Published in final edited form as:

Neurogastroenterol Motil. 2019 April ; 31(4): e13547. doi:10.1111/nmo.13547.

Measurement of novel intestinal secretory and barrier pathways and effects of proteases

Adam L. Edwinston¹ and Madhusudan Grover^{1,#}

¹Division of Gastroenterology & Hepatology, Mayo Clinic, Rochester, MN, USA

Abstract

The epithelial lining of the gastrointestinal (GI) tract in conjunction with the enteric nervous system (ENS) plays an important role in mediating solute absorption and secretion. A dysregulated ionic movement across the epithelium can result in GI diseases that manifest as either watery diarrhea or constipation. Hirschsprung disease is an example of an ENS disorder characterized by absence of enteric ganglia in distal gut resulting in obstructive phenotype. Receptor rearranged during transfection (RET) gene variants are the most commonly recognized genetic associations with Hirschsprung disease. In this issue of *Neurogastroenterology and Motility*, Russell et al. demonstrate that RET mediates colonic ion transport through modulation of cholinergic nerves. They go on to show inhibition of RET can attenuate accelerated transit in a rat model. Normalizing secretory and absorptive defects has been an attractive therapeutic strategy. In addition to the intrinsic regulation of secretory processes, luminal mediators like bile acids, short chain fatty acids, and proteases can affect both secretion and barrier function of the intestinal epithelium. Elevated levels of proteases have been identified in a wide range of GI diseases including irritable bowel syndrome. Proteases are known to cause visceral hypersensitivity and barrier disruption *in vitro* and in animal models. The goals of this review are to describe fundamental concepts related to intestinal epithelial secretion, the utility of Ussing chambers to measure ionic mechanisms and to discuss examples of novel signaling pathways; namely the RET signaling cascade in secretomotor neurons and effects of luminal proteases on barrier and ionic secretion.

Introduction

The intestinal epithelium is a complex environment that serves as a restrictive barrier against luminal antigens, resident microflora and invading pathogens, and as a surface that allows for absorption and secretion, both of which are critical for nutrient acquisition and maintaining homeostasis. Disruption of this barrier caused by stress, either psychological or physical, can cause increased permeability and the potential onset of gastrointestinal (GI) disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD)¹.

#Correspondence: Madhusudan Grover, MD, Assistant Professor of Medicine and Physiology, Enteric NeuroScience Program, Division of Gastroenterology and Hepatology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA, Tel: 507-284-2478, Fax: 507-266-0350, grover.madhusudan@mayo.edu.

Author contributions

Adam L. Edwinston: study concept, manuscript drafting

Madhusudan Grover: study concept, critical revision of the manuscript, study supervision

Declarations: None to disclose for both authors

Impairment of ion transport across the intestinal epithelium can have drastic consequences on GI physiology and function and can explain many of the symptoms associated with IBS, IBD and other diseases of the GI tract. Improper ionic balance can result in both diarrheal and constipation phenotypes through effects on intestinal secretion, and additionally also cause visceral hypersensitivity and hyperalgesia². For these reasons, identifying mediators of ion absorption and secretion in the GI tract have become attractive targets for treating GI diseases. In this issue of *Neurogastroenterology and Motility*, Russell et al. were able to demonstrate Cl^- secretion by stimulation of enteric cholinergic nerves is facilitated by receptor rearranged during transfection (RET) and inhibition of RET to ameliorate the accelerated transit which is commonly associated with many GI diseases³. The identification of RET as a mediator of secretion has underscored how important regulating ion transport in the gut is to maintaining GI physiology, but also highlights the need to identify other regulators of GI secretion and the underlying mechanisms.

Recently, microbiota and other luminal mediators like bile acids and tryptamine have been shown to affect intestinal secretion. Proteases are another important class of luminal molecules that have been studied extensively in pathophysiology of IBS and as mediators of visceral hypersensitivity in animal models⁴. These proteases can break down cell-cell junctions and cleave protease activated receptors (PARs) at the cell surface which reduce barrier integrity and increase secretion into the gut. Intestinal proteases are an attractive target for the treatment of IBS as inhibition of protease activity in animal models has resulted in the resolution of visceral hyperalgesia⁵. However, the effects of proteases on intestinal secretory mechanisms are not well understood. Here we outline some of the fundamental concepts of ionic secretion in the GI tract and how it contributes to both the absorptive and secretory processes. Emphasis is placed on RET signaling and its role in affecting neuronal-mediated secretion and on proteases as potential novel mediators of intestinal secretion in addition to their role in mediating barrier disruption.

Measuring secretion in Ussing chambers

Both active and passive transport of ions contributes to the overall ionic flux across the intestinal epithelium. Assessing ion transport across intestinal and other epithelia has been made possible through the use of Ussing chambers. The discovery of this elegant technique by Hans Ussing in intact frog skin has revolutionized our understanding of secretory and barrier mechanisms across variety of epithelial surfaces. Briefly, a polarized epithelial surface of interest is mounted in a chamber whereby two halves are separated from each other and bathed in solutions of equal volume and ionic composition with an external current passed through the membrane. The delivered current (Short-circuit current, I_{sc}) keeps the surface in a voltage clamp mode ($V=0$) and thus provides a measure of ionic flux⁶. Furthermore, pharmacological activation and blockage of specific secretory channel proteins can allow studying their role in the overall secretory process. To examine nerve-evoked secretory responses, electrical field stimulation (EFS) can be used⁷. Lastly, in mucosa-submucosa or full thickness preparations, application of drugs such as Tetrodotoxin (TTX) can block neuronal pathways and help understand epithelial-specific regulation of secretory mechanisms.

The active ionic transport of Na^+ and Cl^- is the primary driver of I_{sc} across the intestinal epithelium in a site-dependent fashion. An increase in apical anionic secretion or cationic absorption can result in an increase in epithelial I_{sc} and an inverse of those processes can cause decrease in I_{sc} . In the small intestine and throughout the colon, absorption of sodium and chloride ions is facilitated using a pair of transporters present on the apical surface. A sodium-hydrogen exchanger, either NHE2 or NHE3, mediates Na^+ transport, while SLC26A3 (or DRA, Downregulated-in-adenoma) or SLC26A6 controls Cl^- movement by an exchange with HCO_3^- . Na^+ and Cl^- movement across the basolateral membrane is then facilitated by a Na^+/K^+ ATPase transporter and a potassium-chloride co-transporter (KCC1) respectively. Mice deficient in Na^+ transporters and Na^+/K^+ -ATPase activity have increased permeability, fluid accumulation in the intestinal lumen and develop chronic diarrhea due to a lack of ion absorption⁹. The drug atropine which inhibits bicarbonate secretion prevents increases in I_{sc} suggesting muscarinic receptors play a major role in intestinal secretion by regulating $\text{Na}^+/\text{Ca}^{2+}$ exchange¹⁰. In the distal colon however, Na^+ can be absorbed without co-transport of Cl^- . Apical membrane epithelial sodium ion channels (ENaC) allow for Na^+ absorption which is also moved across the basolateral membrane via a Na^+/K^+ ATPase. An example of studying this in Ussing chambers is by the use of diuretic amiloride which blocks ENaC activity and causes a drop in I_{sc} ⁷.

Secretion of chloride ions in the small intestine and colon occurs through apical calcium activated chloride channels (CaCCs) or through cystic fibrosis transmembrane conductance regulator (CFTR) channels via a cAMP or cGMP mediated process¹¹. Activity of CaCCs is dependent on the release and presence of intracellular Ca^{2+} , while the presence of cAMP activates the basolateral $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC1) and CFTR which is the primary means by which Cl^- is secreted into the lumen. An example of studying this in the Ussing chamber is through the use of a CFTR activator, CFTR_{act}-J027, on mouse duodenal tissue. Activation of CFTR resulted in Cl^- secretion and an increase in I_{sc} . Oral administration of CFTR_{act}-J027 was able to normalize whole gut transit time in a loperamide induced mouse model of constipation¹². In another study, nine out of ten patients receiving a CFTR inhibitor had resolution of diarrheal symptoms. Application of the same inhibitor to HT29-CL19A cells mounted in Ussing chambers resulted in a decrease in I_{sc} demonstrating the importance of CFTR channels in secretion¹³. Additionally, bumetanide, an NKCC1 inhibitor, decreases I_{sc} by preventing Cl^- secretion indicating inhibition of NKCC1 could potentially reduce transit and diarrhea by preventing ionic and fluid secretion into the lumen of the colon⁷. This is supported by the observation of intestinal obstructions developing in *Nkcc1*^{-/-} knockout mice and diuretics like bumetanide that block NKCC1 channels have been associated with intestinal dysfunction, primarily constipation¹⁴.

In the accompanying article in this issue by Russell et. al., EFS was used to assess how the inhibition of the receptor RET using GSK3179106 affected the neuronal contribution to I_{sc} . RET is a tyrosine kinase that along with GDNF family receptor α (GFR α) forms a complex that is important for glial cell-derived neurotrophic factor (GDNF) ligand signaling. Functional RET is critical for ENS development. RET signaling is necessary for presynaptic axon terminal maturation, regulating protein expression¹⁵. Conditional knockouts of GFR α 3 in mice have also implicated RET kinase as a mediator of visceral hypersensitivity and nociception¹⁶. In the context of human disease, loss of RET or mutations in RET gene have

been associated with colonic dysmotility seen in Hirschsprung disease patients¹⁷. Aganglionosis is a reported intestinal phenotype in RET-deficient mouse models.

In response to EFS, Russell et al. noted a significant reduction in ion transport when RET was inhibited with GSK3179106 and normalization of fecal output in a rat model of neostigmine induced accelerated transit. GSK3179106 is a gut-restricted pyridine hinge binder small molecule RET kinase inhibitor shown to reduce colonic hypersensitivity in a mouse model of acute irritation stress¹⁸. The observation that GSK3179106 also reduces ionic transports demonstrates RET has a functional role beyond ENS development. Russell et al. went on to show that ion transport after the addition of the acetylcholine receptor agonist carbachol was attenuated when RET was inhibited. This observation provided direct evidence demonstrating RET as a mediator of cholinergic-induced active ion transport³. Cholinergic compounds help invoke smooth muscle contraction and excitation through Ca^{2+} influx resulting in the activation of CaCCs, and Cl^- secretion into the gut, and thus possibly accelerating transit. Drugs such as the acetylcholinesterase inhibitor neostigmine increase the amount of available Ach which has been shown to lead to faster intestinal transit in rats¹⁹. They were also able to show RET inhibition abrogated increased GI motility induced by neostigmine, suggesting that RET not only associates with neurons, but also has a role in influencing cholinergic induced colonic motility *in vivo*³.

Proteases as luminal mediators of intestinal function

Intestinal proteases make up approximately 2% of the genome, serving a variety of functions ranging from digestion, host defense, immune responses, and cellular development^{4, 20}. The pancreas is the major contributor of proteases in the intestinal tract releasing trypsin, elastase and chymotrypsin among other proteases to drive digestion²¹. Epithelial cells, immune cells (mast cells, macrophages and neutrophils), along with other residential cells have all been shown to be important sources of proteases. Additionally, microbiota and dietary sources of proteases add to the complexity of the protease milieu²². A number of protease inhibitors (Serpins, elafin, etc.) have been identified that keep a check on the protease activity⁴. Elevated levels of proteases however have been reported in tissues collected from patients suffering from GI diseases which have been shown to be associated with higher permeability and a decrease in the expression of tight junction (TJ) proteins^{5, 23}. The effects of intestinal proteases on neuro-epithelial secretory mechanisms are less clear. In the sections following, we will describe the effects of proteases on epithelial secretion and barrier and provide contemporary examples on how ussing chambers have been used to study these processes.

Proteases and PAR activation in the GI tract

The activation of protease activated receptors (PARs) is responsible for a range of functions and signaling at the cellular level. To date, four unique PARs have been identified, PARs-1 through 4, each with unique cleavage sites, role and distribution amongst cells²⁴. PAR activation involves irreversible binding by proteases and cleavage of the extracellular N-terminal domain of PARs. The newly formed N-terminus acts as a ligand for the receptor, triggering downstream intracellular signaling²⁵. Activated PARs can couple with G proteins to activate a wide range of signaling pathways responsible for adhesion, growth, motility and

even secretion. Signaling via PAR activation however is not limited to the cell membrane surface. Recent evidence has shown internalization of activated PARs may contribute to pathways responsible for persistent pain in IBS patients and inhibition of PAR-2 endosomal trafficking has been suggested as a means to ameliorate symptoms²⁶.

PARs-1 and 2 are expressed on epithelial cells, neurons, immune cells, smooth muscle cells. Compared to PAR-1, there is a greater density of PAR-2 found in the small intestine and colon²⁷. PAR-4 is also found in the small intestine and colon, while PAR-3 is primarily in the stomach and small intestine²⁸. Both PAR-1 and PAR-2 play important roles in the secretion and absorption of Cl^- in the intestine²⁹. In the mouse distal colon, basolateral activation of PAR-2 stimulates Cl^- and K^+ secretion while blocking Na^+ absorption³⁰. PAR activation has also been shown to affect barrier as administration of 2fAP, a PAR-2 agonist, to the basolateral side of cells caused a decrease in transepithelial resistance (TER) and redistribution/internalization of junctional protein ZO-1²⁷. Interestingly, the location of PAR-2, either apical or basolateral, has been shown to activate distinctly different signaling pathways^{27, 31}. As a consequence, luminal proteases or tissue proteases can act on PARs present both apically and basolateral, eliciting physiological responses which include ion exchange, secretion with possible effects on motility as well as affect intestinal permeability^{32, 33}.

Proteases and barrier integrity

The apical junctional complex that forms between intestinal cells is comprised of tight junctions (TJ) at the top followed by adherens junctions and desmosomes. Integral membrane proteins (claudins, occludin) and junctional complex proteins (Zonula occludens, ZO)s regulate TJs³⁴. Disruption of the TJ can increase migration of luminal and microbial contents into the subepithelial space and eventually into portosystemic circulation. An increase in epithelial permeability can be facilitated by luminal proteases acting on TJ proteins. Opening of TJs provides greater accessibility to PARs located on neurons which can enhance fluid secretion into the intestinal lumen³⁰. In T84 cells, the protease inhibitor (4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF) prevented the IFN- γ mediated cleavage of claudin-2, increased flux of 44kD horseradish peroxidase (HRP) and drop in TER³⁵. Colonic biopsy supernatants collected from IBS and Crohn's disease patients have elevated levels of trypsin and chymotrypsin-like activity respectively, both of which correlate with decreased epithelial expression of occludin³⁶. In another study, colonic biopsy supernatants of IBS patients with elevated proteolytic activity resulted in decreased ZO-1 expression, drop in TER and an increased flux of 4kDa fluorescein isothiocyanate (FITC) conjugated dextran³⁷. A similar result was seen when fecal supernatants from IBS-D patients were administered intracolonic in mice: ZO-1 was redistributed to the cytoplasm, there was an increase in FITC-dextran flux (4 kDa) and an increased phosphorylated myosin light chain (MLC)³⁸. Activation of sodium-glucose transporter SGLT1 results in MLC phosphorylation and drop in TER which can be inhibited by ML-9, an inhibitor of MLC phosphorylation implicating interplay between secretory and barrier regulatory proteins³⁹.

Cleavage of PAR-2 can cause a significant increase in the expression of TNF- α , IL-1 β and INF- γ mRNA in mouse tissues, all of which are Th1 helper cell cytokines⁴⁰. The release of

these Th1 pro-inflammatory cytokines in response to PAR activation can have negative effects on barrier function and secretion⁴¹. TNF- α can directly affect Na⁺ absorption in mouse jejunal tissue by inhibition of NHE3 and also result in MLCK activation and occludin endocytosis⁴². IL-1 β also increases barrier permeability by causing increased levels of MLCK production and promoting MLCK activity⁴³. INF- γ specifically has been shown to cause dose dependent losses in TER in T84 cells, a loss of ZO-1 expression and redistribution of occludin, ZO-1, 2 away from TJs⁴⁴. Chemokines such as CXCL-8 and CXCL-5 are known to cause greater migration and activity of neutrophils, increasing the presence of proteases at the site of injury^{45, 46}. IL-8 alone can regulate the permeability of endothelial cells causing a downregulation of TJ protein expression (ZO-1, occludin and claudin-5)⁴⁷.

Additionally, TJ proteins as well as the mucus layer lining the GI tract serve as potential targets for secreted bacterial proteases. *Helicobacter pylori* and *Campylobacter jejuni* both secrete the serine protease HtrA which is a bacterial stress protein able to cleave E-cadherin, disrupting the epithelial barrier. Breakdown of E-cadherin facilitates greater paracellular migration and access to the basolateral membrane, enabling host cell invasion of the bacteria⁴⁸. Metalloproteases secreted by *Clostridium perfringens* have been shown to breakdown collagen, gelatin and basement membrane type IV collagen, all components of the extracellular matrix. Addition of *C. perfringens* culture supernatant to rat distal colon in Ussing chambers lowered TER⁴⁹. Purified *Pseudomonas* elastase, *Vibrio cholerae* hemagglutinin/protease and MMPs from *Enterococcus faecalis*, all resulted in ZO-1 disruption and drop in TER of MDCK monolayers and mouse colonic strips^{50–52}.

In response to invading pathogens, immune cells produce proteases as a defense mechanism. T84 cells exposed to media conditioned with degranulated mast cells or cultured neutrophils showed a drop in TER, increased flux of horseradish peroxidase (HRP, 44kDa) and TJ protein redistribution of occludin, ZO-1 and perijunctional F-actin. Moreover, when purified neutrophil elastase or trypsin were added to monolayers the effect on barrier was still seen^{53, 54}. Critically, elevated levels of proteolytic activity in GI disease may be a result of activation or dysregulation of macrophages, mast cells, neutrophils as many of these cells are implicated in various GI disorders. Overall, this suggests proteases of host and microbial origin can disrupt barrier function; however, it remains to be seen if specific proteases within the broad families have this effect on barrier and how their activity is (or is not) regulated in disease states.

Proteases and secretory responses

Proteases of both bacterial and host origin play an important role in activating signaling pathways that mediate absorption, secretion and transit. Addition of metalloproteases from *Pseudomonas aeruginosa* and *Serratia marcescens* to FRT cells in Ussing chamber experiments resulted in increased I_{sc} as a direct effect on ENaC channel activation⁵⁵. In the context of IBS, transcript levels for the zymogen trypsinogen IV, an activator of ENaC activity, is upregulated in the small intestine⁵⁶. In IBS-D patients, RNA sequencing data shows an increased expression of GUCA2B and PDZD3 in rectosigmoid tissue. Both of

these genes encode for mucosal ion channels. GUCA2B is responsible for Cl^- and water secretion whereas PDZD3 functions with NHE3 and in signaling with CFTR⁵⁷.

Work done on human bronchial epithelial cells has shown the importance of PAR-2 activation on ion transport. PAR-2 agonist SLIGRL-NH₂ caused an increase in I_{sc} associated with an increase in intracellular Ca^{2+} , with no effect on I_{sc} when PAR-1 was stimulated⁵⁸. Basolateral addition of thrombin or the PAR-1 agonist TFLLR-NH₂ to SCBN cells, a duodenal epithelial cell line, led to the release of intracellular stores of Ca^{2+} , increase in apical chloride secretion and I_{sc} ⁵⁹. Similar observations have been made after the basolateral addition of trypsin to human colonic and mouse jejuni tissue. This induced Cl^- secretion correlated with an increase in intracellular Ca^{2+} levels; however, there was no effect upon luminal addition⁶⁰. In Ussing chamber experiments, addition of indomethacin, a cyclooxygenase inhibitor and agonist of cAMP-dependent Cl^- secretion, prevented the effect of trypsin on I_{sc} while TTX addition did not, demonstrating that epithelial Cl^- release can be independent of a neuronal contribution⁶¹. In contrast to these findings, a separate study demonstrated trypsin added to the mucosal surface of mouse cecum caused an increase in I_{sc} . This could be due to the use of cecal tissue or due to the higher concentration of trypsin (10–100 μM) as compared to the other studies (1 μM)⁶². Submucosal secretomotor neurons in particular, which mediate neurogenic secretion in the intestine, express PAR-2 which upon activation by proteases can induce both acute and long-term excitation of the neurons. Mast cell tryptase induced PAR-2 mediated activation of submucosal neurons has been demonstrated⁶³. These observations indicate changes in I_{sc} and secretion in the human and mouse intestine are driven by protease activation of PARs localized on both the apical and basolateral surface of epithelial cells as well as possibly through effects on submucosal secretomotor neurons.

Conclusions

Understanding the intrinsic and extrinsic regulation of secretion and absorption in the GI tract is a critical to addressing diseases that affect intestinal transit. *Ex vivo* studies on strips of mucosa-submucosa intestinal tissues can help determine the role of specific epithelial and enteric nervous system processes involved in a physiological response. In addition to examining the fundamental role of proteins like RET in experimental tissue from animal models, these studies can utilize biopsies from patients suffering from a spectrum of intestinal disorders and provide individualized assessment of secretory responses. Finally, the regulation of intestinal secretion from luminal mediators is of significant importance. One such illustrated example is the effect of luminal proteases on intestinal barrier function and secretion.

Acknowledgments

Funding: This work was supported by NIDDK K23 DK 103911 to Dr. Grover

Abbreviations:

TJ tight junctions

RET	Receptor rearranged during transfection
GI	Gastrointestinal
ENS	Enteric Nervous System
IBS	Irritable Bowel Syndrome
IBD	Inflammatory Bowel Disease
EFS	Electrical Field Stimulation
PAR	Protease activated receptor
I_{sc}	Short circuit current

References

1. Grover M, Camilleri M, Smith K, et al. On the fiftieth anniversary. Postinfectious irritable bowel syndrome: mechanisms related to pathogens. *Neurogastroenterol Motil* 2014;26:156–67. [PubMed: 24438587]
2. Fuentes IM, Christianson JA. Ion channels, ion channel receptors, and visceral hypersensitivity in irritable bowel syndrome. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society* 2016;28:1613–1618. [PubMed: 27781369]
3. Russell JP, Mohammadi E, Ligon C, et al. Enteric RET inhibition attenuates gastrointestinal secretion and motility via cholinergic signaling in rat colonic mucosal preparations. *Neurogastroenterol Motil* 2018:e13479. [PubMed: 30311722]
4. Vergnolle N. Protease inhibition as new therapeutic strategy for GI diseases. *Gut* 2016.
5. Cenac N, Andrews CN, Holzhausen M, et al. Role for protease activity in visceral pain in irritable bowel syndrome. *The Journal of Clinical Investigation* 2007;117:636–647. [PubMed: 17304351]
6. Clarke LL. A guide to Ussing chamber studies of mouse intestine. *American Journal of Physiology - Gastrointestinal and Liver Physiology* 2009;296:G1151–G1166. [PubMed: 19342508]
7. Krueger D, Michel K, Zeller F, et al. Neural influences on human intestinal epithelium in vitro. *J Physiol* 2016;594:357–72. [PubMed: 26527433]
8. Barrett KE, Keely SJ. CHAPTER 76 - Integrative Physiology and Pathophysiology of Intestinal Electrolyte Transport. In: Johnson LR, ed. *Physiology of the Gastrointestinal Tract* (Fourth Edition). Burlington: Academic Press, 2006:1931–1951.
9. Schultheis PJ, Clarke LL, Meneton P, et al. Renal and intestinal absorptive defects in mice lacking the NHE3 Na⁺/H⁺ exchanger. *Nature Genetics* 1998;19:282–285. [PubMed: 9662405]
10. Dong H, Sellers ZM, Smith A, et al. Na⁺/Ca²⁺ exchange regulates Ca²⁺-dependent duodenal mucosal ion transport and HCO₃⁻ secretion in mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2005;288:G457–G465. [PubMed: 15499079]
11. Camilleri M, Sellin JH, Barrett KE. Pathophysiology, Evaluation, and Management of Chronic Watery Diarrhea. *Gastroenterology* 2017;152:515–532.e2. [PubMed: 27773805]
12. Cil O, Phuan P-W, Lee S, et al. CFTR Activator Increases Intestinal Fluid Secretion and Normalizes Stool Output in a Mouse Model of Constipation. *Cellular and Molecular Gastroenterology and Hepatology* 2016;2:317–327. [PubMed: 27127798]
13. Ren A, Zhang W, Thomas HG, et al. A Tannic Acid-based Medical Food, Cesinex(®), Exhibits Broad-spectrum Antidiarrheal Properties: a Mechanistic and Clinical Study. *Digestive Diseases and Sciences* 2012;57:99–108. [PubMed: 21748285]
14. Flagella M, Clarke LL, Miller ML, et al. Mice Lacking the Basolateral Na-K-2Cl Cotransporter Have Impaired Epithelial Chloride Secretion and Are Profoundly Deaf. *Journal of Biological Chemistry* 1999;274:26946–26955. [PubMed: 10480906]

15. Baudet C, Pozas E, Adameyko I, et al. Retrograde signaling onto Ret during motor nerve terminal maturation. *J Neurosci* 2008;28:963–75. [PubMed: 18216204]
16. Tanaka T, Shinoda M, Feng B, et al. Modulation of visceral hypersensitivity by glial cell line-derived neurotrophic factor family receptor α -3 in colorectal afferents. *Am J Physiol Gastrointest Liver Physiol* 2011;300:G418–24. [PubMed: 21193524]
17. Puri P, Shinkai T. Pathogenesis of Hirschsprung's disease and its variants: recent progress. *Seminars in Pediatric Surgery* 2004;13:18–24. [PubMed: 14765367]
18. Schenck Eidam H, Russell J, Raha K, et al. Discovery of a First-in-Class Gut-Restricted RET Kinase Inhibitor as a Clinical Candidate for the Treatment of IBS. *ACS Medicinal Chemistry Letters* 2018;9:623–628. [PubMed: 30034590]
19. Campbell-Dittmeyer K, Hicks GA, Earnest DL, et al. Increased colonic transit in rats produced by a combination of a cholinesterase inhibitor with a 5-HT4 receptor agonist. *Neurogastroenterol Motil* 2009;21:1197–e108. [PubMed: 19210632]
20. Turk B Targeting proteases: successes, failures and future prospects. *Nat Rev Drug Discov* 2006;5:785–99. [PubMed: 16955069]
21. Johansson KA, Grapin-Botton A. Development and diseases of the pancreas. *Clin Genet* 2002;62:14–23. [PubMed: 12123481]
22. Carroll IM, Maharshak N. Enteric bacterial proteases in inflammatory bowel disease-pathophysiology and clinical implications. *World journal of gastroenterology* 2013;19:7531–7543. [PubMed: 24431894]
23. Tooth D, Garsed K, Singh G, et al. Characterisation of faecal protease activity in irritable bowel syndrome with diarrhoea: origin and effect of gut transit. *Gut* 2014;63:753–760. [PubMed: 23911555]
24. Macfarlane SR, Seatter MJ, Kanke T, et al. Proteinase-Activated Receptors. *Pharmacological Reviews* 2001;53:245. [PubMed: 11356985]
25. Soh UJK, Dore MR, Chen B, et al. Signal transduction by protease-activated receptors. *British journal of pharmacology* 2010;160:191–203. [PubMed: 20423334]
26. Jimenez-Vargas NN, Pattison LA, Zhao P, et al. Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. *Proceedings of the National Academy of Sciences* 2018;115:E7438.
27. Lau C, Lytle C, Straus DS, et al. Apical and basolateral pools of proteinase-activated receptor-2 direct distinct signaling events in the intestinal epithelium. *American Journal of Physiology - Cell Physiology* 2011;300:C113–C123. [PubMed: 21068362]
28. Vergnolle N Clinical relevance of proteinase activated receptors (PARs) in the gut. *Gut* 2005;54:867–874. [PubMed: 15888798]
29. Nguyen TD, Moody MW, Steinhoff M, et al. Trypsin activates pancreatic duct epithelial cell ion channels through proteinase-activated receptor-2. *Journal of Clinical Investigation* 1999;103:261–269. [PubMed: 9916138]
30. Cuffe JE, Bertog M, Velazquez-Rocha S, et al. Basolateral PAR-2 receptors mediate KCl secretion and inhibition of Na⁺ absorption in the mouse distal colon. *J Physiol* 2002;539:209–22. [PubMed: 11850514]
31. D'Andrea M R, Derian CK, Leturcq D, et al. Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. *Journal of Histochemistry & Cytochemistry* 1998;46:157–164. [PubMed: 9446822]
32. Cenac N, Garcia-Villar R, Ferrier L, et al. Proteinase-Activated Receptor-2-Induced Colonic Inflammation in Mice: Possible Involvement of Afferent Neurons, Nitric Oxide, and Paracellular Permeability. *The Journal of Immunology* 2003;170:4296. [PubMed: 12682265]
33. Vergnolle N, Macnaughton WK, Al-Ani B, et al. Proteinase-activated receptor 2 (PAR2)-activating peptides: Identification of a receptor distinct from PAR2 that regulates intestinal transport. *Proceedings of the National Academy of Sciences* 1998;95:7766.
34. Buckley A, Turner JR. *Cell Biology of Tight Junction Barrier Regulation and Mucosal Disease*. Cold Spring Harbor perspectives in biology 2018;10:a029314. [PubMed: 28507021]

35. Willemsen LEM, Hoetjes JP, Van Deventer SJH, et al. Abrogation of IFN- γ mediated epithelial barrier disruption by serine protease inhibition. *Clinical and Experimental Immunology* 2005;142:275–284. [PubMed: 16232214]
36. Coeffier M, Gloro R, Boukhetala N, et al. Increased proteasome-mediated degradation of occludin in irritable bowel syndrome. *Am J Gastroenterol* 2010;105:1181–8. [PubMed: 19997094]
37. Piche T, Barbara G, Aubert P, et al. Impaired intestinal barrier integrity in the colon of patients with irritable bowel syndrome: involvement of soluble mediators. *Gut* 2009;58:196–201. [PubMed: 18824556]
38. Gecse K, Roka R, Ferrier L, et al. Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. *Gut* 2008;57:591–9. [PubMed: 18194983]
39. Turner JR, Rill BK, Carlson SL, et al. Physiological regulation of epithelial tight junctions is associated with myosin light-chain phosphorylation. *Am J Physiol* 1997;273:C1378–85. [PubMed: 9357784]
40. Cenac N, Coelho A-M, Nguyen C, et al. Induction of Intestinal Inflammation in Mouse by Activation of Proteinase-Activated Receptor-2. *The American Journal of Pathology* 2002;161:1903–1915. [PubMed: 12414536]
41. Mahraoui L, Heyman M, Plique O, et al. Apical effect of diosmectite on damage to the intestinal barrier induced by basal tumour necrosis factor- α . *Gut* 1997;40:339–343. [PubMed: 9135522]
42. Clayburgh DR, Musch MW, Leitges M, et al. Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo. *J Clin Invest* 2006;116:2682–94. [PubMed: 17016558]
43. Al-Sadi R, Ye D, Said HM, et al. IL-1 β -Induced Increase in Intestinal Epithelial Tight Junction Permeability Is Mediated by MEKK-1 Activation of Canonical NF- κ B Pathway. *The American Journal of Pathology* 2010;177:2310–2322. [PubMed: 21048223]
44. Youakim A, Ahdieh M. Interferon-gamma decreases barrier function in T84 cells by reducing ZO-1 levels and disrupting apical actin. *Am J Physiol* 1999;276:G1279–88. [PubMed: 10330020]
45. Padrines M, Wolf M, Walz A, et al. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS Lett* 1994;352:231–5. [PubMed: 7925979]
46. Nufer O, Corbett M, Walz A. Amino-terminal processing of chemokine ENA-78 regulates biological activity. *Biochemistry* 1999;38:636–42. [PubMed: 9888803]
47. Yu H, Huang X, Ma Y, et al. Interleukin-8 regulates endothelial permeability by down-regulation of tight junction but not dependent on integrins induced focal adhesions. *Int J Biol Sci* 2013;9:966–79. [PubMed: 24155670]
48. Hoy B, Geppert T, Boehm M, et al. Distinct Roles of Secreted HtrA Proteases from Gram-negative Pathogens in Cleaving the Junctional Protein and Tumor Suppressor E-cadherin. *Journal of Biological Chemistry* 2012;287:10115–10120. [PubMed: 22337879]
49. Pruteanu M, Hyland NP, Clarke DJ, et al. Degradation of the extracellular matrix components by bacterial-derived metalloproteases: Implications for inflammatory bowel diseases. *Inflammatory Bowel Diseases* 2010;17:1189–1200. [PubMed: 20853433]
50. Azghani AO, Gray LD, Johnson AR. A bacterial protease perturbs the paracellular barrier function of transporting epithelial monolayers in culture. *Infection and Immunity* 1993;61:2681. [PubMed: 8500907]
51. Wu Z, Milton D, Nybom P, et al. *Vibrio cholerae* hemagglutinin/protease (HA/protease) causes morphological changes in cultured epithelial cells and perturbs their paracellular barrier function. *Microb Pathog* 1996;21:111–23. [PubMed: 8844654]
52. Steck N, Hoffmann M, Sava IG, et al. *Enterococcus faecalis* metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. *Gastroenterology* 2011;141:959–71. [PubMed: 21699778]
53. Chin AC, Lee WY, Nusrat A, et al. Neutrophil-mediated activation of epithelial protease-activated receptors-1 and -2 regulates barrier function and transepithelial migration. *J Immunol* 2008;181:5702–10. [PubMed: 18832729]

54. Jacob C, Yang PC, Darmoul D, et al. Mast cell tryptase controls paracellular permeability of the intestine. Role of protease-activated receptor 2 and beta-arrestins. *J Biol Chem* 2005;280:31936–48. [PubMed: 16027150]
55. Butterworth MB, Zhang L, Liu X, et al. Modulation of the Epithelial Sodium Channel (ENaC) by Bacterial Metalloproteases and Protease Inhibitors. *PLOS ONE* 2014;9:e100313. [PubMed: 24963801]
56. Kerckhoffs AP, Ter Linde JJ, Akkermans LM, et al. Trypsinogen IV, serotonin transporter transcript levels and serotonin content are increased in small intestine of irritable bowel syndrome patients. *Neurogastroenterol Motil* 2008;20:900–7. [PubMed: 18363639]
57. Camilleri M, Carlson P, Acosta A, et al. RNA sequencing shows transcriptomic changes in rectosigmoid mucosa in patients with irritable bowel syndrome-diarrhea: a pilot case-control study. *Am J Physiol Gastrointest Liver Physiol* 2014;306:G1089–98. [PubMed: 24763552]
58. Danahay H, Withey L, Poll CT, et al. Protease-activated receptor-2-mediated inhibition of ion transport in human bronchial epithelial cells. *American Journal of Physiology-Cell Physiology* 2001;280:C1455–C1464. [PubMed: 11350741]
59. Buresi MC, Schleihauf E, Vergnolle N, et al. Protease-activated receptor-1 stimulates Ca²⁺-dependent Cl[–] secretion in human intestinal epithelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2001;281:G323–G332. [PubMed: 11447011]
60. Kunzelmann K, Schreiber R, König J, et al. Ion transport induced by proteinase-activated receptors (PAR2) in colon and airways. *Cell Biochemistry and Biophysics* 2002;36:209–214. [PubMed: 12139406]
61. Mall M, Gonska T, Thomas J, et al. Activation of ion secretion via proteinase-activated receptor-2 in human colon. *Am J Physiol Gastrointest Liver Physiol* 2002;282:G200–10. [PubMed: 11804840]
62. Ikehara O, Hayashi H, Waguri T, et al. Luminal trypsin induces enteric nerve-mediated anion secretion in the mouse cecum. *J Physiol Sci* 2014;64:119–28. [PubMed: 24421180]
63. Reed DE, Barajas-Lopez C, Cottrell G, et al. Mast cell tryptase and proteinase-activated receptor 2 induce hyperexcitability of guinea-pig submucosal neurons. *The Journal of physiology* 2003;547:531–542. [PubMed: 12562962]