HCO$_3^-$ secretion is not impaired in inflamed mouse anterior proximal colon

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Abstract

One of the characteristics of inflammatory bowel disease (IBD) is an impaired, diminished or absent mucus barrier. For mucus to be effective in restricting commensal bacteria to the intestinal lumen, it must be expanded or hydrated. Mucus is secreted in a compacted state, and bound by H⁺ and Ca²⁺ ions, which shield the protein core of mucus consisting of many negative charges. A proposed mechanism for mucus expansion, is that HCO₃⁻ is secreted along with mucus into the intestinal lumen and it binds Ca²⁺ and neutralizes H⁺ ions. However, a recent study revealed a reduction in expression of the NaHCO₃ cotransporter, NBCe1 in inflamed proximal colon. NBCe1 is thought to be involved in intestinal HCO₃⁻ secretion, therefore reduced secretion of HCO₃⁻ may be the cause of the impaired mucus barrier in IBD. To test this, we investigated whether HCO₃⁻ secretion was compromised in the proximal colon, specifically in the anterior proximal colon (APC), in an animal model of IBD, IL10⁻/⁻ mice infected with Helicobacter typhlonius.

By using the Ussing short circuit technique and pH stat technique, the electrogenic and electroneutral components of HCO₃⁻ secretion were measured. The results reveal that there was no appreciable electrogenic HCO₃⁻ secretion, and the majority of the electrogenic anion secretion, in the APC was a result of electrogenic Cl⁻ secretion. In addition, although, electroneutral HCO₃⁻ secretion was present in the APC, it was very small, it was not stimulated by forskolin, and it was unaffected by inflammation.

The results indicate that, little if any HCO₃⁻ secretion occurs in the anterior proximal colon, and that which does occur does not involve NBCe1. This suggests that the hydration of mucus in the proximal colon does not involve HCO₃⁻ and NBCe1 has some other role, possibly regulation of cell pH.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>*/-</td>
<td>Knockout mouse</td>
</tr>
<tr>
<td>CaCC</td>
<td>Ca(^{2+}) activated Cl(^{-}) channel</td>
</tr>
<tr>
<td>CaCO(_3)</td>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>CaHCO(_3)</td>
<td>Calcium bicarbonate</td>
</tr>
<tr>
<td>cAMP</td>
<td>3′-5′-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial Na(^{+}) channel</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>H(_2)CO(_3)</td>
<td>Carbonic acid</td>
</tr>
<tr>
<td>HCO(_3^{-})</td>
<td>Bicarbonate</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IECs</td>
<td>Intestinal epithelial cells</td>
</tr>
<tr>
<td>I(_{sc})</td>
<td>Short-circuit current</td>
</tr>
<tr>
<td>mM</td>
<td>Millimol.L(^{-1})</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milli litre</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>NaHPO(_4)</td>
<td>Na(^{+}) Phosphate</td>
</tr>
<tr>
<td>NBCe1</td>
<td>Electrogenic Na(^{+})-HCO(_3^{-}) exchanger 1</td>
</tr>
<tr>
<td>NHE1</td>
<td>Na(^{+})-H(^{+}) exchanger 1</td>
</tr>
<tr>
<td>NKCC1</td>
<td>Na(^{+}) -K(^{+})-Cl(^{-}) cotransporter</td>
</tr>
<tr>
<td>pH(_{i})</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>R(_{t})</td>
<td>Transepithelial resistance</td>
</tr>
</tbody>
</table>
Slc26a3  Solute carrier family 20 member 3
Slc26a6  Solute carrier family 20 member 6
TNF    Transepithelial resistance
TNFα  Tumour necrosis factor alpha
TTX    Tetrodotoxin
UC     Ulcerative colitis
VT    Transepithelial voltage
WT     Wild type
ΔIsc    Change in short circuit current
Ω.cm²    Ohms square cm of tissue
µA.cm⁻² Micro amperes per cm of tissue
1. Introduction

1.1 BACKGROUND

Inflammatory bowel disease (IBD) is a debilitating disease that limits the quality of life for the individuals that have it. IBD is a multi-factorial and idiopathic disease, which is characterized by uncontrolled and persistent inflammation of the gastrointestinal (GI) tract. It arises from an abnormal inflammatory response to the resident intestinal microbiota by their own mucosal immune system, and is therefore classed as an autoimmune disease (Abraham & Cho, 2009; Hyun & Mayer, 2006). There are many symptoms of IBD, such as abdominal pains and diarrhoea, where in extreme cases these symptoms can be very disruptive (Sullivan et al., 2009). The most common complication of IBD is anaemia, which is due to rectal bleeding (Stein et al., 2010). IBD patients also have an increased risk of colorectal cancer (Nieminen et al., 2013). The many aspects surrounding IBD are complex and cannot be explained by any single mechanism or agent, but there is an ongoing attempt at unveiling the many distinctive factors that ultimately gives rise to IBD.

Based on the region of effect and continuity of inflammation IBD is usually subdivided into two diseases – Crohn’s disease (CD) and ulcerative colitis (UC). CD usually affects the terminal ileum, caecum and colon but can affect the entire colon in a discontinuous pattern. These discontinuous regions of inflammation are often referred to as ‘skip lesions’. The widespread inflammation can be transmural and is often associated with granulomas, fistulas, and strictures (Bernick & Kane, 2011). In contrast, UC involves a diffuse inflammation affecting the rectum and colon in a continuous pattern (Abraham & Cho, 2009; Barkas et al., 2012). The colon is the main interest in these experiments, due to the association of damage between CD and UC.

Genetic predisposition is important to the development of IBD; however there are additional factors that are also responsible for the disease etiology. A two-hit hypothesis is used to describe the pathogenesis of IBD. The development of IBD has been associated with a genetic basis (the first hit), and is influenced by environmental risk factors (the second hit). Through genome-wide association studies, certain genes have been identified, such as NOD2 (Jess et al., 2005) that play an important role in increasing the susceptibility of an individual to IBD. Many genetic studies have been conducted, which have shown a relationship between certain genes to patients with UC and CD. There is now evidence of more than 163 genomic loci...
associated with IBD. Of those, 110 loci are common to both UC and CD, while only 30 are specific to CD and 23 to UC (Jostins et al., 2012). Effects of certain environmental factors, such as diet (Reif et al., 1997), pathogenic infections and antibiotics (Molodecky & Kaplan, 2010), play a part in triggering IBD. There are many environmental factors that contribute to the development of IBD; however, there are many causative factors, which are not well understood. What is known is that certain microbial agents, including normal or altered intestinal microflora (Fabia et al., 1993), either increase the risk or actually trigger inflammation, which ultimately leads to IBD. The role of environmental factors in the development of inflammation is supported by the many animal models developed to study IBD. One such animal, which is used in this study, is the interleukin 10 knockout (IL-10−/−) mouse. This mouse has a genetic susceptibility to develop colitis. Knockout of IL10 will leave the mouse immunocompromised, but under germ free conditions they do not develop inflammation. However, if they are exposed to bacteria, such as Helicobacter typhlonius, they will develop inflammation. This provides evidence to support a two-hit model, which needs to include both genetic susceptibility and environmental factor. The consequences of environmental and genetics factors that trigger IBD can range from an impaired intestinal barrier, inappropriate response of the mucosal immune system, to alterations in bacterial activity, and an increase infiltration of bacteria into intestinal epithelial cells (IECs).

Bacteria live in symbiosis with humans, where more than $10^{14}$ microorganisms of at least 1000 different species occupy just the Gastrointestinal (GI) tract; most of which are contained within the colon (Gill et al., 2006). Intestinal homeostasis is maintained by a protective physical barrier, the mucus and an immunological defence (Roda et al., 2010). The mucus barrier is vital for bacterial colonisation of the gut, while preventing these commensal bacteria from coming in contact with the IECs. There are two layers of mucus, an inner and outer layer. The outer layer of mucus provides both a habitat and a food supply for bacteria, while in turn, the bacteria aid in digestion and provide an energy source for the IECs (Deuring et al., 2013; Johansson et al., 2011b; Nugent et al., 2001; Hansson, 2012). However, if certain individuals are genetically susceptible, and inflammation is triggered by an environmental factor, this may cause disruption of the microbial flora or dysregulation of the immune system and ultimately lead to the development of IBD (Hansson, 2012). Therefore, the overall objective of this study is to determine the cause of the compromised mucus barrier in intestinal inflammation.
1.1.1 Restricting commensal bacteria to the intestinal lumen

There are several lines of defence that prevent microorganisms from contacting and passing through the epithelium. The primary defence is a physical barrier, the mucus, which prevents bacteria from contacting the IECs. The second level is the epithelial cells, which form a continuous sheet of cells by interconnecting tight junctions. Studies have observed impaired tight junctions in IBD; where tight junctions are regulators of paracellular permeability. Therefore, the impairment of tight junctions will affect the degree of exposure of the intestinal immune system to the commensal bacteria (Yu et al., 2012; Chichlowski & Hale, 2008). This defect can also cause leakage of absorbed fluid (Schmitz et al., 1999) and result in the most debilitating symptom of IBD, diarrhoea.

Following from the mucus barrier and epithelial barrier, a third level of defence exists and it consist of the innate immune cells, macrophages and dendritic cells. If all else fails, the adaptive immune system is then able to remove bacteria, acting as the fourth-line of defence (Hansson, 2012). However, a defective and inappropriately active immune system (possibly as a result of genetic alteration) can still lead to inflammation and possibly IBD. The main feature that defines IBD is an elevated infiltration of innate immune cells (macrophages, neutrophils, natural killer T cells and dendritic cells), which results in increased levels of TNF-α, IFN-γ, interleukin-1β and cytokines (Abraham & Cho, 2009). Also, associated with IBD is a reduction in anti-inflammatory cytokines, for example IL-10, which suppresses production of pro-inflammatory mediators, such as IFN-γ or IL-17A (Wilson et al., 2011).

Up to the point where bacteria can trigger the immune system to cause inflammation is damaging to the intestinal epithelial cells. Therefore, the function of the mucus barrier is vital for restricting commensal bacteria to the intestinal lumen and preventing any subsequent damage. In the colon, mucus is composed of two layers – an outer layer that contains oligosaccharides as a food supply and is accessible to bacteria; and an inner layer. The inner layer is 50-200 µm thick and has a protein composition similar to the outer layer, but is attached to the epithelium and densely packed. The outer layer is important for maintaining the symbiotic relationship between host and bacteria, while the inner layer physically prevents bacteria from passing through (Hansson, 2012; Johansson et al., 2008). Importance of maintaining a mucus barrier can be observed with the mouse model of inflammation, the Muc2 knockout (Muc2−/−) mouse. Where by knocking out Muc2, a component of mucus, impairs the protective capacities of the mucus layer, and leads to an inflammatory response (Lu et al., 2011).
1.2 Mucus

Goblet cells produce mucus and secrete it to the luminal side of the intestinal epithelium. The macromolecular constituents of mucus are mucin glycoproteins. The two classes of mucins include transmembrane mucins (MUC1, 3A, 3B, 4, 12, 13, 15, 16, 17 etc.), which are attached to the enterocytes on the apical surface, and gel-forming mucins (e.g. MUC2). MUC2 is the predominant mucin produced by goblet cells in the intestine (Johansson et al., 2011a; Sheng et al., 2012). MUC2 mucin encodes for the protein that makes up the skeleton of both mucus layers. Mucins are a family of glycosylated protein in which 80% of the mass is glycans (Karlsson et al., 1997). These O-glycans serve as an energy source for commensal bacteria, and also provide mucins with a high water binding capacity (Kim & Ho, 2010).

1.2.1 Mucus Secretion

Mucins are secreted in a compact state, within mucin granules. The mucin protein core contains numerous negative charges, which will cause repulsion due to the electrostatic forces. However, in the granule, positively charged Ca\(^{2+}\) ions and H\(^+\) ‘shield’ the negative charges on the mucin protein core (Fig. 1.1) (Verdugo et al., 1987). On secretion, hydration and expansion of the mucus layer is important for maintaining the barrier against commensal bacteria. Recent evidence from Yang et al., (2013) suggest that expansion and dispersion of mucin requires HCO\(_3^-\) secretion, which was suggested, that in the small intestine, to be CFTR-dependent HCO\(_3^-\) secretion. Although, different intestinal segments contain different transporters, such as Cl\(^-\)/HCO\(_3^-\) exchangers and short-chain fatty acid (SCFA)/HCO\(_3^-\) exchanger (Vidyasagar et al., 2005) (CFTR and Cl\(^-\)/HCO\(_3^-\) exchanger are illustrated in Fig. 1.2) that are involved in secretion of HCO\(_3^-\). All of these transporters may contribute to the secretion of HCO\(_3^-\) for expansion of the mucus. A proposed mechanism by which HCO\(_3^-\) is required for expansion of the mucus, is by binding Ca\(^{2+}\) or neutralizing H\(^+\) to produce CaCO\(_3\), CaHCO\(_3\) and H\(_2\)CO\(_2\) (Fig. 1.1A) (Quinton, 2010b). Removing the Ca\(^{2+}\) and H\(^+\) shielding from the mucin protein core, will allow repulsion from the electrostatic forces of the negative charges, thus allowing the expansion of mucus (Quinton, 2010a). Therefore, if this was the case, then HCO\(_3^-\) is essential in aiding the expansion of mucus (e.g., Chen et al., 2010; Garcia et al., 2009). Fluid secretion can be driven by HCO\(_3^-\) secretion, but Cl\(^-\) secretion can also drive water secretion, to contribute to hydration and expansion of mucus (Geibel, 2005).

In the case of colonic epithelial diseases, the mucus layer is either incorrectly structured, diminished or absent. This could be due to impaired hydration and expansion of mucus as a
result of reduced bicarbonate secretion (Chen et al., 2010; Garcia et al., 2009). If \( \text{HCO}_3^- \) secretion is impaired, \( \text{Ca}^{2+} \) will remain with the mucin, which will prevent the repulsive electrostatic forces of the negative ions and expansion of the mucus layer (Fig. 1.1B) (Chen et al., 2010; Quinton, 2010a). The subsequent disruption of the mucus layer means the physical barrier preventing commensal bacteria from contacting the IECs will be defective and that will allow for opportunistic microorganisms to invade and trigger an immune response (Sheng et al., 2012). If the immune system is activated for extended periods, the chronic inflammation that results could ultimately lead to IBD.

Supporting the notion that bicarbonate secretion is affected by IBD, the intraluminal pH of the gastrointestinal tract differs between IBD patients and healthy patients. The pH of the colonic contents of UC patients has been observed to be reduced, as a result of reduced bicarbonate secretion (Raimundo et al., 1992). Although, there are often differences in the patients diet and the severity of their colitis, that can lead to differences in pH (Ewe et al., 1999). Reduction in pH can also be attributed to impaired absorption of SCFA (Chapman et al., 1994), or elevated concentrations of luminal lactic acid (Hove & Mortensen, 1995). However, evidence of reduced faecal bicarbonate in UC patients (Roediger et al., 1984) suggest a correlation to reduced secretion of bicarbonate and possibly ties in with an altered mucus barrier.
The secretion of mucin coupled with HCO$_3^-$ allows for mucus expansion by removing the Ca$^{2+}$ and H$^+$ ‘shielding’. (B) Cystic fibrosis (CF), where there is no HCO$_3^-$ secretion results in impaired mucus expansion (redrawn from Quinton, 2010a)

Figure 1.1 Model of mucus expansion
1.3 Anion Secretion

Not just the secretion of mucus, but the secretion of many ions are important for regulating the luminal environment. Secretion of anions, such as Cl\(^{-}\) and HCO\(_3\)\(^{-}\), occurs in the oesophagus and throughout the intestine (Boron & Boulpaep, 2005). The secretion of Cl\(^{-}\) is accompanied by paracellular transport of Na\(^{+}\) to the lumen, where the accumulation of NaCl provides an osmotic gradient for water movement (Venkatasubramanian et al., 2010). Fluid secretion provides an aqueous medium for digestive enzymes to diffuse to their substrates and allow water soluble products of digestion to diffuse to the IECs for absorption (Barrett & Keely, 2000). HCO\(_3\)\(^{-}\) is also able to drive water secretion, however, HCO\(_3\)\(^{-}\) alkalinises the luminal pH as well (Sjoblom 2011). This is important, because fermentation by bacteria in the colon produces short chain fatty acids, which reduce the pH (Louis et al., 2007). In order for efficient fermentation to occur, it is thought that HCO\(_3\)\(^{-}\) is secreted to maintain a pH of about 4-5 (McConnell et al., 2008). Other functions of the secreted HCO\(_3\)\(^{-}\) besides maintaining pH and mucus expansion, is that bicarbonate secretion is important for wound healing (Feil et al., 1989) and electrolyte reabsorption (Binder et al., 2005).

1.4 Colonic Epithelium

The colonic epithelium is divided into surface cells and crypt cells (Fig. 1.2). Secretion of Cl\(^{-}\) can occur along the surface and crypt cells, through cystic fibrosis transmembrane conductance regulator (CFTR) (Strong et al., 1994). Secretion of HCO\(_3\)\(^{-}\) differs between these two regions – crypt cells secrete HCO\(_3\)\(^{-}\) electrogenerically whilst surface cells secrete HCO\(_3\)\(^{-}\} electroneutrally (Vidyasagar et al., 2004). Electrogenic ion transport generates a potential difference or transepithelial voltage (V\(_{te}\)) (Lewis, 1996), which is the basis of the Ussing short circuit technique, used to measure electrogenic anion secretion. Whereas, electroneutral ion transport does not generate a potential difference.

1.5 Cl\(^{-}\) Secretion

The secretion of Cl\(^{-}\) occurs electrogenically via CFTR, but there is also evidence of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCC) on the apical membrane (Frizzell & Morris, 1994). The accumulation of Cl\(^{-}\), comes from basolateral uptake, through NKCC1 (Grubb et al., 2000) and Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchangers (Gawenis et al., 2010; Ikuma et al., 2003). The basolateral Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchanger is expressed in the proximal colon and exchanges accumulated HCO\(_3\)\(^{-}\} for Cl\(^{-}\} entry into the cell.
1.6 Bicarbonate secretion

The transporters associated with $\text{HCO}_3^-$ secretion include CFTR, $\text{Cl}^-/\text{HCO}_3^-$ exchangers (slc26a3) on the apical membrane, and Na-$\text{HCO}_3$ cotransporters (NBCe1/NBCn1), NHE1, $\text{K}^+$ channels (KCNQ1/KCNN4) on the basolateral membrane.

The transporters that secrete $\text{HCO}_3^-$ out of the colonic epithelial cells are CFTR and slc26a3. Crypt cells secrete $\text{HCO}_3^-$ in a $\text{Cl}^-$-independent, cAMP-induced manner. $\text{HCO}_3^-$ is therefore secreted electrogenically by CFTR. CFTR is better known for the secretion of $\text{Cl}^-$, which mainly contributes to fluid secretion and mucosal hydration (Venkatasubramanian et al., 2010); however, CFTR is also involved in $\text{HCO}_3^-$ secretion (Seidler et al., 1997). The permeability of CFTR to $\text{HCO}_3^-$ is only about 25% of its permeability to $\text{Cl}^-$ (Kim & Steward, 2009). However, under certain conditions, where the intracellular $\text{Cl}^-$ concentration is low, or $\text{HCO}_3^-$ concentration is high, this may modulate the permeability of CFTR for $\text{HCO}_3^-$ secretion (Shcheynikov et al., 2004). Although, this is still uncertain, as other studies have shown the permeability ratio does not change in low $\text{Cl}^-$ or high $\text{HCO}_3^-$ concentrations (Tang et al., 2009). In cases where $\text{Cl}^-$ is accumulated above equilibrium, so that CFTR is more likely to secrete $\text{Cl}^-$ than $\text{HCO}_3^-$, it may be possible that CFTR operates in parallel with a $\text{Cl}^-/\text{HCO}_3^-$ exchanger (Gray et al., 1993). So, CFTR could supply $\text{Cl}^-$ in the lumen, which can be utilised by the $\text{Cl}^-/\text{HCO}_3^-$ exchanger to take up $\text{Cl}^-$ for $\text{HCO}_3^-$ secretion. There are intracellular pathways, such as the cAMP-dependent pathway, which stimulate secretion of $\text{HCO}_3^-$.

$c\text{AMP}$ stimulates NBCe1 to increase $\text{HCO}_3^-$ uptake (Bachmann et al., 2003), and also increases open probability of CFTR (Liu et al., 2001), thereby increasing the $\text{HCO}_3^-$ conductance across the apical membrane. $c\text{AMP}$ also activates KCNQ1, which provides efflux of $\text{K}^+$ to maintain an electrochemical gradient for $\text{Cl}^-$ and $\text{HCO}_3^-$ secretion by hyperpolarising the cell membrane (Smith & Welsh, 1992). The $\text{Na}^+/-\text{K}^+\text{-ATPase}$ utilizes ATP to drive the transport of ions, by transporting $\text{Na}^+$ out of the cell (accumulated by NBCs and NHEs) in exchange for $\text{K}^+$ entering the cell, which is then recycled via $\text{K}^+$ channels (KCNQ1/KCNN4). Following secretion of an anion ($\text{HCO}_3^-$), a driving force is generated for the movement of a cation ($\text{Na}^+$) and an osmotic gradient for water along the paracellular pathway. This allows for expansion and hydration of the mucus.

In comparison, slc26a3 is found on the apical membrane in colon surface cells and due to the coupling of $\text{HCO}_3^-$ secretion with $\text{Cl}^-$ absorption, with a stoichiometry of 1:1, $\text{HCO}_3^-$ secretion is electroneutral. Slc26a6 on the other hand, is an electrogenic $\text{HCO}_3^-$ transporter (1 $\text{HCO}_3^-$/2 $\text{Cl}^-$).
Cl\(^-\)), however it is exclusively expressed in the small intestine rather than the colon (Schweinfest et al., 2006).

The model focuses primarily on the difference between apical HCO\(_3^-\) transporters in surface and crypt cells. NaK2Cl\(^-\) cotransporter and NHEs are excluded for the reason of space. The slc26a3 is expressed in the surface cells and secrete HCO\(_3^-\) electroneutrally, whereas CFTR is primarily expressed in the crypts cells, which is permeable to HCO\(_3^-\) for electrogenic HCO\(_3^-\) secretion.

Figure 1.2 Simplified cellular model of HCO\(_3^-\) secretion in the colon

The model focuses primarily on the difference between apical HCO\(_3^-\) transporters in surface and crypt cells. NaK2Cl\(^-\) cotransporter and NHEs are excluded for the reason of space. The slc26a3 is expressed in the surface cells and secrete HCO\(_3^-\) electroneutrally, whereas CFTR is primarily expressed in the crypts cells, which is permeable to HCO\(_3^-\) for electrogenic HCO\(_3^-\) secretion.
1.3.2 Bicarbonate Influx

$\text{HCO}_3^- \text{ accumulates within both crypt and surface cells of the colon by one of two ways.}$

Carbonic anhydrase (CA) catalyses the hydrolysis of $\text{CO}_2^-$ to $\text{HCO}_3^-$ (Fig. 2) (Hug et al., 2003; Vidyasagar et al., 2004):

$$\text{H}_2\text{O} + \text{CO}_2 \xleftrightarrow{\text{CA}} \text{H}_2\text{CO}_3 \xleftrightarrow{} \text{H}^+ + \text{HCO}_3^-$$

There are several isoforms of CA expressed in the colon, some of which are expressed as cytosolic isozymes and others as membrane-associated CA isozymes (Pan et al., 2007). Geibel et al., (2000), observed that endogenously generated $\text{HCO}_3^-$ has a minimal role in secretion of $\text{HCO}_3^-$ across the apical membrane in colonic crypt cells. This suggests that the endogenously generated $\text{HCO}_3^-$ in crypt cells might be utilised by other processes; which could possibly include the basolateral $\text{Cl}^-$/\text{HCO}_3^- exchanger, to aid in $\text{Cl}^-$ transport (Gawenis et al., 2010). The focus of this study was to determine if the mucus barrier is impaired and this was suggested to be related to $\text{HCO}_3^-$ secretion. If the generation of $\text{HCO}_3^-$ endogenously is not involved with $\text{HCO}_3^-$ secretion then the impairment of CA in crypt cells is unlikely to be, or to a lesser extent, involved with a reduced $\text{HCO}_3^-$ secretion associated with inflammation.

Therefore, it is more likely that other $\text{HCO}_3^-$ transporters are impaired in inflamed tissue, such as NBCe1.

Besides endogenous production of $\text{HCO}_3^-$, Na-$\text{HCO}_3$ cotransporters transport $\text{HCO}_3^-$ into the colonic epithelial cells. Vidyasagar et al., (2004) demonstrated that $\text{HCO}_3^-$ is required in the serosal bath solution for secretion of $\text{HCO}_3^-$ across the apical membrane, which indicates the importance of $\text{HCO}_3^-$ uptake across the basolateral membrane. Although, the study did not exclude the possibility that $\text{CO}_2$ could play a part in $\text{HCO}_3^-$ secretion, considering the Ringer’s solution that contains $\text{HCO}_3^-$ is usually gassed with 95% $\text{O}_2$ and 5% $\text{CO}_2$, whereas $\text{HCO}_3^-$ free Ringer’s is gassed with 100% $\text{O}_2$. Therefore by bathing the basolateral membrane in $\text{HCO}_3^-$ free Ringer’s, the absence of $\text{CO}_2$ could have been responsible for reduced $\text{HCO}_3^-$ secretion, while $\text{HCO}_3^-$ Ringer’s in the presence of $\text{CO}_2^-$ may contribute to $\text{HCO}_3^-$ secretion. NBCs are classified as electrogenic (NBCe1), which transports $\text{HCO}_3^-$ and $\text{Na}^+$ with a stoichiometry of 2:1; and electroneutral (NBCn1), which transports $\text{Na}^+$ and $\text{HCO}_3^-$ with a stoichiometry of 1:1, into the cell. It is suggested that NBCe1 is expressed in colonic crypt cells at higher levels than NBCn1, however both transporters are found within the two different areas of the colonic cells (Yu et al., 2009). The area of expression may be a result of difference in function between the two NBCs. A recent study by Barmeyer et al., (2013), suggests that NBCn1 in epithelial cells
of the proximal colon regulates intercellular pH (pH_i) to maintain pH_i homeostasis, whereas NBCe1 mediates transcellular transport of HCO_3^−. Given the function of NBCn1 is for regulation of pH_i, then it may be possible that NBCe1, which mediates HCO_3^− secretion, is impaired in inflammation.

A previous study in our lab (Fig. 1.3, Fan and Butt, unpublished data) on the expression of NBCe1 provides support that NBCe1 may be affected by inflammation. Inflammation was induced by infecting IL-10−/− mice with Helicobacter typhlonius and used to investigate the effect of inflammation on the expression of the bicarbonate transporter, NBCe1. In this study it was observed the expression of NBCe1 was reduced in the inflamed proximal colon of this inflammatory mouse model. This study provides evidence that NBCe1 might be related to the reduced HCO_3^− secretion in inflamed tissue, as to whether this is involved with electrogenic or electroneutral HCO_3^− secretion has yet to be determined.

The current understanding of how HCO_3^− secretion is implicated in inflammatory bowel disease is still limited. However, there is increasing evidence that certain HCO_3^− transporters are affected by IBD. There are ongoing studies looking for these defective transporters, such as a reduction in the expression of DRA (slc26a3) protein and mRNA was observed (Yang et al., 1998). Whereas other studies, such as Xiao et al (2012b), which examined the effects on CFTR, found no differences in expression between inflamed and non-inflamed mice.
Figure 1.3 Expression of the NaHCO3 cotransporter

The expression of the NaHCO3 cotransporter NBCe1 is reduced in the proximal colon of IL10−/− mice infected with H. typhlonius. (A) A representative immunoblot showing reduced levels of NBCe1 expression in the proximal colon of IL10−/− animals infected with H. typhlonius. (B) Mean levels of expression of NBCe1 in the proximal colon of WT control and infected mice and IL10−/− infected and control mice. All values X ± sem., n = 4 mice for each treatment. P<0.01 (Fan & Butt, unpublished results)
1.4 Model of IBD

Many animal models have been developed to investigate the cause and effect of IBD. Unfortunately, given our current understanding of IBD and all the environmental, genetics and immunological aspects uncovered, there are currently no available models that truly represent all features of human IBD, although, there are models that have been developed, which have given great insight into the pathology of IBD (Wirtz & Neurath, 2007). In this study, the animal used as a model for inflammation is the IL10−/− mouse infected with *H. typhlonius*.

There are numerous gene knockout IBD models, many of which are involved in suppression of the proinflammatory cytokines (e.g. IL-2−/−, IL-10−/−, STAT-3−/−). GI immune homeostasis is maintained by appropriate proinflammatory mechanisms, along with anti-inflammatory mechanisms. Animal models that exhibit Th1 immune responses are commonly used for the study of CD, whereas Th2-mediated colitis triggers UC (Mizoguchi & Mizoguchi, 2010). For CD, there are pro-inflammatory mediators such as interferon (IFN)-γ or interleukin (IL)-17A, which triggers activation of the Th1 immune response. However, there are several mechanisms that are able to suppress the production of these pro-inflammatory mediators. The key suppressor is the cytokine IL-10, which is well known to suppress IFN or IL-17A (Wilson *et al.*, 2011). However, the knockout of this gene does not necessarily mean the spontaneous development of inflammation, as shown by IL-10−/− mice in germ-free conditions (Balish & Warner, 2002). Instead these mice are more susceptible to develop colitis in response to infectious agents (Wilson *et al.*, 2011). *H. typhlonius* is an example of an infectious agent, which induces colitis in these IL-10−/− mice (Chichlowski *et al.*, 2008).

Members of the *Helicobacter* species are now routinely used to evoke inflammation in immunocompromised hosts and although more research is required to explicitly identify the role of *Helicobacter* in the initiation of IBD in humans, the molecular evidence that exist (e.g. Hansen *et al.*, 2011) provides correlation between various non-pylori *Helicobacter* species (e.g. *H. canadensis, H. canis, H. cinaedi, H. fennelliae, H. pullorum, H. winghamensis*) with human IBD

The IL10−/− mice infected with *H. typhlonius* are frequently used as an animal model of inflammation, and the morphological and histopathological outcomes have been previously assessed (Fig 1.4C, D, E). Inflammation in this study was confirmed through visual appearances of the tissue, where the mucosal layer of the colon was noticeably thicker (in accordance with
a histological study by Dommels et al, 2007) and the caecum was smaller compared with the non-inflamed mice (Fig. 1.4A, B).
Figure 1.4 Comparison of the colon of IL10−/− clean mice with IL10−/− mice infected with H. typhlonius

The gross morphology of the colon and caecum between (A) IL10+/− clean mice and (B) IL10+/− mice infected with H. typhlonius. The morphology of (C) the IL10+/− clean mice proximal colon and (D) IL10+/− clean mice infected with H. typhlonius, under the microscope. (E) Scoring for histological inflammation was developed by adapting and modifying rodent and IL10−/− colitis scores found in literature (Lindstrom & Butt, unpublished observations). ***P<0.001, Kruskal Wallis with Dunn’s post test.
1.5 Hypotheses and Aim

The mucus barrier is a vital barrier, which restricts commensal bacteria to the intestinal lumen. Quinton’s (2010a) proposed a mucus expansion model, where bicarbonate is essential in aiding the process of mucus expansion. However, in IBD, the mucus barrier is compromised. This could be due to compromised hydration as a result of impaired $\text{HCO}_3^-$ secretion, as the evidence of reduced NBCe1 expression suggests.

Here we are investigating whether $\text{HCO}_3^-$ secretion is compromised in an inflammatory model. Specifically, to determine if electrogenic $\text{HCO}_3^-$ secretion is compromised by using the Ussing short circuit ($I_{sc}$) technique, or if electroneutral $\text{HCO}_3^-$ secretion is compromised by using the $pH$ stat technique, as a result of the reduced expression of NBCe1.

We predict that $\text{HCO}_3^-$ secretion from specifically the anterior proximal colon will be significantly reduced in the IL10$^{-/-}$ mice infected with H. typhlonius and this will provide evidence towards mucus barrier impairment in inflamed tissue.
2. Methods

2.1 Animal Model

Male and female homozygous IL10\(^{-/-}\) (IL10\(^{-/-}\) 129S(B6)-Il10\(^{tm1Cgn}\)/J) mice were initially sourced from Jackson Laboratories (Bar Harbor, Maine, USA) and maintained at the University of Otago Hecus Taieri Resource Unit (HTRU) animal facility. They were kept under specific pathogen-free (SPF) conditions and housed in sterilised filter-top micro-isolator cages on corncob bedding (The Andersons Inc., Maumee, Ohio, USA) and shredded paper. Mice had free access to standard rodent chow pellets (Specialty Feeds Pty Ltd., Glen Forest, Western Australia, Australia) and filter-sterilised or autoclaved tap water. The experiments were performed in accordance to the Animal Welfare Act, 1999, ethics number 8/11 and approved by the University of Otago Animal Ethics Committee.

Experiments were performed using either IL10\(^{-/-}\) mice as control (non-inflamed) animals, or IL10\(^{-/-}\) mice infected with \textit{H. typhlonius} as the animal model for inflammation (Wilson et al., 2011). The mice were bred at the Hercus Taieri breeding facility, in which IL10\(^{-/-}\) mice were kept separately from the infected mice, where the infection of \textit{H. typhlonius} is able to spread by using the same bedding of previously infected IL10\(^{-/-}\) mice.
2.2 DISSECTION AND TISSUE PREPARATION

The mice were killed by cervical dislocation, and death was confirmed by feeling for separation in the cervical vertebrae. A small incision was made in the centre of the lower abdomen and extended upwards on both sides of the abdominal wall. The peritoneum was cut away in the same fashion, the entire colon exposed and removed and placed in ice-cold NaCl/HCO₃⁻ Ringer’s.

The proximal colon (Fig 2.2A), which was defined as the section of the colon extending from the junction of the caecum and colon to 1 cm below the striped tissue, was divided into two segments, The anterior proximal colon (APC) and the distal proximal colon (DPC) (Fig 2.2I). The APC was clearly defined by the striped appearance, whereas the DPC was the remaining, non-striped tissue. The faecal contents were flushed out with ice-cold NaCl/HCO₃⁻ Ringer’s, and the remainder of the dissection was done with the tissue bathed in ice-cold NaCl/HCO₃⁻ Ringer’s. The proximal colon was cut open along the mesentery, with the serosal side facing upwards (Fig 2.2B, C). The muscle layer was then be stripped away as described by Clarke (2009). The muscle layer was scored horizontally with a scalpel, and stripped off with forceps (Fig 2.2D). On the serosal side, 0.125cm² plastic rings were glued on with Loctite-454 instant adhesive (Loctite Australia Pty Ltd., Caringbah, NSW, Australia) (Fig 2.2E). The rings were cut out (Fig 2.2F) and mounted in the Ussing chamber (Fig 2.2G, H). For the Ussing chamber experiments, paired tissues from both the APC and DPC were mounted in the chambers for each experiment. Whereas, for the pH stat experiments only the APC were examined, therefore only paired tissue from the APC were mounted in each setting. Each experiment consisted of either four Ussing chambers for Iₛₑ measurements or two pH stat chambers being set up.
**Figure 2.1 Dissection and tissue preparation for the Ussing chamber and pH stat**

A) Proximal Colon separated from the rest of the GI tract; (B) Tissue was cut longitudinally down the mesentery; (C) Tissue was pinned out and a scalpel was used to score a horizontal line across one end of the tissue; (D) Seromuscular layer was removed with forceps; (E) Plastic rings were glued onto the tissue (F) and were cut out; (G) Rings with the tissue are slotted onto the adapter of the Ussing chamber; (H) Complete tissue preparation for mounting into Ussing chamber; (I) The division of the proximal colon into anterior proximal colon (APC) and distal proximal colon (DPC)
2.3 Measurement of Epithelial Transport

For measuring the ion transport by the mice colon, the Ussing short circuit current technique was used for the electrogenic ion transport, while this was combined with the pH-stat technique to measure electroneutral ion transport. The Ussing chamber (Fig 2.2) containing the tissue was mounted between two 10 mL reservoirs, which contained Ringer’s solution to bathe the mucosal and serosal sides of the tissue separately. Different Ringer’s solutions were used to fill the reservoirs depending on the experiment conducted (Table 2.1). The NaCl/HCO₃⁻ Ringer’s and HCO₃⁻ buffered/Cl⁻ free Ringer’s were gassed with 95% O₂ and 5% CO₂ gas mixture. This carbogen mixture maintained a PO₂ of approximately 400mmHg and PCO₂, to preserve a physiological pH of approximately 7.4 (Clarke, 2009). The HCO₃⁻ free/NaCl Ringer’s and buffer-free Ringer’s were gassed with 100% O₂. The reservoirs were surrounded by a water jacket, which maintained the Ringer’s temperature at 37°C. To prevent net hydrostatic pressure gradient from influencing ion transport, the Ringer’s in each reservoir were kept at the same level. The leads passing out from in front of the chamber contained 3% agar with 150mM NaCl and were used to measure the transepithelial voltage (Vᵢₑ), while the leads at the back contain 3% agar with 3M KCl and were used to pass the Iᵢₑ through the tissue. Tissues were short-circuited with a VCC MC2 Dual channel voltage/current clamp (Physiologic Instruments).
Figure 2.2 Ussing chamber

The Ussing chamber contains two 10 mL reservoirs (blue), which are filled with Ringer’s solution. Where the two reservoirs meet at the bottom, is where the epithelium is mounted. The Ringer’s is gassed in the outer sides of the reservoir and drives the movement of the solution in the direction indicated by the arrows. A water jacket (red) surrounds the reservoirs and maintains the Ringer’s temperature at 37°C. The agar leads in the front (green) measures transepithelial voltage ($V_{te}$), whereas agar leads at the back measures the short circuit current ($I_{sc}$).
For the pH stat, the titration manager (Titralab) was used to maintain the pH by administering 5mM HCl or HNO\textsubscript{3} via a burette to maintain the pH at 7.4, and an electrode was used to measure the pH, to determine the amount of acid required for titration (Fig 2.3).

In the case of HCO\textsubscript{3}\textsuperscript{-} measurement, the pH stat technique can be used for measuring electroneutral HCO\textsubscript{3}\textsuperscript{-} secretion. The pH stat is used specifically to measure electroneutral H\textsuperscript{+} or HCO\textsubscript{3}\textsuperscript{-} flux across an epithelium. A pH stat electrode is placed within the mucosal bathing solution, and is able to measure the pH. The electrode then feeds the measurement to a pH stat titrator. The titrator is programmed to maintain a pH at 7.4. Connected to the titrator is a bottle of acid (e.g. HNO\textsubscript{3} or HCl) that is also attached to a burette. The burette is placed within the mucosal reservoir. If the electrode measurement of the mucosal solution exceeds pH of 7.4, the titrator will titrate acid into the mucosal solution to reduce the pH back to 7.4. The measure of secretory HCO\textsubscript{3}\textsuperscript{-} flux \(J_{\text{HCO}_3}\text{sm}\) is the amount of corresponding acid required to maintain the mucosal pH at 7.4.

For the use of the pH stat titrator, the mucosal bathing solution contained buffer- and HCO\textsubscript{3}\textsuperscript{-}-free Ringer’s, whereas the serosal bathing solution contained NaCl/HCO\textsubscript{3}\textsuperscript{-} Ringer’s. The mucosal reservoir was gassed with 100% O\textsubscript{2}, whereas the serosal reservoir was gassed with 95% O\textsubscript{2} and 5% CO\textsubscript{2}, and all drugs were administered on the serosal reservoir so as to not alter the pH of the mucosal solution, which was maintained at 7.4. The measure of HCO\textsubscript{3}\textsuperscript{-} flux was \(\mu\text{M/cm}^2/\text{h}\).
Figure 2.3 The pH stat in combination with the Ussing chamber

The pH stat includes the Ussing chamber, with addition of an electrode, and burette, which is placed at the top of the mucosal reservoir of the Ussing chamber. The electrode is connected to the titration manager. The burette is also connected to the titration manager, but is also connected to a bottle of acid (e.g. HCl or HNO$_3$), which allows titration of acid to the mucosal chamber. The reservoirs were filled with 10 mL Ringer’s and a water jacket kept the temperature set at 37°C. The Ussing clamp setup also is the same, where the agar leads in the front measures transepithelial voltage ($V_{te}$), whereas agar leads at the back measure the short circuit current ($I_{sc}$).
2.4 Ringer's Solution and Pharmacological Agents

The Ringer’s solution’s employed in the Ussing chamber and pH stat experiments are summarized in Table 2.1. All reagents were purchased from Sigma Aldrich Corp., (St Louis, MO, USA). Drugs used for the experiments are summarized in Table 2.2. Concentrated stocks of these drugs were made. The solvents that the drugs were made up in are also summarized on Table 2.2. These drugs were then added as small aliquots of stock solutions to the appropriate side of the tissues. The concentrations listed in Table 2.2 are the final bath concentrations of the drug administered. Control experiments (Fan & Butt, unpublished data) demonstrated that equivalent volumes of the vehicle had no effect. For the use of acetazolamide, it was dissolved in Tris solution and made up to pH 7.4 with Hepes, and this solution was used to prepare a HCO$_3^-$ free/NaCl Ringer’s containing 1 mmol l$^{-1}$ acetazolamide. 4,4’-Diisothiocyanato-2,2’-stibenedisulfonic acid (DIDs) was prepared as a 10 mmol l$^{-1}$ stock in Ringer’s corresponding to the Ringer’s used in the experiment, and this was used to replace the appropriate volume of Ringer’s in the reservoirs, to give a final concentration of 0.5 mmol l$^{-1}$.

### Table 2.1 Ussing chamber and pH stat Ringer’s solution composition (in mmol l$^{-1}$)

<table>
<thead>
<tr>
<th></th>
<th>NaCl/HCO$_3^-$</th>
<th>HCO$_3^-$ free/NaCl</th>
<th>HCO$_3^-$ buffered/Cl$^-$ free</th>
<th>Buffer free</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>120</td>
<td>140</td>
<td>0</td>
<td>140</td>
</tr>
<tr>
<td>Na-Gluconate</td>
<td>0</td>
<td>0</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>K-Gluconate</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>1.25</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Ca-Gluconate</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>(NaH)PO$_4$</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Na-pyruvate</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>25</td>
<td>0</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Hepes/Tris</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gas</td>
<td>95% O$_2$ : 5% CO$_2$</td>
<td>100% O$_2$</td>
<td>95% O$_2$ : 5% CO$_2$</td>
<td>100% O$_2$</td>
</tr>
</tbody>
</table>
Table 2.2 A list of pharmacological agents used in the Ussing chamber and pH stat experiments, their respective concentrations, solvent, mucosal or serosal side of chamber the drug was administered to, and the known effects of the drug on target proteins.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Solvent</th>
<th>Reservoir Added to</th>
<th>Target Protein</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin</td>
<td>1 μM/L</td>
<td>Ethanol</td>
<td>Mucosal &amp; Serosal</td>
<td>COX-2</td>
<td>Inhibits prostaglandin production</td>
</tr>
<tr>
<td>TTX</td>
<td>1 μM/L</td>
<td>Milli Q H₂O</td>
<td>Serosal</td>
<td>Naᵥ</td>
<td>Inhibit neural activity in GIT</td>
</tr>
<tr>
<td>Amiloride</td>
<td>100 μM/L</td>
<td>Milli Q H₂O</td>
<td>Mucosal</td>
<td>ENaC</td>
<td>Inhibits electrogenic Na⁺ channel</td>
</tr>
<tr>
<td>Forskolin</td>
<td>20 μM/L</td>
<td>DMSO</td>
<td>Mucosal &amp; Serosal</td>
<td>AC</td>
<td>Stimulates production of cAMP</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>10 μM/L</td>
<td>Ethanol</td>
<td>Serosal</td>
<td>NKCC1</td>
<td>Inhibits Cl⁻ uptake via Na⁺/K⁺/Cl⁻ transporter</td>
</tr>
<tr>
<td>DIDs</td>
<td>0.5 mM/L</td>
<td>*Ringers</td>
<td>Serosal</td>
<td>NBCe1 &amp; Cl⁻/HCO₃⁻ exchanger</td>
<td>Inhibits HCO₃⁻ uptake &amp; Cl⁻ uptake</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>1 mM/L</td>
<td>HCO₃⁻ free/NaCl Ringers</td>
<td>Mucosal &amp; Serosal</td>
<td>CA</td>
<td>Inhibits endogenous conversion of CO₂ to HCO₃⁻</td>
</tr>
</tbody>
</table>

COX-2, cyclooxygenase 2; Naᵥ, neural current; ENaC, epithelial sodium channel; AC, adenylate cyclase; NKCC1, sodium/potassium/chloride cotransporter; NBCe1, electrogenic sodium/bicarbonate exchanger; Cl⁻/HCO₃⁻ exchanger, chloride/bicarbonate exchanger; CA, carbonic anhydrase. *The type of Ringer’s corresponds to the Ringer’s used in the experiment.
2.5 Tissue Treatment and Data Analysis

After mounting the tissues into the Ussing chambers, basal steady-state of secretion was established by treating the tissue with a series of drugs to inhibit any spontaneous stimulation of secretion (Fig. 3.1A). The spontaneous production of PGE$_2$ can result from damage of the tissue during the dissection, which can lead to increase in secretion (Smith et al., 1982), therefore 1 µM indomethacin was administered to the mucosal and serosal reservoirs of the Ussing chamber to inhibit spontaneous PGE$_2$ production. Disruption of the neurons within the intestinal plexi can also stimulate spontaneous secretion, therefore 1 µM TTX was added to the serosal chamber after indomethacin to inhibit enteric nervous activity (Grubb, 1997). Following this 100 µM amiloride was added to the mucosal chamber to inhibit electrogenic Na$^+$ absorption via ENaC (Bridges et al., 1989; Kato & Romero, 2011).

Once a steady-state of secretion had been reached, after pre-treatment with amiloride, 20 µM forskolin was added to both mucosal and serosal chambers to stimulate anion secretion. Forskolin stimulates anion secretion through stimulating cAMP production (Seider et al., 1997), and in this case, will stimulate the transporter CFTR to secrete Cl$^-$ and/or HCO$_3^-$ . When the $I_{sc}$ response to forskolin had reached a steady-state secretion, 10 µM bumetanide was added to the serosal chamber to inhibit NKCC1 and Cl$^-$ uptake (Isenring & Forbush, 1997). This was followed by 0.5 mM DIDs, after $I_{sc}$ response to bumetanide had reached a steady-state, which inhibits both NBCe1 and basolateral Cl$^-$ /HCO$_3^-$ exchanger (Isenberg et al., 1993). Due to the transient increase in $I_{sc}$ after the administration of DIDs (Brayden et al., 1993), more than one hour was allowed for the inhibitory effect of DIDs on $I_{sc}$ to reach a steady state before the measurement was taken.

Each $I_{sc}$ measurement was taken as the steady state change in $I_{sc}$ before and after forskolin addition ($\Delta I_{sc}$ Forskolin, Fig 2.4). NaK2Cl contranport dependent electrogenic Cl$^-$ secretion was quantified as the bumetanide sensitive $I_{sc}$ ($\Delta I_{sc}$ bumetanide, Fig 2.4), while DIDs sensitive $I_{sc}$ ($\Delta I_{sc}$ DIDs, Fig 2.4) provides a measure of electrogenic HCO$_3^-$ secretion or NKCC1 independent Cl$^-$ secretion. The recorded $I_{sc}$ measurements were converted to µA.cm$^{-2}$ to account for the actual diameter of tissue in which $I_{sc}$ was measured from. The transepithelial resistance ($R_t$) was also calculated by dividing the voltage across the tissue by the $I_{sc}$. By doing this before and after the drugs, the average $R_t$ could be obtained.

In the pH stat experiments, pre-treatment of the tissue involved administering 1 µmol l$^{-1}$ indomethacin into the ice-cold NaCl/HCO$_3^-$ Ringer’s during the dissection and stripping of the
colonic tissue. After mounting the tissue, 1 µmol l⁻¹ indomethacin and 1 µmol l⁻¹ TTX was administered.

The statistical analysis performed were one-way Anova and unpaired Student T-Test between the relevant pairs of data (e.g. non-stimulated forskolin I_{sc} vs. stimulated forskolin I_{sc}, or HCO₃⁻ free DIDs I_{sc} vs. HCO₃⁻ Ringer’s DIDs I_{sc} etc); where P < 0.05 were considered statistically significant.
Figure 2.4 Analysis of Isc
3. Results

3.1 Ussing Chamber

3.1.1 Pharmacological Pre-treatment of tissue

Pre-treatment of tissues with indomethacin and TTX, was done to inhibit any spontaneous secretion resulting from PGE$_2$ production and endogenous enteric nervous activity (Smith et al., 1982; Grubb, 1997). These drugs, resulted in a reduction of $I_{sc}$ (Fig 3.1A), consistent with inhibiting spontaneous anion secretion. Irrespective of the bathing solution, indomethacin and TTX inhibited the initial $I_{sc}$ and the effect was comparable in the APC and DPC (Fig 3.1B).

The $R_t$ was measured before and after forskolin, bumetanide and DIDs, in all protocols. These values were then used to assess the integrity of the tissue. The $R_t$ values are listed in the appendix. The results were similar for each protocol, where the resistance decreased slightly after addition of the drugs, but the actual resistance did not change much across the entire experiment.
Figure 3.1 ΔIsc response to pre-treatment of tissues across all parameters and Rt values in NaCl/HCO₃— Ringer’s

Pre-treating the tissues with indomethacin, TTX and amiloride, showed a reduction in Iᵦ across all parameters. The ΔIᵦ with pre-treating the tissue was not significantly different across all parameters in (A) the APC and NaCl/HCO₃⁻ Ringer’s in (B) the DPC. All values X ± SEM, Unpaired Student’s T Test.
3.1.2 Stimulated Anion Secretion vs. Basal Anion secretion

The cAMP-stimulated electrogenic secretory response in the colon is thought to consist of a combination of electrogenic Cl\(^-\) secretion and electrogenic HCO\(_3\)^\(^-\) secretion. To determine the relative contribution of Cl\(^-\) and HCO\(_3\)^\(^-\) secretion to the secretory response, tissues were stimulated with forskolin and then treated sequentially with serosal bumetanide (100 µmol l\(^-1\)) and serosal DIDs (0.5 mmol l\(^-1\)). To control for spontaneous activity of these transporters a time matched control was included that was not stimulated with forskolin but was treated with bumetanide and DIDs.

**Anterior proximal colon** – In the APC, forskolin induced an increase in I\(_{sc}\) of ∆47.2 ± 6.6 µA cm\(^-2\), consistent with the stimulation of electrogenic anion secretion (Fig 3.2A, C). The subsequent addition of serosal bumetanide to the stimulated tissue caused a reduction in I\(_{sc}\) of ∆-31.4 ± 4.7 µA cm\(^-2\). This was consistent with inhibition of electrogenic Cl\(^-\) secretion. The paired control tissue had a reduction in I\(_{sc}\) of ∆-12.3 ± 2.1µA cm\(^-2\), in response to serosal bumetanide, although, the reduction of control tissue was significantly less (P<0.01) than stimulated tissue. The subsequent addition of serosal DIDs further attenuated I\(_{sc}\) in both stimulated (∆-40.3 ± 4.9 µA cm\(^-2\)) and control (∆-14.4 ± 3.7 µA cm\(^-2\)) tissues, which was again consistent with inhibition of electrogenic anion secretion. The DIDs-sensitive I\(_{sc}\) in the stimulated tissues was significantly greater (P<0.01) than that in the control tissues. This initial experiment indicates that forskolin stimulated an increase in I\(_{sc}\), and associated with that, was an increase in bumetanide-sensitive I\(_{sc}\) and DIDs-sensitive I\(_{sc}\). The results suggest that the increase in I\(_{sc}\) stimulated by forskolin was due to stimulation of NKCC1 dependent Cl\(^-\) secretion, indicated by the effect of bumetanide, and NBCe1 dependent HCO\(_3\)^\(^-\) secretion, indicated by the effect of DIDs. The effect of bumetanide and DIDs on the control tissues indicates that although the tissue was pre-treated with indomethacin and TTX some basal anion secretion remains.

**Distal proximal colon** – Addition of forskolin induced an increase in I\(_{sc}\) of ∆49.4 ± 7.2 µA cm\(^-2\) (Fig 3.2B, D). Serosal bumetanide caused a reduction in I\(_{sc}\) of ∆-57.1 ± 10.3 µA cm\(^-2\), and further attenuation of I\(_{sc}\) of ∆-15.3 ± 3.0 µA cm\(^-2\) was seen upon addition of DIDs. The addition of serosal bumetanide to control tissue induced a reduction in I\(_{sc}\) of ∆-33.2± 5.9 µA cm\(^-2\), and like the APC, was significantly less (P<0.05) than stimulated tissue. The addition of DIDs in control tissue caused a reduction in I\(_{sc}\) of ∆-12.9 ± 5.5 µA cm\(^-2\), which was not significantly different (P>0.05) from stimulated tissue. The overall results in the DPC suggest that forskolin does not stimulate an increase in the DIDs-sensitive I\(_{sc}\) and the bumetanide-sensitive I\(_{sc}\) only accounts for ~50% of the forskolin response. In contrast, in the APC, bumetanide and DIDs had
comparable effects and accounted for ~100% of the forskolin stimulated $I_{sc}$. So the results in the DPC suggest that there is no DIDs-sensitive electrogenic $\text{HCO}_3^-$ secretion, and about 50% of the secretory response is due to electrogenic $\text{Cl}^-$ secretion (i.e., the effect of bumetanide), which suggests there is something else going on. Based on these data, electrogenic $\text{HCO}_3^-$ secretion in the DPC was not pursued, as it appears it does not secrete $\text{HCO}_3^-$ electrogenically.
Figure 3.2 Changes to Isc in response to drug treatments

A) APC and (B) DPC representative Isc trace, showing the effects of indomethacin, tetrodotoxin and amiloride (I/T/A) to bring the Isc to basal steady state of secretion; stimulatory effect of forskolin to increase Isc; and inhibitory effects of bumetanide and DIDs

C) The effect of drugs on Isc in the APC. All values X ± sem., n = 6 mice for each treatment.

D) The effect of drugs on Isc in the DPC. All values X ± sem., n = 8 mice for each treatment.

**P<0.01; ****P<0.0001, One-way ANOVA.
3.1.3 Inflammation has little effect on electrogenic anion secretion in the anterior proximal colon (APC)

In order to determine if inflammation affected the magnitude of the anion secretion, inflamed tissue was compared with non-inflamed tissue. We were particularly interested in the DIDs-sensitive component, which is thought to represent electrogenic \( \text{HCO}_3^- \) secretion. These experiments were conducted by bathing both mucosal and serosal reservoirs with NaCl/\( \text{HCO}_3^- \) Ringer’s. By comparing inflamed to non-inflamed tissue, we were able to assess the effect inflammation has on the stimulatory response to forskolin, and the bumetanide and DIDs-sensitive \( I_{sc} \).

Although there was a trend towards a reduction in the secretory response, the difference in DIDs-sensitive component between non-inflamed (\( \Delta \)-40.4± 5.0 µA cm\(^{-2} \)) and inflamed tissue (\( \Delta \)-28.5 ± 8.6 µA cm\(^{-2} \)) was not significant(Fig. 3.3A, B). The effect of forskolin in non-inflamed tissue (\( \Delta \)47.2 ± 6.6 µA cm\(^{-2} \)) compared with inflamed tissue (\( \Delta \)30.7 ± 10.3 µA cm\(^{-2} \)) was not significantly different (\( P>0.05 \)). Neither was there a significant difference for the bumetanide-sensitive component between non-inflamed (\( \Delta \)-31.4 ± 4.7 µA cm\(^{-2} \)) and inflamed tissue (\( \Delta \)-32.6 ± 7.1 µA cm\(^{-2} \)), which does not seem to be altered at all. This was unexpected because it suggests that electrogenic anion secretion was not impaired in inflamed tissue, despite the expression study showing NBCe1 is reduced. Therefore, the following Ussing \( I_{sc} \) experiments sought to determine whether NBCe1 plays a significant role in electrogenic anion secretion or if another process was occurring that could account for the non-significant difference between inflamed and non-inflamed tissues.
Figure 3.3 Comparing effects of drugs on changing $I_{sc}$ in non-inflamed tissue with inflamed tissue from IL10$^{-/-}$ mice infected with $H. typhlonius$

A) Representative $I_{sc}$ trace, showing the effects of drugs in the APC. By using NaCl/HCO$_3^-$ Ringer’s to bathe the inflamed tissue, the effect of drugs on changing the $I_{sc}$ was seen in (B) the APC to not be significantly different to non-inflamed tissue (Indomethacin, TTX, amiloride, I/T/A). All values X ± sem., n = 8 mice for each treatment. NS P>0.05, One-way ANOVA.
3.1.4 The DIDs-sensitive $I_{sc}$ is Cl$^-$ dependent in the anterior proximal colon (APC)

The lack of a significant effect of inflammation on electrogenic HCO$_3^-$ secretion was unexpected, given that NBCe1 expression is markedly reduced by inflammation and HCO$_3^-$ secretion is thought to be driven by NBCe1 activity. To test whether bumetanide and DIDs inhibit electrogenic Cl$^-$ and HCO$_3^-$ secretion, respectively, the effect of replacing Cl$^-$ and HCO$_3^-$ in the bathing solution was carried out. In Cl$^-$ free Ringer’s, the bumetanide-sensitive $I_{sc}$ should be eliminated, and DIDs-sensitive $I_{sc}$ should not be affected, because it is thought to be due to HCO$_3^-$ secretion, driven by NBCe1. On the other hand, in HCO$_3^-$ free Ringer’s, the DIDs-sensitive $I_{sc}$ should be eliminated and bumetanide-sensitive $I_{sc}$ should not be affected.

In the initial experiment, a paired tissue from the APC was used, however, for one of those tissues, Cl$^-$ was replaced with gluconate in both the mucosal and serosal solutions. The HCO$_3^-$ free experiment, again used paired tissues from the APC, however, for one of those tissues, HCO$_3^-$ was replaced with Cl$^-$ and Hepes in the Ringer’s solution. The Hepes buffered Ringer’s was also gassed with 100% O$_2$.

In HCO$_3^-$ buffered/Cl$^-$ free Ringer’s the bumetanide-sensitive $I_{sc}$ ($\Delta I_{sc} = 39.1 \pm 15.6 \mu A cm^{-2}$) and Hepes buffered Ringer’s ($\Delta I_{sc} = 66.8 \pm 15.9 \mu A cm^{-2}$) (Fig. 3.5A, B) were similar. Although not significant, the difference in the values may have accounted for with an increased Cl$^-$ uptake. The results also showed that the $\Delta I_{sc}$ response to forskolin was not significantly different.
(P>0.05) between NaCl/HCO$_3^-$ Ringer’s (Δ78.2 ± 23.0 µA cm$^{-2}$) and Hepes buffered Ringer’s (Δ91.4 ± 14.1 µA cm$^{-2}$)

These results indicate that a basolateral transporter that is Cl$^-$ dependent and DIDs-sensitive, is involved in electrogenic anion secretion. All of which point towards the Cl$^-$/HCO$_3^-$ exchanger. The DIDs-sensitive $I_{sc}$, which is essentially eliminated in Cl$^-$/free Ringer’s, means that NBCe1 is not involved in the stimulatory response of forskolin. While the absence of inhibiting DIDs-sensitive $I_{sc}$ with HCO$_3^-$ free Ringer’s suggests that HCO$_3^-$ uptake from NBCe1 does not drive Cl$^-$/HCO$_3^-$ exchange as well. However, there are other ways for HCO$_3^-$ to accumulate within the cell. Therefore, what might be happening is that metabolic generation of CO$_2$ is converted to HCO$_3^-$, and this is able to drive the Cl$^-$/HCO$_3^-$ exchanger.
Figure 3.4 The effect of drugs in NaCl/HCO₃—Ringer’s vs. HCO₃—Buffered/Cl—Free Ringer’s (APC)

A) Representative Iₛc trace, showing the effects of drugs in APC. The effect of removing Cl⁻ from both mucosal and serosal bathing solution was compared with NaCl/HCO₃⁻ Ringer’s in (B) the APC, which indicated a reduction in the stimulatory effect of forskolin, and reduction in bumetanide and DIDs inhibitory effect. All values X ± sem., n = 8 mice for each treatment. **P<0.01; ***P<0.001; ****P<0.0001, One-way ANOVA.
Figure 3.5 The effect of drugs in NaCl/HCO₃⁻ Ringer’s vs. HCO₃⁻ free/NaCl Ringer’s

A) Representative $I_{\text{sc}}$ trace, showing the effects of drugs in the APC. Removal of HCO₃⁻ from the mucosal and serosal bathing solution by using HCO₃⁻ free Ringer’s was compared with NaCl/HCO₃⁻ Ringer’s, where (B) the APC indicates that there is no significant difference in the effect of the drugs between the two Ringer’s solutions. All values X ± sem., n = 6 mice for each treatment. NS P>0.05, One-way ANOVA.
3.1.5 Endogenous HCO$_3^-$ generation drives Basolateral Cl$^-$/HCO$_3^-$ exchanger

Endogenous HCO$_3^-$ generation might be what drives the Cl$^-$/HCO$_3^-$ exchange. To investigate if this was the case, acetazolamide was used in Hepes buffered Ringer’s to inhibit carbonic anhydrase, and this was compared with the paired tissues in just Hepes Buffered Ringer’s. 1 mmol l$^{-1}$ acetazolamide was dissolved in Tris solution, which was then used to make up the Hepes buffered Ringer’s. This allowed for immediate exposure of the tissue to acetazolamide when mounted into the Ussing chamber.

There was a slight reduction in forskolin response, with addition of acetazolamide to Hepes buffered Ringer’s (Δ41.8 ± 11.7 µA cm$^{-2}$), which did not change significantly (P>0.05) compared with just Hepes buffered Ringer’s (Δ60.7 ± 12.4 µA cm$^{-2}$) (Fig. 3.6A, B). The bumetanide-sensitive I$_{sc}$ was slightly increased, although not significantly different in Hepes Buffered Ringer’s with acetazolamide (Δ-48.3 ± 12.8 µA cm$^{-2}$) compared with Hepes Buffered Ringer’s (Δ-38.8 ± 6.8 µA cm$^{-2}$). The DIDs-sensitive I$_{sc}$ was in accordance to what was expected, where the complete removal of HCO$_3^-$ with acetazolamide attenuated the effect of DIDs (Δ-8.1 ± 2.1 µA cm$^{-2}$) compared with Hepes Buffered Ringer’s (Δ-55.5 ± 10.9 µA cm$^{-2}$). This indicates that the DIDs-sensitive I$_{sc}$ is dependent on the generation of HCO$_3^-$ through hydration of metabolic CO$_2$, and endogenous HCO$_3^-$ is driving the uptake of Cl$^-$ via Cl$^-$/HCO$_3^-$ exchanger.
Figure 3.6 Effect of drugs on changing Isc

A) Representative Isc trace, showing the effects of drugs in the APC. (B) By removing all forms of HCO₃⁻ generation in the APC, using acetazolamide in HCO₃⁻ free/NaCl Ringer’s, the effect of DIDs in reducing Isc was significantly reduced. All values X ± sem., n = 7 mice for each treatment. *P<0.05, One-way ANOVA.
3.2 pH STAT

The expression study on NBCe1, does suggest an impairment of HCO₃⁻ secretion in inflamed tissue. However, inflammation did not appear to have an effect on electrogenic anion secretion, and in fact, the Ussing short circuit experiments suggests that electrogenic HCO₃⁻ secretion only plays a small role or no role at all in the overall electrogenic anion secretion. There are, however, two mechanisms of HCO₃⁻ secretion thought to occur in the proximal colon, electrogenic and electroneutral. The Ussing Iₛₚ technique is unable to measure electroneutral HCO₃⁻ secretion, therefore, the pH stat technique in combination with the Ussing Iₛₚ technique was used to investigate the electroneutral component.

Immediately following mounting the tissue in the Ussing chamber, the mucosal chamber had a pH of 6.8 ± 0.4 and took 30 – 60 min for the mucosal pH to reach 7.4 and the pH stat to start delivering acid. A 30 min flux period was done for measuring the basal HCO₃⁻ flux. Then, 20 μM forskolin was added to the serosal reservoir only, and 15 min was allowed for the flux to reach a steady state before the stimulated JHCO₃⁻ was measured over a 30 min period. Finally, 0.5 mM DIDs was added to the serosal chamber, a 15 min wait period followed by a 30 min flux measurement was taken.

3.2.1 Electroneutral HCO₃⁻ secretion is not affected in inflamed tissue

The JHCO₃⁻ₚₘ⁺ flux results of the non-inflamed tissue was not significantly (P>0.05) different in basal flux, forskolin or DIDs, in comparison with the inflamed tissue (Fig 3.7A). The basal flux for non-inflamed tissue (1.86 ± 0.68 μM/cm²/h) was not significantly different from inflamed tissue basal flux (1.28 ± 0.16 μM/cm²/h), although there was a much larger variation in non-inflamed JHCO₃⁻ₚₘ⁺ measurements. JHCO₃⁻ₚₘ⁺ measurements for forskolin were also not significantly different between non-inflamed (1.98 ± 0.61 μM/cm²/h) and inflamed tissue (1.49 ± 0.53 μM/cm²/h). Neither was DIDs JHCO₃⁻ₚₘ⁺ response significantly different between non-inflamed (3.00 ±0.73 μM/cm²/h) and inflamed tissue (1.47 ± 0.58 μM/cm²/h). These results suggest that there was no appreciable difference in HCO₃⁻ secretion between inflamed and non-inflamed tissue, indicating that electroneutral HCO₃⁻ secretion was not affected by inflammation.

Although there does appear to be a trend towards a smaller JHCO₃⁻ₚₘ⁺ in inflamed tissue compared with the non-inflamed tissue, which may indicate a reduction JHCO₃⁻ₚₘ⁺ in inflammation. Also, the addition of forskolin does not seem to stimulate an increase in HCO₃⁻ flux in either inflamed or non-inflamed tissues. One of two things could be happening here, either electroneutral HCO₃⁻ is not affected by inflammation, or that tissue damage in the
inflamed tissue, caused by dissection and stripping of the muscle layer, is resulting in an increased flux of $\text{HCO}_3^-$, which leads us to wonder whether there is stimulation of electroneutral $\text{HCO}_3^-$ secretion. The corresponding Ussing $I_{\text{sc}}$ experiments (Fig 3.7B, C) reveal there is no difference in electrogenic anion secretion and shows that the tissue is responsive to drugs administered.
Figure 3.7 pH stat HCO$_3^-$ flux measurements

A) HCO$_3^-$ flux measurements, comparing control tissue (filled bars) with inflamed tissue (empty bars). There is no significant difference in HCO$_3^-$ flux between inflamed and non-inflamed tissue during basal flux, forskolin and DIDs. B) Representative $I_{sc}$ trace of control and inflamed tissue and (C) summary data of Ussing $I_{sc}$ measurements. All values $X \pm$ sem., $n = 8$ mice for each treatment, One-way ANOVA.
3.2.2 There is a small paracellular flux of $\text{HCO}_3^-$

When using the $pH$ stat technique, the mucosal bathing solution differs from the serosal solution. While Na$^+$ and Cl$^-$ have an equal concentration on both sides of the tissue, the mucosal Ringer’s solution does not contain $\text{HCO}_3^-$, whereas the serosal chamber contains 25mM $\text{HCO}_3^-$. This means there is a large gradient for $\text{HCO}_3^-$. Due to this difference in concentration gradient, there may be a significant increase in passive paracellular flux of $\text{HCO}_3^-$. If this flux is increased due to damage associated with preparation of the tissue it may mask any changes associated with active transport. To determine if tissue damage maybe the cause of increased $\text{HCO}_3^-$ flux this experiment looked to see how much the passive flux contributes to the active $\text{HCO}_3^-$ flux.

The only way to differentiate passive flux and active flux was to compare the mucosal to serosal (reversed tissue) and serosal to mucosal fluxes. This assumes that there is no net active absorptive (mucosal to serosal) flux of $\text{HCO}_3^-$, which is reasonable given that there is a lack of transporters on the mucosal side of the epithelium to take up $\text{HCO}_3^-$. Therefore any $\text{HCO}_3^-$ movement that was measured was indicative of paracellular transport of $\text{HCO}_3^-$ either via the tight junctions or via damage caused in preparation of the tissue. The non-reversed tissue would therefore be a measure of the active $J_{\text{HCO}_3^-,\text{sm}}$ secretion and be inclusive of the passive flux.

Paired tissues from the APC were used for the $pH$ stat measurements, where one of the tissues was mounted with the mucosal side of the tissue bathed in buffer and $\text{HCO}_3^-$-free Ringer’s and serosal side bathed in NaCl/$\text{HCO}_3^-$ Ringer’s (control tissue). The corresponding pair was mounted in reverse with mucosal side of the tissue bathed in NaCl/$\text{HCO}_3^-$ Ringer’s and serosal side bathed in buffer- and $\text{HCO}_3^-$-free Ringer’s. However, in the reversed tissue, because the serosal side of the tissue faces the chamber that contains the $pH$ stat electrode, forskolin was only added to the mucosal chamber to avoid changing pH in the serosal chamber and affecting the flux measurements. The measurements, however, were taken at times analogous to the corresponding non-reversed tissue.

The basal flux for reverse tissue ($0.24 \pm 0.24 \mu M cm^{-2} h^{-1}$) did not change significantly ($P>0.05$) compared with the control tissue ($1.26 \pm 0.43 \mu M/cm^2/h$). Whereas, the response of forskolin, was significantly increased in control tissue ($1.71 \pm 0.52 \mu M cm^{-2} h^{-1}$) compared with reversed tissue ($0.30 \pm 0.13 \mu M cm^{-2} h^{-1}$). Although the basal mucosal to serosal flux was not significantly different to the serosal to mucosal flux, on average it was about 20% of the serosal to mucosal
flux (Fig 3.8A). This indicates that the preparation of the tissues was not associated with significant damage and raises the issue as to whether there is significant electroneutral HCO$_3^-$ secretion occurring in the APC. This is reinforced by the fact that forskolin did not appear to alter the secretory flux appreciably.

The Ussing $I_{sc}$ measurements done in parallel to the $pH$ stat measurements, indicate forskolin stimulated electrogenic anion secretion (Fig 3.8B, C).
Figure 3.8 pH stat measurements in combination with Ussing $I_{sc}$ measurements of reversed tissue compared with control tissue

A) HCO$_3^-$ flux measurements, comparing control tissue with reversed tissue. The amount of HCO$_3^-$ secreted in response to forskolin in control tissue is significantly greater than the reversed tissue. B) Representative $I_{sc}$ trace of control and reversed tissue and (C) summary data of Ussing $I_{sc}$ measurements. All values $X \pm$ sem., $n = 4$ mice for each treatment. *P<0.05, One-way ANOVA.
3.2.2 Forskolin does not stimulate active $\text{HCO}_3^-$ secretion

In the experiment comparing inflamed to non-inflamed tissue, there was a similar active $J_{\text{HCO}_3^-}$ between basal flux and addition of forskolin, and an increase in $J_{\text{HCO}_3^-}$ with addition of DIDs. This protocol sought to investigate the effect of forskolin and DIDs on stimulated $\text{HCO}_3^-$ secretion by comparing control and stimulated tissues mounted in the same orientation.

The basal flux in control tissue (6.00 ± 4.03 µM cm$^{-2}$ h$^{-1}$) was not significantly different (P>0.05) compared with stimulated tissue (2.46 ± 1.32 µM cm$^{-2}$ h$^{-1}$) (Fig 3.9A). Neither was the $J_{\text{HCO}_3^-}$ significantly different (P>0.05) in response to forskolin in stimulated tissue (2.26 ± 1.18 µM cm$^{-2}$ h$^{-1}$) compared with the time analogous to control tissue (3.84 ± 2.25 µM cm$^{-2}$ h$^{-1}$). DIDs in control tissue (3.52 ± 1.60 µM cm$^{-2}$ h$^{-1}$) was also not significantly different (P>0.05) compared with stimulated tissue (2.84 ± 0.68 µM cm$^{-2}$ h$^{-1}$).

In the stimulated tissue, there appears to be a similar pattern in the stimulated $J_{\text{HCO}_3^-}$ where forskolin had no effect, while DIDs slightly raised the $J_{\text{HCO}_3^-}$. However, the $J_{\text{HCO}_3^-}$ in the control tissue had an opposite trend, where the time analogous to forskolin addition and addition of DIDs resulted in a steady reduction in $J_{\text{HCO}_3^-}$, although there is also a very large variation in the basal flux measurements. These results suggest that forskolin and DIDs do not have any effect on active $J_{\text{HCO}_3^-}$.

The $I_{sc}$ measurements done in parallel to the $\text{HCO}_3^-$ flux measurements (Fig 3.9B, C), indicate, in the stimulated tissue, there is an increase in electrogenic anion secretion in response to forskolin, and reduction in response to DIDs. These data are comparable to the Ussing $I_{sc}$ experiment (Fig 3.2C).
Figure 3.9 pH stat measurements in combination with Ussing $I_{sc}$ measurements of stimulated tissue compared with control tissue

A) Comparing stimulated to non-stimulated tissue with the $pH$ stat. (B) Example trace of $I_{sc}$ and (C) summary data of $I_{sc}$ measurements in stimulated vs. non-stimulated tissue. All values $X \pm$ sem., $n = 4$ mice for each treatment. *$P<0.05$; ***$P<0.001$, One-way ANOVA
4. Discussion

Maintaining an effective mucus barrier is essential for preventing microorganisms from contacting the intestinal epithelium. $\text{HCO}_3^-$ secretion into the intestinal lumen is thought to be crucial for the expansion of mucus (Yang et al., 2013). However, impairment of the mucus barrier is involved with the development of IBD, where it is suggested that diminished $\text{HCO}_3^-$ secretion prevents expansion of the mucus (Chen et al., 2010; Garcia et al., 2009). The reduction in expression of certain transporters associated with $\text{HCO}_3^-$, should lead to reduced $\text{HCO}_3^-$ secretion. However, whether this involves a reduction in electrogenic or electroneutral $\text{HCO}_3^-$ secretion or both has yet to be determined. Therefore, the present study used the Ussing $I_{sc}$ and $pH$ stat technique to investigate if electrogenic or electroneutral $\text{HCO}_3^-$ secretion is affected by inflammation. The results indicate that in the anterior region of the proximal colon the crypt cells are primarily involved in electrogenic $\text{Cl}^-$ secretion rather than electrogenic $\text{HCO}_3^-$ secretion, and overall electrogenic anion secretion is unaffected in inflamed tissue. The $pH$ stat results also reveal that there is no significant difference in electroneutral $\text{HCO}_3^-$ secretion between non-inflamed and inflamed tissue. Furthermore, this suggests there is low $\text{HCO}_3^-$ secretion in the proximal colon, raising questions as to the role of $\text{HCO}_3^-$ secretion in mucus hydration in the colon.

4.1 Basal $I_{sc}$ and $R_T$

As mentioned by Clarke (2009), pre-treating the epithelium with antagonist is important to prevent any spontaneous secretion. For this study tissues were pre-treated with indomethacin, TTX and amiloride. Indomethacin and TTX inhibited any spontaneous stimulation associated with PGE$_2$, enteric nervous activity, and amiloride inhibited any absorption due to ENaC (Smith et al., 1982; Grubb, 1997; Bridges et al., 1989; Kato & Romero, 2011).

By calculating the $R_T$ before and after every drug administered, the change in resistance could be used to assess the integrity of the tissue (Clarke, 2009). In the NaCl/$\text{HCO}_3^-$ Ringer’s, treatment with forskolin, bumetanide, and DIDS, all reduced $R_T$, and a similar pattern occurred for all Ussing $I_{sc}$ experiments. However, the actual reduction in $R_T$ values, before and after addition of drugs was relatively small in comparison to the effect that the drug elicited on the $I_{sc}$.
4.2 Electrogenic anion secretion in the APC is not affected in inflamed tissue

The results of Ussing $I_{sc}$ experiments, comparing the effect of forskolin on inflamed tissue to non-inflamed tissue for the APC revealed there was no significant difference. This suggests that even with inflammation, forskolin can stimulate anion secretion, and there was no noticeable impairment to secretion driven by NKCC1 or the basolateral $\text{Cl}^-/\text{HCO}_3^-\text{exchanger.}$ The Ussing $I_{sc}$ technique experiments also indicated that stimulated secretion in the APC was attributed to electrogenic $\text{Cl}^-$ secretion. Considering there was a reduced expression of NBCe1 observed in inflamed tissue (Fan & Butt, unpublished results, see figure 1.3), the ion transport properties were not affected by this impairment, which suggests that electrogenic $\text{HCO}_3^-$ secretion may not be important to mucus expansion in the APC.

As figure 3.1A indicates, the addition of forskolin stimulates an increase in $I_{sc}$, which was at first, indicative of an increase in $\text{Cl}^-$ (Greger et al., 1997) and $\text{HCO}_3^-$ secretion (Grubb, 1997; Tuo et al., 2009). This was further validated by the bumetanide-sensitive $I_{sc}$ (Isenring & Forbush, 1997) and DIDs-sensitive $I_{sc}$. However, later experiments showed that DIDs was inhibiting basolateral $\text{Cl}^-/\text{HCO}_3^-$, which was contributing to electrogenic $\text{Cl}^-$ secretion, rather than NBCe1 and electrogenic $\text{HCO}_3^-$ secretion.

What was initially proposed was that the forskolin response was a combination of electrogenic $\text{Cl}^-$ and electrogenic $\text{HCO}_3^-$ secretion. Therefore, it was expected that the total $I_{sc}$ would be reduced upon removal of $\text{Cl}^-$, consistent with CFTR being an electrogenic $\text{Cl}^-$ transporter (Clarke et al., 1992). The results did show the elimination of the bumetanide-sensitive $I_{sc}$ in $\text{Cl}^-$ free Ringer's. However, what was not expected was removal of $\text{Cl}^-$ greatly reduced the DIDs-sensitive component. This gave the first indication that a large component of the DIDs-sensitive $I_{sc}$ was $\text{Cl}^-$ dependent. There is evidence of a chloride/bicarbonate exchanger on the basolateral membrane of surface and crypt cells, which drives the efflux of $\text{HCO}_3^-$ for influx of $\text{Cl}^-$ (Rajendran & Binder, 1999; Ikuma et al., 2003). The $\text{Cl}^-/\text{HCO}_3^-$ exchanger is expressed at high levels on the basolateral membrane of the proximal colon (Alper et al., 1999) and a study (Walker et al., 2002) suggest that the transporter supports anion secretion in the duodenum. However, evidence of the basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchanger playing a significant role in anion secretion is still lacking (Gawenis et al., 2010). The basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchanger drives $\text{Cl}^-$ entry in exchange for $\text{HCO}_3^-$, indicating an alternative role for the accumulated $\text{HCO}_3^-$ in the cell. These data suggest that the basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchanger does play a major role in
Cl\textsuperscript{−} secretion, and in fact can account for up to 50\% of the anion secretion stimulated by forskolin. The transport process would also account for the Cl\textsuperscript{−} dependence in the DIDs-sensitive \( I_{sc} \), as DIDs is also known to inhibit the basolateral Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger (Isenberg \textit{et al.}, 1993).

It has been suggested that NBCe1 provides the HCO\textsubscript{3}\textsuperscript{−} for the basolateral Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger (Walker \textit{et al.}, 2002). If NBCe1 were to drive Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange, then upon removal of HCO\textsubscript{3}\textsuperscript{−} from the serosal bathing solution, NBCe1 will stop, and the basolateral Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger should stop. This was not the case, as removal of HCO\textsubscript{3}\textsuperscript{−} from the bathing solution had no effect on the DIDs-sensitive \( I_{sc} \) stimulated by forskolin. The results were unexpected, where the DIDs-sensitive \( I_{sc} \) was unchanged, even when exogenous HCO\textsubscript{3}\textsuperscript{−} was removed. Therefore, it could be concluded that basolateral uptake of HCO\textsubscript{3}\textsuperscript{−} was not involved in recycling HCO\textsubscript{3}\textsuperscript{−} with Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger for electrogenic Cl\textsuperscript{−} secretion. The basolateral Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger most likely exchanges HCO\textsubscript{3}\textsuperscript{−} generated endogenously, rather than from Na-HCO\textsubscript{3} cotransporter, as observed by Ikuma \textit{et al.}, (2003); where Cl\textsuperscript{−} dependent alkalinisation from the Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger was not altered when serosal bath Na\textsuperscript{+} was removed.

The results of adding acetazolamide were in accordance to what was expected, where the DIDs-sensitive \( I_{sc} \) was significantly reduced with acetazolamide. This indicates that endogenous HCO\textsubscript{3}\textsuperscript{−} is driving Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange. The effect of acetazolamide inhibits the enzyme carbonic anhydrase, preventing the hydrolysis of CO\textsubscript{2} to HCO\textsubscript{3}\textsuperscript{−} and as this inhibits the production of endogenous HCO\textsubscript{3}\textsuperscript{−}, it will consequently prevent Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange by the basolateral Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger. Therefore, DIDs will be ineffective when it acts on the non-functioning basolateral Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger.

Overall, these experiments suggest that in the APC the main anion that is secreted is Cl\textsuperscript{−}, whereas there is little evidence that electrogenic HCO\textsubscript{3}\textsuperscript{−} secretion occurs (Fig 4.1). A large portion of Cl\textsuperscript{−} uptake is due to NKCC1, however, the basolateral Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger also plays a significant role in the uptake of Cl\textsuperscript{−}. This occurs by production of HCO\textsubscript{3}\textsuperscript{−} by the hydrolysis of metabolic CO\textsubscript{2} with carbonic anhydrase. The HCO\textsubscript{3}\textsuperscript{−} generated is recycled by the basolateral Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger, for uptake of Cl\textsuperscript{−} and allows for electrogenic Cl\textsuperscript{−} secretion.

In Cl\textsuperscript{−} free Ringer’s, the DIDs-sensitive component is essentially eliminated. Therefore, the DIDs-sensitive component in electrogenic anion secretion is entirely attributed to Cl\textsuperscript{−} secretion, following uptake from Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger, and not HCO\textsubscript{3}\textsuperscript{−} secretion. Also, the
result should be that in Cl$^-$ free Ringer’s, if HCO$_3^-$ was involved in stimulated secretion, then the $\Delta I_{sc}$ stimulated by forskolin would be equivalent to the DIDs-sensitive $I_{sc}$. However, the DIDs-sensitive component was eliminated, and therefore, not equal to the $\Delta I_{sc}$ stimulated by forskolin, meaning forskolin was stimulating a different transport process that resulted in an increased $I_{sc}$. What forskolin was stimulating is unclear at this point. A possible reason may be due to the accumulation of HCO$_3^-$ in Cl$^-$ free conditions, considering HCO$_3^-$ taken up from NBCe1 or endogenous HCO$_3^-$ generation, cannot leave via the basolateral Cl$^-$/HCO$_3^-$ exchanger. Therefore, when forskolin stimulates secretion, the accumulated HCO$_3^-$ will be secreted by CFTR, resulting in a greater stimulatory response to forskolin.
Endogenous HCO$_3^-$ generation drives basolateral Cl$^-$/HCO$_3^-$ exchange for uptake of Cl$^-$ and electrogenic Cl$^-$ secretion. Hydration of CO$_2$ also produces H$^+$, which exits the cell via NHE1 and is necessary for HCO$_3^-$ generation by CO$_2$ hydration (Jacob et al., 2000). NKCC1 also contributes to Cl$^-$ uptake and electrogenic Cl$^-$ secretion. NBCe1 is expressed in the APC and drives uptake of HCO$_3^-$ across the basolateral membrane, but the function of the HCO$_3^-$ is questionable. Na$^+/K^+$-ATPase maintains the electrochemical gradient, along with K$^+$ channels (KCNQ1/KCNN4). Secretion across apical membrane of both Cl$^-$ and HCO$_3^-$ occurs by CFTR.
4.3 COMPENSATORY INCREASE IN CL\(^-\) SECRETION

In the APC, the I\(_{sc}\) increase in response to forskolin in HCO\(_3\)^- free Ringer’s was not significantly different from that in NaCl/HCO\(_3\)^- Ringer’s. This is not unexpected as others have shown that when one secretory transport pathway is inhibited, another will compensate (Gawenis et al., 2010). There is a suggestion that this is occurring in the experiment reported here. In HCO\(_3\)^- free Ringer’s the bumetanide-sensitive I\(_{sc}\) was 30% greater than that in HCO\(_3\)^- containing Ringer’s. Although this difference was not statistically significant it does point to a compensatory increase in Cl\(^-\) secretion driven by NKCC1.

There is no appreciable electronegic HCO\(_3\)^- secretion in the APC, which means the compensatory increase in Cl\(^-\) secretion is not due to a reduction in HCO\(_3\)^- secretion. A basolateral Cl\(^-\)/HCO\(_3\)^- exchange knockout model shows an elevated secretion by NKCC1 (Gawenis et al., 2010). In which case, the increase in Cl\(^-\) secretion was met with a compensatory increase in uptake of Cl\(^-\), and the transporter responsible was the NKCC1.

This was also observed when acetazolamide was used. When looking at the bumetanide-sensitive I\(_{sc}\), although there was no significant difference, the trend of an increased bumetanide-sensitive I\(_{sc}\) also suggests a ‘compensatory’ increase in NKCC1 activity, similar to the HCO\(_3\)^- free Ringer’s results, and consistent with what is suggested by Gawenis et al., (2010). This is suggested, because in the first protocol, a large DIDs-sensitive I\(_{sc}\) was seen, which means a large portion of the stimulated secretion is due to Cl\(^-\) taken up from the basolateral Cl\(^-\)/HCO\(_3\)^- exchanger. However, when acetazolamide was administered, the stimulatory secretion in response to forskolin was not significantly affected. A possible explanation may be due to an increase in Cl\(^-\) secretion due to a ‘compensatory’ increase in Cl\(^-\) uptake via NKCC1, as the basolateral Cl\(^-\)/HCO\(_3\)^- exchanger is inhibited by acetazolamide.
4.4 ELECTROGENIC ANION SECRETION IN THE DPC

In the DPC forskolin stimulated anion secretion. However, in contrast to the APC, in the DPC, the bumetanide-sensitive $I_{sc}$ was much greater than the DID-sensitive $I_{sc}$, and the DID-sensitive component between stimulated and non-stimulated tissue shows a similar reduction in $I_{sc}$. This indicates that $\text{HCO}_3^-$ secretion is not actually stimulated by forskolin, considering the same amount of $\text{HCO}_3^-$ is being secreted even when the tissue is not stimulated. So, the DID-sensitive $I_{sc}$, after being stimulated by forskolin, is seen as reducing the basal steady-state secretion of $\text{HCO}_3^-$ rather than reducing a stimulated secretion of $\text{HCO}_3^-$. This means that electrogenic $\text{HCO}_3^-$ secretion driven by NBCe1 does not occur in the DPC. The bumetanide-sensitive $I_{sc}$ is stimulated by forskolin, however, it does not account for the entire forskolin response, since the amount of bumetanide-sensitive $I_{sc}$ is not equivalent to the forskolin stimulatory response. This suggests that in the DPC some other secretory process is stimulated.
4.5 Electroneutral HCO$_3^-$ secretion is not affected in inflamed tissue

There was no substantial difference in secretory HCO$_3^-$ flux between inflamed and non-inflamed tissue, indicating that electroneutral HCO$_3^-$ secretion was not affected by inflammation. When using the pH stat technique, the measure of HCO$_3^-$ flux is not necessarily representative of electroneutral HCO$_3^-$ secretion, as any HCO$_3^-$ secreted to the mucosal solution will be detected by the pH stat electrode. However, the Ussing I$_{sc}$ experiments have shown that there is no stimulated electrogenic HCO$_3^-$ secretion in the APC meaning, the measure of HCO$_3^-$ flux in the pH stat may be attributed to electroneutral HCO$_3^-$ flux. Although, to accurately differentiate between electrogenic and electroneutral HCO$_3^-$ secretion using the pH stat, one of two experiments could be conducted. One would be to use a CFTR inhibitor, such as thiazolidinone derivatives, CFTR(inh)-172 (Verkman et al., 2013) to measure only apical Cl$^-$/HCO$_3^-$ exchanger transport, or to use Cl$^-$ free solution in the mucosal reservoir to measure the proportion of HCO$_3^-$ flux, which is electrogenic flux. The latter experiment was conducted, but unexpectedly the fluxes were much larger than those seen in the presence of mucosal Cl$^-$, suggesting that the Cl$^-$ free Ringer’s had a detrimental effect on the tissues.

One possible explanation for the lack of a difference between the inflamed and non-inflamed tissues is the flux that is measured is passive flux occurring as a result of damage to the tissue during preparation. However, this was excluded by mounting the tissue in reverse, so the apical membrane faced the solution containing HCO$_3^-$, and measuring the passive movement of HCO$_3^-$. There is a lack of transporters on the apical membrane that can take up HCO$_3^-$, so any HCO$_3^-$ that moves from the mucosal solution to serosal solution is a result of paracellular transport. Paracellular movement of HCO$_3^-$ has been established in the intestinal tract, and accounts for approximately 25% of basal secretion in the duodenum (Hogan et al., 1997; Allen et al., 1993), whereas the results for this experiment indicated that the passive flux was approximately 30% of the total HCO$_3^-$ flux. The measure of HCO$_3^-$ in the control tissue (non-reversed tissue), was therefore a combination of both active flux and passive flux of HCO$_3^-$ transport, where the active flux was due to HCO$_3^-$ movement through transporters. The forskolin response in reversed tissue, was significantly different from the control tissue, whereas the basal flux shows a trend that suggests an active flux was present. The active flux of HCO$_3^-$ secretion can be taken as the total flux measured in the control tissues minus the passive flux of the reverse tissues. Since the passive flux of HCO$_3^-$ is not equal to or greater
than the active flux, then the dissection was not causing significant damage to the tissue. Therefore, it could be concluded that electroneutral HCO$_3^-$ secretion was not affected by inflammation, and it might be possible that NBCe1, and HCO$_3^-$ secretion may not be as important in the proximal colon compared with other areas of the intestinal tract.

Due to the small effect of forskolin and DIDs in inflamed and non-inflamed tissue, we went on to further investigate the effect of forskolin and DIDs on electroneutral HCO$_3^-$ secretion. The $J_{\text{HCO}_3^- \text{sm}}$ of stimulated tissue does not significantly change in response to forskolin or DIDs. Neither does $J_{\text{HCO}_3^- \text{sm}}$ change significantly in control tissue. DIDs, however, showed a slight increase in HCO$_3^-$ flux, which was not expected because DIDs supposedly inhibits NBCe1 and should result in reducing HCO$_3^-$ secretion. However, DIDs lacks specificity in inhibiting NBCe1 (Huang et al., 2012), and may have a number of other effects, such as inhibiting the basolateral Cl$^-$/HCO$_3^-$ exchanger for one, and it is also known to induce release of intracellular Ca$^{2+}$, which increases Cl$^-$ secretion (Grubb et al., 1994). The addition of forskolin did not change the $J_{\text{HCO}_3^- \text{sm}}$ very much, but this is consistent with Yu et al., (2009), and Xiao et al., (2012a), who also observed very small changes in HCO$_3^-$ flux in response to forskolin. Also, the corresponding Ussing $I_{\text{sc}}$ measurements indicate an increase in electrogenic anion secretion in response to forskolin and reduction in response to DIDs. This means the effect of forskolin and DIDs remained the same, and that the tissue was still responsive during the pH stat flux measurements. However, the $pH$ stat measurements may indicate that HCO$_3^-$ is not stimulated to be secreted electroneutrally by forskolin.
4.6 $\text{HCO}_3^- \text{ secretion's effect on mucus}$

It has been demonstrated that there is reduced expression of NBCe1 in the inflamed proximal colon (Fan and Butt, unpublished results, see figure 1.3). If NBCe1 is involved in colonic $\text{HCO}_3^-$ secretion this should result in a reduced uptake of $\text{HCO}_3^-$. Therefore, this impaired $\text{HCO}_3^-$ uptake may reduce $\text{HCO}_3^-$ secretion, which is proposed to be responsible for reduced mucus expansion (Quinton, 2010) (Fig 1.1B). However, the Ussing $I_{sc}$ experiments have indicated that there is no appreciable stimulation of electrogenic $\text{HCO}_3^-$ secretion in the APC, therefore, the conclusion is, that electrogenic $\text{HCO}_3^-$ secretion is not involved with mucus expansion. The $pH$ stat experiments also indicate that electroneutral $\text{HCO}_3^-$ secretion is not stimulated to be secreted either, which means it is also not involved with mucus expansion. Two conclusions can be made from this; firstly, since there is no stimulation of electrogenic or electroneutral $\text{HCO}_3^-$ secretion, then it suggests that NBCe1 may not be driving $\text{HCO}_3^-$ for secretion, therefore, when NBCe1 expression is reduced in inflammation it would not matter in terms of $\text{HCO}_3^-$ secretion. Secondly, the mucus expansion model where $\text{HCO}_3^-$ is required for removing the $\text{Ca}^{2+}$ and $\text{H}^+$ shielding from mucin is in question, since there is no appreciable stimulated secretion of $\text{HCO}_3^-$, which means, it would not matter if $\text{HCO}_3^-$ secretion is impaired in inflammation or not.
4.8 Future Experiments

4.8.1 Investigate HCO$_3^-$ secretion in other intestinal areas

IBD affects many different areas of the intestinal tract. The results of this study do not appear to implicate a reduced electrogenic or electroneutral HCO$_3^-$ secretion in the APC or DPC of inflamed tissue. However, questions still remain about the electroneutral component of HCO$_3^-$ secretion, and the electrogenic component of the DPC. There is still the possibility that an impairment of HCO$_3^-$ secretion might be involved in other areas of the intestine that could be associated with IBD.

4.8.2 Further investigate the electroneutral HCO$_3^-$ secretion using pH stat

As mentioned, the pH stat does not differentiate between electrogenic and electroneutral HCO$_3^-$ secretion, so in order to accurately measure electroneutral HCO$_3^-$ secretion, one of two experiments could be performed. By using a CFTR inhibitor, such as CFTR(inh)-172 (Verkman et al., 2013), the electrogenic component of HCO$_3^-$ secretion will be inhibited, and this will allow for any HCO$_3^-$ flux measurements by the pH stat to be attributed to electroneutral HCO$_3^-$ secretion. The other option is to prevent electroneutral HCO$_3^-$ secretion by removing Cl$^-$ from the mucosal bathing solution, since the electroneutral HCO$_3^-$ transporter is an apical Cl$^-$/HCO$_3^-$ exchanger. This would allow the measure of electrogenic HCO$_3^-$ flux, and to determine what proportion of the HCO$_3^-$ flux measured in this study, is attributed to electroneutral HCO$_3^-$ flux.
5. 5. Conclusion

These experiments have provided insight into the electrogenic and electroneutral secretion of \( \text{HCO}_3^- \) in the proximal colon of IL10\(^{-/}\) mice. The Ussing short circuit experiments demonstrated that the forskolin stimulated electrogenic anion secretion that occurs in the APC is due to \( \text{Cl}^- \) secretion, and this is driven by a combination of basolateral NKCC1 activity and \( \text{Cl}^-/\text{HCO}_3^- \) exchange. The \( \text{HCO}_3^- \) utilized by the \( \text{Cl}^-/\text{HCO}_3^- \) exchanger is derived from metabolic \( \text{CO}_2 \). It was also unlikely that anion secretion in the crypt cells were affected by inflammation. The trends following the \( \text{pH} \) stat technique, allowed us to speculate the presence of an active flux and a possibility for reduction in electroneutral \( \text{HCO}_3^- \) secretion, although further testing is required. However, as it stands, the NBCe1 transporter may not play a significant role in \( \text{HCO}_3^- \) secretion in the APC, electrogenic and electroneutral \( \text{HCO}_3^- \) secretion are not stimulated to be secreted, and are unaffected by inflammation.
References


## Appendix

### NaCl/HCO$_3^-$—Ringer’s

#### APC

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### NaCl/ HCO$_3^-$—Ringer’s

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### HCO$_3^-$ buffered/Cl$^-$ free Ringer’s

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**HCO₃⁻ free/ NaCl Ringer's APC**

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