A Study of Epithelial Cl- Secretion in the Murine Distal Colon

Trey S Rottgen

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A Study of Epithelial Cl- Secretion in the Murine Distal Colon

Trey S. Rottgen

Dissertation submitted
to the School 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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Morgantown, West Virginia 2018

Keywords: KCNQ, TMEM16A, Ussing chamber, Colon, DSS, ENS

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ABSTRACT

A Study of Epithelial Cl⁻ Secretion in the Murine Distal Colon

Trey S. Rottgen

Colonic epithelium has an on-going basal level of Cl⁻ secretion, which is necessary for maintaining a properly hydrated epithelial mucus layer, and the movement of waste products. However, alterations in the level of Cl⁻ secretion can have significant effects. Loss of colonic Cl⁻ secretion, such as that seen in Cystic Fibrosis or ulcerative colitis can result in intestinal obstruction. While infection with Vibrio cholera or increased activity of the Enteric nervous system (ENS) can result in increased Cl⁻ secretion and resultant diarrhea. In this study, we decided to evaluate both sides of the spectrum via an animal model of inflammatory bowel disease, and study ENS regulation of epithelial Cl⁻ secretion.

Ulcerative colitis (UC) is well-characterized to have a significant attenuation in colonic Cl⁻ secretion. An animal model of UC, which utilizes the epithelial irritant dextran-sulfate sodium (DSS), has also been shown to have significantly blunted epithelial Cl⁻ secretion. More specifically, previous observations have demonstrated the loss of Ca²⁺-activated Cl⁻ secretion. Therefore in our study, we examined Ca²⁺-activated Cl⁻ secretion in murine distal colon mounted in Ussing chamber. A subset of animals underwent a chronic induction model of DSS-colitis. Animals that received DSS demonstrated a loss of Ca²⁺-activated Cl⁻ secretion when stimulated with carbochol in Ussing chamber experiments. Protein isolated from colonic epithelium of DSS-colitis animals demonstrated a significant decrease in the Ca²⁺-activated Cl⁻ channel, TMEM16A. This would indicate that loss of TMEM16A in colonic epithelium following DSS-treatment contributes to the observed loss of Ca²⁺-activated Cl⁻ secretion in functional studies.

ENS activity has previously been observed to contribute to the measured Cl⁻ secretion in colonic epithelium. Therefore, we decided to evaluate the efficacy of KCNQ K⁺ channel activators to hyperpolarize and decrease enteric neuronal signaling. Flupirtine, a pan-KCNQ activator, was able to significantly inhibit ongoing Cl⁻ secretion measured as a function of short-circuit current (I_SC) in Ussing chamber preparations. Application of tetrodotoxin (TTX), following flupirtine, was unable to inhibit Cl⁻ secretion, however reversal of order of application resulted in a significant inhibition of Cl⁻ secretion via TTX. Immunofluorescence studies also demonstrated the co-localization of KCNQ2 and KCNQ3 with axons of enteric neurons positively-labeled with choline acetyltransferase. From these results, we conclude that activation of KCNQ2 and KCNQ3 in enteric neurons is able to inhibit basal Cl⁻ secretion in colonic epithelium.
ACKNOWLEDGEMENTS

First of all, I would like to thank my Wife, Melissa, for all of her support, especially in recent months leading up to this point. She not only makes wonderful food, but also has been a great help going through this process due to her own experiences. I would not be here without her assistance. So, I just want to say thank you so very much for your love and support. And of course being the greatest dog mom Cooper could ever ask for.

I would to thank my family for always being there for me. I especially want to thank my Mom for the tremendous job she did in raising me, and giving me basically all the tools to succeed. She’s the hardest working person I know and always has this calm and collected manner even when things are difficult. I know I am not as good as she is but staying calm under pressure is one of my defining attributes, and I completely contribute that to my Mom. Also, a thanks to my brother for always putting up with me (even though I was annoying). High school and College were so easy because of your tips you always gave to me.

I would like to thank Dr. Greg Dick and Drew Fancher for really opening the door to research. I am forever grateful to them for taking the time to mentor some silly little undergraduate that had way too many questions.

I also would like to thank my labmates, Drew and Emily, for putting up with me the past couple of years. It has been a ton of fun, and I will truly miss working with and seeing you guys on a regular basis.

Of course, I need to thank Dr. Rajendran (Raj) for allowing me to join his lab. Working for him I have learned a tremendous amount about India and more
importantly how to “get things in black”. I would like to thank my committee members: Drs. Stanley Hileman, David Siderovski, Justin Kupec, and HanGang Yu for their helpful advice and professional mentorship.

Thank you, Everyone.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NHE3</td>
<td>Sodium/Hydrogen Exchanger 3</td>
</tr>
<tr>
<td>DRA</td>
<td>Down-regulated in Adenoma</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial Sodium Channel</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Regulator</td>
</tr>
<tr>
<td>FSK</td>
<td>Forskolin</td>
</tr>
<tr>
<td>CaCC</td>
<td>Ca$^{2+}$-activated Cl$^{-}$ Channel</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>Half-maximal Excitatory Concentration</td>
</tr>
<tr>
<td>ENS</td>
<td>Enteric Nervous System</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>IPAN</td>
<td>Intrinsic Primary Afferent Neuron</td>
</tr>
<tr>
<td>Nav</td>
<td>Voltage-sensitive Sodium Channel</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>KCNQ</td>
<td>Voltage-sensitive K$^{+}$ channel</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran-Sulfate Sodium</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>CCH</td>
<td>Carbochol</td>
</tr>
<tr>
<td>TMEM16A</td>
<td>Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} Channel</td>
</tr>
<tr>
<td>ITPR3</td>
<td>IP\textsubscript{3} Receptor</td>
</tr>
<tr>
<td>I\textsubscript{sc}</td>
<td>Short-Circuit Current</td>
</tr>
<tr>
<td>Flu</td>
<td>Flupirtine</td>
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<tr>
<td>TOL</td>
<td>Tolterodine</td>
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<tr>
<td>ICA</td>
<td>ICA-110381</td>
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<tr>
<td>NICO</td>
<td>Nicotine</td>
</tr>
<tr>
<td>XE-991</td>
<td>KCNQ Antagonist</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>Half-maximal Inhibitory Concentration</td>
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A STUDY OF EPITHELIAL Cl^- SECRETION IN THE MURINE DISTAL COLON

ABSTRACT

ACKNOWLEDGEMENTS

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Chapter I

INTRODUCTION
The function of the large intestine (colon) is primarily to absorb water from the lumen, participate in electrolyte homeostasis, and propel waste forward for elimination in the form of feces.

For the average adult, the colon absorbs approximately 1.5-2 liters of water each day [1]. This can increase to as much as 5-6 liters of water absorption in the circumstance of increased volume presentation [2]. However, volumes exceeding this limit or presentation of a large bulk volume will result in diarrhea. Water absorption is heavily reliant on electrolyte transport in the colon, as evidenced by several pathologies that lead to excess luminal electrolytes and resultant water loss [3]. However, the relative contribution of the paracellular versus transcellular pathways is still under investigation [4].

Electrolyte transport within the colon is accomplished via movement through a paracellular and transcellular pathway [1]. Paracellular movement makes up the largest component, however as contents progress distally within the colon, the transcellular component increases [1]. The paracellular component is simply movement of electrolytes down a concentration gradient, but the transcellular movement is performed via a myriad of transporters and ion channels that line the colonic mucosa. Together, the two pathways are able to absorb approximately 100 mmol/L of Na⁺ each day, with an astounding upper limit of 400 mmol/L per day [5].

Elimination of waste following water and electrolyte absorption is driven by a complex series of coordinated muscle contractions that propel the feces forward for elimination via defecation. The muscle contractions are not under
conscious control or even under the control of the central nervous system for the most part. This is primarily accomplished by a nervous system of the gastrointestinal tract known as the enteric nervous system [6].

These separate, but incredibly intermingled tasks are accomplished via an interaction between the absorptive surface or epithelium of the colonic mucosa and the enteric nervous system. The following chapter will provide greater detail on both the epithelial layer and enteric nervous system, as well as elaborate on an ion channels specific to each.

**Colonic Epithelium**

The epithelial layer lining the colonic lumen consists of a simple columnar epithelium intermixed with mucus-secreting goblet cells [7]. The organization of epithelium at the level of the cell consists of an apical membrane that is exposed to luminal contents, and a basolateral membrane that interacts directly with the interstitial space. This kind of organization gives the epithelial cells a type of “polarity” upon differentiation that is simply the localization of different transporters [7]. Epithelial cells are anchored to a basement membrane and other epithelial cells via tight junction proteins [8]. The purpose of a “tight” epithelial layer is to minimize interaction between the “outside” and the host’s immune system [9]. Histological examination of the colonic epithelium reveals a repeating pattern of crypts of Lieberkuhn followed by surface cells [7]. The historical paradigm characterizes these different locations as secretory cells located within the crypts and absorptive cells localized at the surface of the mucosa [10, 11].
Whether this is actually the case is debatable, however this provides an avenue to discuss some general electrolyte transport within the colon.

The primary cation that is absorbed in the colon is the monovalent Na\(^{+}\) ion \([12]\). In the proximal portions and to a lesser degree in distal portions of the colon, Na\(^{+}\) is absorbed via the electroneutral transporter sodium/hydrogen exchanger isoform 3 (NHE3) \([13]\). NHE3 is coupled to the down-regulated in adenoma (DRA) transporter that exchanges Cl\(^{-}\) and HCO\(_3\)\(^{-}\) \([14, 15]\). This mechanism is coupled via pH changes within the cellular microenvironment that absorbs a single Na\(^{+}\) coupled with absorption of a single Cl\(^{-}\), while extruding a proton and single HCO\(_3\)\(^{-}\) molecule \([14]\). Upon moving to a more distal location within the colon, Na\(^{+}\) absorption changes from handling via NHE3 to the electrogenic, epithelial Na\(^{+}\) channel (ENaC) \([16, 17]\). The movement of Na\(^{+}\) through ENaC is mediated by a favorable electrochemical gradient that is generated via Na\(^{+}\)/K\(^{+}\) atpase, which is located on the basolateral membrane \([7]\). The “pump” moves intracellular Na\(^{+}\) to the interstitial space and creates a void by which extracellular Na\(^{+}\) located on the apical side of the membrane can move down its electrochemical gradient \([18]\). Also, the movement of Na\(^{+}\) into the cell and eventual basolateral extrusion via the Na\(^{+}\)/K\(^{+}\) pump causes absorption of water (H\(_2\)O) via paracellular movement or aquaporin proteins located within apical membranes of colonic epithelium \([19, 20]\).

While Na\(^{+}\) absorption is a major component of on-going electrolyte transport within the colon, the presence of an electrogenic anion secretion is present \([21]\). In Ussing chamber studies, the majority of observed short-circuit
current ($I_{SC}$) can be attributed to apical anion secretion. The primary component of this basal anion secretion is comprised of the Cystic Fibrosis transmembrane regulator (CFTR) [22, 23]. The channel is so named due to its association with the disease Cystic Fibrosis. It was characterized to mediate the observed pathology in the early 1990s [24, 25]. The channel is a cAMP-sensitive Cl$^-$ channel that has a single-channel conductance of only 8-12 pS [26, 27]. However, stimulation with basolateral forskolin (FSK) can induce observable currents in either whole-cell patch clamp electrophysiology or Ussing chamber.

At the time of identification of the CFTR channel or lack thereof in CF patients, many researchers observed the presence of a compensatory Cl$^-$ current [28, 29]. This additional Cl$^-$ current seemed to be activated more through Ca$^{2+}$-sensitive pathways instead of intracellular production of cyclic nucleotides [28-30].

The Ca$^{2+}$-activated Cl$^-$ channel (CaCC) was initially observed in *Xenopus oocytes* [31]. The channel demonstrated a slight voltage and time-dependence upon activation at lower Ca$^{2+}$ concentrations. Also, the channel was sensitive to inhibition by niflumic acid with an apparent $K_i$ of 17 µM [31]. Several potential CaCCs have been proposed; however practically all putative candidates did not demonstrate a similar electrophysiological profile to the CaCC initially described in *Xenopus oocytes* [32-34]. More recently though, three separate groups identified a CaCC that resembles the native current in electrophysiological terms [35-37]. Since the identification of this new putative CaCC, it has been named TMEM16A. The initial studies on TMEM16A demonstrated the channel to be localized to salivary and cultured human bronchial epithelial cells [35, 36].
Following these initial observations, another group utilizing a global knock-out mouse of TMEM16A demonstrated an absence of a Ca$^{2+}$-activated Cl$^-$ current in mouse trachea [38].

Similar to the reports of TMEM16A-mediating the Ca$^{2+}$-activated Cl$^-$ current in native respiratory epithelium, the mice were also used to study colonic epithelium [38, 39]. Transepithelial potential [$V_{TE}$] of distal colon administered basolateral carbachol (CCH; cholinergic agonist that increases intracellular Ca$^{2+}$ concentration) was able to significantly hyperpolarize in $Tmem16a^{+/+}$ mice [38]. However, $Tmem16a^{-/-}$ littermates were not able to respond to basolateral CCH administration [38]. Calculated $I_{SC}$ from control animals was approximately 60 µA/cm$^2$, while $Tmem16a^{-/-}$ animals had a calculated $I_{SC}$ of about 10 µA/cm$^2$ [38]. It was not until recently that the tissue-specific [$V_{Il1}$] knock-out mice of TMEM16A confirmed the previously observed results [40]. Similar to the global knockout mice, CCH-stimulated $I_{SC}$ was significantly less in the $Tmem16a^{-/-}$ animals [40].

TMEM16A has been observed in several human colonic epithelial cell lines. The HT-29 and T$_{84}$ cell lines have both demonstrated expression of TMEM16A, characterized by immunoblot [41, 42]. However, a group that employed the use of siRNA targeted against TMEM16A in the T$_{84}$ cell line did not demonstrate a large decrease in ATP-stimulated $I_{SC}$ [43]. The only change in the measured $I_{SC}$ was the initial peak prior to the plateau phase of the trace, which would potentially indicate a minor role for TMEM16A in human colonic epithelium [43]. However, ATP-stimulated $I_{SC}$ in T$_{84}$ cells has previously been characterized to be
mediated more through adenosine receptors, instead of Ca\(^{2+}\)-increasing purinergic receptors [44].

**Characterization of the Ca\(^{2+}\)-activated Cl\(^-\) Channel TMEM16A**

Considerable research has also focused on the physical characterization of TMEM16A [45-47]. One of the initial findings suggested that the final quaternary structure of TMEM16A existed as a dimer in the plasma membrane [48]. This was demonstrated with TMEM16A proteins that were fused to either GFP or mCherry [48]. The different TMEM16A conjugates were able to undergo fluorescence resonance energy transfer [FRET], which indicated a close proximity of the two proteins [48]. However, it was not until a couple of years later that the actual sequence of amino acids important for this interaction was identified [49]. Mutants of TMEM16A lacking an \(\alpha\)-helix that corresponded to residues 161-179 were not able to form functional channels, hence the lack of observed Cl\(^-\) currents in transfected HEK 293 cells [49].

Shortly after this discovery, a group was able to identify several residues that were important for the voltage-dependence of the channel, as well as amino acids that participate in the Ca\(^{2+}\)-sensitivity of TMEM16A [50]. The residues that confer a voltage-dependence of the channel were found to be located within the first intracellular loop and consisted of four repeating glutamic acid residues [\textit{EEEE}] [Fig. 1] [50]. Alanine substitution at these residues shifted the half-maximal activation of the channel from 64 \(\pm\) 0.9 mV to a more depolarized potential of \(\approx\)160 mV at 1 \(\mu\)M Ca\(^{2+}\) [50]. Also, the residues important for Ca\(^{2+}\)-
sensitivity were found to be located directly adjacent to the glutamic acid residues and consisted of a glutamic acid, alanine, valine, and lysine [EAVK] [Fig. 1] [50]. Deletion of these particular residues was able to shift the Ca\(^{2+}\)-sensitivity drastically from 1 µM (which was able to increase open-probability at very hyperpolarized potentials) to 25 µM Ca\(^{2+}\) (which could increase open-probability only marginally) [50]. While EAVK residues are undoubtedly important for channel gating, it has also been shown that glutamic acid residues [E702/705] are also essential for Ca\(^{2+}\)-sensitivity of TMEM16A [Fig. 1] [51]. This was demonstrated by mutants of these two residues having a Ca\(^{2+}\)-sensitivity several orders of magnitude less than their respective wild-type [WT] channel [[Ca\(^{2+}\)], 20 µM WT vs. 2 mM E702/705 M] [51]. The importance of these residues was confirmed by another group that mutated these same amino acids and obtained a channel with significantly less Ca\(^{2+}\)-sensitivity [52]. This group was able to demonstrate cooperativity between these residues and the amino acids initially found to confer sensitivity [EAVK] [52]. Three other acidic moieties [E650, E730, D734] have been shown to contribute to the Ca\(^{2+}\)-sensitivity of TMEM16A [51]. Also, Scudieri et al. developed TMEM16A chimera proteins by substituting residues from TMEM16B [another member of the protein family, also exhibiting Ca\(^{2+}\)-stimulated Cl⁻ secretion] into the sequence of TMEM16A to determine potential domains necessary for Ca\(^{2+}\)-binding [53]. The group was able to determine from their results that the third intracellular loop of TMEM16A participates in conferring Ca\(^{2+}\)-sensitivity to the channel [53]. This was made
obvious by the deletion of these residues resulting in a shift of the EC$_{50}$ of Ca$^{2+}$ from 0.25 µM to 2 µM at a holding potential of +100 mV [53].

While the previously mentioned work was essential for identifying residues necessary for Ca$^{2+}$-binding and voltage-sensitivity [50, 51, 53], it was not until the crystal structure was elucidated that researchers could more clearly visualize the interaction of the previously mentioned residues [and several others] in Ca$^{2+}$-binding and channel gating of TMEM16A [54]. In 2014, Brunner et al. were the first group to generate a crystal structure of TMEM16 from Nectria haematococca [nhTMEM16] [54]. The conserved protein from Nectria haematococca only functions as a lipid scramblase, however, the protein is still sensitive to Ca$^{2+}$ and demonstrates increased scramblase activity with increasing concentrations of Ca$^{2+}$ [54]. The results of the study were able to demonstrate that nhTMEM16 does in fact associate as a dimer in the plasma membrane [54]. This study also described a Ca$^{2+}$-binding segment that was embedded within the hydrophobic membrane [54]. This research group was also the first one to postulate the possible mechanisms of ion conductance by either a single pore or double-barreled architecture [46, 54]. Following the initial observations obtained from the crystal structure of nhTMEM16, two different groups were able to resolve the crystal structure of TMEM16A from murine origin [47, 55, 56]. This new information about the channel was able to illuminate important residues for interaction with conducting anions as well as illustrate that each monomer of TMEM16A was able to bind two individual Ca$^{2+}$ ions [55]. Also, the greater resolution with the murine TMEM16A allowed for an accurate description of how
Ca\(^{2+}\)-binding mediates Cl\(^-\) conductance [56]. This is accomplished via a hinge mechanism that is dependent on a glutamic acid residue [E654] interacting with two Ca\(^{2+}\) ions that allow for opening of a single pore within the channel [56].

As evidenced by the tremendous amount of research that has been conducted on the characterization of TMEM16A there is still much to be done in the future. Hopefully, the previous observations can be utilized to further characterize the channel in regard to potential protein-protein interactions, or the development of novel antagonists that allow for the pharmacological study of the channel in isolation. Also, some preliminary evidence indicates a potential role for the channel in several pathologies. Whatever the case, research will continue on the CaCC, TMEM16A.

**Enteric Nervous System**

The enteric nervous system (ENS) in humans contains about as many neurons as the entire spinal cord, which is approximately 500 million neurons [57]. Due to the ENS containing so many neurons, it has previously been referred to as the "mini-brain of the gut". Almost every neurotransmitter that has been identified in the central nervous system (CNS) has also been observed in the ENS [58]. The complexity of the ENS is so vast that the majority of the gastrointestinal tract can function with local control from ENS neurons, instead of extrinsic signals from the CNS [59, 60]. The ENS has the ability to regulate local blood flow in the tissue, motility of the tract, and electrolyte secretion via control of epithelial tissue [61]. The ENS is primarily localized to two major layers: the submucosal plexus and the myenteric plexus [6]. The submucosal plexus is
located just beneath the submucosa, while the myenteric plexus is actually located between the two smooth muscle layers of the GI tract (circular and longitudinal).

Whether located within the myenteric or submucosal plexus, there is a wide variety of neurons that contribute to the proper functioning of the gastrointestinal tract, however, they can be generally subdivided into afferent and efferent limbs of the ENS. Intrinsic primary afferent neurons (IPANs) have axons that sample the luminal contents for noxious stimuli that could cause harm to the mucosal layer, as well as, exhibiting an ability to respond to inflammatory mediators [62-64]. Integration of these signals can lead to a reflexive arc utilizing other ENS neurons or the stimulation of a centrally-mediated arc to increase motility and secretion to dilute and eliminate any potential hazard [61]. While the IPANs are sensory in function, the ENS is not without effector neurons such as secretomotor neurons [6]. Axons originating from the secretomotor neurons can stretch either in an ascending or descending longitudinal fashion as to coordinate an overall complex response of rhythmic muscle contractions [6, 65]. Some of these axons may even project to the mucosa to synapse on epithelium [66]. With this type of patterned distribution, the secretomotor neurons are able to increase/decrease contraction of the smooth muscle of the GI tract and in turn increase or decrease motility, as well as, signal to the epithelial layer to modify on-going electrolyte transport depending on the current synaptic inputs [6, 66].

As far as signaling within the ENS, many different neurotransmitters are utilized regardless of whether the neuron is a secretomotor neuron or IPAN [58].
However, cholinergic fibers (acetylcholine-releasing, ACh) comprise approximately 60% of the total fibers within the ENS [67]. In many cases, ACh is released along with small neuropeptides such as vasoactive intestinal peptide or calcitonin-gene related peptide, which contribute to the overall observed response [58]. ACh that is released from secretomotor neurons mediates its effect through muscarinic receptors located on smooth muscle or the basolateral surface of the epithelium. The binding of ACh to a muscarinic receptor leads to a transient increase in intracellular Ca$^{2+}$, which may mediate a contraction of smooth muscle or an increase in Cl$^{-}$ secretion from epithelium, depending on the localization of release [68, 69].

While the released neurotransmitter is the actual molecule that can modulate the activity of its target, it is universally understood that ion channels located on a neuron can regulate the release of said neurotransmitter [70, 71]. Previous studies have shown that blockade of voltage-sensitive Na$^{+}$ channels (Na$_V$) with tetrodotoxin (TTX) can prevent release of neurotransmitter via inhibition of the propagating action potentials [72]. Neurotransmitter release can also be inhibited in this same fashion from enteric neurons [73, 74]. However, the generation of an action potential and eventual release of a neurotransmitter is dependent on the cell reaching a threshold potential.

Hyperpolarization via activation of K$^{+}$ channels in neurons can prevent synaptic inputs from reaching a threshold potential. More specifically though, activation of KCNQ (K$_V$7) channels or the “M” current in CNS neurons is able to cause a significant hyperpolarization and inhibition of neurotransmitter release.
A previous group has also shown that activation of $K_v7$ channels on neuronal cell membranes of the ENS can cause a dramatic hyperpolarization and prevent IPANs from responding to the noxious stimulus bradykinin [76]. The group was also able to show that distension with supraphysiological pressures was unable to generate an action potential when neurons were hyperpolarized via activation of KCNQ channels.

**Characterization of KCNQ Channels**

KCNQ channels are voltage-sensitive $K^+$ channels ($K_v$) [77, 78]. All members of the family consist of six transmembrane domains [79, 80]. The fourth of which is the S4 domain and confers the voltage-sensitivity to the KCNQ family, as well as, all voltage-sensitive $K^+$ channels [81-83]. The group is composed of five different isoforms, which are simply KCNQ1-KCNQ5 [77, 84-86]. Similar to many other $K^+$ channels [87-89], the KCNQ family associates in the membrane as a tetramer to form the functional channel [90, 91]. These channels have been characterized as slowly deactivating with visible currents at hyperpolarized potentials [92]. The family of channels are also frequently referred to as the “M” current. This is due to early observations that stimulating the $M_1$ receptor would inhibit the visible current [93, 94]. However, stimulation of receptors for angiotensin II, bradykinin, or purines will cause inhibition of the current or really any other Gαq activation pathway [95-97]. The KCNQ family has also been characterized as contributing to resting membrane potential in many different cell types [98-101]. The channels have been functionally identified and have been shown to play a major role in a vast array of tissues, including vestibular hair
cells of the inner ear, cortical neurons, cardiac repolarization, and K⁺ recycling in respiratory and colonic epithelium [102-106]. Several pathologies, which include a form of deafness, a familial type of epilepsy, as well as the long QT syndrome in the heart have been linked to mutations in one variant or another [102, 107, 108]. The aforementioned diseases also demonstrate the functional importance of KCNQ channels in cellular membranes.

While several different isoforms of the KCNQ family exist, only a couple have real functional significance in neuronal tissue. KCNQ2 (Kᵥ7.2) and KCNQ3 (Kᵥ7.3) are the primary contributors to the M current in neuronal tissue, however, in some peripheral circuits KCNQ5 (Kᵥ7.5) has been identified [101, 109, 110]. Early observations of the neuronal M current demonstrated that the current, as previously mentioned, could be inhibited via activation of muscarinic receptors [94]. The mechanism of inhibition would later be shown to be PIP₂ depletion in the cellular membrane [111]. It was also shown that the native M current could dampen the excitability of neurons and therefore reduce the amount of action potentials [112, 113].

In 1998, Biervert et al. was able to clone the open-reading frame of one of these channels, KCNQ2 [114]. The study was able to show that not only was this in fact a voltage-sensitive K⁺ channel, but mutations in the channel were directly linked to a neonatal seizure disorder through the cloning of a mutant isoform from a patient [114]. In that same year, Wang et al. was able to not only clone KCNQ2 independently, but also clone KCNQ3 [77]. In vitro transcribed cRNA of KCNQ2 and KCNQ3 was injected into *Xenopus oocytes* and voltage-clamped for
functional characterization [77]. KCNQ2, as previously described, had observable currents, however, injection of KCNQ3 alone did not produce currents above that of background [77]. Co-expression of the two isoforms increased the observed currents dramatically. Also, the co-expression of the two channels seemed to affect sensitivity to the inhibitor tetraethylammonium (TEA), since the previous K_d for KCNQ2 alone was much less than during co-expression of both channels (0.16 ± 0.02 mM vs. 3.5 ± 0.7 mM) [77]. More importantly though, the study was able to show that co-expression of the two channels resulted in a profile that was similar to the native M current in half-activation (-45.4 mV vs. -46.7 mV, M vs. KCNQ2/3) and pharmacological inhibition to XE-991 (0.98 ± 0.15 µM vs. 0.6 ± 0.1 µM, M vs. KCNQ2/3) [77]. This was a major discovery in identifying the putative neuronal M current channels.

Animal models utilizing genetic manipulations have been very useful in determining the importance of KCNQ2 and KCNQ3 in neuronal physiology. In 2005, a group utilized a dominant-negative knock-in of KCNQ2 [112]. M currents were significantly less than wild-type littermates. Also, spike-frequency was significantly increased in neurons expressing the KCNQ2 dominant-negative. Unfortunately, a genetic deletion model of KCNQ2 resulted in death of peri-natal mice within only a few hours of birth [115]. Less has been done with KCNQ3, however, one group did develop a model utilizing a substitution of glycine to valine at position 311 [116]. This specific mutation is derived from a family that was well characterized to have benign familial neonatal convulsions. Animals homozygous for this mutation demonstrated a significant attrition rate as
compared to heterozygotes. Elicited current upon a step protocol demonstrated a significantly blunted response to depolarizing potentials.

Following some of these observations that clearly indicated KCNQ2/3 were important to overall neuronal excitability, it was shown that the channels associated with ankyrin-G in axon initial segments (AIS) [117, 118]. This interaction was shown to be mediated via several c-terminal residues. Substitution of those residues in KCNQ2 (E810A, S811A, D812A) and KCNQ3 (E837A, T838A, D839A) (Fig. 2) resulted in loss of localization to the AIS in cultured hippocampal neurons [118]. Shah et al. developed a small peptide that was able to disrupt this interaction in brain slices undergoing patch clamp electrophysiology [119]. Application of the ankyrin-binding-peptide (ABP) did result in an overall depolarization of hippocampal neurons. More interestingly though, the disruption in the AIS led to a dramatic reduction (7.8 ± 0.8 mV) in the threshold potential for generation of an action potential [119].

Another residue that may have less importance for channel localization but definitely has significance in terms of modulation of channel activity is the residue important for binding of several pharmaceuticals such as retigabine and flupirtine. Both have been shown to be potent activators of native M current, as well as expressed KCNQ2 and KCNQ2/3 subunits [120, 121]. The substitution of a single tryptophan (W236 Q2/ W265 Q3) (Fig. 2) can result in complete loss of sensitivity to retigabine [122, 123]. The proposed mechanism is that binding to the tryptophan can stabilize the activation gate, which would increase activation, as well as, slow deactivation of the channel (Both phenomenon have been
observed in electrophysiology experiments) [123]. Flupirtine has not been scrutinized in this same way. However, the drug shares substantial homology to retigabine and is assumed to promote channel activation in a similar manner.

**Inflammatory bowel Disease**

This group of diseases are characterized as inflammation along the gastrointestinal tract (GI), primarily occurring in the distal portions of the small intestine and all throughout the colon [124]. Inflammatory bowel disease (IBD) is primarily two different diseases, which are Crohn's disease and ulcerative colitis. Crohn's disease while normally localized to the small and large bowel can potentially manifest in any portion of the GI tract. Also, Crohn's disease can elicit inflammation throughout the entire thickness of the bowel, ranging from the epithelial layer to the underlying longitudinal smooth muscle [125]. This can result in the formation of strictures or even bowel perforations. However, ulcerative colitis (UC) is only localized to the large bowel. It is clinically characterized as inflammation beginning within the rectum and moving proximally. In severe cases of pancolitis (inflammation of the entire colon), "inflammatory backwash" can move into the distal ileum of the small bowel causing inflammation. As far as inflammation within the tissue, UC is typically isolated to the mucosa (epithelium) and submucosa [126]. Besides differences in the location of the two diseases, the two pathologies also have a different profile when it comes to the inflammatory response. Crohn's Disease typically has a cytokine profile that resembles a Th\(_1\) response, while UC is more typically a Th\(_2\) response [125, 126]. While this information has provided avenues for the development of new
therapies, the actual etiology of either disease is still unknown and under intense research.

Since tissue from a patient can only be obtained upon manifestation of the disease, many animal models have been utilized to more fully understand the pathogenesis of IBD [127]. One of the more common models utilized for IBD research is the dextran-sulfate sodium (DSS)-induced colitis model [128-130]. The model has been shown to have consistent results as well as offering ease of replication. Animals that undergo DSS exposure are simply administered their normal drinking water supplemented with 2-5% DSS [128]. The size of the dextran has been shown to impact the severity of colitis that develops within animal. The typical size that is utilized within the literature is 36,000-50,000 daltons [131, 132]. The overall histopathological features that develop are similar to IBD with a large inflammatory infiltrate in the lamina propria, crypt distortion, and crypt abscesses [133]. However, selection of the host has been shown to impact the elicited inflammatory response. C57Bl/6 mice when exposed to DSS typically develop Th₁-type response (Crohn’s disease), but Balb/c mice when administered DSS develop more of a Th₂ inflammatory response, which is more similar to the clinical pathology of UC [126, 134, 135].
Specific Aims:

Specific Aim 1: To determine if the observed absence of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} secretion in DSS-colitis is the result of the down-regulation of the Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channel, TMEM16A. To evaluate this, functional evidence will be collected utilizing the Ussing chamber to measure epithelial Cl\textsuperscript{-} secretion from mounted colonic epithelium from either control or DSS-colitis mice. Also, protein expression/localization of TMEM16A will also be evaluated using immunoblot and immunohistochemistry techniques.

Specific Aim 2: To determine if activation of KCNQ channels in enteric neurons can inhibit apical Cl\textsuperscript{-} secretion via hyperpolarization and resultant inhibition of neurotransmitter release. Mounted colonic epithelium from murine distal colon will have flupirtine (KCNQ activator) applied to the basolateral membrane in the presence or absence of other inhibitors of the ENS. Also, co-localization via immunofluorescence of KCNQ2 and KCNQ3 with choline acetyl transferase will be carried out to determine the presence of and potential contributors to the observed effect in functional studies.
References


Figure 1. Schematic of TMEM16A channel protein with identified residues necessary for voltage and Ca\(^{2+}\)-sensitivity. TMEM16A channel protein consists of 10 transmembrane domains [TMD]. The intracellular loop between TMD-2 and TMD-3 contains the voltage-sensitive \(\text{EEEE}\) residues, as well as, the \(\text{EAVK}\) residues that participate in Ca\(^{2+}\)-sensitivity of the channel. The E702 and E705 residues located within the seventh TMD also participate in Ca\(^{2+}\)-sensitivity of the channel. The third intracellular loop (shown in pink), and the residues E654, E730 and D734 have also been implicated to affect Ca\(^{2+}\)-sensitivity of the channel.
Figure 2. Schematic of KCNQ2 and KCNQ3 channels with labeled residues important for drug binding and channel localization. Tryptophan (W 236 Q2/ W 265 Q3) labeled in yellow is necessary for binding of retigabine and the homologous drug, flupirtine. The orange box correlates to residues important for localization to axon initial segments (AIS). Deletion or substitution of these residues results in a complete loss of localization. The S4 domain is important voltage-sensitivity of the channel (hence labeling with the positive signs).
Chapter II

Dextran Sulfate Sodium (DSS)-induced Chronic Colitis Attenuates Ca^{2+}-activated Cl^{-} Secretion in Murine Colon by Down-regulating TMEM16A

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Running Title: Decreased TMEM16A Expression in DSS-induced Colitis

Key Words: Ussing Chamber, Colonic epithelium, Cl^{-} channels, Muscarinic Signaling, Colitis,
Abstract. Attenuated Ca$^{2+}$-activated Cl$^{-}$ secretion has previously been observed in the model of dextran-sulfate-sodium (DSS)-induced colitis. Prior studies have implicated dysfunctional muscarinic signaling from basolateral membranes as the potential perpetrator leading to decreased Ca$^{2+}$-activated Cl$^{-}$ secretion. However, in our chronic model of DSS-colitis, Chrm3 transcript (1.028 ± 0.12 vs. 1.029 ± 0.27, $p > 0.05$) and CHRM3 protein expression (1.021 ± .24 vs. .928 ± .09, $p > 0.05$) were unchanged. Therefore, we hypothesized that decreased CCH-stimulated Cl$^{-}$ secretion in DSS-induced colitis can be attributed to a loss of Ca$^{2+}$-activated Cl$^{-}$ channels (CaCC) in apical membranes of colonic epithelium. To establish chemically-induced colitis, Balb/C mice were exposed to 4% DSS for 5 alternating weeks to stimulate a more moderate, chronic colitis. Upon completion of the protocol, whole thickness sections of colon were mounted in the Ussing chamber under voltage-clamp conditions. DSS-induced colitis demonstrated a complete inhibition of basolateral administration of CCH-stimulated Cl$^{-}$ secretion that actually displayed a reversal in polarity (15.40 ± 2.22 µA/cm$^2$ vs. -2.47±0.25 µA/cm$^2$). Western blot of potential CaCCs, quantified by densitometric analysis, demonstrated no change in bestrophin-2 (BEST2) and cystic fibrosis transmembrane regulator (CFTR), while Anoctamin-1 (ANO1, TMEM16A) was significantly down-regulated (1.001 ± 0.13 vs. 0.510 ± 0.12, $p < 0.05$). Our findings indicate that decreased expression of TMEM16A in DSS-induced colitis contributes to the decreased Ca$^{2+}$-activated Cl$^{-}$ secretion in murine colon.
Introduction

Chloride secretion in secretory epithelium has been primarily subdivided into cAMP-stimulated and Ca$^{2+}$-activated through the use of different pharmacological agonists (9, 13). Aberrations in Cl$^-$ secretion have been observed in several pathological conditions, which include Cystic Fibrosis, Vibrio cholera infection, and inflammatory bowel disease (8, 14, 37). Decreased Cl$^-$ secretion in ulcerative colitis (UC) or animal models of UC (dextran-sulfate sodium; DSS) has been previously reported (10, 12). More specifically, Ca$^{2+}$-activated Cl$^-$ secretion has been shown to be decreased in the DSS-induced colitis animal model (3, 16, 19). Previous studies focused on this phenomenon concluded the attenuation to be attributable to dysfunctional muscarinic signaling from the basolateral surface of colonic epithelium (1, 18). However more recently, it has been reported to be possible perturbations in ion channel function/expression in colonic epithelium (16).

Ca$^{2+}$-activated Cl$^-$ channels (CaCCs) have been implicated to be involved in epithelial differentiation, salivary secretion and mucociliary clearance (28, 30, 35). However initial attempts to characterize CaCCs proved difficult, as potential candidates did not match the biophysical properties of the native protein (15). In the past decade a novel CaCC, anoctamin 1 (ANO1, TMEM16A), was identified by three separate groups (6, 34, 40). Young et al demonstrated that injected siRNA to *Tmem16a* significantly inhibited pilocarpine (muscarinic agonist)-stimulated salivary secretion in mice (40), while Caputo et al was able to achieve a similar knockdown of Ca$^{2+}$-activated Cl$^-$ secretion with siRNA targeted to
TMEM16A in primary human bronchial epithelial cells (6). Following these seminal discoveries, several groups utilizing a global gene knock-out of Tmem16a demonstrated the absence of Ca\(^{2+}\)-activated Cl\(^{-}\) secretion in secretory epithelium isolated from trachea and colon (28, 30). However, the global deletion of the gene resulted in tracheal malacia and premature death of the animal (29). More recently, a tissue-specific knock-out of Tmem16a was developed. Isolated distal colon from these mice demonstrated an absence of Ca\(^{2+}\)-activated Cl\(^{-}\) secretion following CCH administration (4).

While TMEM16A has been identified as the primary mediator of Ca\(^{2+}\)-activated Cl\(^{-}\) secretion in colonic epithelium, it has not been studied in DSS-induced colitis. Thus, the goal of this study was to identify as to whether the previously described decreased Ca\(^{2+}\)-activated Cl\(^{-}\) secretion in colonic epithelium was attributable to decreased expression of TMEM16A in a DSS-induced chronic colitis. Our results indicate that TMEM16A protein expression is down-regulated in a chronic model of DSS-induced colitis and contributes to the observed loss in Ca\(^{2+}\)-activated Cl\(^{-}\) secretion.
Materials and Methods

Animals. Non-fasting male Balb/cAnNCrl mice (17 weeks old, Charles River, NY) were provided normal chow for the entirety of the project. Prior to the beginning of the 10-week project, mice (7 weeks) were randomly selected for either control or DSS groups, respectively. Balb/C mice selected for the DSS protocol were provided 4% DSS for 5 days (with 9 days of normal drinking water in between) for 5 alternating weeks, beginning with the first week. Animals utilized for the acute protocol were assigned to either control or DSS groups. DSS groups were administered 4.5% DSS in the drinking water for five (5) consecutive days followed by one day of normal drinking water. Both groups were then euthanized on the seventh day of the protocol. All animals used in the study were provided food and water ad libitum. All animals were anesthetized with intraperitoneal (i.p.) injection of Fatal Plus (Patterson Veterinary, SC). The experimental protocols used in this study were approved by the West Virginia University Institutional Animal Care and Use Committee.

Histology and Immunohistochemistry. Distal colon segments were isolated from euthanized mice via bilateral pneumothorax. Isolated colonic segments were measured immediately upon removal with a constant temperature of 22-23°C. Distal colon segments were then rinsed with phosphate-buffered saline (PBS) to remove any residual content and debris. Colonic segments were then opened longitudinally along the mesenteric border. An approximate 3 mm piece of segment was then cut from the intact colon and immediately placed in 10% neutral-buffered formalin for tissue fixation. Fixed tissue was then given to the
West Virginia University Translational Pathology Department for paraffin-embedding and haemotoxylin and eosin (H&E) staining. Prepared slides consisted of 5 µm thick sections mounted on glass slides, followed by H&E staining for visualization of tissue architecture from the respective treatment groups. Immunohistochemistry was also performed on control and DSS tissue by the West Virginia University Translational Pathology Department to determine the localization of TMEM16A in whole thickness sections of mouse distal colon. Anti-TMEM16A antibody (Alomone, Jerusalem, Israel) was used at a dilution of 1:100. Sectioned tissue was incubated at 37°C for a period of one hour in primary antibody. Following the initial step, tissue was incubated in goat anti-rabbit horseradish-peroxidase conjugated secondary antibody (1:1000) for one hour. Finally, a chromogenic substrate was added for visualization of immune-stained slides. All images were captured with the AxioImager (Carl Zeiss, Germany) on a 20x magnification.

**Electrophysiology.** Distal Colons were removed from euthanized mice via bilateral pneumothorax and rinsed with ice-cold PBS to clear residual contents. Segments were opened longitudinally along the mesenteric border to expose the epithelium. Full thickness tissue was then mounted on a pin slider with an oval aperture of 2.8 w x 11 l mm with a cross-sectional area of 0.30 cm² and placed in the EasyMount Ussing chamber (Physiological Instruments, San Diego, CA) for electrophysiological study as previously described (26). Mounted tissue was bathed on both sides with Ringers’ solution (in mM: 115 NaCl, 25 NaHCO₃, 2.4 K₂HPO₄, 0.4 KH₂PO₄, 1.2 CaCl₂, 1.2 MgCl₂ and 10 glucose), while also being
continuously voltage-clamped at 0 mV to abolish any developing electrochemical gradient. I_{SC} was continuously monitored with a 5 mV pulse applied to the tissue every 5 s to measure resistance (Ω). Transepithelial voltages (V_{TE}) were calculated using Ohm’s law.

\[ \Delta V = (\Delta I_{SC} \times R_{TE}) \]

Tissues were continuously maintained at 37°C and gassed with 5% CO₂-95% O₂ (pH:7.4). Mounted tissue was incubated for 30 minutes with 5 µM indomethacin and 1 µM tetrodotoxin to inhibit prostaglandin synthesis and potentially activated enteric neurons, respectively. Following equilibration, 10 µM amiloride was added apically to inhibit minor electrogenic Na⁺ absorption (ENaC) in the distal colon. Upon reaching a steady-state, tissue was administered 100 µM carbachol (CCH) basolaterally to activate Gαq muscarinic receptors to indirectly activate apical Ca²⁺-activated Cl⁻ secretion via intracellular increases in Ca²⁺ (11, 22). Fifteen minutes following CCH addition, 10 µM forskolin (FSK, adenylate cyclase inhibitor that increases intracellular cAMP) was basolaterally added to assess tissue viability. All representative traces and presented group data are designated as positive deflections indicating apical anion secretion/cation absorption or negative deflections representative of cation secretion/anion absorption.

**Colonic Epithelial Cell Isolation.** Distal colons were isolated from euthanized mice via bilateral pneumothorax and immediately rinsed with ice-cold phosphate-buffered saline (PBS) to clear luminal contents. The distal colon was then
opened longitudinally along the mesenteric border. Tissue was then cut into approximately 2 mm long pieces and submerged in 40 mL of ice-cold PBS with 5 mM EDTA in a 50 mL Falcon tube. The pieces of tissue in PBS-EDTA were then incubated at 37°C with gentle rocking for 30 minutes. Following this incubation, colonic tissue was vigorously shaken to disperse colonic crypts and surface epithelium in solution. Supernatant was then loaded into 1.5 mL microcentrifuge tubes and spun at 1x10^3 g for 5 minutes to pellet suspended cells. Isolated epithelial cells were used in downstream applications, such as RT-qPCR and Immunoblotting.

**RNA Isolation and RT-qPCR analyses.** Isolated colonic epithelium were resuspended in Qiazol (Qiagen, Valencia, Ca). Total-RNA was isolated using the MicroRNEasy kit, as per manufacturer’s instructions (Qiagen, Valencia, Ca). Concentrated RNA was immediately quantified using the Nanodrop 1000 (Thermo, Waltham, MA). RNA was then stored at -80°C until further application. Real-time PCR was carried out using a one-step kit that included 10 minutes of reverse transcription of total-RNA (50 ng/well) followed immediately by quantitative PCR utilizing a dsDNA-binding dye (New England Biolabs, Ipswich, MA). Custom primers (400 nM/primer/well) designed using the NCBI primer-blast application were utilized to amplify specific targets (Table-1). Determination of differential expression was achieved using the 2^(-ΔΔCt) method, (21). β-Actin threshold cycles for each sample were obtained and then subtracted from threshold cycles of transcripts of interest to obtain a ΔCt value. The ΔCt value
from control samples was then subtracted from DSS-colitis samples to obtain a 
\( \Delta \Delta C_t \) value. Fold changes of transcripts of interest were then calculated as 
\( 2^{-\Delta \Delta C_t} \). This method was utilized for comparison of all quantitative PCR values 
presented within the text and figures.

**Protein Isolation and Western Blotting.** Pelleted colonic epithelium were 
resuspended in 350 µl of RIPA (radioimmunoprecipitation assay) buffer containing 
full protease inhibitor cocktail (cOmplete, Mini, Sigma-Aldrich, St. Louis, MO). Tissue was homogenized using glass homogenizers with a final sonication step 
to fully liberate transmembrane proteins. Protein concentration for each individual 
sample was determined using the Pierce BCA protein quantification kit (Thermo, 
Waltham, MA). Samples were then immediately frozen at -80°C and maintained 
there until further use. On the day of immunoblotting, protein samples were 
removed from the -80°C freezer and allowed to thaw on ice. Samples were 
diluted to 1 µg/µl with the addition of 4x Lammelli buffer (Biorad, Hercules, CA) 
and remaining volume with diH₂O. Following preparation, protein samples were 
warmed at 37°C in a water bath for 15 minutes and then loaded at 20 µg/well 
onto handcast tris-glycine gels (SureCast, Invitrogen, Waltham, MA). Running 
conditions consisted of 85V until passing the stacking buffer at which point the 
gel was completed at 135V. Gels were transferred to PVDF membranes for 90 
minutes at a constant 20V. Upon completion, PVDF membranes were blocked in 
5% non-fat dry milk (Kroger, Cincinnati, OH) in tris-buffered saline with 0.1% 
tween-20 (TBST) for a period of 1 hour at room temperature. PVDF membranes
were then incubated in primary antibody: TMEM16A 1:500, CFTR 1:200, BEST2 1:500, M3R 1:200 (Alomone Labs, Jerusalem, Israel), IP3R 1:500, β-Actin 1:1000 (Cell Signaling, Danvers, MA) overnight at 4°C. In the morning, membranes were washed 5x in TBST for 5 minutes and then placed in goat anti-rabbit horseradish-peroxidase conjugated secondary antibody (1:10000 dilution) for 2 hours at RT. Membranes were washed 5x in TBST and imaged on the G-Box (Syngene, Cambridge, United Kingdom). All target proteins were quantified using the imageJ software (National Institute of Health, Bethesda, MD) with normalization to β-Actin.

Statistics. All presented results represent mean ± standard error (SE) from five different animals, unless otherwise denoted. Statistical tests that were performed on the presented data sets were either unpaired student’s t-test or one-way ANOVA with Tukey post-hoc analysis using GraphPad Prism 6.0 (San Diego, CA). p < 0.05 was used for determination of significance.

Stock Solutions. DSS (MP Biomedicals, Burlington, MA) 4% in the drinking water. Ussing chamber stock solutions were: 5 mM Indomethacin (Indo, dissolved in ethanol; Sigma-Aldrich, St. Louis, MO), 1 mM Tetrodotoxin (TTX, dissolved in acetic acid; Tocris Pharmaceuticals, Minneapolis, MN), 10 mM Amiloride (Amil, dissolved in DMSO; Sigma-Aldrich, St. Louis, MO), 100 mM Carbochol (CCH, dissolved in deionized water; Sigma-Aldrich, St. Louis, MO), 10 mM Forskolin (FSK, dissolved in DMSO; Tocris Pharmaceuticals, Minneapolis, MN), and 3 µM Ionomycin (IONO, dissolved in DMSO; Tocris Pharmaceuticals,
Minneapolis, MN). All stock solutions were diluted 1:1000 in the chamber to obtain [µM].

Results

Induction of Chronic DSS-colitis in Balb/c mice. Model accuracy was evaluated using several parameters previously utilized to demonstrate consistent induction of DSS-colitis (19). Colon length in the DSS-colitis group exhibited a consistent shortening as compared to control mice (Fig. 1A). Group data of measured colonic lengths from control mice were significantly longer than their DSS counterparts (8.75 ± 0.276 cm vs. 6.05 ± 0.221 cm, p < 0.001) (Fig. 1B). Histological studies were also conducted to examine proper colitis induction. The whole thickness of tissue isolated from control mice exhibited normal histological architecture (i.e. stacked test tubes) with intact surface epithelium and minimal inflammatory cells located in the lamina propria (Fig. 1C, top). Colonic mucosa from DSS-colitis animals exhibited substantial widening of the lamina propria as a result of an influx of inflammatory cells (Fig. 1C, bottom). Crypts in the colitic colon were also distorted with an obvious enlargement of the underlying muscularis (Fig. 1C, bottom), which could be the result of increased proliferation. However, this could also be the result of colonic contraction and without a marker for proliferation cannot be excluded. Transcript abundance of acute-phase reactants was quantitated to further demonstrate active inflammation within the distal colon. Quantitative PCR (qPCR) demonstrated a significant increase in cytokine transcript abundance of TNFα (1.008 ± 0.033 vs. 8.293 ± 0.732, p < 0.0001) and IL-6 (0.980 ± 0.017 vs. 10.530 ± 1.045, p < 0.0001) from DSS-colitis
samples (Fig. 1D). Besides the prototypical inflammatory cytokines, Th2-mediated cytokines were selected for study for their relevance to the clinical pathology, UC. IL-17 mRNA abundance was not significantly increased (1.000 ± 0.754 vs. 2.964 ± 0.676), however, transcript abundance of IL-4 (1.506 ± 0.747 vs. 7.109 ± 2.229, p < 0.05), IL-10 (1.062 ± 0.144 vs. 11.310 ± 2.262, p < 0.01), and IL-33 (0.998 ± 0.028 vs. 4.340 ± 1.367, p < 0.05) were all significantly increased in the DSS-colitis mice (Fig. 1D).

**Effect of Chronic DSS-induced Colitis on Ca^{2+}-activated Cl^{-} Secretion.**
Control mucosa demonstrated a robust increase in Ca^{2+}-activated Cl^{-} secretion, which was measured as positive deflections in short-circuit current (I_{SC}) (ΔI_{SC}, 15.40 ± 2.22 μA/cm², n = 5) (Fig. 2F). This was paralleled by a calculated hyperpolarization of transepithelial potential (ΔV_{TE}, -1.64 ± 0.21 mV, n=5) (Fig. 2E) following 100 μM CCH application (Fig. 2A/C). DSS-treated mucosa did elicit a response upon application of CCH, however, this response was significantly smaller in magnitude and opposite in apparent charge (Fig. 2B/D). The decrease in measured I_{SC} (ΔI_{SC}, -2.47 ± 0.25 μA/cm², n=5) (Fig. 2F) most likely indicates activation of Ca^{2+}-activated K^{+} channels, which are known to reside in the apical membrane of colonic epithelium (31, 32, 36, 42). This altered response to basolateral CCH could potentially be related to decreased synthesis of prostaglandin E_{2}, which has previously been shown to uncouple CCH-administration from apical Cl^{-} secretion (7). Also, calculated V_{TE} paralleled this response with a slight depolarization due to increased positive charge accumulating on the luminal side of the chamber (ΔV_{TE}, 0.296 ± 0.07 mV, n = 5)
Application of basolateral 10 µM Forskolin (FSK) was administered to assess the tissues ability to respond to another physiological agonist (Fig. 2C/D).

**Effect of Acute DSS-induced Colitis on Ca²⁺-activated Cl⁻ Secretion.**

Following our initial observations in the Ussing chamber that recapitulated previously published work with the acute model of DSS, along with western blot quantification of TMEM16A that demonstrated a decrease in expression (1.000 ± 0.13 vs. 0.065 ± 0.01, \( p < 0.05 \)), we decided to utilize the acute model (16, 19). This was done to establish that changes in CCH-induced \( I_{SC} \) were in fact due to aberrations in intracellular Ca²⁺ responses. However, we first needed to establish that our acute model of DSS-induced colitis was in agreement with previously published literature (16). As previously seen with our controls from the chronic model, control mucosa elicited a robust increase in \( I_{SC} \) (\( \Delta I_{SC} \), 29.42 ± 6.42 µA/cm², \( n = 6 \)) when exposed to basolateral 100 µM CCH (Fig. 3A/C). Also as expected, colonic mucosa isolated from animals that received DSS demonstrated a drop in baseline \( I_{SC} \) (\( \Delta I_{SC} \), -2.43 ± 0.17 µA/cm², \( n = 6 \)) following basolateral 100 µM CCH (Fig. 3B/C). To then establish that Ca²⁺ mediates the CCH-induced changes in \( I_{SC} \) in control and DSS mucosa, 3 µM ionomycin (calcium ionophore, IONO) was added prior to basolateral CCH administration. 3 µM IONO induced an increase in measured \( I_{SC} \) in control mucosa (\( \Delta I_{SC} \), 15.30 ± 2.24 µA/cm², \( n = 4 \)) (Fig. 3D/E), which was followed by basolateral addition of 100 µM CCH. Administration of CCH was only able to minimally increase \( I_{SC} \) (\( \Delta I_{SC} \), 0.90 ± 1.09 µA/cm², \( n = 4 \)) (Fig. 3D/E). And when compared to mucosa that was treated with
CCH prior to IONO, the measured increase in $I_{SC}$ was significantly less ($\Delta I_{SC}$, $29.42 \pm 6.42 \mu A/cm^2$ vs. $0.90 \pm 1.09 \mu A/cm^2$, $p<0.01$) (Fig. 3F). Colonic mucosa from DSS-colitis mice was then incubated with 3 µM IONO, which caused a drop in baseline $I_{SC}$ ($\Delta I_{SC}$, $-3.21 \pm 0.73 \mu A/cm^2$, $n = 4$) (Fig. 3G/H). Following this, 100 µM CCH was administered to the basolateral surface of the tissue. CCH was only able to elicit a small drop in measured $I_{SC}$ ($\Delta I_{SC}$, $-0.31 \pm 0.49 \mu A/cm^2$, $n = 4$) (Fig. 3G/H). Overall, this demonstrated a similar pattern to control mucosa of CCH-induced $I_{SC}$ being attenuated when following IONO administration ($\Delta I_{SC}$, $-3.21 \pm 0.73 \mu A/cm^2$ vs. $-0.31 \pm 0.49 \mu A/cm^2$, $p<0.01$) (Fig. 3I). Taken together, these results would indicate that CCH-induced changes $I_{SC}$ are Ca$^{2+}$-mediated in both control and DSS-exposed mucosa.

**Effect of Chronic DSS-induced Colitis on Cl$^-$ Channel Transcript Abundance.** RT-qPCR analysis was utilized to assess transcript abundance of several Cl$^-$ channels known to be expressed in distal colonic epithelium (28, 37, 41). Two Ca$^{2+}$-activated Cl$^-$ channels ($Tmem16a$, $Best2$) as well as the cystic fibrosis transmembrane regulator ($Cftr$) were selected for analysis. $Tmem16a$ mRNA abundance was significantly decreased in epithelium isolated from DSS-colitis mice ($1.045 \pm 0.15$ vs $0.325 \pm 0.04$, $p < 0.01$) (Fig. 4A), which may indicate a potential decrease in translated channel protein. However, $Best2$ ($0.993 \pm 0.03$ vs. $0.301 \pm 0.32$, $p < 0.001$) and $Cftr$ ($1.042 \pm 0.209$ vs. $0.096 \pm 0.013$, $p < 0.01$) were also significantly decreased as compared to control animals (Fig. 4B/C).
Effect of Chronic DSS-induced Colitis on Cl⁻ Channel Protein Expression in Mucosal Lysates. Western blot of epithelial lysates focused on different Cl⁻ channels implicated to elicit a response to increasing intracellular levels of Ca²⁺ in colonic epithelium. Western blot technique was carried out on lysates to assess potential differences in protein expression between control and DSS mice. As previously seen with the RT-qPCR data, TMEM16A was significantly down-regulated in DSS-colitis (1.001 ± 0.13 vs. 0.510 ± 0.12, p < 0.05) (Fig. 5A/B). However, western blot quantification of BEST2, another CaCC, from the lysates demonstrated no difference between control and DSS groups (0.952 ±0.04 vs. 0.930 ± 0.031, p > 0.05) (Fig. 5A/C). CFTR was also quantified via western blot technique. This was to address previous observations that have described a mechanism by which CFTR is Ca²⁺-sensitive (5, 38). Protein expression for CFTR was also unaltered (1.074 ± 0.09 vs. 1.053 ± 0.07, p > 0.05) (Fig. 5A/D) despite the mRNA transcript abundance being significantly decreased, similar to Best2 (Fig. 4B/C).

Transcript and Protein Abundance of Gαq Muscarinic Signaling Pathway. Previous work has shown that the decreased CCH-stimulated IₘSC in DSS-colitis was attributable to aberrations in signaling of pro-secretory stimuli (1, 18). To assess this from the standpoint of activation by CCH, mRNA abundance, as well as protein expression, of the M₃ receptor (CHRM3), a prototypical Gαq G-protein coupled receptor, was quantified in the distal colon. mRNA transcript abundance for Chrm3 was unchanged between control and DSS-colitis groups (1.028 ± 0.12 vs. 1.029 ± 0.27, p > 0.05) (Fig. 6A). Western blot quantification of CHRM3 was
also unchanged between the different experimental groups (1.021 ± 0.24 vs. 0.928 ± 0.09, p > 0.05) (Fig. 6B/C). While signal transduction is initiated through CHRM3, canonical Gαq signaling also includes the production of the second messenger inositol triphosphate (IP₃), which leads to the release of Ca²⁺ via binding to the inositol triphosphate receptor (ITPR3). While Itpr3 transcript abundance was not significantly decreased (0.988 ± 0.09 vs. 0.816 ± 0.05, p > 0.05), protein expression of ITPR3 was significantly down-regulated (1.000 ± 0.09 vs. 0.426 ± 0.13, p < 0.01) in DSS-colitis mice as compared to their parallel controls (Fig. 6A, B, D).

**Immunohistochemistry of TMEM16A in Distal Colon of Control and DSS Mice.** Immunohistochemistry (IHC) of TMEM16A was performed to follow-up the finding of decreased protein expression with immunoblotting (Fig. 5A/B) and to determine the exact localization of that protein. IHC performed with anti-TMEM16A antibody on control tissue demonstrated staining of the surface epithelium with additional staining of the upper crypts (Fig. 7A). However, DSS-exposed tissue that was incubated with anti-TMEM16A antibody demonstrated substantially less membrane staining of the epithelium, with more generalized staining of the cytoplasm (Fig. 7C). This generalized staining, especially in DSS-colitis, is most likely sub-apical vesicles that have not been properly trafficked to the plasma membrane, however, the staining could also be potential splice variants of TMEM16A located in cellular organelles. Control peptide experiments incubated with the anti-TMEM16A antibody were run in parallel for both groups. The addition of peptide to either Control or DSS-colitis tissue was devoid of
staining indicating specificity of the antibody, instead of possible non-specific binding to similar epitopes (Fig. 7B/D).

Discussion

Utilizing the previously described model of colitis in mice (20, 39), five alternating weeks of 4% DSS administration was able to induce epithelial dysplasia, crypt distortion and increased inflammatory infiltrate in the lamina propria, similar to the histopathological features observed in UC (2). Previously, acute induction of DSS-colitis had resulted in decreased CCH-stimulated Ca$^{2+}$-activated Cl$^{-}$ secretion in murine distal colon (3, 16). However, these losses in Ca$^{2+}$-activated Cl$^{-}$ secretion have been attributed to dysfunctional muscarinic receptor signaling (1), or membrane depolarization related to decreased functioning of basolateral K$^{+}$ channels (16). These previous works were also conducted prior to the discovery of the novel CaCC, TMEM16A (6, 34, 40). TMEM16A exhibits a similar electrophysiological profile to that found in cultured and native human bronchial epithelium (6). Also, global and tissue-specific knockout studies of TMEM16A in mice have demonstrated an absence of Ca$^{2+}$-activated Cl$^{-}$ secretion (4, 28).

In this study, our findings demonstrate a significant attenuation in Ca$^{2+}$-activated Cl$^{-}$ secretion in the colitic colon that is in part due to the down-regulation of TMEM16A. This conclusion is evidenced by the use of a Ca$^{2+}$ ionophore (ionomycin) that demonstrates a complete lack of Ca$^{2+}$-activated Cl$^{-}$ secretion (Fig. 3), as well as significantly decreased mRNA abundance and
protein expression of TMEM16A in the DSS-induced colitic colon (Figs. 4 & 5). In addition, other potential canonical and non-canonical CaCCs were also examined (5, 41). Transcript abundance was decreased for both Best2 and Cftr (Fig. 4), however, the protein expression of these Cl⁻ channels in whole cell lysates was unchanged in the colitic colon, as compared to control littermates (Fig. 5). Also, transcript abundance and protein expression of CHRM3 were unchanged in DSS-induced colitis (Fig. 6), which is in agreement with a more recent publication (16). Taken together, our results indicate that decreased expression of TMEM16A following DSS administration substantially blunts Ca²⁺-activated Cl⁻ secretion in murine distal colon.

While TMEM16A expression was substantially down-regulated, the protein expression of ITPR3 was also significantly decreased in the DSS-colitis cohort (Fig. 6). And while this could contribute to the attenuation in CCH-stimulated Cl⁻ secretion in DSS-colitis, a previous observation with a tissue-specific knockout of TMEM16A in colonic epithelium demonstrated decreased CCH-stimulated Ca²⁺ release as measured by fura-2 (33). Schreiber et al. did put forth one possibility that TMEM16A may provide a physical tether for ITPR3 to the plasma membrane and loss of TMEM16A could disrupt this architecture (33). Also, a prior study has demonstrated a physical interaction of TMEM16A and ITPR3 in nociceptive neurons (17). Taken together with our findings, the diminished expression of TMEM16A in colonic epithelium could cause a destabilization of ITPR3 in endoplasmic reticular membranes, leading to a premature degradation of the receptor. This notion is supported by the fact that mRNA abundance of ltpr3
(0.988 ± 0.09 vs. 0.816 ± 0.05, p > 0.05) is not significantly decreased in DSS-colitis (Fig. 6). This would potentially indicate a non-genomic mechanism of decreased protein expression, possibly a protein-protein interaction between ITPR3 and TMEM16A.

Though it is most likely that the down-regulation of TMEM16A is a result of transcriptional regulation, it is uncertain as to what mechanism mediates this regulation of the channel. Several groups have previously shown that IL-4 and IL-13, Th2 immune mediators, were able to significantly increase Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} secretion in bronchial epithelium (6, 43). Another group incubated IL-4 and IL-13 on immortalized colonic epithelium (T\textsubscript{84}), and while IL-13 did not seem to have an effect, IL-4 was able to substantially inhibit the CCH-stimulated $I_{SC}$ in a dose-dependent manner (23). Another study incubated IL-10 on T84 monolayers and was able to demonstrate a similar dose-dependent inhibition of CCH-stimulated $I_{SC}$ (24). Together, this would provide support that Th\textsubscript{2} signaling in colonic epithelium may oppose the effects that are observed in respiratory epithelium. Also, it is worth noting that our results demonstrate a significant increase in transcript abundance of several prototypical Th\textsubscript{2} cytokines (Fig. 1D), which could lead to the decreased expression of TMEM16A.

Previous studies have demonstrated the importance for TMEM16A in mucociliary clearance in murine airways (28, 30). Furthermore, poor hydration of the mucus layer between secretory epithelium and resident bacteria has been shown to promote an environment of bacterial overgrowth, leading to an increased interaction of non-host with host (25, 27). This potentially highlights the
impact that TMEM16A may have on colonic inflammation in UC. Utilizing these previous observations, we speculate that decreased TMEM16A may exacerbate colonic inflammation via an increase in interaction between resident immune cells and the microbiota. From a clinical perspective an increase in Ca\(^{2+}\)-activated Cl\(^{-}\) secretion could decrease this interaction and help to ameliorate further inflammation induced via this mechanism.

In conclusion, our findings demonstrate a significant decrease in Ca\(^{2+}\)-activated Cl\(^{-}\) secretion in a DSS-induced colitic colon. The CaCC, TMEM16A, is significantly decreased in the DSS-induced colitis cohort of mice, while other Cl\(^{-}\) channels (BEST2, CFTR) were unchanged at the protein level. The significant decrease in ITPR3 may be the result of a loss of TMEM16A stabilization through protein-protein interactions. Also, we speculate that the loss of TMEM16A in colonic epithelium may contribute to the inflammatory response.
**Acknowledgements**

This study was supported by R01DK018777 (V.M. Rajendran) from the National Institute of Health/Diabetes and Digestive and Kidney Diseases.

**Conflict of Interest**

On behalf of all the authors, the corresponding author states that there is no conflict of interest.
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Figure Legends

Figure 1. **Evaluation of DSS-colitis in Balb/C Mice.**  
A. Representative images of excised colons from either control or DSS-colitis cohorts.  
B. Group data of measured colons from either control or colitic mice. Represented data is means ± SE of different groups, collected from 6 different animals in each respective group ($p < 0.001$).  
C. Haemotoxylin and eosin staining of formalin-fixed colonic tissue. Each image represents a different animal from their corresponding treatment. DSS-colitis (C, Bottom) demonstrates crypt dysplasia, leukocyte infiltration, and thickening of underlying serosa (20x, AxioImager).  
D. RT-qPCR analyses of multiple cytokines involved in the development of colitis. Asterisks (*) indicate significance of each individual cytokine transcript as compared to control. $^*p < 0.0001$ – compared to control; $^{*}$ $p < 0.01$ – compared to control; $^#p < 0.05$ – compared to control.

Figure 2. **Effect of DSS-colitis on transepithelial potential and Cl− secretion.**  
A. Representative trace of calculated transepithelial potential ($V_{TE}$) in control mice following application of 100 µM carbochol (CCH) and 10 µM Forskolin (FSK).  
B. Representative trace of calculated $V_{TE}$ in DSS-colitis mice following application of 100 µM CCH and 10 µM FSK.  
C. Representative trace of elicited short-circuit current ($I_{SC}$) in control mice following basolateral administration of 100 µM CCH and 10 µM FSK.  
D. Representative trace of elicited $I_{SC}$ in DSS-colitis mice following basolateral administration of 100 µM CCH and 10 µM FSK.  
E. Summarized data of calculated $\Delta V_{TE}$ following CCH administration in either
control (open bars) or DSS-colitis (closed bars) mice. DSS-colitis demonstrates a complete inhibition with an actual reversal in polarity as compared to control. F. Group data of ΔI_{SC} from both control (open bars) and DSS-colitis (closed bars) groups following CCH-administration. DSS-colitis, in parallel to calculated ΔV_{TE}, demonstrates an elicited current that is in opposition to that of the control cohort. Bar graphs of summarized data represent means ± SE of 5 different animals. *p < 0.001 - compared to control.

Figure 3. **Effect of Ionomycin on CCH-induced Changes in I_{SC} from Control and Acute DSS-Colitis Mice.** A. Representative trace of measured short-circuit current (I_{SC}) following basolateral 100 µM carbochol (CCH) administration to control mucosa. B. Representative trace of I_{SC} from DSS-colitis mucosa following basolateral 100 µM CCH administration. C. Group data of ΔI_{SC} following CCH-administration in control and DSS mice. D. Representative trace of I_{SC} from control mucosa that was stimulated with 3 µM ionomycin (IONO) and followed by 100 µM CCH. E. Group Data of ΔI_{SC} with 3 µM IONO followed by 100 µM CCH in control mucosa. F. Group data of CCH-induced ΔI_{SC} with and without 3 µM IONO administration. G. Representative trace of I_{SC} from mucosa exposed to DSS administered 3 µM IONO and 100 µM CCH. H. Group data of ΔI_{SC} from DSS-colitis in response to 3 µM IONO followed by 100 µM CCH administration. I. Group data of DSS-colitis CCH-induced ΔI_{SC} with and without 3 µM IONO administration. All presented bar graphs are summarized data that represent the means ± SE. *p < 0.01 – compared to control.
Figure 4. **Effect of DSS-colitis on transcript abundance of Cl⁻ channels in murine colon.** Transcript abundance was determined for (A) *Tmem16a*, (B) *Best2*, and (C) *Cftr* in control (open bar) or DSS-colitis mice (closed bar). Bar graphs of summarized data represent means ± SE of 5 different animals normalized to an endogenous control, *Actb*. *p < 0.001* - compared to their respective control.

Figure 5. **Effect of DSS-colitis on Cl⁻ channel protein expression in murine colon.** A. Representative images of protein expression of the different characterized Cl⁻ channels from epithelial lysates. B. TMEM16A protein expression normalized to β-actin for quantification of control and DSS-colitis groups. TMEM16A expression (closed bars) is significantly decreased in DSS-colitis mice as compared to control. C,D. Summarized data of western blot quantification of BEST2 and CFTR normalized to β-actin in control and DSS-colitis mice. Neither protein was significantly altered from the control cohort. Bar graphs of summarized data represent means ± SE of 5 different animals from each respective group. *p < 0.05*-compared their respective control.

Figure 6. **Characterization of Gαq muscarinic signaling pathway.** A. Transcript abundance of *Chrm3* and *Itpr3* from control and DSS-colitis groups. mRNA abundance of either transcript was not significantly different from the control cohort (*P > 0.05*). B. Representative images of western blots for CHRM3
and ITPR3 with corresponding loading controls for normalization. C. Summarized data of CHRM3 protein expression normalized to β-actin. CHRM3 expression in DSS-colitis was unaltered as compared to control counterparts. D. Group data of ITPR3 protein expression normalized to β-actin from control and DSS-colitis. ITPR3 expression was significantly down-regulated in DSS-colitis. Bar graphs of summarized data represent means ± SE of 5 different animals from each respective group. *p < 0.01-as compared to control.

Figure 7. Immunohistochemistry of TMEM16A in colonic tissue sections. A. Immunohistochemistry of TMEM16A in control tissue demonstrates pronounced expression and localization of the protein to surface epithelium and upper crypts (top, left). B. DSS-colitis tissue stained for TMEM16A exhibits less staining in epithelial cells and a more diffuse signal in the lamina propria (Bottom, left). C,D. Both cohorts of tissue were run in parallel with a control peptide specific for the anti-TMEM16A antibody. Both images demonstrate a lack of staining when co-incubated with control peptide. Images were captured at 20x magnification on the AxioImager (Carl Zeiss, Germany).
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Table 1. Custom qPCR primers for determination of transcript abundance.

All primers were designed utilizing the NCBI primer-blast application with the indicated reference sequence (ref seq).
Figure 1.

A

Control DSS

B

Length (cm)

C

CON DSS

D

Relative mRNA abundance (Arbitrary Units)

* * $

TNFα IL-6 IL-4 IL-10 IL-17 IL-33

CON DSS
Figure 2.

A

B

C

D

E

F

Calculated $V_{TE}$ (mV)

Short-Circuit Current ($\mu$A/cm$^2$)

CON DSS

$\Delta V_{TE}$ (mV)

$\Delta I_{sc}$ ($\mu$A/cm$^2$)

100 µM CCH

10 µM FSK

0 µM CCH

10 µM FSK
Figure 3.

A

B

C

D

E

F

G

H

I

3 µM Ionomycin

CCH-Induced \( \Delta I_{SC} \) (µA/cm²)

3 µM Ionomycin

CCH-Induced \( \Delta I_{SC} \) (µA/cm²)
Figure 4.
Figure 5.

A

Con DSS Con DSS
140 kDa
TMEM16A

β-Actin

Con DSS Con DSS
260 kDa
CFTR

Con DSS Con DSS
140 kDa
BEST2

β-Actin

C

CON DSS
1.2
0.8
0.4
0.0
BEST2 (Arbitrary Units)

D

CON DSS
1.2
0.8
0.4
0.0
CFTR (Arbitrary Units)
Figure 6.

A

B

C

D

CON DSS

Relative mRNA abundance

Con

DSS

CHRM3

ITPR3

(225 kDa)

(70 kDa)

(40 kDa)

β-Actin

β-Actin

CHRM3

ITPR3

(40 kDa)

(40 kDa)

(225 kDa)

(70 kDa)

(40 kDa)
Figure 7.

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Chapter III

Activation of KCNQ (Kv7) K⁺ Channels in Enteric Neurons Inhibits Chloride Secretion in the Murine Distal Colon

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Running Title: Kv7 Activation Inhibits Colonic Secretion via Enteric Neurons

Key Words: Ussing Chamber, Colonic epithelium, KCNQ, Kv7 channels, Enteric neurons,
**Abstract.** The Enteric nervous system (ENS) regulates blood flow, motility and electrolyte transport along the entire gastrointestinal tract. KCNQ K⁺ channels have been characterized to contribute to the resting membrane potential in visceral neurons and are also present in the ENS. In this study, we wanted to evaluate the efficacy of KCNQ activators on the ENS from the standpoint of anion secretion in mouse distal colon. Anion (i.e. Cl⁻) secretion was measured as a function of short circuit current (Isc) in intact mouse distal colon mounted under voltage clamp conditions in an Ussing chamber. Pharmacological approaches were utilized to assess the effect of activation of KCNQ channels in enteric neurons on apical Cl⁻ secretion. Immunofluorescence studies were conducted to identify the isoform and location specific expression of KCNQ channels. Immunofluorescence studies indicated the presence of Kv7.2 and Kv7.3 channels that co-localized with choline acetyl transferase in enteric neurons of mouse distal colon. Flupirtine, a KCNQ activator markedly inhibited Isc (i.e. Cl⁻ secretion). However, application of flupirtine following tetrodotoxin resulted in a significantly blunted response. A similar effect was observed when tissue was pre-incubated with the muscarinic antagonist tolterodine. Serosal nicotine stimulated Cl⁻ secretion in control colon. However, tissues that were pretreated with flupirtine prior to application of nicotine remained unresponsive to the ganglionic agonist. Our data indicate that activation of KCNQ (Kv7) channels of enteric neurons inhibits Cl⁻ secretion. Also, activators of KCNQ channels could have therapeutic benefit in diarrheal pathologies associated with an overall hyperexcitable ENS.
Introduction

The enteric nervous system (ENS) is comprised of approximately 400-600 million neurons [1]. The ENS participates in a multitude of functions that include regulation of motility, local control of blood flow during digestion, and influence over electrolyte transport [2-5].

Numerous pathologies have been identified that cause a dysregulation of one or all of the previously mentioned regulatory processes [6-8]. While Hirschprung’s disease is the most notable of ENS pathologies, and results in an absolute loss of enteric neurons, several diseases have been identified that elicit an overall hyperexcitable ENS [9, 10]. For example, patients with irritable bowel syndrome with diarrhea (IBS-D) administered alosetron demonstrated delayed colonic transit times, indicating increased serotonergic signaling and ENS overactivity [9]. While IBS-D is considered more of a disease of the developed world, infections with rotavirus or vibrio cholera can also cause excitation of enteric neurons, which could increase epithelial secretion and contribute to the overall pathology of each of those diseases [8, 11].

K⁺ channels are responsible for setting the resting membrane potential in a vast array of cells due to the large permeability of K⁺ as compared to other predominant ions [12]. The KCNQ (Kᵥ7) family, which are voltage-sensitive K⁺ channels (Kᵥ), have been described to regulate resting membrane potential in several different cell types [13-15]. The Kᵥ7 family has been described as small, slow-deactivating K⁺ channels with a hyperpolarized threshold for half-maximal activation [16]. A couple members of the Kᵥ7 family, Kᵥ7.2 and Kᵥ7.3, have been
characterized as making up the native M current, and more importantly contributing to resting membrane potential in neurons [17-20]. Disruptions in these genes have led to the development of seizures and hyperalgesia in rodent models [19, 21, 22]. Previous observations have also shown Kv7.3 to co-immunolocalize with sensory afferents in the gastrointestinal tract, as well as, participate in visceral nociception [18, 23].

While the Kv7 family has been shown to be present in neurons located within the gastrointestinal tract, it has not previously been studied from the aspect of epithelial electrolyte transport. Therefore, the goal of this study was to evaluate the efficacy of Kv7 activators on enteric neurons and the resultant effect on mucosal electrolyte transport. Our results indicate that Kv7.2/3 activation causes an immediate and sustained hyperpolarization of enteric neurons that results in decreased epithelial secretory function via decreased neurotransmitter release.
Material and Methods

Animals. Male Balb/c mice (10-13 weeks, Charles River, NY) were provided normal chow and water ad libitium for the entirety of the project. All animals were anesthetized via 5% inhalational isoflurane (Patterson Veterinary, SC) for isolation of the whole colon for downstream applications. Mice were then euthanized via cervical dislocation. All experimental protocols utilized in this study were approved by the West Virginia University Institutional Animal Care and Use Committee.

Cell Culture. The colonic adenocarcinoma $T_{84}$ cell line (ATCC, Manassas, VA) was maintained and passaged in a T75 flask (Corning, Corning, NY) that was kept at 37°C and 5% CO$_2$- 95% O$_2$ gas. Cells were maintained in DMEM/F12 (ThermoFisher, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Hampton, NH) with 1% pencillin-streptomycin (Mediatech, Manassas, VA). Upon passage, cells were seeded close to confluence on 1.12 cm$^2$ transwell plates (Corning, Corning, NY) for use in Ussing chamber monolayer studies. $T_{84}$ cells maintained on the transwell plates were checked for confluence each day. Upon reaching confluency, cells were then maintained in monolayers for 18 days for proper development of tight junctions, as well as, differentiation of the colonic epithelial cell line. Media was replaced every other day with fresh culture media until utilization in Ussing chamber studies.
**Electrophysiology.**

*Native tissue.* Whole colon was removed from the anesthetized mouse and rinsed with ice-cold phosphate-buffered saline (PBS) to remove any residual contents. Whole colon was then pinned near the caecum in a sylgard plate filled with ice-cold PBS containing 5 µM indomethacin. The colon was then opened longitudinally along the mesenteric border of the tissue. The sylgard dish was then placed under a dissecting microscope for the isolation of distal colon segments. Whole-mount distal colon was then placed on an oval 0.04 cm² aperture for mounting in the EasyMount Ussing chamber (Physiological Instruments, San Diego, CA) for electrophysiological study, as previously described [24]. Mounted tissue was then bathed with bilateral addition of 5 mL of Ringers’ physiological saline solution (in mM: 115 NaCl, 25 NaHCO₃, 2.4 K₂HPO₄, 0.4 KH₂PO₄, 3 KCl, 1.2 CaCl₂, 1.2 MgCl₂ and 10 glucose) to each hemi-chamber. Tissues were allowed to equilibrate in open-circuit conditions for a period of 15 minutes prior to voltage-clamping. All mounted tissues were continuously voltage-clamped at 0 mV to abolish any developing electrical gradient. Short-circuit current (I_{sc}) was continuously monitored with a 5 mV pulse applied every 5 seconds to determine transepithelial resistance. Tissues were maintained in the chamber at 37°C and continuously gassed with 5% CO₂- 95% O₂ (pH 7.4).

*T₈₄ monolayers.* The day of experiments, PBS was warmed in a water bath to 37°C for washing of T₈₄ monolayers maintained on transwell plates. Inserts of T₈₄ cells were removed from their respective plates and gently washed with warmed
PBS until clear. Monolayers were then mounted in the EasyMount Ussing chamber and bathed on both sides by Ringers’ physiological saline solution. Monolayers were voltage-clamped at 0 mV for the duration of the experiment with a 5 mV pulse applied every 5 seconds. However, all monolayers were allowed to equilibrate and come to a steady-state prior to the application of any drugs.

All representative traces presented within this article are designated as positive $I_{SC}$ indicating either secretion of an anion or absorption of a cation from the mucosal surface, while negative deflections of $I_{SC}$ indicate cation secretion or anion absorption from the mucosal side of the tissue.

**Immunofluorescence.** Whole colon was removed from euthanized mice and rinsed with ice-cold PBS to remove any residual contents. Tissues were pinned on a sylgard plate and opened longitudinally along the mesenteric border. Colon was then pinned along the borders with the mucosa facing up. A shallow, longitudinal cut was made to remove mucosa from the underlying myenteric plexus and smooth muscle. Mucosa was then carefully teased away, starting from the cut and moving proximally along the colon. Upon isolation of the myenteric plexus and longitudinal smooth muscle, tissues were placed in 10% neutral-buffered formalin for 2 hours at RT. Following fixation, tissue was placed in 30% sucrose solution dissolved in PBS and stored at 4°C until further processing. The day of immunostaining, tissue was removed from sucrose solution and washed 3x with PBS for a period of 1 hour to rehydrate the tissue.
Tissue was then incubated with 1% Triton-X100 PBS for a period of 4 hours to fully permeabilize the membranes. Samples were then washed with PBS 3x for 10 minutes and placed in 5% goat serum to block for 2 hours. Following blocking, tissue was placed in the different primary antibodies (Kv7.2 1:100, Kv7.3 1:100, Alomone Labs, Jerusalem, Israel, ChAT 1:100 Millipore, Burlington, MA) diluted in blocking solution overnight at 4°C with gentle shaking. In the morning, tissue was washed with PBS at RT 5x for 5 minutes. Upon completion of washing, tissue was incubated with secondary antibody (anti-rabbit Alexa Fluor 488 1:500, anti-goat Alexa Fluor 546 1:500) for 4 hours in the dark at RT. Tissue was removed and washed with PBS 5x for 5 mins to dilute out any residual secondary antibody. Samples were then mounted on slides with mounting media (SlowFade Diamond, ThermoFisher, Waltham, MA) and imaged for immunolocalization accordingly. All images were captured on LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany) with a 63x oil-immersion objective.

**Percent Inhibition and Dose-Response.**

*Percent inhibition.* The percent inhibition of the different drugs was calculated by obtaining the maximal response from all applied agents and then dividing the measured change in I_{SC} by the maximal response to obtain a percent value. Data was transformed into a percent due to variability in baseline measurements between mounted tissues. Percent inhibition was calculated as follows:

\[
\text{Maximal response: } X_{\text{baseline Isc}} - Y_{\text{remaining Isc}}
\]
Percent Inhibition: \((X_{\text{Isc prior to drug application}} - Y_{\text{steady-state Isc after drug application}})\)

Maximal response

*Dose-Response.* This was performed to calculate a half-maximal inhibitory concentration (IC$_{50}$) for flupirtine (general Kv7 agonist) and ICA 110381 (specific agonist of Kv7.2/3). Pharmacological agents were diluted from their respective stock concentrations to a range of $10^{-4}$ M to $10^{-1}$ M, which were then diluted 1:1000 (5 µL into 5 mL) within the chamber. Different concentrations were added sequentially, starting with $10^{-4}$ M and increasing by a factor of 10 for each addition. Tissue was allowed to reach a steady-state prior to the application of the next administered dose.

*Statistics.* All presented data represent mean ± standard error (sem) from five different animals, unless otherwise denoted within the text. Statistical tests that were performed on the presented data was student’s unpaired t-test using Graphpad Prism 5.0 (San Diego, CA). \(p < 0.001\) was used for the determination of significance.

*Stock solutions.* Indomethacin was prepared as a 5 mM stock solution in ethanol. Ussing chamber stocks are as follows: 1 mM tetrodotoxin (TTX, dissolved in acetic acid, Tocris Pharmaceuticals, Minneapolis, MN), 10 mM tolterodine (TOL, dissolved in H$_2$O, Tocris Pharmaceuticals, Minneapolis, MN), 50 mM flupirtine (FLU, dissolved in DMSO, Tocris Pharmaceuticals, Minneapolis,
MN), 50 mM ICA 110381 (ICA, dissolved in DMSO, Tocris Pharmaceuticals, Minneapolis, MN), 50 mM nicotine (NICO, dissolved in H₂O, Tocris Pharmaceuticals, Minneapolis, MN), 10 mM forskolin (FSK, dissolved in DMSO, Tocris Pharmaceuticals, Minneapolis, MN), and 50 mM XE-991 (dissolved in H₂O, Tocris Pharmaceuticals, Minneapolis, MN).

**Results**

**Inhibition of I_{SC} in Whole-Mount Colon with the Kv7 Activator Flupirtine.**

Previous studies have shown that inhibition of the ENS with tetrodotoxin (TTX), a voltage-sensitive Na⁺ channel (Naᵥ) inhibitor, was able to significantly attenuate the on-going basal Cl⁻ secretion in Ussing chambers [25]. This is accomplished via inhibiting propagation of action potentials and eventual release of neurotransmitters [26]. With this in mind, flupirtine, a Kv7 activator that hyperpolarizes neurons and ultimately decreases release of neurotransmitter, could be applied to indirectly inhibit on-going Cl⁻ secretion. As demonstrated by the representative trace (Fig. 1A), basolateral 100 µM flupirtine application was able to significantly decrease the observed I_{SC} from the mounted tissue. However, basolateral application of 1 µM TTX only slightly decreased I_{SC} (Fig. 1A). Group data of 100 µM flupirtine application prior to 1 µM TTX administration demonstrated a similar pattern of flupirtine being responsible for a significant majority of the percent of inhibited I_{SC} (FLU vs. TTX, 94.23 ± 1.50 % vs. 5.77 ± 1.50 %) (Fig. 1C). However, administration of 1 µM TTX prior to flupirtine resulted in a significant decrease in measured I_{SC}, while flupirtine only slightly decreased
I\textsubscript{SC} (Fig. 1B). Group data of TTX administration prior to flupirtine also demonstrated a similar pattern with TTX conferring a majority of the percent-inhibited I\textsubscript{SC} in Ussing chamber experiments (TTX vs. FLU, 77.92 ± 6.6 % vs. 22.08 ± 6.6 %, \( p < 0.001 \)) (Fig. 1D). Comparison of order of administration demonstrated flupirtine following TTX application was significantly less than when administered prior to TTX (22.08 ± 6.58 % vs. 94.23 ± 1.50 %, Fig. 1E).

Dose-Response of I\textsubscript{SC} to Basolateral Flupirtine Application. Following the initial finding of flupirtine decreasing I\textsubscript{SC} in colonic epithelium, we decided to more fully characterize the drug by determining a half-maximal inhibitory concentration (IC\textsubscript{50}). Initial administration of 100 nM flupirtine was unable to elicit any effect on the tissue, as demonstrated by the representative trace (Fig. 2A). However, increasing doses of flupirtine, from 1 \( \mu \text{M} \) up to highest concentration of 100 \( \mu \text{M} \), were able to inhibit the on-going I\textsubscript{SC} (Fig. 2A). The data as a whole demonstrated a significant inhibition with the addition of 10 \( \mu \text{M} \) flupirtine, which accounted for a 49.44 ± 5.9 % decrease from the previous baseline current. 100 \( \mu \text{M} \) flupirtine application was also able to further decrease I\textsubscript{SC} (37.48 ± 3.8 %), however not as robustly as the previously administered 10 \( \mu \text{M} \) dose, which would indicate reaching an asymptote of effect (Fig. 2B). Utilizing a non-linear fit to the calculated percent of inhibition presented in Fig. 2B, we were able to calculate an IC\textsubscript{50} for flupirtine of 9.37 ± 0.42 \( \mu \text{M} \).
Inhibition of on-going $I_{SC}$ in Colonic Epithelium with the Muscarinic Antagonist Tolterodine. As described previously, NaV channels are located within ENS neurons, so inhibition of those channels with TTX would inhibit neurotransmitter release (i.e. acetylcholine) onto epithelial cells. To further establish that basolateral flupirtine may be acting on ENS neurons, we decided to use the muscarinic antagonist tolterodine to inhibit acetylcholine activation of muscarinic receptors on epithelial cells. As expected and in concordance with literature [27], basolateral application of 10 µM tolterodine was able to inhibit a significant amount of basal $I_{SC}$ (80.35 ± 3.72 %, Fig. 3C), as illustrated by the representative trace (Fig. 3A). Addition of 100 µM flupirtine was able to further decrease $I_{SC}$ (19.65 ± 3.72 %, Fig. 3A/C). However, the addition of 100 µM flupirtine prior to 10 µM tolterodine application demonstrated a significant inhibition of $I_{SC}$ (99.59 ± 0.41 %, Fig. 3B/D), more similar to flupirtine administration prior to 1 µM TTX (Fig. 1D). Unexpectedly though, addition of 10 µM tolterodine to the basolateral surface led to a paradoxical increase in $I_{SC}$ (-15.2 ± 8.56 % Fig. 3B/D), as opposed to the decrease in $I_{SC}$ observed with flupirtine application. Comparison of order of administration demonstrated that flupirtine administration prior to tolterodine resulted in a significant increase in percent of inhibition (99.59 ± 0.41 % vs. 19.65 ± 3.72 %, Fig. 3E).

Pre-Incubation with Flupirtine Inhibits Basolaterally-Administered Nicotine-Induced $I_{SC}$. Increased firing of ENS neurons has previously been shown to elicit increases in measured $I_{SC}$ [4]. In line with this notion, we decided to activate ENS
neurons via basolateral addition of 100 µM nicotine. As illustrated by the representative trace (Fig. 4A), basolateral administration of nicotine dramatically increased measured $I_{SC}$. Tissue that was pre-incubated with 100 µM flupirtine did exhibit the expected decrease in $I_{SC}$. However, addition of 100 µM nicotine was not able to stimulate $I_{SC}$ (Fig. 4B). Tissues that received nicotine-only were able to elicit large increases in $I_{SC}$, while tissues that were pre-incubated with flupirtine were practically unresponsive to nicotine (93.22 ± 18.86 µA/cm$^2$ vs. 0.26 ± 1.12 µA/cm$^2$, Fig. 4C).

**Application of Flupirtine and TTX in T$_{84}$ Monolayers Does Not Inhibit $I_{SC}$**.

T$_{84}$ monolayers have been used to study the colonic epithelium for several decades [28, 29]. The T$_{84}$ cell line was originally isolated from a colonic adenocarcinoma, and consists of a homogenous population of secretory epithelium [30]. Therefore, application of any pharmacological agents that interact with ENS neurons to exert their effect would not demonstrate an efficacy in the T$_{84}$ cell line. To establish this within our lab, we first mounted T$_{84}$ monolayers in the Ussing chamber, and upon reaching a steady-state applied 1 µM TTX to the basolateral surface of the monolayer. TTX was without effect (Fig. 5A/C), however to confirm that monolayers were functioning as a tight epithelium 10 µM forskolin (FSK) was applied basolaterally. As expected, 10 µM FSK was able to increase $I_{SC}$ dramatically (24.58 ± 2.88 µA/cm$^2$, Fig. 5A/D). Following this outcome, another group of monolayers were administered 100 µM flupirtine to the basolateral side of the chamber. As previously seen with TTX application,
100 µM flupirtine was unable to decrease measured $I_{SC}$, however, there was a very modest increase in baseline $I_{SC}$ (0.57 ± 0.23 µA/cm², Fig. 5B/C). $T_84$ monolayers were once again administered 10 µM FSK, which significantly increased $I_{SC}$ (24.61 ± 4.21 µA/cm²) to comparable levels of $T_84$ monolayers exposed to TTX (Fig. 5D). Both groups were then exposed to 50 µM XE-991 (Kv7 antagonist), which demonstrated a significant inhibition of FSK-stimulated $I_{SC}$ (Fig. 5A/B).

**Kv7.2 and Kv7.3 Exhibit Co-Localization with Choline Acetyl Transferase in ENS Neurons.** Since previous work has shown that Kv7.2/3 are crucial to the native M current in neurons and may contribute to the observed effect of flupirtine on $I_{SC}$, we decided to immuno-label for the two different channels in whole mount tissue. Choline acetyl transferase (ChAT) was used as a marker of enteric neurons due to the high abundance of cholinergic neurons in the ENS, and potential co-localization of the ion channels of interest. ChAT consistently labeled axons of enteric neurons, as illustrated by Fig. 6A/D. Kv7.2 and Kv7.3 (Fig. 6B/E) consistently co-localized with enteric neuronal axons that were positively immuno-stained for ChAT (Fig. 6C/F).

**ICA 110381, a Specific Activator of Kv7.2/3, Inhibits $I_{SC}$ in a Dose-Dependent Manner.** Following identification of Kv7.2 and Kv7.3 in ENS neurons, we decided to use a specific activator of the heterotetramer, Kv7.2/3. ICA 110381 has previously demonstrated selectivity for Kv7.2/3 over the other members of the
KCNQ family of K⁺ channels [31]. Basolateral application of 100 µM ICA was able to significantly inhibit the I_{SC} (Fig. 7A). Similar to before with flupirtine, administration of 1 µM TTX following ICA was practically unable to decrease I_{SC} (Fig. 7A). ICA 110381 was able to account for 96.88 ± 0.94 % of the total inhibited I_{SC}, while 1 µM TTX only inhibited 3.12 ± 0.94 % of the current (Fig. 7B). A dose-response curve was also generated for ICA 110381 (Fig. 7C). Concentrations ranging from 1 µM to 100 µM were able to decrease I_{SC}. The use of a non-linear fit of the grouped data allowed for the calculation of an IC₅₀ for ICA 110381, which was 10.55 ± 0.75 µM (Fig. 7D).

**Discussion**

Our findings in this study indicate that hyperpolarization of enteric neurons with the Kv7 activator flupirtine is able to decrease resultant mucosal secretion via decreased neuronal firing. This conclusion is evidenced by the fact that flupirtine administration prior to TTX leads to substantial inhibition of baseline I_{SC}, however the simple reversal of that order leads to a significant inhibition of I_{SC} by TTX with a much smaller contribution from flupirtine (Fig. 1). As not to rely on this as our only evidence, we then characterized the ability of flupirtine to inhibit nicotine-stimulated I_{SC}, which is mediated through nicotinic receptors on post-ganglionic fibers [32]. Control tissue administered basolateral nicotine demonstrated a significant increase in I_{SC}, however pre-incubation with flupirtine left the tissue practically unresponsive, owing to ENS neurons being substantially hyperpolarized (Fig. 4). Also, to more fully implicate the ENS in mediating the
effects of flupirtine, T₈₄ monolayers [30] did not exhibit a decrease in Iₛₐₑ upon basolateral administration (Fig. 5). We then wanted to try and characterize the specific Kᵥ7 channels responsible for mediating the effects of flupirtine. Immunofluorescence studies demonstrated that the canonical M current channels, Kᵥ7.2 and Kᵥ7.3, co-localized with axons that stained positively for ChAT (Fig. 6). Finally, the use of the Kᵥ7.2/3 activator, ICA 110381, demonstrated a similar profile to flupirtine administration (Fig. 7). In total, our results would indicate that the decrease in Iₛₐₑ from basolateral application of flupirtine is ENS-mediated via hyperpolarization of enteric neurons through activation of Kᵥ7.2/3.

Kᵥ7.1, another member of the Kᵥ7 family, has been localized and functionally described in epithelial tissues [33-35] and could be thought to participate in the observed response in native tissue. However, activation of Kᵥ7.1 in epithelial tissues leads to an increase in K⁺ recycling via the basolateral membrane and an increase in apical Cl⁻ secretion [36]. These previous observations would agree with our results that flupirtine was not able to decrease Iₛₐₑ in T₈₄ monolayers, but actually resulted in a small increase in Iₛₐₑ [37] from activation of presumably of Kᵥ7.1 (Fig. 5). To further demonstrate the importance of Kᵥ7 in apical Cl⁻ secretion, XE-991, a non-specific Kᵥ7 inhibitor, was administered to the basolateral membrane of T₈₄ monolayers, which demonstrated a significant decrease in FSK-mediated Iₛₑ.

The effect of flupirtine on cholinergic neurons was also studied, since cholinergic fibers make up approximately 60% of neurons within the ENS [38]. As
expected, tolterodine (muscarinic receptor antagonist) application was able to partially inhibit on-going mucosal Cl⁻ secretion [39, 40]. Flupirtine was able to further decrease measured $I_{SC}$. This would indicate that non-cholinergic neurotransmitters also participate in modulating mucosal electrolyte transport, which has also been previously described [25]. Application of flupirtine prior to tolterodine would then be expected to inhibit all ENS neurons, and the application of the muscarinic receptor antagonist following this would have no effect or only very modestly decrease $I_{SC}$. However, tolterodine application following flupirtine administration unexpectedly increased $I_{SC}$ (Fig. 3B/D). This could be the result of a subpopulation of interneurons that are less sensitive to flupirtine. Application of tolterodine would block the remaining excitatory stimulation on inhibitory secretomotor neurons, which would remove the “brake”, and lead to the observed increase in $I_{SC}$.

Previous studies have shown that several gastrointestinal disorders result in an overall increase in excitability of ENS neurons [8-10, 41]. However, current clinical options are limited for these pathologies, with many observed adverse effects [42, 43]. Unfortunately, flupirtine, which had been available for nearly three decades in several European countries, was recently removed from the market due to issues of hepatotoxicity [44]. Development of novel $K_{V7}$ channel activators, with a greater safety profile, could yield therapeutic potential in patients that have been unresponsive to traditional treatments for an over-active ENS. Since $K_{V7}$ channel activation causes a sustained hyperpolarization of enteric neurons that synapse on the epithelium, as well as the smooth muscle,
targeted treatment could help to alleviate associated symptoms. Also, our immunofluorescence studies demonstrate localization of Kv7.3 in gastrointestinal smooth muscle (Fig. 6), which along with previous observations of Kv7 channels in smooth muscle may further enhance the efficacy of a Kv7 activator by reducing motility of gastrointestinal tract [45]. In all, Kv7 activators could potentially be utilized clinically for the treatment of gastrointestinal disorders attributed to hyper-excitability of the ENS.

In summary, our results demonstrate that activation of Kv7 channels via flupirtine in enteric neurons significantly inhibits mucosal electrolyte transport in the colon. As demonstrated through immunofluorescence studies and a specific pharmacological activator, this mechanism is mediated through Kv7.2/3 located within axons of enteric neurons. Also, this discovery may have clinical significance as a potential therapeutic agent to decrease ENS hyper-excitability in certain gastrointestinal pathologies and associated-outcomes.
References


**Figure Legends**

Figure 1. **Basolateral 100 µM Flupirtine Inhibits I_{sc} in the Murine Distal Colon.** A. Representative trace of basolateral application of 1 µM tetrodotoxin
(TTX) on full thickness tissue preparation in the Ussing chamber. TTX was able to decrease \( I_{sc} \), however addition of basolateral 100 \( \mu \)M flupirtine (Flu) following the TTX-induced plateau only modestly decreased \( I_{sc} \). 

**B.** Representative trace of basolateral application of 100 \( \mu \)M Flu in the Ussing chamber. Measured \( I_{sc} \) substantially decreased from the previous baseline, however application of 1 \( \mu \)M TTX only slightly decreased measured \( I_{sc} \). 

**C.** Group data of percent inhibition of maximal response of 1 \( \mu \)M TTX prior to 100 \( \mu \)M Flu. 

**D.** Group data of percent inhibition of maximal response of 100 \( \mu \)M Flu prior to 1 \( \mu \)M TTX application. 

**E.** Group data of percent inhibition of maximal response of 100 \( \mu \)M Flu prior to or following TTX application. All presented bar graphs are summarized data of means \( \pm \) SE from five different animals (n=5). Plotted points on each bar graph are representative of individual experiments. *\( p < 0.001 \) – compared to control.

**Figure 2. Dose-Response of Basolateral application of Flupirtine in Murine Distal Colon.** 

**A.** Representative trace of a dose-response curve of Flu. The initial administered concentration of Flu was 100 nM and increased sequentially by a log order of 10 until reaching a final concentration of 100 \( \mu \)M Flu. Each arrow within the trace indicates time of administration of an individual dose of Flu. 

**B.** A dose-response curve of percent inhibition of maximal response was generated from multiple experiments (n=5) to determine an approximate half-maximal inhibitory concentration (IC\(_{50}\)) in the murine distal colon.
Figure 3. **Tolterodine and Flupirtine Inhibit Cholinergic-induced I_{sc} in the Distal Colon.**  
A. Representative trace of basolateral application of 10 \( \mu \text{M} \) tolterodine (TOL) followed by basolateral 100 \( \mu \text{M} \) Flu on whole-mount distal colon in the Ussing chamber.  
B. Representative trace of basolateral application of 100 \( \mu \text{M} \) Flu followed by 10 \( \mu \text{M} \) TOL in the Ussing chamber.  
C. Group data of percent inhibition of maximal response of 10 \( \mu \text{M} \) TOL prior to Flu application.  
D. Group data of percent inhibition of maximal response of 100 \( \mu \text{M} \) Flu prior to application 10 \( \mu \text{M} \) TOL.  
E. Group data of percent inhibition of maximal response of 100 \( \mu \text{M} \) Flu prior to or following 10 \( \mu \text{M} \) TOL application. All presented bar graphs represent summarized data of means ± SE from five different animals (n=5). Plotted points on each bar graph represent individual experiments that contribute to the overall mean and SE. *\( p < 0.001 \)

Figure 4. **Flupirtine Inhibits Nicotine-Induced Increases in I_{sc}.**  
A. Representative trace of basolateral 100 \( \mu \text{M} \) nicotine (NICO)-induced increases in measured I_{sc} in the distal colon.  
B. Representative trace of 100 \( \mu \text{M} \) NICO-induced increases in I_{sc} that was pre-incubated with 100 \( \mu \text{M} \) Flu.  
C. Group data of NICO-induced \( \Delta I_{sc} \) in tissue that was pre-incubated with or without 100 \( \mu \text{M} \) Flu prior to basolateral 100 \( \mu \text{M} \) NICO administration. All presented bar graphs represent summarized data of means ± SE from five different animals (n=5). Plotted points on each bar graph represent individual experiments that contribute to the overall mean and SE. *\( p < 0.001 \)
Figure 5. **Flupirtine Does Not Decrease $I_{sc}$ in $T_84$ Monolayers.**  
A. Representative trace of measured $I_{SC}$ in $T_84$ monolayers that were treated with basolateral 100 µM Flu. Monolayers were then stimulated with 10 µM forskolin (FSK) that was inhibited with 50 µM XE-991 upon reaching a steady-state.  
B. Representative trace of measured $I_{SC}$ in $T_84$ monolayers that were incubated with 1 µM TTX. Monolayers were then stimulated with 10 µM FSK and then inhibited with 50 µM XE-991.  
C. Group data of measured $\Delta I_{SC}$ following either 100 µM Flu or 1 µM TTX.  
D. Group data of 10 µM FSK-induced $\Delta I_{SC}$ from $T_84$ monolayers incubated with Flu or TTX. All presented bar graphs represent summarized data of means ± SE from five different animals (n=5). Plotted points on each bar graph represent individual experiments that contribute to the overall mean and SE. *$p < 0.001$.

Figure 6. **$K_v7.2$ and $K_v7.3$ Co-Localize with Choline Acetyl Transferase in Enteric Neurons of the Murine Distal Colon.**  
A/D. Axons of enteric neurons that stained positive for choline acetyl transferase (ChAT).  
B/E. Positive immunostaining for $K_v7.2$ and $K_v7.3$ in murine distal colon.  
C/F. Merged images of panels A/B and D/E demonstrate co-localization of $K_v7.2$ and $K_v7.3$ with axons positively-stained for ChAT.

Figure 7. **The Specific $K_v7.2/3$ Activator, ICA 110381, Inhibits $I_{sc}$ in the Murine Distal Colon.**  
A. Representative trace of measured $I_{SC}$ that has been administered 100 µM ICA 110381(ICA) to the basolateral surface. This was then
followed by 1 µM TTX application, which was also applied to the basolateral surface. **B.** Group data of percent inhibition of maximal response of tissues that received basolateral 100 µM ICA prior to 1 µM TTX. **C.** Representative trace of a dose-response curve that was sequentially administered 100 nM ICA, increasing by a factor of ten each time, with a final basolateral concentration of 100 µM ICA. Each arrow indicates the time of administration of each corresponding concentration of ICA. **D.** A dose-response of percent inhibition of maximal response from multiple experiments (n=5) was generated to determine the IC_{50} of ICA. All presented bar graphs represent summarized data of means ± SE from five different animals (n=5). Plotted points on each bar graph represent individual experiments that contribute to the overall mean and SE.
Figure 1.
Figure 2.

A

100 µM Flu
10 µM Flu
1 µM Flu
100 nM Flu

Short-Circuit Current (µA/cm²)

B

Percent Inhibition of Maximal Response

Flupirtine [M]
Figure 3.

A

Short-Circuit Current (µA/cm²)

10 µM TOL

100 µM Flu

10 min

B

Short-Circuit Current (µA/cm²)

100 µM Flu

10 µM TOL

5 min

C

Percent Inhibition of Maximal Response

TOL-sensitive Flu-sensitive

D

Percent Inhibition of Maximal Response

E

Percent Inhibition of Maximal Response

After TOL Before TOL
Figure 4.

A

B

C

Short-Circuit Current

5 min

100 µM NICO

100 µM FLU

100 µM NICO

Nicotine-induced ∆Isc

Flupirtine

(-) (+)
Figure 5.

A

B

C

D

10 μM FSK

1 μM TTX

50 μM XE-991

10 μM FSK

100 μM Flu

50 μM XE-991

TTX-sensitive Flu-sensitive

FSK-Induced

∆I_{SC} (µA/cm²)

TTX-exposed Flu-exposed

FSK-induced ∆I_{SC} (µA/cm²)
Figure 6.
Figure 7.

A

100 µM ICA

1 µM TTX

20 min

Short-Circuit Current (µA/cm²)

B

Percent Inhibition of Maximal Response

ICA-sensitive

TTX-sensitive

C

100 µM ICA

10 µM ICA

1 µM ICA

100 nM ICA

5 min

Short-Circuit Current (µA/cm²)

D

Percent Inhibition of Maximal Response

ICA [M]
Chapter IV

Conclusions and Proposal of Future Work
As previously presented, I have demonstrated that the absence of Ca\textsuperscript{2+}-activated Cl\(^{-}\) secretion in DSS-treated animals is a result of decreased expression of TMEM16A in colonic epithelium. I have also shown that activation of KCNQ2/3 and the resultant hyperpolarization in enteric neurons can inhibit mucosal Cl\(^{-}\) secretion. Each of these are novel findings and contribute to their respective fields. The following discussion will elaborate on their greater implications with a specific focus on inflammatory bowel disease.

**TMEM16A and Dextran-Sulfate Sodium-Induced Colitis**

As previously discussed, the DSS-induced colitis is a commonly utilized animal model of inflammatory bowel disease (IBD) [1]. It has previously been shown that the colitis that develops significantly blunts apical Cl\(^{-}\) secretion in the distal colon [2]. More specifically though, agonists that elicit increases in intracellular Ca\textsuperscript{2+} and resultant Cl\(^{-}\) secretion have been shown to be completely abolished. Stimulation with agonists that normally increase \(I_{\text{SC}}\) actually lead to a decrease following DSS treatment. Previous studies that focused on this phenomenon attributed these changes to a dysfunctioning ENS or alterations in the basolateral K\textsuperscript{+} conductance [3, 4]. However, these studies were prior to the identification of TMEM16A, a CaCC. Our group was the first to reexamine this previously observed effect of DSS-induced colitis, and we were able to determine that TMEM16A was in fact down-regulated in the DSS-induced colitis model. Our functional data demonstrated that CCH-induced Cl\(^{-}\) secretion in DSS animals was not only abolished, but it had reversed in its direction of polarity, similar to previous reports. Also, the use of a Ca\textsuperscript{2+} ionophore (ionomycin) was able to
reproduce a similar $I_{SC}$ profile to that of CCH, indicating this mechanism is mediated via Ca$^{2+}$. However, most important of all the findings was the quantification of TMEM16A via immunoblot. Immunoblot analysis demonstrated a significant reduction in total TMEM16A in colonic epithelium. Immunohistochemistry also demonstrated that the remaining protein was not properly trafficked to the cellular membrane in colonic epithelium.

While this seems to answer the question as to why Ca$^{2+}$-activated Cl$^-$ secretion is absent in DSS-exposed animals, it does not solve the entire riddle. The mechanism by which TMEM16A is down-regulated in colonic epithelium following DSS-exposure is still unknown. However, several groups have previously demonstrated that Ca$^{2+}$-activated Cl$^-$ secretion could be decreased following incubation with certain cytokines in immortalized cell lines of colonic epithelium [5, 6]. Both cytokines (IL-4 and TGF-β) that decreased Ca$^{2+}$-activated Cl$^-$ secretion are more typically represented in a Th$_2$-mediated immune response. Since, the utilization of the Balb/c mice in our study typically respond in more of a Th$_2$ response (and our qPCR results would indicate as such), this may be the reason for loss of TMEM16A protein expression. With this as our working hypothesis, it would be interesting to repeat this study in C57Bl/6 mice, which typically demonstrate a Th$_1$ response upon DSS exposure, and determine if they would demonstrate a protection against loss of TMEM16A. Also, colon specific-knockout of TGF-β or IL-4 in combination with DSS-induction could potentially provide some protective mechanism by preventing signaling through either cellular pathway.
While signaling through a Th₂ immune response may be a likely cause for down-regulation of TMEM16A, the most important question to answer is, ‘Does down-regulation of TMEM16A have any role in the development of or continuance of inflammation in DSS-colitis, or for that matter IBD?’. The evidence for this is much less than is present to even support the previously proposed mechanism whereby TMEM16A is down-regulated in colonic epithelium. However, it has been shown previously that Ca²⁺-activated Cl⁻ secretion decreases in the first couple days of DSS-exposure even though physical symptoms such as loose stools with copious amounts of mucus do not manifest until about day 5 of a one week exposure [1]. Also, animals treated with DSS have disrupted mucus barriers that typically prevent interaction between the host and the microbiome [7]. It has been demonstrated that activation of TMEM16A is crucial for release of Muc5AC, however that study was conducted in human bronchial epithelial cells [8]. Muc5AC and similar proteins like it are necessary for maintaining the mucosal mucus barrier. Applying this knowledge, it is plausible that down-regulation of TMEM16A in DSS-colitis or potentially IBD could cause a decrease in Muc2 protein (more common in the intestine) secretion and overall disruption in the mucus barrier lining the colonic lumen. This would eventually lead to increased interaction between the microbiome and the host, which would elicit an inflammatory response. While this is highly speculative, it does provide a plausible route of further investigation.
KCNQ2/3 and the Enteric Nervous System

The ENS has a vast supply of neurons along the entire gastrointestinal tract. Previous research has demonstrated that the ENS can control local blood flow, motility of the tract, and influence electrolyte transport [9]. As far as ENS control over electrolyte transport, previous studies have demonstrated that application of TTX (Na\textsubscript{V} inhibitor) or atropine (muscarinic antagonist) inhibits ongoing Cl\textsuperscript{−} secretion via inhibition of I\textsubscript{SC} [10, 11]. Our study was similar in setup, however, we focused on KCNQ channels and their contribution to resting membrane potential in enteric neurons. Activation of KCNQ channels with basolateral flupirtine was able to inhibit the observed I\textsubscript{SC} in full thickness tissue from murine distal colon. This was achieved via inhibition of neurotransmitter release from enteric neurons that were substantially hyperpolarized. Application of TTX following flupirtine was practically unable to decrease the measured I\textsubscript{SC}. Also, pre-incubation with flupirtine prior to stimulation with nicotine resulted in an unresponsiveness of the tissue. Application of flupirtine on the T\textsubscript{84} colonic epithelial cell line further implicated that effect was mediated through the ENS, since the monolayers were unresponsive to the drug. However, flupirtine is a non-specific activator of KCNQ channels, therefore the use of immunofluorescence was employed to demonstrate the presence of KCNQ2 and KCNQ3 in enteric neurons.

The previously mentioned work seems to indicate that activation of KCNQ channels in enteric neurons can lead to inhibition of neurotransmitter release. Unfortunately, functional evidence of the exact channels that contribute to the
observed response is lacking. While the use of a specific activator of KCNQ2/3 was utilized in the study, channel activators are prone to selectivity issues. A specific inhibitor to the channel would be of clear benefit to the study and future research. However, the only available KCNQ inhibitors are linopiridine and XE-991, which are both global KCNQ inhibitors. This means that a specific channel can not be identified from inhibition with either of the drugs. Also, KCNQ1 has been characterized to reside on the basolateral membrane of colonic epithelium and inhibition causes decreased apical Cl\(^-\) secretion due to loss of a favorable electrochemical gradient [12, 13]. So, application of either of the inhibitors causes a decrease in \(I_{SC}\), when one might expect an increase due to increased firing of enteric neurons. Hopefully in the future better pharmacological tools (inhibitors) can be developed that will allow for this functional characterization, or the development of a conditional knock-out model with an enteric neuron promoter.

The Enteric Nervous System And Inflammatory Bowel Disease

While seemingly disconnected, the two previously presented studies have a potential common thread of IBD. Research has primarily focused on the immune response that is present within the tissue, as well as, the changes in electrolyte transport across the epithelial layer. However, it has been shown that inflammation is able to elicit a hyper-excitability of enteric neurons [14, 15]. It would be of personal future interest to evaluate the effects of DSS administration on the ENS from the standpoint of electrolyte transport and epithelial integrity. I would be curious to see if oral administration of KCNQ activators is capable of
attenuating the deleterious effects of DSS-colitis and for that matter IBD as a whole. Also, utilization of animal models with dysfunctional enteric KCNQ channels with DSS administration would be interesting to characterize the severity of colitis as well as alterations in epithelial transport. Hopefully in the near future, activators truly specific to KCNQ2/3 will become available and allow for this evaluation.
References


