determined by two-way ANOVA with Tukey's post-hoc test. Lines and error bars represent means $\pm$ SEM.

A **B**

Supplemental Figure 2. Dietary Na⁺ depletion induces electrogenic Na⁺ absorption and K⁺ secretion. (A) Short circuit current (I_{SC}) measured in mucosae from normal (open circles) and dietary Na⁺-depleted (closed circles) rat distal colon mounted under voltage clamp conditions. 100 μ M amiloride (AMIL) was added to the mucosal bath where indicated. (B) ENaC γ and BK α mRNA abundance measured by qRT-PCR using total RNA isolated from normal and dietary Na⁺-depleted rat distal colon. Lines and error bars represent means $\pm$ SEM, * p <0.01, as determined by Student's unpaired t-test.

CHAPTER 5

TITLE: Aldosterone up-regulates NKCC1 expression and enhances electrogenic Cl⁻ secretion in rat proximal colon.

RUNNING TITLE: Aldosterone enhances proximal colon NKCC1 and Cl⁻ secretion.

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AJN and VMR conceived of and designed the experiments. AJN performed all experiments and wrote the manuscript. AJN and VMR edited and prepared the manuscript in its current version.

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ABSTRACT:

Colonic electrolyte transport is regulated by aldosterone, which markedly enhances luminal K^+ secretion throughout the colon. Previous studies demonstrated that the basolateral K^+ loader, NKCC1, was transcriptionally regulated by aldosterone in the distal colon specifically to support K^+ secretion. Although NKCC1 also is required to support luminal Cl^- secretion throughout the gastrointestinal tract, its function in the distal colon appears restricted to the K^+ secretory pathway. Major segmental variability exists between the proximal and distal regions of the colon, especially concerning the effect of aldosterone on various electrolyte transport systems. Experiments were therefore designed to determine whether aldosterone also up-regulates NKCC1 in the proximal colon to support K^+ secretion. Using dietary Na^+ depletion as a model of secondary hyperaldosteronism, we found that while NKCC1 protein expression was enhanced, electrogenic K^+ secretion was not inhibited by either bumetanide (NKCC1 inhibitor) or Ba^{2+} (non-selective K^+ channel blocker). In contrast, basal and stimulated Cl^- secretion, as well as CFTR Cl^- channel protein expression was significantly enhanced by aldosterone. These data suggest that the function of NKCC1 and its regulation by aldosterone differs between proximal and distal regions of the colon. Development of therapeutic strategies in treating pathologies related to aberrant colonic K^+ and/or Cl^- transport – such as colonic pseudo-obstruction or ulcerative colitis – may benefit from the findings of this study.

NEW AND NOTEWORTHY: This study describes a novel mechanism by which aldosterone differentially regulates K^+ and Cl^- transport in the proximal versus distal colon. In the proximal colon, NKCC1 expression is enhanced by aldosterone and correlates with increased Cl^- secretion and CFTR expression. This differs from the distal colon, where NKCC1 is enhanced along with

BK channels to support K^+ , but not Cl^- secretion. These findings may be relevant to pathologies involving aberrant colonic K^+ and/or Cl^- transport.

KEY WORDS: Mineralocorticoid, Na^+ depletion, large intestine, Ussing chamber

INTRODUCTION:

Aldosterone is a critical regulator of colonic epithelial ion transport, serving to enhance Na^+ and water absorption and K^+ secretion (1-3). The effects of aldosterone on colonic transport processes are known to vary substantially by anatomical region within the colon. For example, aldosterone completely inhibits electroneutral NaCl absorption and stimulates electrogenic Na^+ absorption through the epithelial Na^+ channel (ENaC) in the distal colon (4-6), but it *enhances* electroneutral (Na^+/H^+ exchanger 3; NHE-3)-dependent Na^+ absorption in the proximal colon (7,8). With respect to K^+ transport, aldosterone enhances K^+ secretion in both distal and proximal regions of the colon (1,9,10), although there may be differences in the secretory pathways and channels/transporters involved (11). In addition, apical membrane H^+/K^+ ATPases, which mediate active K^+ absorption, are found specifically in the distal colon (12,13) and are also transcriptionally up-regulated by aldosterone (14).

In the distal colon, expression of the apical membrane large conductance K^+ (BK) channel is increased by aldosterone (15-17), correlating to BK-mediated K^+ secretion as measured by isotopic flux assays (18) or electrophysiological recordings (16,19). Aldosterone-induced apical K^+ secretion is supported by basolateral K^+ uptake via Na^+/K^+ ATPase and the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC1), NKCC1 (17). $^{86}\text{Rb}^+$ flux experiments have established that net K^+ secretion is also enhanced by aldosterone in the proximal colon (9,20). Still, the mechanism of aldosterone-induced K^+ secretion in the proximal colon is not known, apart from recent functional and immunohistochemical evidence *against* a role for aldosterone in the regulation of BK channels or BK-mediated K^+ currents in rat proximal colon (11).

Recently, NKCC1, which facilitates basolateral K^+ uptake, was shown to be functionally up-regulated by aldosterone via the prevention of its proteasomal degradation in colonic epithelial

cells *in vitro* (21,22). Our recent studies, however, have demonstrated that aldosterone transcriptionally enhances NKCC1 expression both *in vivo* and *ex vivo*, specifically to support K^+ secretion through the apical membrane BK channel in rat distal colon (23). However, it is unknown whether or not aldosterone controls NKCC1 expression and/or function within the proximal region of the tissue. The present study was therefore initiated to test the hypothesis that aldosterone up-regulates NKCC1 to support enhanced K^+ secretion in rat proximal colon. Our results indicate that NKCC1 expression is upregulated by aldosterone, but this does *not* support electrogenic K^+ secretion. Rather, basal and agonist-stimulated Cl^- secretion is enhanced by aldosterone concurrently with NKCC1 upregulation, highlighting a novel aspect of the segmental variability in aldosterone function within the colon.

METHODS:

Animals

Male Sprague-Dawley rats were housed 2 per cage and maintained in 12-hour light/dark cycles in temperature-controlled housing. Rats were randomly assigned to groups receiving either standard rodent chow or Na^+ -deficient diet (MP Biomedicals #02960364) for 6-7 days to induce secondary hyperaldosteronism, as has been described previously (2,10,24,25). All rats were allowed free access to water throughout. On the day of experiments, rats were rapidly anesthetized with 5% isoflurane and maintained under a deep plane of anesthesia with 3% isoflurane delivered via nose cone. Colons were removed by cutting away mesenteric attachments and excising tissue from the caecum to the rectum and subsequently flushed with ice cold saline prior to use in downstream experiments. After tissue removal, rats were euthanized by cutting the diaphragm. All

procedures were approved by West Virginia University's Institutional Animal Care and Use Committee prior to the start of the project.

Ussing chamber experiments

After flushing, the colon was placed on a dissection stage and submerged in ice cold HCO_3^- Ringer's solution containing the following, in mM: 140 Na^+ , 119.8 Cl^- , 25 HCO_3^- , 5.2 K^+ , 1.2 Ca^{2+} , 1.2 Mg^{2+} , 2.4 HPO_4^{2-} , 0.4 H_2PO_4^- , and 10 glucose. The solution was continuously bubbled with 5% CO_2 balanced with oxygen to maintain pH at 7.41. The colon was opened with surgical scissors along the mesenteric border and placed mucosal side facing downward. A lateral incision was made with a razor blade just beneath the longitudinal and circular smooth muscle layers, which were peeled away to leave a submucosa/muscularis mucosae/mucosa preparation.

Segments of proximal colon, identified by striation patterns in the tissue, were then mounted on 1.1 cm^2 plastic sliders for use in an Ussing-style recording chamber (Physiologic Instruments, San Diego CA). The recording chambers were equipped with one pair of voltage-sensing AgCl pellet electrodes and one pair of current-injecting AgCl wire electrodes, each connected to the chamber bath via agar salt bridge (3.5% agar in 3M KCl). The chamber bath itself was temperature controlled contained 5mL of bubbled HCO_3^- Ringer's solution in each chamber half. A multichannel voltage clamp (VCC MC8, Physiologic Instruments) was operated automatically by computer software to measure short-circuit current (I_{SC}), a function of electrogenic ion transport. By convention, positive I_{SC} values correspond to anion secretion and/or cation absorption. Intermittent bi-polar 200 ms, 5mV pulses were applied to monitor trans-epithelial conductance (G_{TE}) at 30 second intervals.

For $^{86}\text{Rb}^+$ flux experiments, 2 μCi of isotope was added to either the mucosa or serosal chamber bath in tissue pairs matched by conductance values within 20% of each other (26).

Following a 20-minute equilibration period, 500 μL samples were taken from the chamber bath opposite to where the isotope was added (i.e., the “sink” side), as well as 10 μL from the isotope bath (“hot” side) to be used in the calculation of basal K^+ transport. Samples were mixed with scintillation cocktail solution (Sigma Aldrich, #03999-5L) and counted using a Tri-carb 4910TR liquid scintillation counter (Perkin Elmer, Waltham MA). Unidirectional $^{86}\text{Rb}^+$ flux rates were calculated from the measured counts per minute (cpm) values using the following equation:

$$K^+ \text{ flux (J) } (\mu\text{Eq}/\text{cm}^2 \cdot \text{hr}) = (5 \times B - [A \times 0.9]) / ([S/5.2] \times 1.1 \times 0.25)$$

“A” and “B” represent cpm/mL values measured from the beginning and end of the flux period, respectively. 5 is the total volume (in mL) of the chamber bath and 0.9 is dilution factor accounting for removal of 500 μL of bath volume upon sample collection. “S” represents the cpm/ μEq (in 1 mL) measured from the standard obtained from the “hot” bath of the chamber. 5.2 is the K^+ concentration in the bath ($\text{mM} = \text{mMol/L} = \mu\text{Eq/mL}$), 1.1 corresponds to the slider aperture (in cm^2) and 0.25 is the time (in hours) for the flux period (26). Net K^+ fluxes were calculated by subtracting the serosal to mucosal ($S-M$) from the mucosal to serosal ($M-S$) fluxes from paired tissues.

Mucosal Scraping

Proximal colon sacs were made by filling with ice cold buffer containing (in mM): 15.4 NaCl, 8 HEPES/Tris (pH 7.5), 5 ethylenediaminetetraacetic acid (EDTA), 0.5 dithiothreitol (DTT), and 0.5 phenylmethylsulphonyl fluoride (PMSF) and incubated in the same solution on ice for 45 minutes. The drained sacs were opened along the mesenteric border. The proximal mucosal

layer was scraped off using glass slides and frozen immediately in liquid nitrogen until use. Scraped mucosae were used for RNA isolation and protein extraction.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from frozen colonic mucosa (50 mg) using the Trizol method and quantitated with NanoDrop spectrometer (Thermo Sci. ND2000C). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using a One-Step reaction kit (NEB #E3005) per the manufacturer's protocol. Briefly, custom gene-specific primers were designed using the published DNA sequence (NCBI database) and synthesized by Thermo Scientific. Primer sequences are listed in Table 1. qRT-PCR reactions performed on a 96-well plate were read using a CFX96 real time PCR machine (BioRad). mRNA abundance was determined using the delta-delta C_t method, with β -actin as a reference housekeeping gene and represented in the figures as fold change in mRNA transcript expression in the experimental (Na^+ -depleted, "aldo") relative to the control (normal) animals.

Western blot analysis

Proteins were extracted from frozen colonic mucosae (50 mg) by homogenizing in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris base, 0.5% sodium deoxycholate, 0.1% SDS, 1.0 % Triton-X100) plus protease inhibitor cocktail (Thermo #A32965), followed by a brief sonication (Fisher Sonic Dismembrator Model 100). Samples were centrifuged for 20 minutes at 12,000 x g and the supernatant was mixed with 4x Laemmli buffer (Life Technologies #NP0007) and heated to 90°C for 5 minutes. Samples were then chilled on ice before adding β -mercaptoethanol (5% w/v), and the aliquoted samples were flash frozen and stored at -80°C. Proteins (~20 μg) were

resolved on 8% Tris-glycine polyacrylamide gels and transferred on to PVDF membranes. Membranes were incubated in blocking buffer (3% BSA in tris-buffered saline plus 0.1% Tween-20 (TBS-T) for 1 hour at room temperature, followed by overnight incubation at 4°C in primary antibody solution. Table 2 provides the source and dilutions used for all primary antibodies. The membranes were then washed in TBST (5 minutes x5) and incubated in TBST + 3% BSA containing goat anti-rabbit HRP-conjugated secondary antibody (Thermo #31462; 1:20,000 dilution) for 1 hour at room temperature before being washed again in TBST (5 min x 5). Immune complexes were detected using enhanced chemiluminescence (West Dura, Thermo #34075). Images were captured using a G:BOX (Syngene) and band intensities were quantified by densitometry using Image J software and normalized to β -actin as a loading control.

Immunofluorescence microscopy

Segments of whole proximal colon were fixed overnight at 4°C in 4% paraformaldehyde. The next day, tissues were washed in PBS, flash frozen in isopentane cooled with liquid nitrogen and embedded in tissue freezing medium. 10 μ m sections were cut with a cryostat and mounted on charged glass slides. 40 minutes of heated antigen retrieval was performed by placing the slides in 10 mM citrate + 0.05% Tween-20 buffer and microwaving on very low power for 45 minutes before allowing gradual cooling to room temperature. Sections were then permeabilized in PBS + 5% goat serum + 0.1% Triton-X for 15 minutes. Afterward, primary antibody solution containing PBS + 0.01% Triton-X (PBST) + 5% goat serum with a 1:500 dilution of NKCC1 primary antibody (Cell Signaling #14581) (23,27) was placed on the sections overnight at 4°C. The next day, tissues were washed 5 times for 5 minutes each in PBST and subsequently incubated in PBST + 5% goat serum containing goat anti-rabbit AlexaFluor 488 secondary antibody (Thermo #11008; 1:2,000 dilution) for 1 hour at room temperature. After washing an additional 5 times for 5 minutes each,

slides were mounted with SlowFade Diamond antifade mountant with DAPI (Thermo #36971) and sealed with clear fingernail polish. IF images were captured using a Zeiss 710 confocal microscope at 20x magnification (Carl Zeiss, Oberkochen, Germany).

RESULTS:

Aldosterone (dietary Na⁺ depletion) enhances net K⁺ secretion in the proximal colon.

Preliminary experiments were conducted to confirm that net K⁺ secretion in the rat proximal colon was enhanced by aldosterone. Unidirectional ⁸⁶Rb⁺ fluxes were measured as described above using tissues from normal or dietary Na⁺-depleted rats. Dietary Na⁺ depletion is a well-established model of secondary hyperaldosteronism that has been extensively characterized (24). Throughout this study, dietary Na⁺-depleted rats are referred to as “aldo” (i.e., hyperaldosteronaemic). *Table 3* depicts the results from ⁸⁶Rb⁺ flux experiments using normal and aldo proximal colonic tissues. Net K⁺ secretion was found to be enhanced in aldo rats via an increase in the serosal-to-mucosal flux ($p < 0.01$ for both net and S-M fluxes). Short circuit current (I_{SC}) and transepithelial conductance (G_{TE}) were also higher in aldo tissues as well. These data are consistent with previous demonstrations (10,20) and provide the basis for further investigation into the role of NKCC1 in these processes.

NKCC1 protein expression is enhanced in “Aldo” proximal colon.

Western blot analysis of normal and aldo rat proximal colon revealed a ~2-fold increase in NKCC1 protein abundance (Fig. 1 A-B) ($p < 0.05$), but no significant change in mRNA expression was detected by qRT-PCR (Fig. 1C) ($p = 0.32$). Confocal immunofluorescence microscopy images qualitatively reflect the results from western blot analysis (Fig. 2), as NKCC1-specific labelling

was more prominent in aldo proximal colon sections, particularly in the crypt region. The observed ~2-fold increase in NKCC1 protein abundance (Fig. 1) mirrors the response previously described in the distal colon using the same model system (23). However, the discrepancy here between changes in NKCC1 mRNA transcript and protein abundance in the proximal colon suggests that the upregulation of NKCC1 in this region may not be the result of a transcriptional mechanism, as was previously demonstrated in the distal colon. While the nature of this phenomenon was not further explored here, a similar pattern has been observed in cultured epithelial cells in response to aldosterone and is likely attributable to changes in the proteasomal pathway (21,22), providing a possible explanation for the findings.

Electrogenic Ba^{2+} - or bumetanide-sensitive K^+ secretion is not present in normal or aldo rat proximal colon.

After establishing that aldo rats exhibit increased net K^+ secretion as well as increased NKCC1 protein expression, experiments were next initiated to delineate the role of NKCC1 in supporting electrogenic K^+ secretion. Here, electrogenic K^+ secretion was measured as I_{SC} (luminal cation secretion being a negative by convention) because $^{86}Rb^+$ was taken off the market during the course of these studies. Agents that inhibit electrogenic K^+ secretion in the Ussing chamber produce positive deflections in I_{SC} , corresponding to the inhibition of “negative” current. This method of quantifying electrogenic K^+ secretion has been widely utilized (16,28).

Surprisingly, neither mucosal Ba^{2+} (non-selective K^+ channel blocker) nor serosal bumetanide (NKCC1 inhibitor) inhibited basal K^+ secretion (Fig. 3). Mucosal Ba^{2+} actually induced a slightly but significantly more *negative* deflection in I_{SC} (Normal vs. Aldo: -1.5 ± 2.6 vs. $-12.7 \pm 3.7 \mu A/cm^2$; $p < 0.05$) (Fig. 3A,D), possibly corresponding to an inhibition of mucosal

Cl⁻ secretion, although G_{TE} was not also noticeably affected (Fig. 3B). Previous studies in our own lab using the distal region of the colon have shown a maximal effect of mucosal Ba²⁺ addition within 5 minutes of application (16,23), indicating the lack of a response here was not likely due to the requirement of a longer time period.

Subsequent application of bumetanide to the serosal chamber bath produced a relatively small and transient decrease in I_{SC} in aldo tissues that was not as pronounced in normal tissues (Normal vs. Aldo ΔI_{SC} (initial): -2.1 ± 0.8 vs. -10.3 ± 2.2 $\mu A/cm^2$; $p < 0.01$) (Fig. 3A,E), possibly corresponding to an initial reduction in Cl⁻ secretion. However, this effect was again very minimal and unaccompanied by a parallel decrease in G_{TE} (Fig. 3B). In addition, the response to bumetanide was not sustained, as the steady-state change in I_{SC} was not significantly different between normal and aldo tissues (Normal vs. Aldo ΔI_{SC} (plateau): 8.7 ± 5.1 vs. 1.3 ± 3.3 $\mu A/cm^2$; $p = 0.26$) (Fig. 3E). The relatively small effect of serosal bumetanide in both normal and aldo rat tissues may be the result of an inability of the drug to completely penetrate the submucosa, which was still intact in these preparations. Distal colonic tissues can be prepared to contain only mucosa and muscularis mucosae, allowing greater access to the basolateral membranes, and this is reflected in a more robust response to bumetanide. However, anatomical differences make this type of tissue preparation extremely difficult, if not impossible, in the proximal colon.

Mucosal BK and IK channel expression is not enhanced by aldosterone in rat proximal colon.

We next sought to determine whether mucosal (apical) membrane K⁺ channels were detectable in proximal colon tissue lysates and whether or not their expression was altered by aldosterone. Perry et. al. recently reported the presence of large conductance K⁺ (BK) channels in patch clamp recording from proximal colonocytes (11). In their study, hyperaldosteronism induced

by dietary K^+ loading (an alternative model to dietary Na^+ depletion) did not increase the number of functional K^+ channels in the proximal colon detectable by patch clamp, western blot or immunohistochemistry. This was in sharp contrast to the effect observed in the distal colon.

Expression of intermediate conductance K^+ (IK) channels, which are also present in the proximal colon in rat, was not quantified in that study. Further, because the models of dietary Na^+ depletion and K^+ loading are not equivalent, quantification of both BK and IK channel expression was performed here. Neither BK nor IK abundance was found to be altered in aldo animals (Fig. 4), as detected by Western blot (Fig. 4A-C) or qRT-PCR (Fig. 4D,E), corroborating the findings of Perry et. al. Although Ba^{2+} -sensitive K^+ secretion was not observed in either normal or aldo proximal colon, both BK and IK channels were detected at the mRNA and protein level. Therefore, BK or IK channels either do not facilitate mucosal K^+ secretion in the proximal colon, or there exists some structural/functional difference between proximal and distal tissues, rendering these channels insensitive to mucosal Ba^{2+} . Even if some other yet-unidentified K^+ channel was involved (e.g., of a voltage-gated family), a concentration 3mM Ba^{2+} would almost certainly be sufficient to inhibit its activity.

Basal, Ca^{2+} - and cAMP-stimulated Cl^- secretion is enhanced by aldosterone in the proximal colon.

NKCC1 is known to support aldosterone-induced K^+ secretion, specifically, in the distal colon (17,23,29) and other tissues (22,30). In the distal colon of aldo rats, basal I_{SC} is negative in the presence of mucosal amiloride (ENaC inhibitor) (31,32), indicating the presence of basal K^+ secretion and minimal Cl^- secretion, if any. These electrical properties are supported by isotopic flux data (2). However, in the rat proximal colon, basal I_{SC} is *increased* by aldosterone (Table1, Fig. 3) (7). Therefore, Cl^- secretion ostensibly predominates over the simultaneous K^+ secretion

that is also apparently present, as indicated by $^{86}\text{Rb}^+$ flux experiments. Indeed, the most well-defined role of NKCC1 throughout the gastrointestinal tract is that of supporting basal and stimulated Cl^- secretion to the lumen (33,34–36). Thus, we next assessed whether the aldosterone-induced increase in NKCC1 expression correlated to an increased capacity for Cl^- secretion.

Aldo proximal colonic tissues once again exhibited higher basal I_{SC} (i.e., Cl^- secretion) compared to tissues from normal rats (Normal vs. Aldo I_{SC} : 45.7 ± 7.3 vs. $75.1 \pm 8.0 \mu\text{A}/\text{cm}^2$; $p < 0.05$) (Fig. 5 A-C). The secretory responses to carbachol (CCH; cholinergic agonist) and forskolin (FSK; adenylyl cyclase activator) were also enhanced by aldosterone (Normal vs. Aldo ΔI_{SC} (CCH): 110.2 ± 29.8 vs. $229.2 \pm 14.5 \mu\text{A}/\text{cm}^2$; $p < 0.01$) (Fig. 5D); (Normal vs. Aldo ΔI_{SC} (FSK): 114.6 ± 16.1 vs. $157.5 \pm 9.2 \mu\text{A}/\text{cm}^2$; $p < 0.05$) (Fig. 5E). These data suggest that aldosterone increases both basal Cl^- secretion and the capacity to respond to either Ca^{2+} - or cAMP-inducing secretory agonists (e.g., neurotransmitters or paracrine signals).

CFTR channel protein expression is also enhanced by aldosterone in rat proximal colon.

Most, if not all, luminal Cl^- secretion in the gastrointestinal tract is mediated by apical membrane-localized Cystic Fibrosis transmembrane conductance regulator (CFTR) Cl^- channels (37–40). TMEM16A, a Ca^{2+} -activated Cl^- channel, is also likely involved (40–42), but does not appear to mediate Cl^- efflux across the apical membrane. Because basolateral NKCC1 protein expression and basal/stimulated Cl^- secretion were all enhanced by aldosterone in the proximal colon, we sought to determine if the expression of either of these two Cl^- channel proteins was increased in conjunction.

Western blot analysis revealed that CFTR protein, and in particular the mature, glycosylated form (“c” band) (43) was increased ~1.5 fold in aldo rat proximal colonic mucosae

(Fig. 6A,D). Similar to NKCC1, the mRNA transcript abundance was not significantly increased either (Fig. 6E). Interestingly, TMEM16A transcript (Fig. 6E) but not protein (Fig. 6B,D) was detected in any of these samples. The TMEM16A antibody used here has been validated previously by our lab and others (⁴⁴), and as a positive control, distal colonic mucosae tissue lysates were probed alongside proximal colon samples. TMEM16A was detected only in the distal colon (Fig. 6C), suggesting possible regional variability in the functional role of TMEM16A.

DISCUSSION:

Our findings indicate that the expression of the basolateral NKCC1 co-transporter was enhanced by aldosterone (dietary Na⁺ depletion) in rat proximal colon. Further, this was correlated with an augmented capacity for electrogenic Cl⁻ secretion, rather than K⁺ secretion. This is in stark contrast to the effects of aldosterone on K⁺ and Cl⁻ transport processes in the distal colon – where K⁺, but not Cl⁻ secretion is enhanced – despite similar effects concerning the expression of NKCC1. A parallel increase in the apical membrane CFTR Cl⁻ channel was observed, whereas BK and IK channel expression was found to be unaltered by aldosterone, which is also contrary to the effects seen in the distal colon. Segmental variability within the two regions of the colon has been well-documented in terms of the effects of corticosteroid hormones, including their influence of Na⁺, K⁺, and Cl⁻ transport. Here, we have identified an additional novel example of heterogeneity in the regulation of colonic ion transport systems by aldosterone.

The results of this study are clinically relevant because colonic K⁺ and Cl⁻ transport is a critical aspect of many pathologies. For example, in patients with end-stage renal disease (ESRD), the colon provides an alternative outlet for K⁺ which can prevent life-threatening hyperkalemia (45,46). On the other hand, colonic pseudo-obstruction (47) and ulcerative colitis (UC) (12,48,49)

are characterized by excessive fecal K^+ losses which contribute to the manifestation of diarrhea. Modulation of colonic ion transport via drugs that act upon the mineralocorticoid (i.e., aldosterone) receptor has even been used clinically in at least one case of pseudo-obstruction (Ogilvie's syndrome) (50). Other diarrheal pathologies such as enteroviral or enterobacterial infection primarily affect Cl^- secretion independently of K^+ secretion (51,52). However, given that such variability exists between colonic regions in terms of aldosterone-regulated K^+ and Cl^- transport, results from this and related studies may help to determine the most appropriate course of action in managing these types of pathologies in patients with unique circumstances (e.g., partial colectomy).

The overall function of aldosterone signaling in the colon is to promote salt ($NaCl$) and fluid retention, while also driving the removal of excess K^+ from the body (53). Thus, an increase in $NKCC1$ expression which may be tied only to Cl^- secretion appears counterproductive because up-regulation of this pathway would, in theory, hinder fluid absorption. However, Cl^- absorption in the proximal colon is known to be electroneutral (mediated primarily by DRA) and occurs mostly in surface absorptive cells (7). On the other hand, $NKCC1$ and $CFTR$ are known to be expressed primarily within the crypt region (36). $^{36}Cl^-$ flux studies in the past have shown an increase in net Cl^- absorption within the proximal colon of aldo rats compared to controls. However, the magnitude of Cl^- flux did not match that of Na^+ (7,9), even though the transport of these two ions is functionally coupled through Na^+/H^+ and Cl^-/HCO_3^- exchangers. The increase in basal I_{SC} (i.e., Cl^- secretion) measured in those studies as well as ours, along with the augmented response to secretagogues (i.e., those potentially derived from basal neurogenic input) may account for the discrepancy in net Na^+ versus Cl^- fluxes. That is to say, a portion of the Cl^- absorbed through surface epithelial cells may ultimately be “recycled” back to the lumen via secretion occurring

within the crypt. Despite this possible explanation, the physiological significance of having an enhanced capacity for Cl^- secretion under hyperaldosteronaemic conditions remains unclear.

Another puzzling observation is that although the net secretion of K^+ is enhanced by aldosterone in the proximal colon, as indicated by $^{86}\text{Rb}^+$ flux experiments (*Table 3*) (20), the nature of this lumenally-directed K^+ transit pathway has yet to be identified. In particular, our data indicate that at least *electrogenic* K^+ secretion in both normal and aldo proximal colon is insensitive to Ba^{2+} – a non-selective K^+ channel blocker – because no effect on I_{SC} was observed. Other experiments from our lab indicate that basal and cAMP-stimulated K^+ secretion in normal proximal colon is not inhibited by other K^+ channel inhibitors either (e.g., iberiotoxin, Tram-34, paxilline) as measured by I_{SC} and ^{86}Rb fluxes (unpublished observations). The apparent insensitivity to K^+ channel inhibitors/blockers, especially Ba^{2+} , raises the possibility that luminal K^+ secretion in the proximal colon is not facilitated by a channel, but rather through some other secretory mechanism. Perry et. al. examined BK channel activity in normal and aldo (K^+ -loaded) rat proximal colonocytes by whole-cell and single channel patch clamp recording (11). Although this approach allows for fine resolution of the biophysical properties of K^+ channels for the purpose of identifying specific channels, vectorial transport functions cannot be measured using this technique. Nonetheless, our data are congruent with the findings from that study, as BK (and IK) channels were detectable, but aldosterone had no apparent effect on their expression. It is possible that the function of these K^+ channels in the proximal colon is unrelated to luminal K^+ secretion. For example, they may be involved only in regulating colonocyte membrane potential so as to influence other ion transport pathways through modulation of electrochemical driving forces.

A recent study using transgenic mice revealed an important role for BK and its accessory subunit, LRRC26, specifically in goblet cells of the colon (54). Ablation of functional BK channels

in goblet cells led to goblet cell deterioration and resulted in increased susceptibility to DSS-induced colitis, which is known to depend on goblet cell function and mucous barrier integrity. Another study concluded that a population of goblet cells was responsible for K^+ secretion in the colon (55). Importantly, goblet cells also express NKCC1 (36,56). Luminal Cl^- secretion from crypt secretory cells is also believed to be tied to mucous and HCO_3^- secretion from goblet cells (57). The new light shed on the importance of goblet cell K^+ channels by Gonzalez-Perez et. al. may be a clue related to the apparently Ba^{2+} -insensitive K^+ secretory mechanism described here. It may also point to a potential setting in which enhanced NKCC1 expression by aldosterone may be of practical importance: if goblet cells and mucous production in the proximal colon are stimulated by aldosterone, then NKCC1 expression may likely increase there, permitting enhanced Cl^- (or HCO_3^-) secretion through those goblet cells or adjacent crypt secretory cells. At the same time, luminal K^+ “secretion” may be occurring through degranulation and dumping of large intracellular, K^+ -rich fluid volumes to the luminal space instead of via facilitated diffusion through apical membrane K^+ channels. This may explain why K^+ channel inhibitors appear ineffective at blocking secretion. These predictions are purely speculative, however, as there have been no reports to our knowledge concerning the regulation of goblet cells by aldosterone. Many experiments will need to be performed to appropriately explore this possibility.

In conclusion, here we have presented novel evidence of the up-regulation of NKCC1 and Cl^- secretory capacity by aldosterone in the rat proximal colon. This effect appears to be unrelated to electrogenic K^+ secretion, which differs from the effects of aldosterone on K^+ and Cl^- transport in the distal colon. Segmental differences must be accounted for when dealing with aldosterone-induced changes to colonic K^+ and Cl^- transport processes.

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TABLES:

<i>Gene Target and Accession number</i>	<i>Primer Sequence</i>	<i>Amplicon length</i>
<i>NKCC1 (Slc12a2)</i> <i>NM_001270617.1</i>	F: 5'- TGGCAAGACTTCAACTCAGC - 3' R: 5'- GGTATCAACAAGGTCAAACCTCC - 3'	177
<i>BKα (Kcnma1)</i> <i>NM_001393699.1</i>	F: 5'- ATGTCTACAGTGGGTACGG - 3' R: 5'- TGGGTGGTAGTTCTTTATGG - 3'	464
<i>IK (Kcnn4)</i> <i>NM_001270701.1</i>	F: 5'- TGGCTGAGCACCAAGAGC - 3' R: 5'- TACAGCACCCACTTGCAACC - 3'	197
<i>CFTR (Cftr)</i> <i>NM_031506.1</i>	F: 5'- TTCTTCAGCTGGACCACACC - 3' R: 5'- TGGAAGCTTGTTCCCTGTCC - 3'	106
<i>TMEM16A (Ano1)</i> <i>NM_001107564.1</i>	F: 5'- TTATGGCCCTCTGGGCTCG - 3' R: 5'- CACCTCCTCTTCCTCGAAGC - 3'	102
<i>β-actin (Actb)</i> <i>NM_031144.3</i>	F: 5'- AGATCAAGATCATTGCTCCTCC - 3' R: 5'- AGTAACAGTCCGCCTAGAAGC - 3'	165

Table 1: Primer sequences used in qRT-PCR analysis.

<i>Target</i>	<i>Host Species</i>	<i>Supplier</i>	<i>Dilution (Application)</i>
<i>NKCC1</i>	Rabbit	Cell Signaling	1:500 (WB) 1:500 (IF)
<i>BKα</i>	Rabbit	Alomone	1:250 (WB)
<i>IK</i>	Rabbit	Gift from Dr. Michael Kashgarian	1:3,000 (WB)
<i>CFTR</i>	Rabbit	Alomone	1:250 (WB)
<i>TMEM16A</i>	Rabbit	Alomone	1:250 (WB)
<i>β-actin</i>	Mouse (HRP-conjugated)	SCBT	1: 1,000 (WB)

Table 2: Primary antibody product and usage information.

⁸⁶ Rb ⁺ Fluxes							
Condition	<i>n</i>	J_{M-S} (μEq/cm ² ·hr)	J_{S-M} (μEq/cm ² ·hr)	J_{Net} (μEq/cm ² ·hr)	<i>n</i>	I_{SC} (μA/cm ²)	G_{TE} (mS/cm ²)
Normal	6	0.6 ± 0.1	1.1 ± 0.1	-0.4 ± 0.1	12	54.2 ± 9.2	13.4 ± 1.0
“Aldo”	8	0.8 ± 0.1	3.0 ± 0.5	-2.1 ± 0.4	16	88.0 ± 8.2	17.3 ± 1.1
<i>p</i>		0.2623	0.0071 [*]	0.0033 [*]		<0.0111 [*]	<0.0191 [*]

Table 3: ⁸⁶Rb⁺ fluxes in normal and dietary Na⁺-depleted (“aldo”) rat proximal colon.

Unidirectional ⁸⁶Rb⁺ fluxes were measured across seromuscular-stripped rat proximal colon under voltage-clamped conditions. Mucosal-to-serosal (M-S) and serosal-to-mucosal (S-M) fluxes were measured over a 15-minute period. Net fluxes were calculated by subtracting S-M from M-S fluxes using tissues pairs matched by conductance, as described in *Methods*. Flux values are given as mean ± SEM. I_{SC} and G_{TE} values represent the averages recorded over the 15-minute flux period and are given as mean ± SEM. * Indicates significance compared to control (untreated), as determined by unpaired Student’s t-test.

FIGURES AND LEGENDS:

Figure 1 –Dietary Na⁺ depletion (aldosterone) enhances NKCC1 protein, but not mRNA expression in rat proximal colon. A: Western blot performed using mucosal homogenates from

normal and Na⁺-depleted (“aldo”) rat proximal colon. Standard molecular weights and primary antibody targets are indicated to the left and right of the blot images, respectively. *B*: NKCC1 band intensity was quantified using densitometry analysis in ImageJ and normalized to actin band intensity as a loading control for each sample (*n*=3 separate animals). *C*: qRT-PCR analysis of NKCC1-specific mRNA transcript abundance between normal and aldo rat proximal colon. Data are represented as fold change relative to control (Normal). Lines and error bars represent mean ± SEM. **p*<0.05 compared with control (Normal) using unpaired Student’s t-test.

Figure 2 – Immunofluorescent labelling of NKCC1 is enhanced by dietary Na⁺ depletion in rat proximal colon. NKCC1-specific labelling (green) in normal (*A*) and Na⁺-depleted (aldo) (*B*) proximal colon sections. Nuclei are labelled with DAPI (blue). Images were captured at 20x magnification. Scale bar = 50 μ m.

Figure 3 – Dietary Na⁺ depletion does *not* enhance Ba^{2+} - or bumetanide-sensitive electrogenic K⁺ secretion in rat proximal colon. Short-circuit current (I_{SC}) (*A*) and trans-epithelial conductance (G_{TE}) (*B*) recordings from normal (open circles) and Na⁺-depleted (aldo) (closed circles) proximal colon treated sequentially with mucosal 3 mM Ba^{2+} and serosal 200 μ M bumetanide (BUMET) where indicated. Basal (*C*), Ba^{2+} sensitive (*D*) and bumetanide-sensitive (*E*) I_{SC} from normal and aldo proximal colon. Ba^{2+} -sensitive and bumetanide-sensitive I_{SC} values

were calculated as the change in I_{SC} (ΔI_{SC}) from before and after the addition of drug to the chamber bath. Lines and error bars represent mean $\pm$ SEM. $n = 5$ tissues from 3 separate animals. * $p < 0.05$ and ** $p < 0.01$ compared with control (Normal) using unpaired Student's t-test.

Figure 4 – Dietary Na⁺ depletion does *not* enhance BK or IK channel expression in rat proximal colon. *A*: Western blots performed using mucosal homogenates from normal and Na⁺-depleted (aldo) rat proximal colon tissues. Standard molecular weights and primary antibody targets are indicated to the left and right of the blot images, respectively. BK α (*B*) and IK (*C*) band intensities were quantified using densitometry analysis in ImageJ and normalized to actin band

intensity as a loading control for each sample ($n=3$ separate animals). qRT-PCR analysis of BK-specific (*D*) and IK-specific (*E*) mRNA transcript abundance between normal and aldo rat proximal colon. Data are represented as fold change relative to control (Normal). Lines and error bars represent mean $\pm$ SEM.

Figure 5 – Dietary Na^+ depletion enhances Ca^{2+} - and cAMP-stimulated Cl^- secretion in rat proximal colon. Short-circuit current (I_{SC}) recordings from normal (open circles) and Na^+ -depleted (aldo) (closed circles) proximal colonic mucosae treated with serosal 100 μM carbachol (CCH) (*A*) or serosal 10 μM forskolin (FSK) where indicated. Basal (*C*), carbachol (Ca^{2+})-stimulated (*D*) and forskolin (cAMP)-stimulated I_{SC} from normal and aldo proximal colon. ΔI_{SC} values were calculated as the change from baseline to post-drug peak I_{SC} for carbachol and from

baseline to post-drug plateau I_{SC} for forskolin. Lines and error bars represent mean $\pm$ SEM. $n = 6$ tissues from 3 separate animals for carbachol and forskolin experiments, totaling 12 tissues from 6 separate animals for basal I_{SC} measurements. $^*p < 0.05$ and $^{**}p < 0.01$ compared with control (Normal) using unpaired Student's t-test.

Figure 6 – Dietary Na⁺ depletion enhances CFTR Cl⁻ channel expression in rat proximal colon. *A-B*: Western blots performed using mucosal homogenates from 3 normal and 3 Na⁺-depleted (aldo) rat proximal colons. Standard molecular weights and primary antibody targets are

indicated to the left and right of the blot images, respectively. *C*: Representative blot showing TMEM16A labelling in normal distal, but not proximal colon homogenates as a positive control for antibody specificity. *D*: Band intensities were quantified using densitometry analysis in ImageJ and normalized to actin band intensity as a loading control for each sample ($n=3$ separate animals). *E*: qRT-PCR analysis of CFTR- and TMEM16A-specific mRNA transcript abundance between 3 normal and 3 aldo rat proximal colon. Data are represented as fold change relative to control (Normal). Lines and error bars represent mean $\pm$ SEM. * $p<0.05$ compared with control (Normal) using unpaired Student's t-test.

CHAPTER 6: GENERAL DISCUSSION

The work presented in this dissertation sheds new light on several regulatory mechanisms that govern colonic epithelial ion transport. Furthering our understanding of these mechanisms may prove useful in developing therapeutic strategies to treat conditions related to aberrant colonic ion transport, such as IBS-D or IBD, as managing electrolyte and fluid absorption is a primary goal in alleviating problematic symptoms associated with these conditions (1,2). Regulation of colonic electrolyte transport is a vastly complex matter, as discussed in Chapter 1. The studies undertaken in this project have been centered around the characterization of two major, but separate control mechanisms in the regulation of colonic electrolyte transport: regulation by submucosal neurons of the enteric nervous system, and regulation by the mineralocorticoid hormone, aldosterone. Associated with each of these sub-projects is its own set of implications for disease treatments and opportunities to expand upon the work that we have done. As such, each will be discussed separately.

6.1 – A novel role for K_v7 channels in controlling ENS-dependent epithelial transport.

The enteric nervous system (ENS) is a powerful regulator of epithelial transport throughout the G.I. tract, including in the colon (3,4). It is capable of sensing chemical and mechanical stimuli and generating a reflexive response completely independent of external inputs, although physiologically, many points of connection exist between the ENS and other neural networks (5). In general, input to the epithelium from the ENS triggers a secretory response involving enhanced secretion of Cl^- and reduced absorption of Na^+ for the purpose of driving water secretion to the lumen (via osmosis) and flushing out potentially harmful substances (6,7). Unfortunately, this

process often proceeds inappropriately in response to everyday stress and sometimes without any provocation, leading to pathological conditions such as IBS-D. Many pharmacotherapies target the ENS by suppressing neuronal activity (8), but existing treatments are commonly associated with adverse side effects. Our studies have identified a novel target (K_v7 channels) in developing treatment strategies for ENS-driven pathologies such as IBS-D.

Voltage-gated K_v7 channels (*KCNQ* gene family) have been extensively characterized in central and peripheral neural populations, as well as in other non-neuronal cell types (9,10,11). These channels open at relatively negative (hyperpolarized) membrane potentials to permit K^+ efflux from the cell (12). This suppresses excitability by hyperpolarizing the membrane, making the generation of action potentials (in neurons) more unlikely. Consequently, K_v7 -activating drugs have been used for the treatment of chronic pain and epilepsy (13) – both of which are related to excessive neuronal firing. However, it was not previously known whether K_v7 channels were expressed by, or had a functional role within, neurons of the ENS.

6.1.1 – Brief summary of findings.

We have demonstrated that at least two K_v7 channel subtypes ($K_v7.2/3$) are expressed by neurons of the submucosal plexus (Chapter 2, Fig. 10; Chapter 3 Fig. 1), and that their activation suppresses Cl^- secretion (see Chapter 2) and enhances basal Na^+ absorption (see Chapter 3) by dampening stimulatory ENS input to the colonic epithelium. Each of these outcomes would, in theory, lead to an increase in the rate of water absorption by enhancing the osmotic gradient across the mucosal barrier (14). This, coupled with other reports of K_v7 channel activators suppressing contractility (11) and afferent sensory neuron activity (15), make this class of drugs an attractive target for treatment of IBS-D. Furthermore, K_v7 activation appears to modulate epithelial transport

specifically in the colon (Chapter 3, Fig. 7), potentially minimizing any widespread or “off-target” effects that may arise with drugs acting throughout the GI tract.

The findings from these two studies build upon our current understanding of ENS function. Specifically, the expression of K_v7 channel subtypes in submucosal neurons had not yet been demonstrated, nor had the effects of Flupirtine or other K_v7 activators been evaluated with respect to epithelial ion transport. The novel observation that ENS-localized K_v7 activation inhibits neurogenic secretion fits nicely with the channels’ known role in suppressing neural activity (10,16,17). In terms of the resultant effects on epithelial Cl⁻ and Na⁺ transport, the results are in line with published literature that has characterized the regulation of transport proteins and channels in response to ENS-derived neurotransmitters (3,18,19,20).

While we did not fully characterize the epithelial intracellular signaling events in response to neuronal K_v7 activation, it stands to reason that such events would proceed as has been described by others. Namely, Ca²⁺ and/or cAMP second messenger systems would likely diminish as a result of reduced activation of basolateral receptors, causing a fall in secretion and a rise in absorption. Thus, the novel aspect of these studies is that we have identified a “new” upstream target in the suppression of neurogenic secretion in the colon, and not an entirely novel ion transport mechanism altogether. This is perhaps very advantageous with respect to therapeutic development because many of the downstream mechanistic details are already well-established. In addition, K_v7 activating drugs are known to be well-tolerated by patients (13,21).

6.1.2 – Remaining questions and future directions.

Does inhibition of ENS-localized K_v7 channels lead to increased water absorption the colon as a result of the induced changes in ion transport?

Transport of solutes allows for the movement of water in biological systems, as water cannot be transported directly. We predict that decreased Cl^- secretion and enhanced Na^+ absorption together would likely drive an increase in overall water absorption in response to ENS-localized $\text{K}_{\text{v}}7$ activation, but we have not yet demonstrated a change in water absorption directly. Thus, the most direct and logical next step in determining the efficacy of $\text{K}_{\text{v}}7$ activators in treating diarrhea is to determine the effects on water transport in the colon. This can be done through *in vivo* perfusion studies using either tritiated polyethylene glycol (^3H -PEG) or non-absorbable fluorescent solutes (e.g., FITC-dextran) as described previously (22,23). These experiments would involve cannulating the large intestine and passing solution through at a slow rate, while collecting from the distal end to calculate change in solute concentration (and thus change in water content). For a less invasive approach, stool wet/dry weight could also easily be measured in animals treated with flupirtine or vehicle to observe the effects in a truly unadulterated context.

IBS-D is commonly associated with stress and symptoms may precipitate in stressful situations (24). $\text{K}_{\text{v}}7$ activators may also alleviate stress-induced Cl^- secretion, as much of this is driven by ENS activity. Because these drugs generally depress neuronal activity, we predict that a secretory response to stressful stimuli would be blunted in animals treated with $\text{K}_{\text{v}}7$ activators. A combination of behavioral assays to evaluate stress sensitivity (e.g., social interaction test or chronic mild stress models) (25), as well as *ex vivo* and *in vivo* experiments to determine electrolyte and water transport will provide insight into the utility of such drugs in alleviating symptoms of IBS-D, and in particular, stress-related IBS-D.

Are K_V7 channels expressed in specific populations of ENS neurons?

We have shown that the effects of flupirtine are apparently specific to the colon, as the ileum was not responsive to treatment in the Ussing chamber experiments detailed in Chapter 2. Within the colon, there are at least two major populations of secretomotor neurons: cholinergic and VIP-ergic (26,27). There are also nitrgic (nNOS) neurons, although they are far fewer in number (27). RNA scope analysis, or simple dual labelling via immunohistochemistry can be used to determine whether K_V7 channels are expressed within any of these sub-populations by identifying cells expressing *KCNQ* genes in conjunction with *Choline acetyltransferase* (cholinergic neuron marker), VIP or *neuronal nitric oxide synthase* (nNOS, a nitrgic neuronal marker). It is also possible that other non-secretomotor populations express K_V7 channels as well, including interneurons, sensory afferents or even glia. RNA scope may provide the starting point for identifying the relevant subpopulations, while immunofluorescent labelling can be used to confirm these findings in native tissue. Characterizing the expression pattern of K_V7 channel subtypes in ENS neurons will inform future initiatives in therapeutic development by revealing the neurochemical and histological landscape in which K_V7 activating drugs operate within the gut.

Are these novel effects of K_V7 channel activators translatable to the human condition?

Many model systems for studying human colonic ion transport exist. However, the majority of these rely on culture of epithelial cells or tissues, and thus lack innervation by an ENS or ENS-like neural structure. Only recently have there been reports of co-culture systems using human tissue-derived organoid monolayers and neuronal progenitor cells together in culture (28,29). Even so, many limitations likely exist in this context, as development of both the

epithelium and the nervous tissue is driven artificially. Therefore, we propose that anatomical and functional characterization be performed using intact human tissue derived from surgical resection. Often, in patients with adenocarcinoma, clean margins of tissue must be excised when removing tumors. These margins provide a unique opportunity to examine ion transport or probe the neural networks using the techniques described above. Doing so will provide the all-important next step in the development of this drug class in treating human pathologies.

Another undertaking aside from wet lab experiments that will be useful in downstream applications is examination of patient records for individuals prescribed flupirtine in the clinic. Flupirtine was used for many years in the treatment of chronic pain and epilepsy, and patient data is likely still available. To our knowledge, there has not been an analysis of G.I. symptom reporting (i.e., incidence of diarrhea) in patients with pre-existing diarrheal conditions (i.e., IBS-D). Should there be a link between clinical flupirtine usage and alterations in G.I. symptoms, this will provide invaluable insight into drug development. If a positive relationship does indeed exist in the patient record, this may very well expedite the timeline of bringing K_v7 activators to market for treatment of IBS-D.

6.2 – Characterization of the role of NKCC1 in aldosterone-stimulated K^+ secretion in colonic epithelium.

Much attention has been directed toward delineating K^+ transport pathways in the colon because the colon plays a supportive, but critical role in maintaining body K^+ homeostasis (30,31). The combined efforts of the kidney and colon balance dietary K^+ intake to K^+ excretion/secretion. Because even small fluctuations in extracellular (i.e., blood) K^+ concentrations can have life threatening consequences, K^+ transport systems in these tissues have been studied extensively.

Aldosterone is known to play a central role in stimulating K^+ secretion and exerts its effects primarily by influencing the transcription of genes related to electrolyte transport, including the channels and transporters themselves or regulatory factors (kinases, ubiquitin ligases, etc.) (32,33,34,35). Within the colon, isotopic flux and short-circuit current (I_{SC}) experiments have revealed that, as in the kidney, increased circulating aldosterone triggers both short- and long-term stimulation of pathways involved in K^+ secretion. However, some underlying details had not yet been elucidated.

6.2.1 – Brief summary of findings.

Past studies had focused primarily on identifying the aldosterone-regulated channel proteins that facilitate K^+ efflux across the apical membranes of transporting epithelial cells (e.g., $BK\alpha$) (32,36). We have built upon this by demonstrating for the first time that 1) aldosterone transcriptionally regulates a key basolateral K^+ co-transporter, NKCC1, specifically to support enhanced and sustained BK-mediated K^+ secretion (see Chapter 4), and 2) that there is segmental heterogeneity with respect to this phenomenon, as this effect is only seen in the distal region of the colon (see Chapter 5).

Aldosterone was already known to regulate various transporters and channels, including NKCC1 and $BK\alpha$, via mechanisms other than direct transcriptional regulation. For example, NKCC1 is regulated by controlling the number of functional transporters that are active and localized to the basolateral membrane. Aldosterone inhibits a ubiquitin ligase called Nedd4L which, in turn, prevents the targeting of NKCC1 for degradation via the proteasomal pathway (34,37), thus increasing the pool of available NKCC1 protein. Aldosterone had also been shown to activate protein kinase C directly (38), which may well have downstream control over various

transport pathways. Here, we demonstrate *transcriptional* control of aldosterone over NKCC1 in the distal colon, and perhaps more importantly, the specificity of increased NKCC1 function to the K^+ transport pathway. This is important because in many tissues, including in the colon under normal physiologic conditions, NKCC1 is considered to support Cl^- secretion primarily (39). Thus, we have identified a new context in which NKCC1 function may be considered.

Interestingly, NKCC1 expression was also increased by aldosterone in the proximal colon, although not at the mRNA transcript level. Here, NKCC1-dependent K^+ secretion was also not apparently increased, whereas Cl^- secretion was. This suggests that there may be an inherent difference in the regional response to circulating aldosterone. One possible explanation for this is that aldosterone may signal through the glucocorticoid receptor in the proximal colon, resulting in increased NKCC1 protein abundance through non-transcriptional regulation, such as alterations in protein turnover, etc. There have been other reports of aldosterone possibly signaling through both mineralocorticoid and glucocorticoid receptors in the colon, each with its own set of outcomes regarding epithelial transport (40). Supporting this possibly is the observation that apical membrane K^+ channel expression is not affected by aldosterone in the proximal colon, nor is the amount of Ba^{2+} -sensitive I_{SC} , which should reflect the basal activity of any/all apical membrane K^+ channel(s) expressed here. These characteristics are in sharp contrast to those of the distal colon, where mineralocorticoid signaling predominates. Throughout the small intestine – a tissue which cannot signal through the mineralocorticoid receptor – NKCC1 primarily supports Cl^- secretion as well.

In spite of this, data from our lab and others have repeatedly shown increased overall rates of luminal K^+ flux as measured by radioisotope tracer experiments. These two findings – that net K^+ (^{86}Rb) flux is enhanced, but that luminal Ba^{2+} (or any selective K^+ channel blocker) does not

seem to inhibit either isotope flux or I_{SC} , seem at first to be immiscible observations. However, a recent study reported that K^+ secretion is largely confined to the goblet cells of the colon (41) – cell type in which NKCC1 is also highly expressed. It may be that in animals with elevated circulating aldosterone, goblet cells take up a greater amount of K^+ across the basolateral membrane due to the increase in NKCC1. Subsequently, this K^+ may be released to the luminal via degranulation (the process by which goblet cells secrete mucus), rather than via diffusion through an apical membrane channel protein. Experiments to determine whether this actually occurs will be discussed below.

Colonic K^+ secretion is only about 5% of that of the kidney (30). However, in certain pathological conditions, the capacity for K^+ secretion becomes very important. In patients with renal failure, the colon serves as a secondary outlet for K^+ from the body and can prevent life-threatening hyperkalemia (42,43). Conversely, some diarrheal diseases such as colonic pseudo-obstruction (Ogilvie's syndrome), ulcerative colitis, and *V. cholera* infection are characterized by excessive K^+ secretion in the colon (44,45,46,47). In at least one patient with pseudo-obstruction, the use of spironolactone (a mineralocorticoid receptor antagonist) attenuated excessive K^+ secretion and stool production (48), suggesting K^+ secretion may also drive water secretion in certain contexts. Our discovery – that NKCC1 is under transcriptional control by aldosterone to specifically support K^+ secretion in the distal colon – may be useful in developing mineralocorticoid-based treating diarrheal conditions such as pseudo-obstruction, colitis or renal failure.

6.2.2 – Remaining questions and future directions.

What transport pathway mediates aldosterone-induced K^+ secretion in the proximal colon?

We and others have demonstrated a major difference between aldosterone-induced K^+ secretion in the proximal versus distal segment of the colon. The distal colon is quite similar to the aldosterone sensitive distal nephron (ASDN) of the kidney in that BK channels mediate luminal K^+ secretion as a means of voiding the body of excess K^+ . In the proximal colon, it is clear from our own studies, as well as from work by Sandle's group (49) that apical membrane BK channels or IK channels are not up-regulated by aldosterone, despite the obvious increase in lumenally-directed K^+ flux measured by ^{86}Rb tracer experiments (Chapter 5, Table 1; 40,50). This enhanced secretion is also apparently insensitive to Ba^{2+} , leaving two possible explanations: aldosterone-induced K^+ secretion in the proximal colon is 1) mediated by a yet undiscovered, Ba^{2+} insensitive K^+ channel, or 2) luminal K^+ secretion is *not* mediated by a channel at all, but rather through some other mechanism.

As mentioned above, a recent report in mice showed that goblet cells are the principal locus of K^+ secretion in the colon (41). During degranulation, a large volume of intracellular content (high in K^+) is released to the extracellular environment – in this case, toward the lumen. This mechanism may explain the apparent increase in K^+ secretion observed in the proximal colon of hyperaldosteronaemic animals. Some preliminary evidence supports this possibility, as a protein called CLCA1, a putative goblet cell marker, is significantly increased in hyperaldosteronaemic animals, both in measured protein abundance and number of cells apparently expressing CLCA1 (Appendix Fig. 1). Thus, goblet cell number may increase as a result of elevated aldosterone.

To determine whether goblet cell degranulation contributes to luminal K^+ secretion, ^{86}Rb flux experiments should be performed under voltage clamped conditions in the presence and absence of an inhibitor of exocytosis. Unfortunately, little information is available surround the key mediators of degranulation in intestinal goblet cells, specifically. A potential candidate

exocytosis/degranulation inhibitor is the Botulinum family of toxins – inhibitors of vesicular release that work via proteolytic cleavage of VAMP/SNAP proteins. These proteins are expressed in goblet cells as well (51). Experiments using dietary Na^+ -depleted mice exhibiting intestinal/goblet cell-specific knockout for key K^+ channels (i.e., BK or IK) would also shed light on this issue because the use of transgenic animals circumvents any technical problems related to pharmacological studies attributable to drug diffusion etc., which can be problematic in Ussing chamber experiments.

Can inhibition of colonic K^+ secretion ameliorate diarrheal symptoms?

Enhanced Cl^- secretion and reduced Na^+ absorption are the archetypal causes of water secretion (20). However, excessive K^+ secretion is associated with several different diarrheal pathologies, including ulcerative colitis, colonic pseudo-obstruction and enterobacterial infection (44,45,46). Previous studies from our lab demonstrate that K^+ secretion mediated by the BK channel is augmented in the DSS model of ulcerative colitis and that blockage of BK channel with iberiotoxin can inhibit ^{86}Rb flux and water secretion *in vitro* (52). We also have supporting evidence that BK channel expression is enhanced in UC patients, and that blockage of BK channels can reduce water secretion in perfused DSS-treated rat colon (Appendix Fig. 2). Whether or not inhibition of BK channels in UC patients can meaningfully reduce stool output is still unknown.

Future studies should be direct at confirming the expression pattern of BK or other K^+ channels in patients with UC, as well as determining whether any of the associated subunits are also affected. In addition, potential changes in the expression and/or function of NKCC1 have not been explored in the context of UC or other inflammatory bowel diseases, particularly concerning K^+ secretion. There is evidence that constitutive K^+ secretion and/or lack of K^+ absorption is a

characteristic of UC (53), indicated by high stool K^+ content and low trans-epithelial potential (cation retention within the gut lumen. Pharmacological and transgenic approaches should be used to abrogate NKCC1/BK-mediated K^+ transport in DSS-induced colitis, as colonic epithelial-specific knockouts are now available. If inhibition of this pathway at either point (NKCC1-mediated uptake or BK-mediated efflux) has a significant effect on water transport, follow-up *in vivo* studies using intestinal perfusion experiments may support the manipulation of this pathway as a therapeutic option in the treatment of UC.

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APPENDIX

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B

C

Figure 1 – Goblet cell marker, CLCA1, expression is increased in Na⁺-depleted (“Aldo”) rat proximal colon. *A*: Representative western blot showing CLCA1 protein from 3 normal and 3 aldo rat proximal homogenates. Standard protein ladder and molecular weights are given in the extreme left lane and to the left of the image, respectively. *B*: Densitometric quantification of band intensities from blot shown in panel A, normalized to actin as a loading control. Lines and error bars represent mean $\pm$ SEM; * $p < 0.05$ as determined by unpaired Student’s t-test. *C*: Immunofluorescent labelling of CLCA1 in normal and aldo rat proximal colon tissue sections. CLCA1 is shown in red, nuclei are labelled with DAPI and shown in blue. Scale bar = 50 μ m.

Figure 2 – BK α expression is enhanced in colitis and its inhibition reduces net water secretion. *A*: Representative western blot showing BK α protein from 3 normal and 1 UC patient colonic surgical resection homogenates. Na⁺/K⁺ ATPase and GAPDH are shown as reference loading controls. *B*: Representative western blot showing BK α protein from 3 normal and 3 DSS-

treated rat colon homogenates. β -actin is shown as a reference loading control. *C*: Net water movement measured via *in vivo* intestinal perfusion in normal and DSS-treated rats (n=3 each). *D*: Effect of Tram-34 (IK channel inhibitor) and Iberiotoxin (IBTX; BK channel inhibitor) on net water secretion in DSS-treated rat colon measured via *in vivo* intestinal perfusion. Lines and error bars represent mean $\pm$ SEM; * $p < 0.05$ versus control as determined by unpaired Student's t-test (*A*) or one-way ANOVA with Tukey's post-hoc (*B*).