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The Actions of Ursodeoxycholic Acid and its Derivatives on Colonic Epithelial Transport and Barrier Function

A thesis presented to the Royal College of Surgeons in Ireland in part fulfilment of the degree of Doctor of Philosophy

By

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August 2014

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I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree (Ph. D), is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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List of Abbreviations

6α-MUDCA 6 alpha methyl ursodeoxycholic acid

ABST apical bile salt transporters

ACh acetyl choline

ADP adenosine diphosphate

Amph B Amphotericin B

ANOVA analysis of variance

ATP adenosine triphosphate

BA bile acid

BSEP bile salt export pump

CA cholic acid

CACC Ca^{2+} activated chloride channels

cAMP cyclic adenosine monophosphate

CCh carbachol

CD Crohn's Disease

CF Cystic Fibrosis

CFTR cystic fibrosis transmembrane conductance regulator

cGMP cyclic guanosine monophosphate

CDCA chenodeoxycholic acid

Con control

DAG diacylglycerol

DAPI 4'-6-Diaminidino-2-phenylindole

DC dendritic cells

DCA deoxycholic acid

DTT dithriothreitol

EDTA ethylenediaminetetraacetic acid

EGF epidermal growth factor

EGFR epidermal growth factor receptor

ELISA enzyme linked immunosorbent assay

ERK extracellular regulated kinase

FSK forskolin

FXR farnesoid X receptor

GI Gastrointestinal

GPCR G protein coupled receptors

h hour(s)

HEPES hydroxyethyl piperazine ethansulafonic acid

HPLC High Performance liquid chromatography

IBD inflammatory bowel disease

IEC intestinal epithelial cell

I_K K^+ currents

IL interleukin

IP_3 inositol-1, 4, 5- trisphosphate

I_{sc} short circuit current

JNK c-Jun NH2 terminal kinase

KCNN4 intermediate- current calcium activated potassium channel4

KCNQ1 voltage dependent potassium channel

LPS lipopolysaccharide

LCA Lithocholic acid

MAPK mitogen activated protein kinase

MDP muramyl dipeptide

MHC major histocompatibility complex

min minute(s)

miRNA microRNA

mRNA messenger RNA

muc2 mucin 2

NBCE1 Na^+/HCO_3^- co-transporter 1

NFκB Nuclear factor kappa beta

NHE Na^+/H^+ exchanger

NKCC1 Na-K-2 Cl cotransporter

OST Organic solute transporter

PBS phosphate buffered saline

PD potential difference

PI3K phosphoinositide-3 kinase

PIP_2 phophatidylinositol-4, 5-bisphosphate

PKA protein kinase A

PKC protein kinase C

PLC phospholipase C

PMSF phenylmethylsulfonimide fluoride

PPS physiological perfusion solution

PSC Primary Sclerosing Cholangitis

s second(s)

SCFA short chain fatty acids

SDS PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis

SGLT Na/glucose co-transporter

siRNA small interfering RNA

SRC steroid receptor co activator

ST_a heat-stable enterotoxin from E coli

STAT signal transducers and activators of transcription

TBST Tris Buffered Saline / Tween 20

TDCA taurodeoxycholate

TER transepithelial resistance

TG thapsigargin

TGFα transforming growth factor alpha

TI terminal ileum

TLR Toll-like receptor

TMEM16A transmembrane protein 16A

TPeA tetrapentylammonium chloride

UC ulcerative colitis

UDCA ursodeoxycholic acid

Abstract

Background: Intestinal disorders, including infectious diseases (ID), inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), are common in the European population and on a global scale and are typically associated with inflammation and/or dysregulated intestinal fluid movement, leading to the clinical endpoint of diarrhoea. Although the health and economic burden of diarrhoeal disease is tremendous, there is still a lack of safe and effective drugs for their treatment. Insults to the intestinal epithelium are critical in the pathogenesis of many of the above-mentioned intestinal disorders as these cells govern the transport and barrier functions of the intestine and also participate in initiation of inflammatory responses. As such, these cells represent excellent targets for the development of new drugs to treat intestinal diseases. Ursodeoxycholic acid (UDCA), the therapeutic component of bear bile, used in traditional Chinese medicine, also has a large evidence base and is commonly used in conventional medical practice to treat cholestatic disorders and hepatic inflammation. Other bile acids (BAs) are also known to exert regulatory effects on colonic epithelial transport, cell growth and immunity. Little however is known, to date, about the effects of UDCA and its metabolites on colonic epithelial transport function and immunity.

Methods: T_{84} cells, a colonic epithelial cell line used as a reductionist model of chloride (Cl^-) secretion, were cultured until they reached confluency and a trans-epithelial resistance of >1000 ohms.cm^{-2} was achieved. These polarised monolayers of T_{84} cells, resected colonic mucosa from C57 black mice, Sprague-Dawley rats and surgically resected sections or endoscopic biopsy specimens of human colon were mounted in Ussing chambers and changes in short circuit (I_{sc}) measured. This technique is reflective of changes in Cl^- secretion. Confocal imaging was used to visualise localisation of transport proteins. Western blotting and surface biotinylation were used to determine protein expression and abundance. Intracellular calcium (Ca^{2+}) was measured using Fura/ 2AM fluorescence and MetaFluor imaging. ELISA measurements were used to assess cytokine levels.

Results: The acute effects of UDCA on Cl^- secretion in colonic epithelial cell model, in treated mice and on *ex vivo* rat and human colonic tissues were examined.

UDCA, unlike other dihydroxy BAs, did not alter basal Cl^- secretion in the T_{84} colonic epithelial cell line. UDCA in fact exerted potent inhibitory actions in T_{84} cell monolayers, significantly attenuating responses to both Ca^{2+} and cyclic-AMP (cAMP) -dependent secretory agonists (Carbachol (CCh) and Forskolin (FSK) respectively, $p < 0.001$, $p < 0.001$, $n = 18$ [UDCA]: 500 μM, bilateral addition).

UDCA inhibited Cl^- secretion across colonic epithelial cells through attenuating activity of basolateral Ca^{2+}-dependent potassium (K^+) channels and the sodium/ potassium ATP-ase pump (Na^+/ K^+ ATPase; $p < 0.001$, $n = 7$, $p < 0.001$, $n = 7$, respectively). UDCA inhibited channel/ pump activity without altering surface expression or localisation of these proteins. UDCA also stimulated an acute rise in intracellular Ca^{2+}. This was due to influx of extracellular Ca^{2+} through membrane store operated Ca^{2+} channels (SOCCs) and L-type Ca^{2+} channels. In turn, this Ca^{2+} influx attenuated subsequent CCh-induced release of Ca^{2+} from intracellular stores, with a consequent inhibition of CCh-induced Cl^- secretion. This anti-secretory feedback mechanism involved activation of extracellular regulated kinase/ mitogen activated protein kinase (ERK-MAP kinase) and appeared independent of protein kinase C (PKC). This pathway appeared to be a key mechanism by which UDCA inhibited Ca^{2+}-dependent secretion. UDCA also attenuated cAMP-induced Cl^- secretory responses, without altering intracellular levels of this 2nd messenger.

Paradoxical to these anti-secretory actions *in vitro*, secretagogue-induced Cl^- secretion was enhanced in the stripped colons of mice treated with UDCA *in vivo*. This appeared due to its rapid metabolism by colonic bacteria to lithocholic acid (LCA), a pro-secretory BA. Increased LCA levels were noted in the caecal contents of mice treated with UDCA, when compared to control, supporting this hypothesis.

6α-methylursodeoxycholic acid (6α- MUDCA), a 6-methylated derivative of UDCA, a metabolically stable analogue that is completely resistant to bacterial dehydroxylation, retained the anti-secretory effects of UDCA noted *in vitro*, inhibiting Cl^- secretion across voltage-clamped colonic tissues and T_{84} cell monolayers. Caecal LCA levels in 6α-MUDCA treated mice were similar to controls and significantly less than UDCA-treated animals.

UDCA also exerted specific and unique actions on transport function in intact human tissue, stimulating an initial rise in basal Cl^- secretion involving activation of muscarinic receptors.

Finally, UDCA was also noted to exert both anti-inflammatory and cytoprotective effects on T_{84} cells and human colonic epithelium. UDCA reduced toll like receptor (TLR)-stimulated cytokine release and was also found to maintain epithelial barrier integrity as demonstrated by trans-epithelial resistance measurement and through inhibition of apoptosis.

Conclusion: UDCA has predominantly anti-secretory and anti-inflammatory effects in the colonic epithelium. Through direct delivery, modification of its structure or targeting of the molecular targets identified in these studies, these beneficial effects could be utilised to treat inflammatory and secretory colonic diseases.

Chapter 1

General Introduction

1.1 Structure, anatomy and functions of the colon

The digestive tract, also called the alimentary canal or gastrointestinal (GI) tract, consists of a long continuous tube that extends from the mouth to the anus. It includes the mouth, pharynx, oesophagus, stomach, small intestine, and large intestine. The large intestine extends for approximately 1200 cm and consists anatomically of the caecum and appendix, ascending, transverse, descending and sigmoid colon, the rectum and anus. The primary function of the digestive system is to prepare nutrients for absorption and utilization by cells of the body. More broadly, the functions of the GI tract can be divided into 4 basic processes: digestion, motility, transport, and barrier function (Hall, 2006). Studies in this thesis focus on the colon, which forms the main part of the large intestine (

Figure **1.1**).

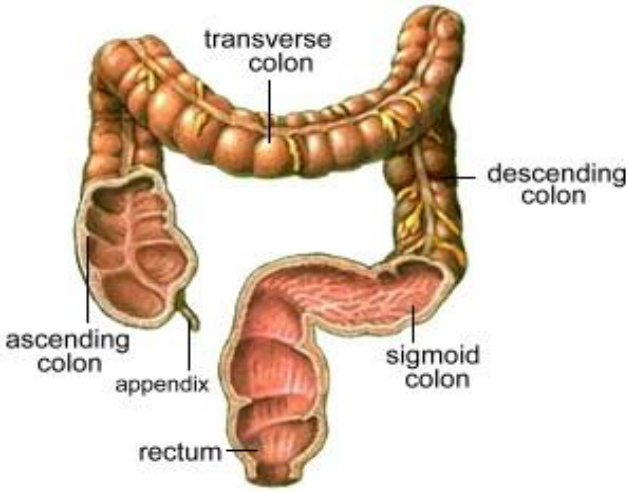


Figure 1.1: The human colon. The colon is comprised of 4 anatomically distinct sections. The terminal ileum enters the caecum which is the most proximal section of the large bowel. The ascending colon extends up the right hand side of the abdomen to become the transverse colon which extends across the midline to become the descending colon after the splenic flexure. The descending colon then loops around the pelvis to form the so-called sigmoid colon, which distally becomes the rectum and anus (Feagan, 2007,Eutamene, 2003,Willis, 1996)**.**

1.1.1 Functional Anatomy of the Colon

Although the colon is divided anatomically into 4 regions, functionally it is more easily classified into the proximal and the distal colon, which operate distinctly for the purposes of fluid transport regulation. The primary function of the proximal colon

(ascending, transverse and descending colon) is fluid absorption, while that of the distal colon (sigmoid colon and rectum) is storage of faeces (Feagan, 2007).

1.1.2 The colonic wall

The colonic wall, like the rest of the GI tract is composed of 4 types of tissue; epithelium, smooth muscle, neural and connective tissue, organised into concentric layers, which are in turn termed the mucosa, submucosa, muscle layer (intramural muscularis) and serosa (Figure 1.2).

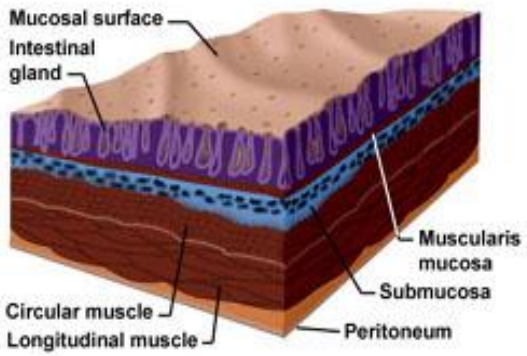


Figure 1.2: The colonic wall. The colonic wall, like the rest of the GI tract consists of the mucosa, submucosa, intramural muscularis (consisting of both circular and longitudinal muscle layers) and lies adjacent to the peritoneum.

The mucosa is the principal interface of the colon with the external environment. It consists of the epithelium, lamina propria, and the muscularis mucosa. Epithelial cells differ throughout the GI tract, depending on function. For example in the mouth, anus and proximal oesophagus, there is a squamous epithelium which has protective functions. In the stomach and small intestine columnar epithelia predominate which secrete mucins and enzymes to protect the mucosa and aid digestion. In the colon, columnar epithelia predominate which are primarily involved in barrier function and fluid and electrolyte transport. The cells of the colonic epithelium may be divided into columnar epithelial cells, goblet cells, which produce mucins, and enterochromaffin or endocrine cells. Columnar and goblet cells comprise approximately 95% of the total surface area of the colon, while enterochromaffin cells comprise the remaining 5%. Underlying the epithelial layer is the lamina propria. This layer consists of connective tissue, which contains the blood

and lymphatic vessels that transport substances, such as nutrients, oxygen, and hormones, to and from the colon. It also contains nerves and immune cells which are important in regulating epithelial function.

Underlying the lamina propria is the muscularis mucosa, which supports the mucosa and forms a barrier between it and the submucosa. The intramural muscularis consists of circular and longitudinal smooth muscle and is responsible for peristalsis. The intramural plexus is divided into the myenteric and sub-mucosal plexuses and it is these nerve networks that control contractions of the intestinal wall, and which co-ordinate motility with intestinal transport. The connective tissue serosa surrounds the entire intestinal tract and is apposed to the peritoneum (Reed, 2009).

1.1.3 The colonic crypt.

Colonic enterocytes can be broadly divided into crypt and surface cells. Crypt cells are not well differentiated and have the highest proliferative capacity. Production and delivery of new epithelial cells from stem cells is one of the main functions of the crypts (Bjerknes, 2005). In contrast, surface cells are much more differentiated (Figure 1.3). Cells migrating from the crypt to the villi undergo apoptosis at the tip of the villi in a process called cell shedding. In humans, cells are shed by a mechanism involving loss of contact with the extracellular matrix and survival signals from β integrins, resulting in the onset of apoptosis (Bullen, 2006).

1.1.4 Cell types within colonic crypts

Stem cells: The human GI tract undergoes continuous self-renewal throughout life (Stappenbeck, 1998). Stem cells, specialized epithelial cells characterized by the ability for self-maintenance, provide the basis for ongoing cell replacement. These cells divide asymmetrically, producing one stem cell, which remains multi-potent and undifferentiated, and one daughter cell, which is committed to differentiation (Bach, 2000). Committed daughter cells undergo several additional rounds of cell division while migrating from the proliferative to the differentiated compartment. Upon differentiation, cells lose the ability to divide and eventually die. The mature gastrointestinal system therefore comprises, at all times, both undifferentiated, pluripotent stem cells, and differentiated, functional, epithelial cells.

In the colon, stem cells are located at the base of the crypts, with proliferation occurring in the lower third of these epithelial folds. Epithelial cells migrate in ordered cohorts towards the luminal surface, differentiating to form one of 3 cell types: colonocytes, goblet cells, and enteroendocrine cells. At the villus tip, cells enter a death program characterized by senescence and/or apoptosis, and are sloughed into the lumen (Merritt., 1997). Figure 1.3 illustrates the anatomy of the colonic epithelium.

Colonocytes and Goblet cells: More than 80% of all epithelial tissue in the colon is comprised of colonocytes (Chang,1971). A primary function of the simple columnar colonic epithelium is to transport fluid and electrolytes to and from the body. The epithelium has the capacity for both absorption and secretion and dysregulation of these processes may result in diarrhoeal disease. Epithelial cells also form a protective barrier that is important in innate immunity. Goblet cells create mucus, trefoil proteins, and other factors that help protect the intestinal mucosa from injury and facilitate tissue repair (Deplancke, 2001). In the adult mouse descending colon, cells of this lineage form about 16 % of the total crypt cell population (Karam, 1999). Specific markers, such as Tff3 and Muc2, for intestinal goblet cells, have been identified and targeted ablation of these have yielded new insights into goblet cell function (Itoh, 1999, van Klinke, 1999). For example, mice deficient in Muc2, the most abundant murine intestinal mucin, display aberrant intestinal crypt morphology, with altered cell maturation and migration (Velcich, 2002).

Endocrine cells: Endocrine cells are found scattered as individual cells within the gut epithelium from the stomach to the colon and represent the largest population of hormone-producing cells in the body (Rehfeld, 1998). Unlike many hormone-producing cells in different glands that differentiate early in life and turnover slowly, enteroendocrine cells actively self-renew and differentiate throughout life, from a large reservoir of stem cells. As mature enteroendocrine cells migrate to the tips of the villi, they presumably undergo apoptosis and are extruded into the lumen (Simon, 1995). In the colon, peptide YY, glucagon-like peptide (GLP)-1, cholecystokinin, and neurotensin are co-expressed in some enteroendocrine cells. Likewise, serotonin-expressing cells often co-express substance P but never the 4 hormones listed

above, leading to the hypothesis that there are 2 major branches for enteroendocrine cell differentiation in the colon (Roth, 1992).

In summary, crypt stem cells give rise to all 4 cell types of the colonic epithelium. Math1 expression restricts cells to the secretory lineage (Yang, 2001), neurogenin 3 restricts cells to the endocrine lineage (Jenny, 2002), whereas the transcription of specific hormones is regulated by several late-acting transcription factors, such as Pax4 (Larsson, 1998), Pax6 (Trinh, 2003), and BETA2 (Mutoh, 1997).

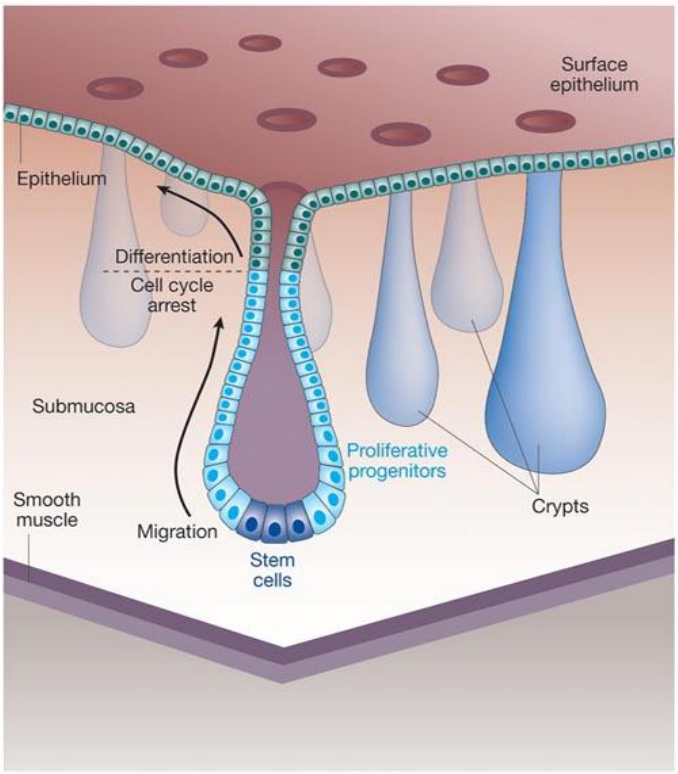


Figure 1.3: The anatomy of the colonic epithelium. The colonic epithelium creates invaginations into the submucosa. These are described as crypts while the surface cells constitute the surface epithelium. At the base of the crypts, undifferentiated stem cells are present which proliferate and migrate along the crypt to the surface epithelium, thereby creating a constant source of replacement for surface cells (Figure from Reya, 2005).

1.1.5 Colonic crypts and fluid transport.

The traditional paradigm of colonic fluid and electrolyte transport suggests that there is spatial separation of absorptive and secretory processes to surface and crypt cells, respectively. However, more recent studies of isolated micro-perfused colonic

crypts revealed constitutive Na^+-dependent fluid absorption, with secretion being regulated by neuro-humoral agonists (Robert, 2001). Epithelial cells differentiate from a secretory to an absorptive phenotype as they migrate along the crypt. Other studies also support the notion that crypt function is more dynamic than previously suggested. For example, the cholinergic agonist, carbachol (CCh), rapidly (within 10 min) reduced cell volume along the entire crypt/villus axis and promoted Na^+/H^+ exchanger 3 (NHE_3) internalization into early endosomes. In contrast, CCh induced membrane recruitment of NKCC1 and cystic fibrosis transmembrane regulator (CFTR) in all crypt and villus enterocytes, NKCC1 in all goblet cells, and NBCE1 in all villus enterocytes. These observations support regulated vesicle trafficking in regulation of Cl^- secretion by goblet cells and Cl^- and HCO_3^- secretion by villus enterocytes during the transient phase of cholinergic stimulation. In essence, these findings suggest that there is a degree of functional plasticity and synchronization of ion transport function along the crypt-villus axis, allowing the epithelium to express either a predominantly secretory or absorptive function in a given situation, and this is likely to be relevant in intestinal disease (Jakab, 2011).

1.2 Fluid transport and the colonic epithelium

The main functions of the intestinal epithelium are essentially 3-fold: it absorbs nutrients from digested food, it acts as a protective barrier preventing entry of noxious substances from the lumen into the body, and it performs a key role in the regulation of fluid and ion transport. In the healthy intestine, fluid absorption normally predominates, which allows conservation of the large volumes of fluid that enter the intestine each day. This fluid absorption is driven predominantly by active transport of sodium. However, the colon is also capable of secreting water, with the principal driving force being secretion of Cl^- ions. This process is essential for appropriate hydration of the mucosal surface and for flushing noxious substances away from the mucosa. This finely-tuned balance between absorptive and secretory processes can be disrupted in disease states, such as infectious diseases, inflammatory bowel disease, coeliac disease, or other malabsorptive diarrhoeas, causing overexpression of secretion or under expression of absorption, leading to the clinical manifestation of diarrhoea. While some diarrhoeal diseases are characterised as being malabsorptive

and others as being secretory, in truth it is likely that most diarrhoeal diseases involve dysregulation of both processes.

1.2.1 Water transport

The colonic epithelium is considered to be a tight epithelium with a high electrical resistance and low paracellular water permeability (Ma, 1999). Water transport is driven by the establishment of osmotic gradients and is paracellular, through tight junctions, or through a family of specialised water channels called aquaporins. Although they are expressed in the epithelium, it is still unclear how much water transport occurs through these channels in the colon (Takata, 2004). Water transport in the small intestine can also occur trans-cellularly through sodium-dependent glucose co-transporters (SGLTs), with up to 5 L of water being absorbed in this manner each day (Keely, 2009).

1.2.2 Colonic fluid absorption

Under physiological conditions, the caecum and proximal colon absorb around 1.5 L of electrolyte-rich fluid per day, which accounts for roughly 90% of the salt and water entering the proximal colon (Debongnie, 1978). The result is a daily faecal electrolyte excretion of less than 5 mM Na^+, 2 mM Cl^-, 9 mM K^+ (Warth, 2000), and approximately 5 mEq (milli- equivalents) HCO_3^- (Caprilli, 1986). This is accomplished by several different processes, including electrogenic and electroneutral absorption of Na^+ and Cl^-, active absorption of K^+ via luminal K^+ pumps, and absorption of short-chain fatty acids (SCFAs) produced by the luminal bacterial flora (Ikuma, 1998, Vidyasagar, 2005, Musch., 2009).

Electroneutral Absorption of NaCl: The majority of NaCl absorption occurs between meals and is driven electroneutrally by the combined activities of Na^+/H^+ and Cl^-/HCO_3^- exchange. Five isoforms of the Na^+/H^+ exchangers (NHE) have been identified to date (Donowitz, 1996). In the colon, electroneutral absorption of NaCl occurs through NHE 1, 2 and 3 (Zachos, 2005), though it is widely accepted that NaCl absorption occurs predominantly through coupling of NHE3 and the Cl^-/HCO_3^- exchanger SLC26A3 (alias downregulated in adenoma, or DRA) (Binder, 1987).

Electrogenic Na^+ absorption: This occurs through the epithelial sodium channel, ENaC. This amiloride-sensitive channel has 3 subunits; α, β and γ, the expression of which can be regulated through ubiquitination and degradation mediated by E3 ubiquitin protein ligases, such as Nedd 4-2 (Zhou , 2005). In the colonic environment there are multiple factors which may acutely regulate Na^+ absorption through ENaC. These include G protein-coupled receptors (GPCRs), tyrosine kinase receptors, second messengers and protein kinases. In addition to this, a relationship exists between regulation of Na^+ absorption and Cl^- secretion. For example, activation of Cl^- secretion by cyclic AMP (cAMP) can result in inhibition of amiloride-sensitive Na^+ currents (Ecke, 1996), while CFTR activation has also been shown to inhibit ENaC currents (Ji, 2000).

Sodium-coupled glucose transporters and Na^+ absorption: Secondary active glucose transport occurs through at least 4 members of the SLC-5 gene family. These include SGLT-1 and SGLT-2, which play important roles in intestinal absorption and renal reabsorption of glucose. Genetic disorders of SGLTs include glucose-galactose malabsorption and familial renal glycosuria. SGLT-1 plays a central role in oral rehydration therapy, which is commonly used to treat secretory diarrhoea in conditions such as cholera infection. Interestingly, SGLTs are not only present in the small intestine and kidneys but also in other areas of the body, such as the hippocampus in the central nervous system. Na^+ and sugar co-transport by SGLT-1 is referred to as secondary active transport because the driving force for the process, i.e., Na^+ gradients, are maintained by active transport through the Na^+/K^+ -ATPase pump. Quite simply, the direction and rate of glucose transport by SGLT-1 are functions of the direction and magnitude of Na^+ gradients established across the plasma membrane by Na^+/K^+-ATPase pumps (Takata, 1996).

Active K^+ absorption: Active K^+ absorption occurs in the colon through the activity of the apical H^+/K^+ -ATPase pump (Sorenson 2010). This is in contrast to the passive absorption of K^+ which occurs in the small intestine (Keely, 2009).

SCFA absorption: Absorption of SCFAs, which are produced by bacterial fermentation of carbohydrates, provides energy to the colonic epithelium and

regulates Na^+ absorption. SCFAs are absorbed by colonic epithelial cells and stimulate Na^+- dependent fluid absorption via a cyclic AMP-independent process which involves Na^+- H^+ , Cl^- and HCO_3^- exchange (Keely, 2009, Binder, 2010).

1.2.3 Colonic Fluid secretion

In addition to absorption, the colonic epithelium also secretes fluid and electrolytes**.** As previously mentioned, secretion and absorption are not strictly compartmentalized, but secretion is believed to take place primarily in the crypts (Welsh, 1982). Electrolyte secretion is the driving force behind fluid secretion in the colon and the primary ions involved are Cl^-, HCO_3^- and K^+ (Kunzelmann, 2002, Field, 2003). Many of the mechanisms involved in electrolyte secretion have been well-characterized (Rajendran, 1998, Barrett, 2000, Sorensen, 2010).

Bicarbonate secretion: HCO_3^- is secreted into the intestinal lumen in order to neutralise HCl from the stomach and the organic acids produced by bacterial fermentation in the large intestine (Binder, 2005). Several mechanisms for HCO_3^- secretion are present in the colon, including a Cl^--dependent mechanism linked to apical Cl^-/HCO_3^- exchange, a cAMP-dependent CFTR-mediated pathway, and also an apical membrane SCFA/HCO^{3-} exchange mechanism (Vidyasagar, 2004).

K^+ secretion: It is generally assumed that a mechanism for K^+ exit is essential to maintain cells in the hyperpolarized state, thus promoting Cl^- secretion. Recent studies have shown that an isoform of the Ca^{2+}-activated K^+ channel, KCNN4, is involved in K^+ secretion in the colon (Barmeyer, 2010). Thus, agonists which promote Ca^{2+}-activated Cl^- secretion can also increase K^+ secretion in the colon (Sorensen, 2010). cAMP induces both active Cl^- and active K^+ secretion in mammalian colon (Bachmann, 2011).

Cl^- secretion: Cl^- secretion is the primary driving force for fluid secretion in the colon and the mechanism underlying this process has been well-elucidated. The energy for Cl^- secretion is derived from the activity of the Na^+/K^+-ATPase pump on the basolateral membrane. This transporter pumps out $3Na^+$ in exchange for $2K^+$ thereby maintaining intracellular Na^+ at a low level and thus creating a favourable gradient for Na^+ entry. This occurs via NKCC1 co-transporters, which also bring in 2 Cl^- along

with 1 K^+. Both Na^+ and K^+ are recycled via Na^+/K^+-ATPase pumps and K^+ channels, respectively. Thus, the net activity of these basolateral transporters is to accumulate Cl^- intracellularly above its electrochemical equilibrium creating a gradient for its exit across regulated channels in the membrane. The CFTR Cl^- channel is the most abundant Cl^- channel in intestinal epithelia but other types of Cl^- channel are also known to exist (e.g. the Ca^{2+}-activated Cl^- channel, TMEM16A). Cl^- secretion into the lumen creates an electrical gradient for cations, particularly Na^+, to follow by a paracellular route through the tight junctions that hold the cells together at the apical membrane. This net movement of salt into the lumen creates the osmotic driving force for fluid secretion to occur (Figure 1.4).

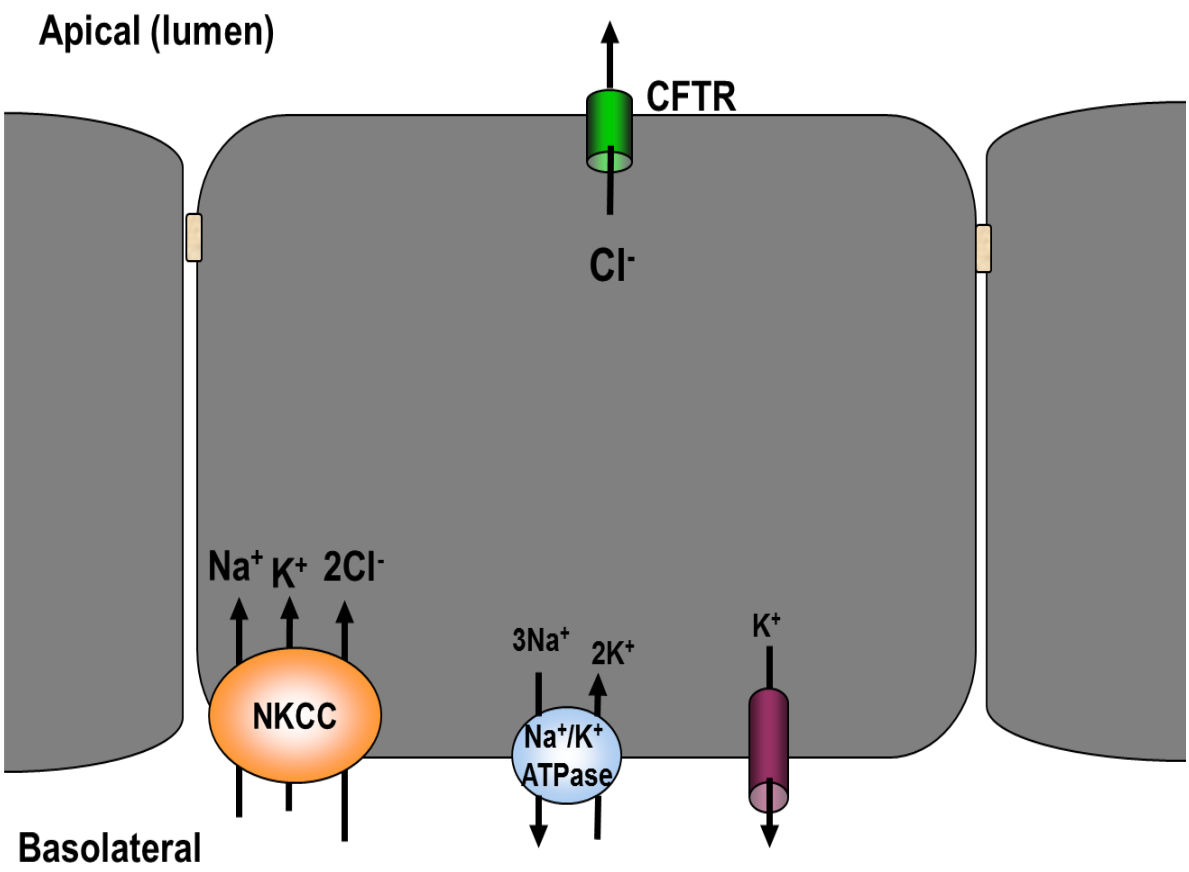


Figure 1.4: Cl^- secretion in the colon. The energy for Cl^- secretion is derived from Na^+/K^+ ATPase pump activity, which pumps Na^+ from the cell in exchange for K^+. This driving force allows Cl^- to enter the cell through the $Na^+/K^+/Cl^-$ co-transporter (NKCC1), which is secreted, apically through Cl^- channels, most notably CFTR. K^+ is recycled through channels in the basolateral membrane.

1.2.4 Transport proteins of the Cl^- channel secretory pathway

i) Na^+/K^+-ATPase pumps: Na^+/K^+-ATPase pumps generate the electrochemical gradient for Na^+ and K^+ movement across the basolateral plasma membrane. The energy for this process is derived from the hydrolysis of ATP to ADP (Barrett, 2000, Kato, 2011). The Na^+/K^+-ATPase pump, which consists of 2 subunits, the catalytic α subunit and the regulatory β subunit, pumps 3 Na^+ from the cell in exchange for 2 K^+ ions with each cycle of ATP cleavage. These subunits can exist in various isoforms and in colonic epithelia, the α1 and β1 isoforms are preferentially expressed (Shanbaky,1995). Several studies have suggested that Na^+/K^+-ATPase pump activity can be regulated through altered trafficking of the α subunit to the cell membrane (Chow, 1995, Bystriansky, 2007). Mitochondrial ATP production is central to pump activity and reduced ATP production or availability can alter pump activity, which may account for the reduced pump activity that occurs in conditions of hypoxia (Papandreou, 2006).

Recently, a number of membrane associated regulatory proteins for the Na^+/K^+-ATPase have been identified. These include the FXYD family of which there are 7 members, several of which are expressed in the colon (Saito, 2010).These proteins can alter pump activity without altering surface expression (Garty, 2006). Intracellular regulators, such as translationally controlled tumour protein (tctp) and sorting nexin 6 (SN6), are also emerging as important regulators of the pump (Yoon, 2006). Another novel modulator of the Na^+/ K^+-ATPase is MONaKA, which has been shown to inhibit pump activity in brain and cultured astrocytes, and which studies from our group have shown to be also expressed in colonic epithelia (Ward 2011; Mao, 2005).

ii) Basolateral K^+ channels: These channels mediate K^+ efflux across the basolateral membrane and, by hyperpolarising the cell, create a driving force for Cl^- secretion to occur. Regulation of K^+ channels can involve many factors, including phosphorylation (Ewald, 1985), changes in cytosolic Ca^{2+} concentrations (Gamper, 2005), and ubiquitination (Rajan, 2005). There are 2 main types of basolateral K^+ channel involved in colonic Cl^- secretion, the cAMP-dependent channel, KCNQ1, and the Ca^{2+}-activated channel, KCNN4 (Matos, 2007). Basolateral K^+ channels have also been reported to be coupled to apical H^+/K^+ activity in rat distal colon via

intracellular pH (Ikuma, 1998), which would result in trans-epithelial K^+ movement. Na^+ absorption is also dependent on K^+ channel function, as Na^+/K^+-ATPase activity in pumping Na^+ across the basolateral cell membrane requires K^+ recirculation (Turnheim, 2002).

Ca^{2+}-dependent K^+ channels have been identified in single-channel recordings from the human intestinal epithelial cell line, T_{84}, and are inwardly rectifying, with a K^+ conductance of 14–32 pS (Devor, 1997, Devor, 1996). The pharmacological and biophysical properties of the epithelial Ca^{2+-}dependent K^+ current are almost identical to those of the intermediate conductance Ca^{2+-}activated K^+ channel gene, IK1 (Kondoh, 1997). The molecular identity of cAMP-dependent K^+ conductances became clearer when it was observed that in colonic epithelial cells it could be inhibited by blockers of KCNQ K^+ channels, such as chromanol 293B (Warth, 1996). However, the properties of heterologously expressed KCNQ channels are distinct from those of cAMP-sensitive epithelial K^+ currents. For example, KCNQ1 currents are relatively slowly activating and voltage-dependent, whereas native epithelial currents are instantaneous and have a linear I-V relationship (Buschmann , 2000). These apparent discrepancies may have been resolved, however, with the discovery that KCNQ1 co-assembles with an accessory subunit, KCNE3, in colonic epithelial cells. The properties of K^+ currents formed by co-assembly of KCNQ1/KCNE3 closely resemble those of native epithelial cAMP-sensitive K^+ currents (Buschmann , 2000).

<u>Ca^{2+}-activated K^+ channels:</u> The KCNN4 protein is part of a potentially heterotetrameric voltage-independent potassium channel that is activated by intracellular Ca^{2+}. Activation of KCNN4 causes membrane hyperpolarization, which promotes Cl^- secretion. Observations by Kumar *et al,* indicate that mucosal KCNN4 channels are capable of driving agonist-induced anion secretion mediated via CFTR and CaCCs (Kumar, 2010).

<u>KCNQ1:</u> KCNQ channels conduct the cAMP-regulated K^+ current present in colonic crypt cells. In the colon, KCNQ1 is thought to assemble with a member of the KCNE family, KCNE3, which shows overlapping mRNA distribution in intestinal tissue. On expression with KCNE3, an instantaneously activating current is seen with a linear

current – voltage relationship. As mentioned above, the KCNQ1 + KCNE1 current is inhibited by chromanol 293B and enhanced by cAMP (Buschmann, 2000). The channel-blocking activity of TEA has also been studied (Buschmann, 2000), showing that the sensitivity of the homo-multimeric channels is: KCNQ2 > KCNQ4 > KCNQ1 > KCNQ5 > KCNQ3.

iii) NKCC1: NKCC1 is a co-transporter that is expressed on the basolateral membrane of epithelial cells all along the GI tract. It was first identified as a 175-kDa protein in the intestine of the winter flounder (Musch, 1982) and constitutes the main basolateral Cl^- uptake pathway into enterocytes. Transport through NKCC1 is electroneutral as it co-transports 2 Cl^- with 1 Na^+ and 1 K^+ (Barrett, 2000). NKCC1-deficient mice exhibit significantly reduced agonist-stimulated I_{sc} in colon (Flagella , 1999, Bachmann , 2003). NKCC1 is phosphorylated by protein kinase A (PKA), making it sensitive to intracellular cAMP levels (Kurihara, 2002). Consistent with the concept of the crypt as the primary site of secretion, NKCC1 is most predominantly expressed at the crypt base (Reynolds, 2007). There is substantial evidence that NKCC does not merely passively respond to secretion-related events, but that it is actively regulated by a number of pathways. In mice lacking the main apical Cl^- exit pathway CFTR, NKCC activity is enhanced by increases in intracellular cAMP and this occurs without changes in its expression level. Phosphorylation and intracellular Ca^{2+} levels have been shown to regulate colonic NKCC (Del Castillo, 1995). Furthermore, protein kinase C (PKC) activation inhibits NKCC in T_{84} cells (Matthews, 1993), and opposes the action of cAMP on the co-transporter (Matthews, 1995).

(iv) CFTR:

CFTR is a glycoprotein expressed at the apical membrane of colonic epithelial cells. It provides the main pathway for Cl^- exit into the lumen. This channel has been noted to be defective in cystic fibrosis (CF), resulting in a significant reduction in Cl^- secretion across airway and intestinal epithelia of CF patients (Lamprecht, 2005). The importance of CFTR to normal colonic function has been demonstrated by studies using biopsies from both normal and CF patient rectum. These studies have noted that both cAMP and Ca^{2+}-dependent Cl^- secretion are markedly absent in CF tissue. In turn, this leads to the clinical manifestation of meconium ileus or intestinal obstructive syndrome, where hard faeces become impacted in the dehydrated

intestinal lumen. Opening of CFTR is primarily regulated by cAMP-dependent PKA, but protein kinase G and protein kinase C (PKC) are also involved. CFTR, like the other transporters already discussed is also regulated by rapid trafficking from sub-apical membrane vesicles to the cell surface (Kunzelmann, 2002).

(v) Ca^{2+} activated Cl^- channels:

Strong evidence suggests that there is a Ca^{2+}-dependent channel involved in Cl^- secretion in respiratory epithelia, and more recently Ca^{2+}-activated channels have been implicated in Cl^- secretion in the intestine. However, controversy still exists as to the contribution of these channels to Cl^- secretion in the intestine and to their relevance for colonic epithelial transport. While cAMP-dependent Cl^- secretion by CFTR has been well examined, detailed analysis of epithelial Ca^{2+}-dependent Cl^- secretion has been hampered by a lack of a molecular counterpart, which has remained elusive. However, one candidate is TMEM16A, as it has been shown that TMEM16A knockout mice lack Ca^{2+}-activated Cl^- secretion in the colon (Ousingsawat, 2009). Molecular identification of TMEM16A has provided a fundamental step in understanding Ca^{2+}-dependent Cl^- secretion in epithelia, but its physiological role in native epithelial tissues remains largely obscure. Data from our group has implicated EGF in regulation of Ca^{2+}-dependent Cl^- conductances and TMEM16A expression in intestinal epithelia by a mechanism involving sequential activation of PI3K and PKCä (Mroz, 2012).

1.2.5 Intracellular Regulation of Cl^- secretion

The activity of the proteins constituting the Cl^- secretory pathway is regulated by an array of extracellular factors that typically act by binding to cell surface receptors, the most important of which with respect to regulation of Cl^- secretion are the G protein-coupled receptors (GPCRs). The G_qPCRs and G_sPCRs sub-types of GPCR are particularly relevant to regulation of Cl^- secretion in the colon since they elevate levels of intracellular Ca^{2+} and cAMP, the more prominent of the pro-secretory 2nd messengers. Figure 1.5 and Figure 1.6 depict the intracellular mechanisms underlying Ca^{2+} and cAMP-dependent Cl^- secretion in colonic epithelial cells. However, it is also clear that crosstalk exists between the 2 pathways. In fact synergism exists between cAMP- and Ca^{2+}-dependent mechanisms of Cl^- secretion. In Ca^{2+}-activated Cl^- secretion, stimulation of G_qPCRs (with CCh or other Ca^{2+}-

dependent agonists) causes activation of phospholipase C (PLC) through coupling of G_qPCRs with $G\alpha$ protein (G_q/ 11) subunits (See Figure 1.5). PLC activation then leads to degradation of PIP_2 into DAG and IP_3. These events lead to the activation of PKC and increased levels of Ca^{2+} by release from intracellular stores. This release of Ca^{2+} then may stimulate both apical Ca^{2+}-dependent Cl^- channels and basolateral Ca^{2+}-activated K^+ channels (KCNN4). IP_3 production also causes generation of IP_4 which downregulates Cl^- secretion possibly through inhibiting CaCCs, thereby providing an intrinsic shut off mechanism for the process (Dutta, 2008). Activation of PKC, downstream of DAG production and elevations in intracellular Ca^{2+}, can have both positive and negative actions on Cl^- secretion (Hirota, 2006).

Activation of G_sPCRs, with associated increases in intracellular cAMP levels, increase Cl^- secretion through activation of CFTR, KCNQ1 and NKCC1 (Hirota, 2006). Activation of adenylate cyclase (AC) increases intracellular cAMP levels leading to parallel activation of PKA and EPac1 (See Figure 1.6). EPac is an acronym for the exchange proteins activated directly by cyclic AMP, a family of cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs) that mediate PKA-independent signal transduction properties of cAMP (Holz, 2006). PKA increases Cl^- secretion through activation of CFTR. EPac 1 however, activates Rap2, and subsequently PLC, to cause release of DAG and IP_3 through degradation of PIP_2. In turn, this causes release of Ca^{2+} from intracellular stores and PKC-dependent activation of Cl^- channels. The overall result is Cl^- secretory responses that are enhanced over those mediated by CFTR alone (Hoque, 2010).

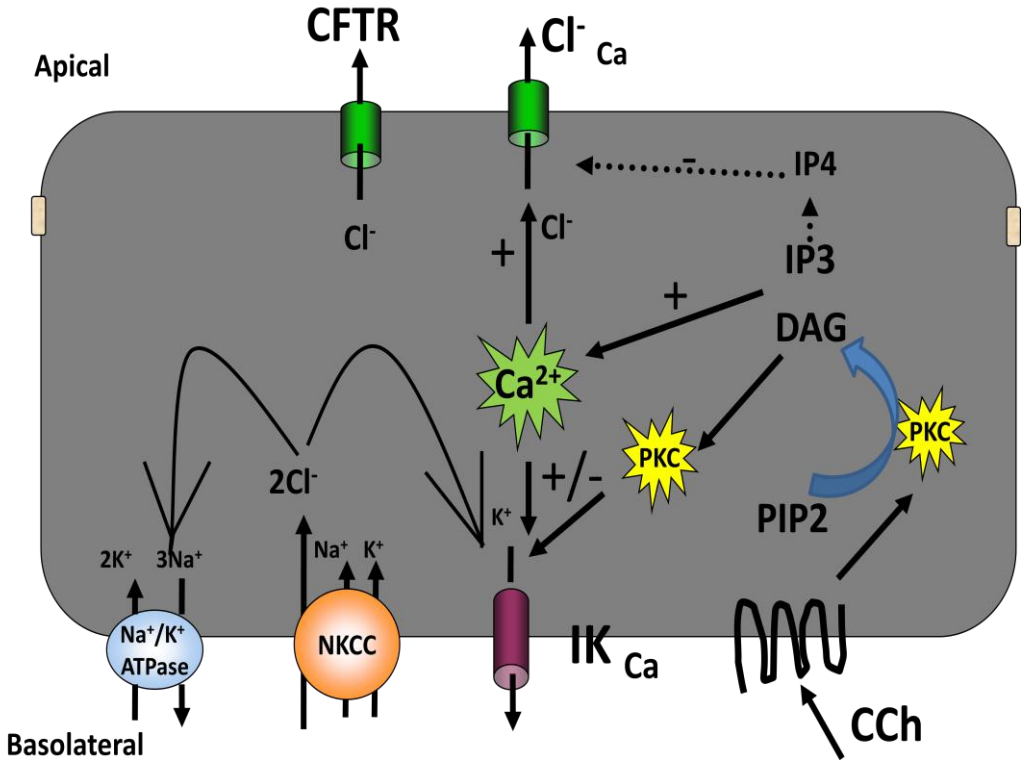


Figure 1.5: Signalling pathways involved in Ca^{2+}-dependent colonic epithelial Cl^- secretion. Stimulation of G_qPCRs (e.g. with the muscarinic agonist, CCh) causes activation of phospholipase C (PLC) through coupling of G_qPCRs with G_α protein (G_q/11) subunits. PLC activation leads to degradation of PIP_2 into DAG and IP_3. These events lead to the activation of PKC and increased levels of Ca^{2+} by release from intracellular stores. IP_3 production also causes generation of IP_4 which downregulates Cl^- secretion, possibly through inhibiting CaCCs, while PKC can have both positive and negative actions on Cl^- secretion (Hirota, 2006).

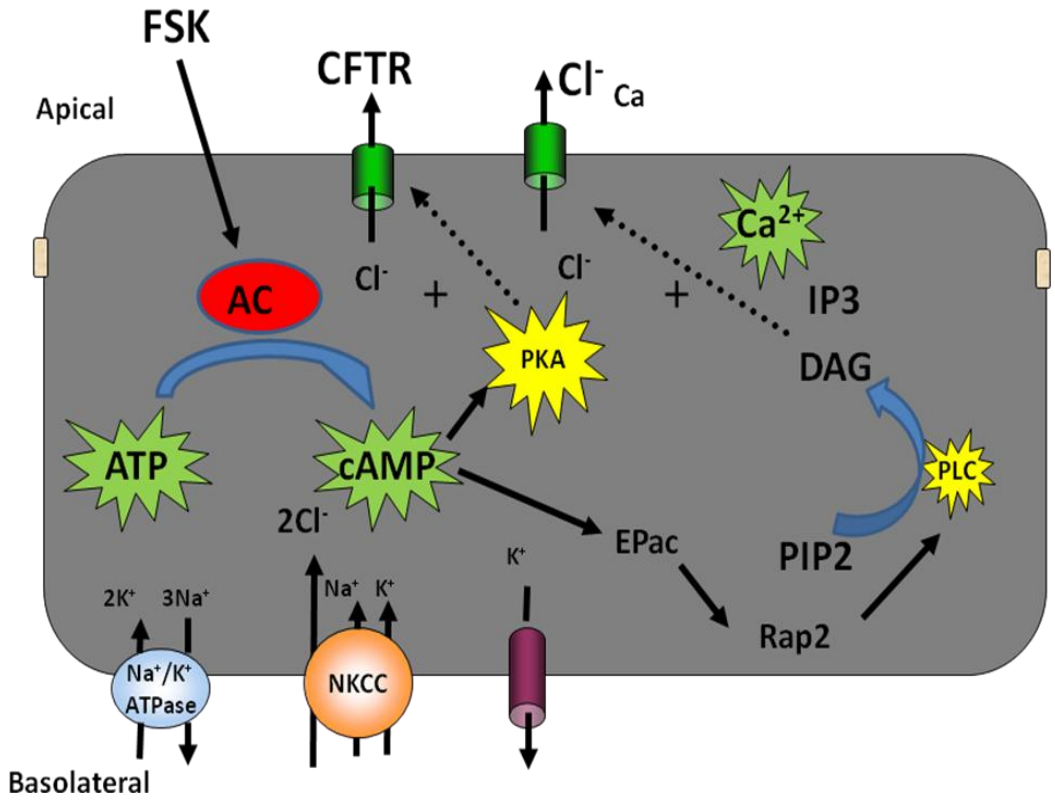


Figure 1.6: Signalling pathways involved in cAMP-dependent colonic epithelial Cl^- secretion Increases in intracellular cAMP levels stimulate Cl^- secretion through activation of CFTR, KCNQ1 and NKCC1. Activation of adenylate cyclase, increases intracellular cAMP levels leading to parallel activation of PKA and EPac1 (Holz et al., 2006). PKA increases Cl^- secretion through activation of CFTR. EPac 1 activates Rap2, and PLC, to cause release of DAG and IP_3 through degradation of PIP_2. In turn, this causes release of Ca^{2+} from intracellular stores and PKC-dependent activation of Ca^{2+} dependent Cl^- channels. The overall result is Cl^- secretory responses that are enhanced over those mediated by CFTR alone (Hoque, 2010).

1.2.6 Extracellular regulation of Cl^- secretion

Typically, Cl^- secretion across intestinal epithelial cells is regulated by an array of endogenous factors that can act by paracrine, autocrine, and endocrine means (Figure 1.7). A partial list of such factors is shown in Table 1.1. It should also be kept in mind that exogenous factors found within the colonic lumen can also have important roles to play in regulating colonic transport function.

Table 1.1 Extracellular regulators of Cl^- secretion

Extracellular factor	Second messenger	Effect on secretion
Eicosanoids (e.g. prostaglandins)	cAMP and Ca^{2+}	+ (Collins, 2009)
Histamine	Ca^{2+}	+ (Schultheiss , 2006)
Acetylcholine	Ca^{2+} via muscarinic receptors	+ (Yajima, 2011)
Substance P	Ca^{2+}	+ (Wapnir, 2002)
Neuropeptide Y	cAMP and Ca^{2+}	- (Strabel, 1995, Cox, 1988)
Serotonin	cAMP	+ (Stoner, 2001)
Guanylin	cGMP	+(Morel, 2003)
NO	cGMP	+(Hennig, 2008)
VIP	cAMP	+(Barrett, 1990)
STa	cGMP	+(Guarino, 1987)

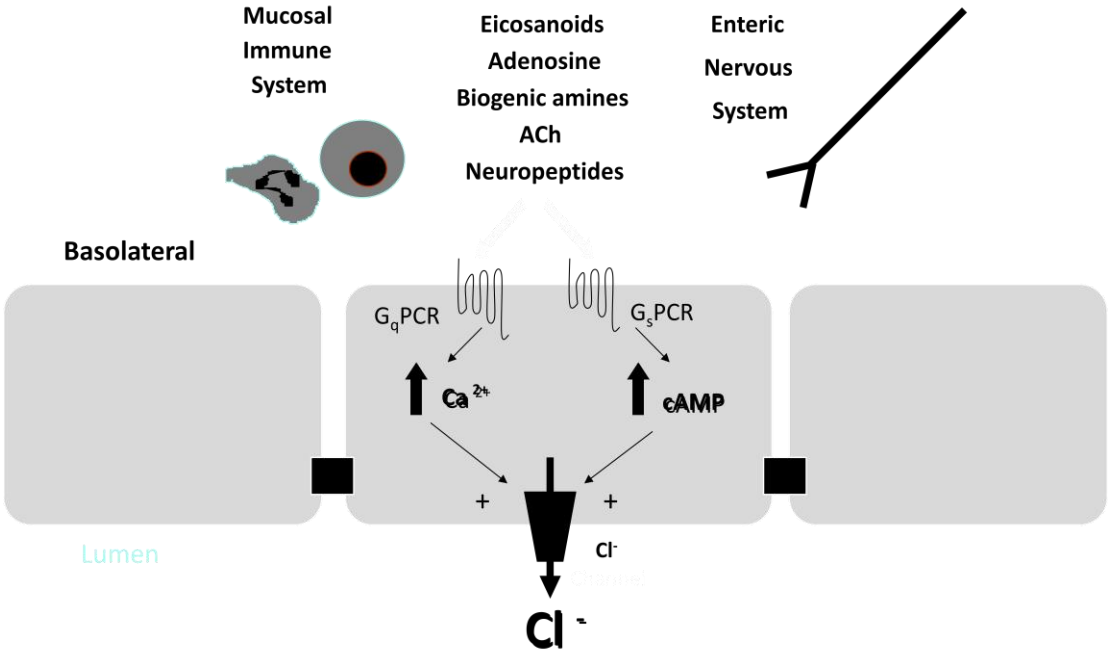


Figure 1.7: Regulation of colonic epithelial Cl^- secretion. Typically, Cl^- secretion is promoted across intestinal epithelial cells by hormones and neuro-immune mediators released from nerves and immune cells in the underlying lamina propria. This diverse group of agonists typically act by binding to and activating GPCRs on the surface of the cell. Activation of G_qPCRs leads to elevation of intracellular Ca^{2+}, while activation of G_sPCRs results in accumulation of intracellular cAMP. In turn, the accumulation of intracellular second messengers activates the ion transport machinery of the epithelial cell to induce Cl^- secretion.

i) <u>Hormonal factors</u>

Hormones, in contrast to local paracrine factors, are released at some distance from their target cells. There are a number of gut hormones that can regulate fluid secretion, such as gastrin, cholecystokinin, and secretin, all of which are released in response to food ingestion (Farack, 1987, Gaginella, 1978). Aldosterone, a mineralocorticoid, also plays an important role in promoting colonic absorptive function through inducing ENaC expression (Eutamene, 2003, Moreto, 2005). Interestingly, in addition to their long-term genomically-mediated effects, hormones can also have rapid actions on epithelial secretory responses (McNamara, 2000). For example, aldosterone can acutely inhibit KCNN4, thus rapidly down regulating Ca^{2+}- dependent secretory responses. Estradiol has also been shown to acutely inhibit colonic Cl^- secretion through inhibition of KCNQ1 channels (O'Mahony, 2007).

ii) Immune factors

Cells of the innate immune system are located adjacent to the epithelium in the lamina propria, and many mediators, such as histamine and eicosanoids, released from these cells have important roles in regulation of Cl^- secretion (Keely, 2009). This makes sense given that increased fluid secretion provides a medium by which to wash away noxious substances and pathogens before they can infect the epithelium. These responses mainly involve activated mast cells and neutrophils and the consequent release of histamine, prostaglandins, leukotrienes, reactive oxygen species, and adenosine (Barrett, 2000), all of which can promote secretory responses. However, it is important to note that activated immune cells, particularly lymphocytes, can also release cytokines that inhibit epithelial transport processes. For example, IFNγ inhibits epithelial secretion through down regulation of Na^+/K^+-ATPase pumps and NKCC1 (Bertelsen, 2004b).

iii) Neuronal factors

The myenteric and intramural (sub-mucosal) plexuses which comprise the enteric nervous system (ENS) form a complex network innervating the entire GI tract and connecting to the central nervous system forming the Brain-Gut axis (Drossman, 2005). Signals from the enteric nervous system control intestinal blood flow, motility and also transport processes (Hubel, 1985). Motor neurons in the ENS fall into 2 broad categories: excitatory or inhibitory. They receive input from other ENS neurons (intrinsic nerves), as well as from sympathetic postganglionic nerves. Local paracrine messengers from non-neural cells such as enterochromaffin cells, mast cells, and other inflammatory cells can have a profound influence on the excitability of these secreto-motor neurons. Excitatory motor neurons promote contraction of the gastrointestinal tract smooth muscle and secretion of mucus as well as promoting fluid secretion within the lumen. The primary excitatory neurotransmitters involved in Cl^- secretion include acetylcholine (ACh), substance P and serotonin. Inhibitory motor neurons release neurotransmitters that suppress contractile activity and secretion. These include vasoactive intestinal polypeptide, nitric oxide, and Adenosine-5'-triphosphate. Acetylcholine (ACh) acts via increases in intracellular Ca^{2+}, and vasoactive intestinal polypeptide (VIP) increases intracellular cAMP (Kunzelmann, 2002). Clinical examples of elevated secreto-motor neuron activity

include inflammatory bowel disease, in which inflammatory mediators excite the secreto-motor neurons to release neurotransmitters and increase secretion, manifesting as diarrhea. Neuropathic conditions that diminish the functional or structural integrity of the ENS may be associated with diminished secreto-motor neuron activity and may manifest as constipation. Neurotensin is another neurotransmitter which is important in colonic secretion and it mediates its functions by binding to two G-protein-coupled receptors, neurotensin receptor-1 (NTR1) and neurotensin receptor-2 (NTR2); NTR1 facilitates most of the intestinal responses of neurotensin, Neurotensin has been shown to stimulate Cl^- secretion in the colon previously. Peptide YY (PYY), somatostatin, vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), and galanin have all been implicated in autonomic dysmotility in the gut and neuropeptide Y in particular has been shown to inhibit Cl^- secretion (Hubel, 1985, Riegler, 1999, Hyland, 2005, Cox, 1988).

iv) <u>Luminal Factors</u>

There is an array of luminal factors that can influence epithelial fluid and electrolyte transport in the intestine. For example, food boluses themselves are responsible for initiating neuronal reflexes which promote fluid secretion. This is necessary in order to lubricate the passage of the bolus so that it does not cause damage to the epithelium (Hansson, 2012). Other important luminal factors include BAs, which when present at high concentrations can acutely promote secretion, and SCFAs, which are produced by bacteria from dietary fibre and some of which (phorbol esters) have been shown to be anti-secretory through an inhibition of NKCC and basolateral K^+ conductance, resulting in a down regulation of cAMP induced Cl^- secretion (Matthews, 1998). On the other hand, bacterial enterotoxins, such as *E.coli* heat stable enterotoxin (ST_a) and cholera toxin, can induce Cl^- secretion through elevation of intracellular cyclic nucleotides, while helminths have been shown to limit Ca^{2+}-induced secretory responses (Hirota, 2006).

1.3 Epithelial cells and their role in barrier function and innate immunity

In addition to their role in regulating fluid and electrolyte transport, epithelial cells also contribute to intestinal barrier function and play important roles in regulating innate immune responses. Disruptions in epithelial barrier function are well-known to be involved in the pathogenesis of intestinal inflammation, and it is therefore important to develop our understanding of the mechanisms involved, so that new targets for therapeutic intervention can be identified. There are many factors that contribute to epithelial barrier function and innate immunity, as outlined in the following sections.

1.3.1 Epithelial Barrier Function

Epithelial barrier function is a key component in the arsenal of defense mechanisms required to prevent infection and inflammation. The intestinal barrier consists of the mucous layer, antimicrobial peptides, secretory IgA, and the epithelial junctional adhesion complex (McGuckin, 2009). Consumption of non-pathogenic bacterial species can contribute to barrier function by decreasing paracellular permeability, providing innate defense against pathogens and enhancing the physical impediment of the mucous layer. In turn, this can help to protect against infections, prevent chronic inflammation, and maintain mucosal integrity.

Colonic epithelial cells themselves constitute a resistant barrier with their plasma membranes being anchored to their neighbouring cells through structures known as tight junctions. Tight junctions also act as a fence to prevent mixing of membrane domain-specific proteins and lipids (Yeaman, 1999) Tight junctions display ionic selectivity and vary in tightness depending on intestinal region. The permeability of tight junctions is determined by factors such as the number of tight junction strands or changes in the relative expression of different claudins (Furuse, 2001). Many extracellular factors can alter the permeability of tight junctions, such as bacterial toxins, bile acids, and cytokines (Kreisberg , 2011).

The mucous layer provides one of the first lines of defence for the epithelium, providing protection by shielding the epithelium from potentially harmful pathogens

and molecules, while acting as a lubricant for intestinal motility (Ohland, 2010). Goblet cells express rod-shaped mucins, which are abundantly glycosylated and either localized to the cell membrane or secreted into the lumen to form the mucous layer (McCool, 1994). MUC-2, as previously mentioned, is the predominant glycoprotein found in small and large bowel mucus. Mucus thickness can vary from 50 to 800 μM, but the 30 μM -thick area closest to the epithelium is essentially sterile in healthy individuals (Swidsinski , 2007). Mucus is the first barrier that intestinal bacteria encounter and pathogens must penetrate it to reach the epithelium during infection. Microorganisms have developed diverse methods to degrade mucus, such as reduction of mucin disulfide bonds (*Helicobacter pylori*), protease activity (*Pseudomonas aeruginosa, Candida albicans, and Entamoeba histolytica*), and glycosidase activity (mixed oral and intestinal microbial communities), in order to promote invasion and/or uptake of mucus-derived nutrients (Windle, 2000). Interestingly, the colonic mucous layer has been shown to be thinner in areas of inflammation, allowing increased bacterial adherence and infiltration (Swidsinski, 2007). UC patients also exhibit reduced mucus thickness, particularly in areas of active inflammation, which is likely a consequence of the disease (Swidsinski, 2007).

Another important component of innate intestinal barrier function is the antimicrobial peptides, of which there are 2 main families; defensins and cathelicidins. The latter family consists of cationic, α-helical antimicrobial peptides constitutively expressed by gastrointestinal epithelial cells, which are involved in host defense against pathogens (Kelsall, 2008). The only microbial or inflammatory stimulus that appears to induce cathelicidin expression is butyrate, which is produced by the enteric micro-flora (Schauber , 2003). Defensins are classified into α-defensins, expressed primarily by small bowel Paneth cells (HD-5 and -6), and β-defensins expressed by epithelial cells throughout the intestine (Wehkamp, 2004). These small (3–5 kDa), cationic peptides display antimicrobial activity against a wide variety of bacteria, fungi, and some viruses, and are constitutively expressed to prevent pathogens from reaching the epithelium. Lowered defensin production has been associated with the development of IBD and with increased susceptibility to bacterial infections (Wehkamp, 2004).

Another type of secreted protein that makes an important contribution to intestinal barrier function is secretory IgA. Approximately 80 % of all plasma cells in the body are found in the intestinal mucosa, and more IgA is produced here than any other immunoglobulin isotype (40–60 mg/ kg/ day in humans). Peyer's patches are the main site for generation of IgA+ B cells. IgM+ B cells are recruited into Peyer's patches, where they are activated and proliferate through interactions with local T cells and/or dendritic cells. Although both IgG and IgA are found in intestinal mucosal secretions, IgA is the more abundant isotype in healthy individuals and is historically considered to be the primary element of the mucosal immune response to microbial antigens (Holmgren, 2005).

In the luminal mucous layer, secretory IgA protects the intestinal epithelium against colonization and/or invasion by binding to antigens on pathogens or commensals. This surrounds the microorganism with a hydrophilic shell that is repelled by the epithelial glycocalyx, thus providing immune exclusion of bacteria (Macpherson, 2008). The antigen-complexed IgA can also bind the receptor FcαRI (CD89) constitutively expressed on immune cells, such as neutrophils, interstitial dendritic cells, monocytes, and some macrophages (Zhu, 2001). Immune exclusion not only protects the epithelium from invading pathogens, it is also important in maintaining gut homeostasis by preventing overgrowth of the enteric micro-flora (Suzuki, 2007). Furthermore, IgA can protect against intracellular pathogens by binding and neutralizing viral or bacterial components during transcytosis across the epithelium. The immune complexes are then secreted apically and invasion is inhibited (Fernandez, 2003).

1.3.2. Innate Immunity

The innate immune system provides immediate, but non-specific, protection against pathogens. It has no immunological memory. Phagocytosis is an important feature of cellular innate immunity, which is performed by macrophages, neutrophils, and dendritic cells that engulf pathogens or particles. The innate immune system responds to common structures shared by a vast majority of pathogens. These highly conserved soluble and membrane bound proteins are collectively called Pattern-Recognition Receptors (PRRs), and it is the PAMP/PRR interaction that triggers the innate immune system and are recognized by the toll-like receptors, or

TLRs (Marshak-Rothstein, 2006). In addition to the cellular TLRs, an important part of the innate immune system is the humoral complement system that opsonizes and kills pathogens through the PAMP recognition mechanism.

1.3.3 Epithelial Toll-like receptors and intestinal innate immunity

When it is under threat and barrier function is compromised, the intestinal epithelium secretes cytokines to activate innate immune responses, mainly to attract neutrophils. Regulation of cytokine secretion is largely under the control of Toll-like receptors (TLRs). TLRs, as previously mentioned, are a type of pattern recognition receptor (PRRs) and recognize motifs that are shared by pathogens, but which are distinguishable from host molecules. Altogether, 13 TLRs (TLR-1 to TLR-13) have been identified in humans and mice (Beutler, 2004). In humans, TLR-1, -2, -4, -5, and -6 are cell membrane associated and respond primarily to bacterial surface-associated PAMPs. The second group, TLR-3, -7, -8, and -9 are expressed on the surface of endosomes, where they respond primarily to nucleic acid based PAMPs from viruses and bacteria. Upon ligand binding, TLRs activate 2 major signalling pathways: the NFκB and the MAPK (p38 and JNK) pathways (Marshak-Rothstein, 2006) as demonstrated in Figure 1.8. Primary human intestinal epithelial cells express constitutive TLR-3 and -5 and low levels of TLR-2 and -4. TLR expression on intestinal epithelial cells is generally low with many of the receptors expressed basolaterally preventing interaction with PAMPs in the lumen. Nevertheless, epithelial cells are responsive to certain TLR ligands and low level recognition of commensal bacteria by TLRs enhances protection from intestinal epithelial injury through induction of tissue protective factors. Expression of TLR-4 is increased in UC while NOD-2 mutations are common in CD (Lavelle, 2010).

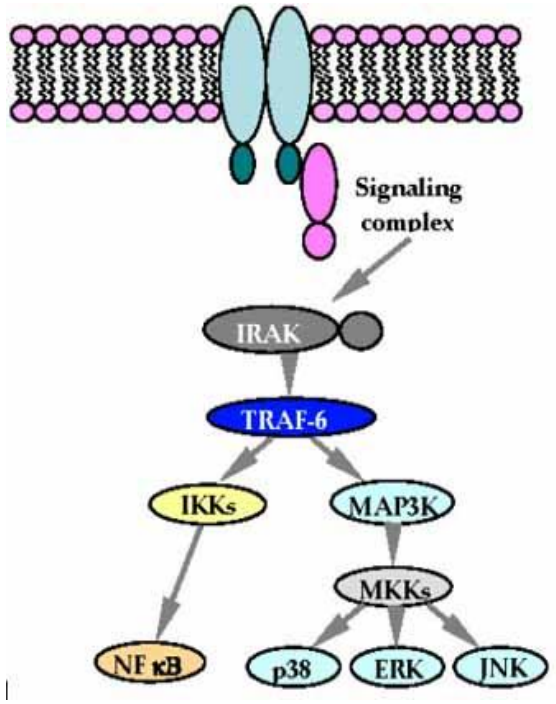


Figure 1.8: Major pathways activated by Toll-like receptors. There are a number of major signalling pathways involved in mediating responses to TLR activation; these include the NFκB and the MAP-3 kinase/JNK pathway (Alvarez, 2005).

Stimulation of TLRs by their pathogen-derived activators (Table 1.2) initiates signalling cascades that involve a number of proteins, such as MyD88, TRIF, and IRAK (Kanzler, 2007). These signalling cascades lead to the activation of transcription factors, such as AP-1, NF-κB, and IRFs, thereby inducing the secretion of pro-inflammatory and effector cytokines that direct the adaptive immune response. TLRs also have an important role in adaptive immunity by activating antigen presenting cells (APCs). The cytokine signalling cascade stimulated by TLR activation begins a complex series of interactions. Among the more important of these is T-cell differentiation and regulation. TLRs on dendritic cells in particular, are essential in the T-helper-1 (Th1) versus Th2 pathways. An important early component of the Th1 response is activation of cytotoxic T cells. Dysregulation of these signalling pathways has severe consequences, and can underlie many autoimmune diseases and conditions of chronic pathological inflammation, such as IBD (Cario, 2010).

Table 1.2 TLRs and their Pathogen-Derived Activators

(Adapted from (Lee et al., 1993))

PAMP	PRR	Pathogen
Pam3CSK4, Zymosan	TLR-1, -2, -6	Gram Positive Bacteria
LPS	TLR-4	Gram negative Bacteria
Flagellin	TLR-5	Bacteria
dsDNA	TLR-3	Virus
ssRNA	TLR-7, -8	Virus
CpG DNA	TLR-9	Bacteria

1.3.4 LPS stimulation of TLR-4

The signal transduction pathway for TLR-4 activation by LPS (lipopolysaccharide) serves as a representative example of the surface bound TLRs. LPS binds to the CD-14 (Cluster of Differentiation-14) receptor, which then transfers it to TLR-4. TLR-4 homodimerizes and forms a complex with the protein, MD-2. Cells require both MD-2 and TLR-4 in order to recognize LPS. TLR4 activation engages a set of MyD88 (Myeloid Differentiation Primary-Response Protein-88) adaptor family members, including TIRAP, TRIF, TRAM (TIR domains containing adapter proteins). This pattern of activation is general for cell surface TLRs, but the subsequent intracellular signal cascades are unique for each TLR, resulting in responses that are appropriate to each threat (Sakaguchi, 2008).

1.3.5 Peptidoglycan stimulation of TLR-2

TLR-2 is activated by bacterial LAM (lipoarabinomannan), BLP (bacterial lipoprotein), and PGN (peptidoglycans). LAM and PGN activate TLR-2 through the CD-14 receptor, similar to the process followed by TLR-4 and with similar downstream effects. BLP can induce both apoptosis and NF-κB activation through activation of TLR-2. TLR-2 is also responsible for recognition of the yeast cell-wall particle, zymosan, which also acts through the CD-14 receptor to influence TLR-2. Phagocytosed TLR-2 vesicles can induce production of the pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α), through stimulation of the NF-κB pathway (Dziarski, 2005).

1.3.6 Endosomal TLRs

Viral nucleic acids contain PAMPs that are recognized by intracellular TLRs, which are located on endosomal membranes. The TLRs found on endosomes are TLR-3, TLR-7, TLR-8, and TLR-9. TLR-3 activates immune cells in response to double-stranded viral RNA. Stimulation of TLR-3 triggers TRIF activation which, in turn, activates the IRF3 transcription factor through TBK1, ultimately leading to production of IFN-γ. TRIF also activates RIP-1 (Receptor-Interacting Protein-1) and TRAF6, which may activate the NF-κB pathway. Small anti-viral compounds activate immune cells via the TLR-7/MyD88-dependent signalling pathway. TLR-7 binds MyD88 and activates IRAF and TRAF6. TRAF6 then activates TANK (also known as I-TRAF). TANK interacts with TBK1 and IKK-β to activate IRF3. TLR-7 or TLR-8 may also activate IRF7 through interaction of MyD88, BTK and TRAF6, thus inducing anti-viral responses by producing interferon-α (IFN-α) (Horton , 2010).

1.3.7 NOD-like receptors

NOD-like receptors (NLRs, also known as CATERPILLERs) constitute a recently identified family of intracellular pattern recognition receptors (PRRs)(Robertson and Girardin, 2012). NLRs are characterized by a tripartite-domain organization with a conserved nucleotide binding oligomerization domain (NOD) and leucine-rich repeats (LRRs). The general domain structure consists of C-terminal LRRs involved in microbial sensing, a centrally located NOD domain, and an N-terminal effector region, comprised of protein-protein interaction domains, such as the CARD, pyrin, or BIR domains. NLRs have been grouped into several subfamilies on the basis of their effector domains: NODs, NALPs, CIITA, IPAF, and NAIPs. NODs and IPAF contain CARD effector domains, whereas NALPs and NAIPs contain pyrin (PYD) effector domains and 3 BIR domains, respectively. NOD-like receptor mutations, and in particular NOD-2 mutations, have been associated with the pathogenesis of Crohn's disease (Cario, 2010).

Regardless of the initiating stimulus and mechanisms involved, one of the primary outcomes of epithelial TLR activation is cytokine release. Cytokine release in the colon plays a key role in the induction and maintenance of gut inflammation (Fantini, 2008) and has also been shown to be involved in the development and growth of

colitis-associated colorectal cancers. Thus, given their central roles in the pathogenesis of inflammatory responses, down-regulation of TLR-induced signalling mechanisms in the colon represents a logical approach for the development of new treatments for IBD (Cario, 2010). However, before this goal can be achieved, a greater understanding of how endogenous and exogenous factors regulate epithelial TLR signalling and cytokine release is required.

1.4 Bile Acids

As outlined in previous sections of this Introduction, there are many endogenous and exogenous factors that interact with epithelial cells to regulate their transport and barrier function. However, one family of molecules that plays a central role in these processes are bile acids. Bile acids (BAs) are a family of steroidal molecules derived from cholesterol and which are biosynthesised in the pericentral hepatocytes of the liver. In recent years, there has been a renaissance in BA research with emergence of data describing their wide ranging biological effects, extending well beyond their traditional roles in facilitating fat digestion and absorption.

1.4.1 The Enterohepatic Circulation of Bile Acids

The primary BAs chenodeoxycholic acid (CDCA) and cholic acid (CA) are synthesised in the liver from cholesterol in a complex series of enzyme-catalysed reactions (Hylemon, 2009). In short, these include (i) 7-hydroxylation (via CYP7A1), (ii) oxidation/isomerisation to 3-oxo forms, (iii) 12-hydroxylation (via CYP8B1), (iv) side chain oxidation to C27 acid, (v) saturation to form A/B cis-fused rings, (vi) reduction of 3-oxo to 7 OH, and (vii) oxidative cleavage of the side chain to the C24 acid. After conjugation to glycine or taurine, Bas are actively transported across the canalicular membrane from the hepatocyte by the bile salt export pump (BSEP, ABCB11), along with phospholipids and cholesterol. The ratio of conjugated BAs, cholesterol and phospholipids in bile is tightly regulated to ensure that cholesterol is solubilised into mixed micelles of conjugated BAs, cholesterol and phospholipids (Vlahcevic, 2003). Excess biliary cholesterol secretion relative to conjugated BAs can result in cholesterol saturated bile and an increased propensity to cholesterol gallstone formation. BAs are stored in the gallbladder and are released into the duodenum in response to alimentary hormones.

In the small bowel, conjugated BAs activate specific pancreatic lipases aiding solubilisation of dietary lipids, sterols and fat-soluble vitamins and form mixed micelles that enable nutrient uptake into enterocytes. In the terminal ileum, conjugated BAs are actively transported into ileocytes by the apical bile salt transporter (ASBT), also known as the ileal bile acid transporter (IBAT). Inside the ileocyte, BAs are bound by the intestinal bile acid-binding protein (I-BABP) and can activate the nuclear bile acid receptor, farnesoid X receptor (FXR), to induce the synthesis of fibroblast growth factor 19 (FGF19, also known as FGF15 in mice). BAs exit the basolateral side of ileal enterocytes via the heterodimeric organic solute transporter (OST α/β) (Hofmann, 2008, Dawson, 2010), and are transported back to the liver, along with FGF19, via the portal circulation. At the liver, conjugated BAs are actively taken into hepatocytes, primarily by the Na^+/taurocholate co-transporting polypeptide (NTCP). FGF19 binds to FGF receptor 4, which in turn down-regulates the gene encoding cholesterol 7α hydroxylase (CYPA71), thereby negatively regulating BA synthesis.

Although the enterohepatic circulation is extremely efficient (See Figure 1.9), with each cycle a small proportion (approximately 5 %) of circulating BAs enter into the colon. Once here, they are metabolized by resident bacteria, which are capable of enzymatically deconjugating glycine and taurine, C7-dehydroxylation, and epimerization or oxidation of key hydroxyl groups on the BA molecule, to produce secondary BAs. Deoxycholic acid (DCA) is the primary metabolite of CA and is normally the predominant colonic BA, while ursodeoxycholic acid (UDCA) and lithocholic acid (LCA) are produced by metabolism of CDCA. The concentrations of each of these BAs within the colon have been previously measured, as outlined below in **Table 1.3** (de Kok, 1999).

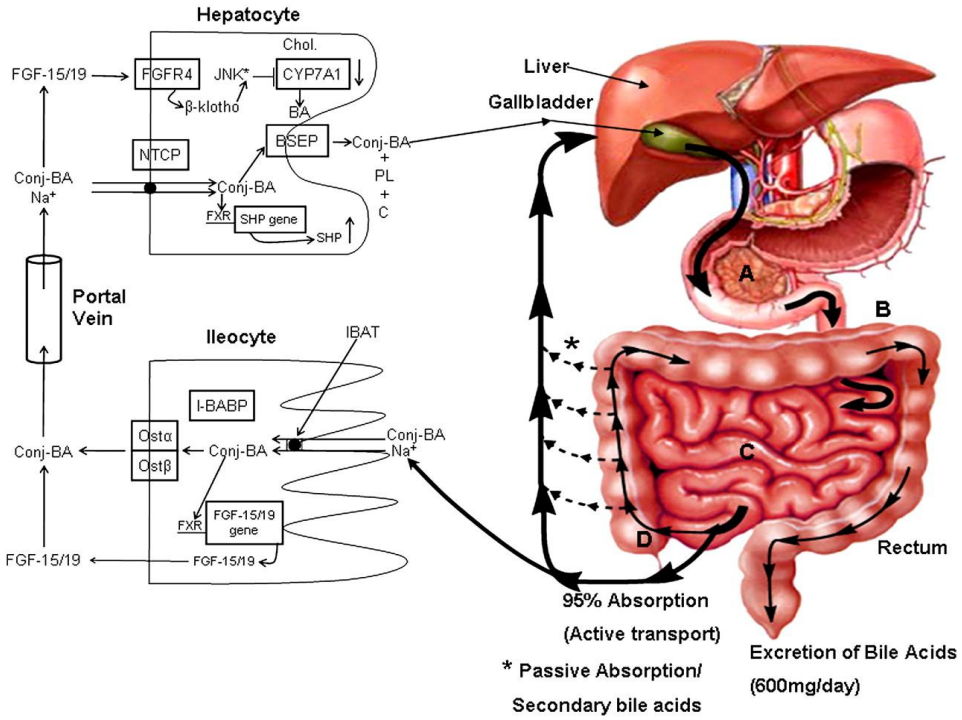


Figure 1.9: The enterohepatic circulation. BAs undergo a process of enterohepatic circulation in which they are released into the small intestine from the gallbladder and are then reabsorbed from the distal intestine and returned to the liver. They can then be recycled and secreted again into the bile. For a description of the molecular mechanisms involved, please see the text. (Source of figure: Hylemon, 2009).

Table 1.3 Bile Acid Concentrations in Faecal Water from Healthy Human Controls (de Kok, 1999)

Colonic Bile Acid	Median Concentration (μM)[1]
Lithocholic Acid (LCA)	13 (4 - 43)
Deoxycholic Acid (DCA)	104 (46 - 210)
Cholic Acid (CA)	11 (0 - 58)
Chenodeoxycholic Acid (CDCA)	4 (0 -17)
Ursodeoxycholic Acid (UDCA)	13 (0 - 39)
Total Bile Acids	184 (88 - 393)
Primary Bile Acids[2]	149 (69 - 384)
Secondary Bile Acids[3]	17 (0 - 82)

[1] Median values with 10th and 90th percentiles in parentheses.

[2] Secondary bile acids = LCA + DCA + UDCA.

[3] Primary bile acids = CA + CDCA

1.4.2 Bile acids as signalling molecules

In the past, BAs were considered solely as detergent molecules, important in solubilisation of cholesterol in the gall bladder and for facilitating absorption of cholesterol, fat-soluble vitamins, and lipids from the intestine. However, during the past 2 decades, it has become increasingly apparent that BAs are also important regulatory molecules. BAs have been discovered to activate specific nuclear receptors, including the farnesoid X, pregnane X, and vitamin D receptors (Cui, 2012, Fujino, 2004, He, 2011, Han, 2010, Wang, 1999). A dedicated GPCR for BAs, known as TGR-5, has also been identified in the liver and intestine (Hofmann, 2008, Poole, 2010, Cipriani, 2011). BAs can also promiscuously interact with other GPCRs, with some of their actions involving activation of the muscarinic M_3 receptor (Raufman, 2003). Interestingly, BAs have also been shown to transactivate receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFr) (Feldman, 2009). Furthermore, due to their detergent properties, BAs can also induce membrane perturbations which, in turn, initiate intracellular signalling cascades (Freel, 1983). The signalling pathways activated by BAs are varied, including Ca^{2+}, cAMP, PKCs, and MAPKs. BAs activate these signalling pathways within seconds to minutes and this action provides mechanisms by which BAs may rapidly alter cellular

function. However, each of these pathways can also activate transcription factors and thereby regulate nuclear gene transcription to alter cell function on a more long-term basis. Indeed, activation of cell signalling pathways by BAs can alter the expression of numerous genes that encode proteins involved in the regulation of bile acid, glucose, fatty acid, and lipoprotein synthesis, metabolism, and transport. BAs also appear to function as nutrient signalling molecules, primarily during the feed/fast cycle. Indeed, BAs have been shown to be involved in regulating multiple physiological processes, a number of which are outlined in Table 1.4.

Table 1.4: Physiological Effects of Bile Acids

Process	Site	Mechanism
Cholesterol elimination	Liver, biliary tree, intestine	Conversion to BAs. Increased biliary cholesterol secretion. Solubilisation in mixed micelles. Faecal excretion.
Stimulation of bile flow	Liver , biliary tree	Secretion of osmotically active substances. Potentiation of HCO_3 secretion. Detachment of phosphatidyl choline from canaliculi.
Stimulation/inhibition of fluid and electrolyte secretion	Intestine	Effects on both cAMP and Ca^{2+} activated secretion.
Stimulation of intestinal motility	Large Intestine	Activation of neural reflexes.
Enhanced lipid absorption	Small Intestine	Mixed micelle formation.
Cleaning absorptive surfaces	Small intestine	Surface activity of BA anions.

Bile Acid Transport:

For enterohepatic circulation of BAs, both the ileal cells and hepatocytes are involved and four transporters are required –two apical and two basolateral. The ileal

bile acid transporter, also known as the apical bile salt transporter (ASBT) mediates sodium dependent co-transport of conjugated BAs. Basolateral transport out of the ileal cell into the portal circulation is mediated by OSTα/β. Passive uptake of conjugated BAs may also occur in the small intestine (Amelsberg, 1993). BAs are then transported to the liver. Hepatocyte uptake is mediated by Na^+-taurocholate co-transporting polypeptide (NTCP), a basolateral sodium-dependent co-transporter. Uptake may also be mediated by one or more of the multiple sodium-independent anion transporters belonging to the organic anion transporter (OATP) family. Conjugated BAs are transported from the hepatocyte back into blood by MRP4, a co-transporter of BAs and glutathione. Extrusion of BAs into the canaliculus is mediated by the ATP-energized pump, BSEP (Amelsberg, 1993).

Bile Acid Receptors:

FXR: A growing body of evidence suggests that the nuclear receptor for BAs, the farnesoid X receptor (FXR), is a critical mediator of epithelial responses to BAs. This has led to a great deal of interest in therapeutically targeting the FXR, and indeed, synthetic agonists of the receptor have been shown to exert protective effects in an animal model of colitis (Gadaleta, 2011). Mroz *et al* has recently published work noting that FXR agonists are anti-secretory. These effects appear to be due to inhibition of multiple components of the epithelial Cl^- secretory pathway, with both basolateral Na^+/K^+ ATPase activity, and apical CFTR Cl^- channel currents being attenuated upon treatment (Mroz, 2014).

TGR5: TGR5 is a membrane associated BA receptor. TGR5 is expressed on enteric nerves and enterochromaffin cells (ECs), where its activation regulates small intestinal and colonic motility. TGR5 is also expressed on colonic epithelial cells and its activation decreases basal secretory tone and inhibits cholinergic-induced secretory responses (Ward, 2013). FXR and TGR5 have distinct ligand binding characteristics (Pellicciarri, 2007) with FXR agonists, such as OCA, being poor agonists of TGR5. Again in recently published data the Keely group has also shown that TGR5 activation with INT-777, a poor agonist of FXR, exerts actions on colonic Cl^- secretion that are distinct to those described for FXR agonists (Ward, 2013).

1.4.3 Bile Acids and Colonic Epithelial Physiology

i) Bile Acid Effects on Epithelial Transport:

BAs have long been recognised as important regulators of epithelial fluid and electrolyte transport in the colon. In conditions of bile acid malabsorption, increased levels of BAs, in particular DCA and CDCA, are present in the colon, where they are well-known to promote fluid and electrolyte secretion, thereby causing diarrhoea associated with such conditions (Mekjian, 1971). These pro-secretory effects of BAs have been extensively investigated in a variety of species and cell culture models and it is clear that strict structure-activity relationships exist for their actions, with only dihydroxy BAs being effective (Keely, 2007, Chadwick, 1979, Dharmsathaphorn, 1989b, Devor, 1993). Furthermore, in addition to their well-established roles in promoting colonic epithelial secretory function, dihydroxy BAs have also been shown to inhibit absorption, an effect that also contributes to the onset of diarrhoea (Black, 1988). It is thought that these pro-secretory and anti-absorptive actions of high concentrations of colonic BAs serve to dilute the luminal contents and therefore prevent BAs from achieving levels that can cause damage to the epithelium, thereby compromising barrier function.

ii) Bile Acid Effects on Intestinal Barrier Function:

In addition to regulating epithelial fluid and electrolyte transport, there is significant evidence that BAs also have important roles to play in regulating epithelial barrier function and in regulation of innate immune responses. BAs regulate epithelial barrier function through multiple actions, including their complex roles in regulating the balance between cell growth and death (Amaral, 2009, Yamaguchi, 2004), regulation of TJ permeability (Chen , 2012), cell migration and restitution (Milovic, 2001, Henrikson, 1989), and mucus secretion (Barcelo, 2001) . Furthermore, it appears that colonic BAs can regulate the synthesis and secretion of pro-inflammatory cytokines from epithelial cells and therefore have roles to play in the initiation of innate immune responses (Hofmann, 2008, Muhlbauer, 2004). The roles of BAs in regulating epithelial transport and barrier function and cytokine secretion are considered in more detail in subsequent chapters of this thesis.

1.4.4 UDCA: The Therapeutic Bile Acid

UDCA has properties distinct from those of other BAs. While the majority of secondary BAs are recognised as being toxic and carcinogenic, UDCA has been shown to have numerous beneficial effects, in particular in treatment of liver disease. UDCA has in fact been used since ancient times in Chinese medicine for treatment of liver maladies. Following oral administration, UDCA absorption in the gut varies from 30–60% (Lazaridis, 2001). UDCA at 8–10 mg/ kg per day causes an enrichment of approximately 40% in biliary BAs (Hofmann, 1994), and in humans, its plasma half-life is 3.5–5.8 days (Ward, 1984). UDCA is currently licensed for use in cholestatic liver disease and gallstone dissolution, and it has undergone clinical trials for many other forms of cholestasis (e.g. (total parenteral nutrition (TPN)-induced and pregnancy-induced cholestasis (Lazaridis, 2001)).

UDCA has also been widely investigated as a potential chemo-preventive agent in colorectal cancer. It has been noted to reduce colon carcinogenesis in mouse models, reducing tumour incidence by up to 50% (Earnest, 1994). UDCA has also been shown to decrease the incidence of colorectal cancer in patients with UC and PSC (Pardi, 2003, Tung, 2001). Despite its extensive clinical usage, the exact mechanisms by which UDCA, or its metabolites, act on the colonic epithelium are not well known. There are, however, many proposed mechanisms of action through which UDCA is thought to elicit its pharmacological actions in other systems. These include expansion of the hydrophilic BA pool leading to displacement of the more cytotoxic hydrophobic BAs, with this displacement being thought to occur at the level of the ileum or at the hepatocyte membrane (Stiehl, 1999). It is unlikely that this is the only mechanism by which UDCA is protective, since it is also known to increase secretion of BAs, promote canalicular transport in the liver, thereby improving bile flow in cholestatic conditions. UDCA has also been shown to have immunomodulator properties and effects on apoptosis, which are also likely to explain some of its therapeutic benefits. Such actions of UDCA are discussed in more detail in Chapter 6.

1.5 Relevance to Disease

It is clear from the discussion above that regulation of epithelial transport and barrier function is highly complex, involving many diverse factors that act through a myriad of extracellular and intracellular mechanisms. It is also clear that disruptions to the ability of epithelial cells to appropriately transport fluid and electrolytes or to prevent entry of pathogens can have serious consequences for health.

1.5.1 Global Impact of Diarrhoeal Disease

It has been well-documented that intestinal disorders associated with diarrhoea are responsible for approximately 1 million child deaths in the under-5 age group globally on an annual basis (See Figure 1.10). Only 44% of children in low income countries receive recommended treatments, with there being little change in these trends since 2000 (UNICEF/WHO, 2009, Wardlaw, 2010, Rudan, 2010). Diarrhoeal diseases are also manifest in the developed world and are a principal feature of many intestinal disorders, including inflammatory bowel disease, infectious diseases, malabsorptive conditions, metabolic disorders, and irritable bowel syndrome. In the elderly, 7–14% of the population complains of “chronic diarrhoea” (Talley, 1992). Using a definition based on excessive stool frequency without abdominal pain, estimates in the Western population are in the order of 4–5% of the general population (Chang, 2009). The American Gastroenterology Association (AGA) estimated in 2002 that the direct cost of chronic diarrhoeal diseases was in the region of $16 billion, annually (Sandler, 2002).

Despite this tremendous burden of disease on both a national and international level, there still remains an incomplete understanding of the molecular mechanisms that are involved in the pathogenesis of diarrhoeal diseases, with a resulting lack of molecular-targeted therapies. For example, in the U.S. market at present, there are no drugs or therapeutics available for diarrhoea which specifically target epithelial fluid and electrolyte transport processes. Thus, there exists substantial gaps in our understanding and therapeutic capability for many diarrhoeal disorders.

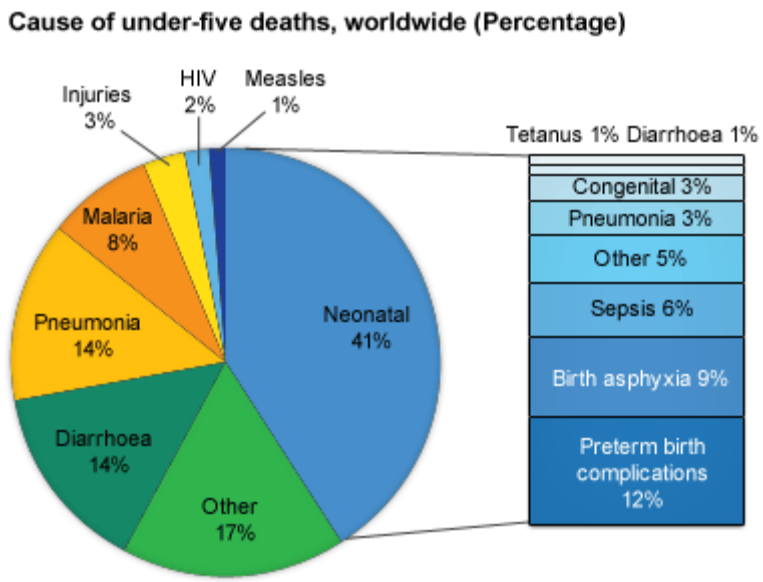


Figure 1.10: Diarrhoea is a significant cause of child mortality. Diarrhoea remains one of the leading causes of child death in the developing world accounting for 14% of deaths in children under the age of 5 globally (Rudan, 2010).

1.5.2 Definition of Diarrhoea

Diarrhoea may be defined in terms of stool frequency, consistency, volume or weight, with patient conceptions of diarrhoea being often focussed on consistency (Wenzl, 1995). Faecal consistency is determined by the water-holding capacity of stool and perhaps best defines the concept of diarrhoea. However, quantification of consistency in clinical practice is difficult, so other criteria, e.g. frequency of defecation or stool weight, may be used. Stool weight of > 200 g/ day is often considered to be the upper limit of normal (Fine, 1999). However, this can be misleading, since stool weights vary greatly, and in non-Western diets “normal” often exceed this volume. Conversely, distal colonic pathology may not increase stool weight beyond 200 g/ day. When defining duration of diarrhoea, there is no firm consensus in the literature. An arbitrary time point of 4 weeks is often referred to when defining chronicity (Fine, 1999). Thus, to be pragmatic, chronic diarrhoea may be defined as abnormal passage of loose/liquid stools, > 3 times/ day, for a period of > 4 weeks, ± daily stool weight of > 200 g/ day. However, there is a potential for confusion since there may be a discrepancy between medical and “lay” concepts of diarrhoea, and this should be clarified at initial appraisal (Madoff, 1992). Faecal incontinence may be misinterpreted as diarrhoea and functional symptoms can sometimes be difficult to distinguish from organic pathology by history alone,

although features such as nocturnal motions and the presence of incontinence can be helpful in differentiating pathologies (Fine, 1999).

Many gastrointestinal diseases can result in disruption to the regulation of intestinal fluid and electrolyte transport, leading to diarrhoea. These include inflammatory, infectious, neoplastic, vascular, and endocrine disruptions. Despite the wide base of evidence for a final common pathway of altered epithelial secretion in many diarrhoeal diseases (Surawicz, 2010), there are few, if any, therapies which target the molecular machinery involved in epithelial secretion. This therapeutic gap is the focus of research in our laboratory, and in order to set the clinical relevance of our research, a number of the most common and relevant diarrhoeal disease states are outlined below.

1.5.3 Irritable Bowel Syndrome (IBS)

IBS is a common functional gastrointestinal disorder that may be triggered by enteric pathogens and has also been linked to alterations in the microbiota and the host immune response. It is commonly divided into diarrhoea-predominant, constipation-predominant, and mixed subtypes. It remains largely a clinical diagnosis based on a number of outlined criteria (Manning and ROME I, II and III criteria) (Drossman, 2010), and the exclusion of other pathologies. The prevalence of IBS worldwide has been estimated to be in the region of 11% (Lovell, 2012). There is an integrated conceptual framework involved in understanding the pathophysiology of IBS which involves a combination of visceral hypersensitivity, abnormal motility and secretion, and dysfunctional communication within the brain-gut axis (Jeffery, 2012). Satisfactory treatments for refractory diarrhoea-predominant IBS are in short supply and its treatment remains problematic.

The gut microbiome is thought by many investigators to play a significant role in the pathogenesis of IBS (Salonen, 2010). A complex ecosystem exists within the gut lumen which is vital in the maintenance of the dynamic nature of intestinal barrier function and antigen recognition. These organisms are capable of influencing gut signalling, affecting gas production, fluid secretion, and enteric nerve and immune cell function. Thus, alterations in the microbiome may influence gut homeostasis to produce intestinal symptoms (Talley, 2011). The well-described entity of post-

infectious IBS supports this hypothesis. Approximately 25% of patients with IBS have an onset of symptoms post enteric infection. It has been shown in one study that enteric infection carries a 7-fold increase in the risk of developing functional bowel problems in at-risk populations (Ghoshal, 2011). Those who are particularly susceptible are female, smokers, patients suffering from psychological stress at the time of infection, and other pathogenic factors (Marshall, 2009). There is a recognised subgroup of IBS where an altered gut microbiome has been recognised, although this is not always the case. In this subgroup, alterations in the luminal gut flora may alter BA levels present and consequently alter their effects on luminal fluid secretion. In turn, alterations in BA levels can also affect bacterial survival and Islam et al have demonstrated that BAs are important regulators of caecal microbiota composition in rats (Islam, 2011).

1.5.4 Infectious Diseases

Many diarrhoeal conditions are caused by release of enterotoxins from microorganisms. Common causes of infectious diarrhoea in the Western world include *Salmonella, Campylobacter, Shigella*, *Clostridium difficile, Yersinia* and *Escherichia coli*. Other causes include *Vibrio cholera,* amoebiasis, tuberculosis, cytomegalovirus, schistosomiasis, and *Herpes simplex* (especially in the immunosuppressed) (Csutora , 2006). In some cases, the mechanisms underlying diarrhoea have been well elucidated. For example, cholera toxin (CT) binds to receptors on the apical surface of epithelial cells, resulting in sustained increases in intracellular cAMP which, in turn, inhibits absorption and stimulates profuse chloride and fluid secretion. There is evidence that CT activates a secreto-motor neural reflex, demonstrated by the fact that CT induced diarrhoea is reduced by drugs such as tetrodotoxin and hexamethonium. The reflex requires presence of the myenteric plexus and utilises secreto-motor neurones containing VIP and AC (Jodal, 1993). In the case of heat stable enterotoxin (ST_a), large increases in intracellular cGMP occur through binding of the toxin to a surface receptor with intrinsic guanylyl cyclise activity. Toxin release is not the only mechanism by which bacterial infection can cause diarrhoea, and non-toxigenic bacteria, such as Salmonella, can also evoke diarrhoea. Proposed mechanisms involved include stimulation of gene expression, leading to heightened secretory responses to endogenous agonists, and reductions in barrier integrity. There is also data to suggest a role for Cl^--driven fluid secretion in

causing the adherent mucous-gel layer to be shed, possibly as a rate-limiting innate defence mechanism (Keely, 2011).

1.5.5 Inflammatory Bowel Disease

IBD includes 2 idiopathic relapsing and remitting inflammatory disorders of the gastrointestinal tract; ulcerative colitis (UC) and Crohn's disease (CD). UC affects only the colon and rectum, while Crohn's disease may involve any part of the GI tract from mouth to anus (Csutora, 2006). Diarrhoea is a common feature amongst patients with IBD.

Over recent decades, the incidence of IBD, particularly Crohn's has been steadily increasing and now affects 2 in every 1000 people in Europe and the USA (Shivananda, 1996, Hou, 2009, Tamboli, 2008). Onset is more common in early adulthood and the chronic waxing and waning nature of both diseases means that they together represent a substantial burden of illness, not only within the hospital service but also in the community. Multidisciplinary care is essential for these patients given the wide ranging consequences of the disease.

While the precise initiating factor in IBD is unknown, it is known that altered immune regulation leads to a prolonged mucosal inflammation which is amplified and perpetuated by recruitment of leucocytes from the intestinal blood supply. Up-regulation of nuclear transcription factors, such as NFκB, is likely to underlie the subsequent release of cytokines, eicosanoids, and other local mediators of inflammation (including reactive oxygen species, platelet activating factors, nitric oxide, proteases, and growth factors).

In UC, cytokine release from non T-helper lymphocytes generates a largely humoral response, while in CD; cell-mediated responses are induced by T-helper 1 cells. Defective intestinal mucous and abnormal intestinal epithelial permeability are also likely to play a role by increasing the access of luminal dietary antigens and bacterial products to the mucosa. Multi Drug Resistant (MDR) protein a has also been implicated in altering barrier function in mouse studies of colitis (Panwala, 1998), where it may be responsible for the efflux of toxic substances and regulating the

expression/ function of transport proteins, such as CFTR (Valverde, 1995). Disruption of electrolyte absorption (electrogenic Na^+ absorption, electroneutral NaCl, and Na^+/ nutrient co-transport) (Sundaram, 1998) also occurs in IBD (McCole, 2005). Thus, while reduced absorptive function may be the predominant contributor to diarrhoea associated with IBD, the loss of secretory function may have implications for barrier function and/or maintenance of crypt sterility (Diaz-Granados, 2000).

Ulcerative colitis: UC usually begins in the rectum and can either remain restricted to this area, or it can spread proximally. In severe pancolitis, the distal ileum is also occasionally involved (known as backwash ileitis). In the colon of UC patients there is extensive inflammation with hyperaemia, granularity, surface pus and blood, and even extensive ulceration. Microscopically there can be chronic and acute inflammatory infiltrates, occurrence of crypt abscesses and cryptitis, with the consequent distortion of crypt architecture. The mucosa may be oedematous. In long-standing colitis, dysplastic changes may occur in cells, where nuclei become crowded and enlarged, losing their polarity with an increased risk of development of carcinoma. The onset of disease may be gradual with a chronic relapsing, remitting course, and features depend on the site and severity of disease.

Acute severe colitis is most common in patients with pancolitis or subtotal disease. This causes profuse diarrhoea (more than 6 bowel motions/day with associated blood, mucus, peridefecatory pain, fever, malaise, and weight loss). Patients are often thin, anaemic, fluid depleted, febrile and tachycardic. Complications include toxic megacolon and/or perforation. Moderate disease is commonly left sided and symptoms include rectal bleeding, diarrhoea, malaise, and abdominal pain. Proctitis, where inflammation is limited to the rectum, causes rectal bleeding, mucus discharge, tenesmus, and pruritus ani, although the patients' general health is usually well-preserved.

Crohn's Disease: This disease entity may affect any part of the gut. Typically, there are "skip" lesions with discontinuous segments of gut being inflamed. Typical changes include lymphoid follicular enlargement, aphthoid ulceration, with progression to deep fissuring ulcers that give a cobblestone appearance to the

mucosa, fibrosis, structuring, and fistulation. Inflammation and fibrosis predispose to the formation of strictures, or narrowing of the mucosa. This may cause the patient to present with obstructing symptoms and can cause microperforation of the gut wall, leading to abscess formation. CD is by nature, a more heterogenous condition than UC and manifestations depend on site and severity of disease.

In ileal and ileocaecal CD pain is the predominant feature. Diarrhoea also commonly occurs and possible mechanisms involved include mucosal inflammation, bacterial overgrowth, and bile acid malabsorption (BAM). There are a number of reasons why patients may experience BAM in IBD. Since the terminal ileum is the main site for BA reabsorption from the intestine, patients who have undergone terminal ileal resection or who have severe inflammation in this area lose their capacity for absorbing BAs. This leads to increased passage of BAs into the colon, where they induce secretion of water and electrolytes, thereby causing diarrhoea. As intestinal adaptation occurs postoperatively, cholegenic diarrhoea often improves. However, in the interim, symptomatic treatment with antidiarrhoeal agents, such as codeine and loperamide, or with bile salt-binding resins, such as cholestyramine, may help.

Prognosis: Intestinal complications of IBD include malnutrition, commonly in CD (Han, 1999), and intestinal cancer with a cumulative risk of 20% for colorectal cancer over 30 years (Bernstein, 2001). Mortality in UC resembles that of the general population while in CD it is increased 2-fold. Causes of death in patients with severe IBD include sepsis, pulmonary embolism, surgery, and immunosuppressive therapy. The prevalence, chronicity, and early onset of IBD mean that it represents a substantial burden to healthcare resources along with its impact on patient quality of life

1.5.6 Current Therapeutic Options for Diarrhoeal Diseases and IBD

Given the many different diseases associated with diarrhoea, accurate clinical judgement is required when considering therapeutic options. As a general rule, some degree of diagnostic testing is indicated in patients with chronic diarrhoea, and treatment should be tailored according to the underlying cause. However, empiric therapy may be warranted in certain situations, such as when a diagnosis is strongly suspected, or when co-morbidities limit diagnostic evaluation. Symptomatic therapy

is indicated when the diagnosis has been made but definitive treatment is unavailable, when diagnosis is elusive, and as a temporary measure during evaluation. A variety of medications can help relieve symptoms, including loperamide, anticholinergic agents, anti-inflammatory agents, and intraluminal adsorbents (such as clays, activated charcoal, bismuth, fibre, and bile acid-binding resins) (Fine, 1999).

Other anti-diarrhoeals: Loperamide is a cheap and widely available over the counter anti- diarrhoeal and is effective for short term treatment of many diarrhoeal conditions, including travellers' diarrhoea (Butler, 2008), but is also somewhat effective for diarrhoea-predominant IBS (Talley, 2003). It is an opioid-receptor agonist and like morphine, decreases the tone of the longitudinal smooth muscles but increases the tone of circular smooth muscles of the intestinal wall. This slows transit through the intestine, allowing more water to be absorbed. Loperamide also decreases colonic mass movements and suppresses the gastro colic reflex (Awouters, 1993). The use of loperamide in children under 3 years is not recommended (Li, 2007) . Loperamide use should be avoided in both adults and children in the presence of high fever or if the stool is bloody (dysentery) (Butler, 2008). Treatment is not recommended for patients that could suffer detrimental effects from rebound constipation. If there is a suspicion of diarrhoea associated with organisms that can penetrate the intestinal walls, such as *E. coli* O157:H7 or *Salmonella*, practice guidelines would advise against the use of anti-motility agents (Thielman, 2004). Loperamide treatment is also not used in symptomatic *C. difficile* infections, as it increases the risk of toxin retention and may precipitate toxic megacolon (Kato, 2008).Other over the counter anti-diarrhoeals include bismuth subsalicylate (*Pepto Bismol*) which can retard the expulsion of fluids into the digestive system by irritated tissues, by "coating" them. There is also some evidence that salicylic acid from hydrolysis of the drug is antimicrobial for *E. coli* (Sox, 1989), however there have been no large scale randomised studies and it should be only used with caution and under medical supervision for any chronic diarrhoeal state.

The pharmacological strategies currently available for the treatment of IBD include corticosteroids, aminosalicylates, and antibiotics. For more definitive management of

severe disease, immunomodulatory agents, such as thiopurines, and more recently biologics, such as anti-TNF antibodies, can be used.

Corticosteroids: These drugs are indicated in patients with active IBD but should not be used in the longer term. These drugs have their actions by activating intracellular glucocorticoid receptors. The main drawback of steroid therapy lies in the multitude of side effects, particularly associated with prolonged use (Faubion, 2001).

Aminosalicylates: These agents are indicated for use in mild–moderately active UC and in preventing relapse in patients with inactive UC. These drugs reduce leucocyte migration, activation of NFκB, IL-1 synthesis, and degradation of prostaglandins. They also act as TNF antagonists and as antioxidants. In relation to the epithelium, they reduce apoptosis, induce heat shock proteins, and reduce major histocompatibility complex (MHC) class II expression. Though considered the "safest" of drugs used in colitis, these agents still carry a range of systemic side effects.

Metronidazole: Metronidazole is indicated in perianal and ileocolonic Crohn's disease and in preventing recurrence after ileal resection. Treatment is generally prolonged and is associated with unpleasant taste, peripheral neuropathy, inability to drink alcohol, and limited efficacy.

Immunomodulatory agents: Thiopurines, such as azathioprine, are pro-drugs which undergo conversion to 6-MP (6 mercaptopurine). While the mechanism of action has not been fully elucidated, they are thought to inhibit immune activation through inhibition of T cell DNA synthesis. These drugs are indicated in steroid-refractory or dependent IBD. Their most serious side effects are related to effects on immune and blood cells (agranulocytosis). Opportunistic infection, cholestatic hepatitis, pancreatitis, skin malignancy, and lymphoma are also problematic and these drugs require regular monitoring by blood testing for the duration of their use (Pearson, 1995). Cyclosporine and methotrexate have also been used in treatment of IBD with variable responses and with multiple side effects (Feagan, 1995).

Anti-TNF-α therapy: Biologics, in the form of infliximab and adalimumab, have revolutionised the management of IBD and represent an enormous advance in treatment of patients with severe or refractory disease. However, there are still some concerns as to long-term efficacy and safety. Therapeutic effects of infliximab can be impressive, with mucosal healing occurring in up to 70% of patients (Colombel, 2011). Furthermore, co-prescription with 6-MP/azathioprine has been shown to improve efficacy (D'Haens, 2010). However, serious infections can be associated with treatment and include TB, Salmonella, cellulitis, and pneumonia. Infliximab use can also cause infusion reactions or be associated with delayed hypersensitivity. There are also associations between drug use and malignancies and in young patients it has been particularly associated with the occurrence of T cell lymphomas, which are notoriously difficult to treat and almost universally fatal (Hanauer, 2002).

Surgery: Finally, surgery can offer a cure for UC but there is always a risk of recurrence of CD after resection. In addition, surgery often entails long-term nutritional complications and can be associated with permanent disfigurement, as is the case with colostomy or ileostomy.

In conclusion, while they have their place in the treatment of disease, all of the treatments currently available for IBD and diarrhoeal diseases in general, have drawbacks, whether it is lack of efficacy, significant systemic side-effects, or expense. Thus, there remains a true clinical need for novel treatments.

1.6 Aims of Thesis

Despite their prevalence and the huge burden that they pose to healthcare systems, effective and safe therapeutic options for diarrhoeal diseases are still lacking. Recent years have seen the emergence of bile acids as an important family of “enterocrine” hormones that regulate diverse aspects of intestinal epithelial function. This has sparked a great deal of interest in targeting BAs for therapeutic purposes. UDCA is a well-studied therapeutic agent, primarily used to treat cholestatic liver diseases. Less is known about the actions of UDCA In the colon, and while the pro-secretory, pro-inflammatory and largely detrimental effects of several colonic bile acids have been well-documented in certain disease states, the role of UDCA has not been well-defined. With this in mind, the overall goal of this thesis was to investigate the effects of UDCA on key aspects of colonic epithelial physiology, and to examine the possibility that UDCA could be targeted for treatment of diarrhoeal or inflammatory intestinal diseases.

In particular, the aims of this thesis were to investigate:

i) the role of UDCA in regulating colonic epithelial secretory function
ii) signalling pathways and molecular targets involved in mediating the effects of UDCA
iii) The role of UDCA in regulating epithelial barrier function and TLR-driven cytokine secretion
iv) The potential for targeting UDCA in treatment of diarrhoeal diseases

Chapter 2:

Materials and Methods

2.1 Materials:

Table 2.1 delineates all of the BAs and BA derivatives used for experiments included in the thesis with concentration ranges used, empiric formulae and source. Table 2.2 provides a comprehensive list of all reagents used in Ussing chamber experiments, concentrations used and source. The specific uses of reagents are described in more detail in the relevant sections of the methods chapter. Table 2.3 outlines a number of the pharmacological inhibitors used for kinase inhibition with references for concentrations used.

Table 2.1: Bile Acids and bile acid derivatives used

Bile Acid (derivative)	Concentration	Empiric formula	Source
Ursodeoxycholic Acid (UDCA)	50–1000 μM	$C_{24}H_{39}O_4 \cdot Na$	Calbiochem
Lithocholic Acid (LCA)	50–1000 μM	○ $C_{24}H_{40}O_3$. Na	Calbiochem
Chenodeoxycholic Acid (CDCA)	50–1000 μM	$C_{29}H_{46}O_6$	Sigma Aldrich
Deoxycholic Acid (DCA), Cholic Acid (CA)	50–1000 μM	$C_{24}H_{40}O_4$ ○ $C_{24}H_{40}O_5$	Sigma Aldrich
Tauroursodeoxycholic Acid (TUDCA)	50–1000 μM	$C_{26}H_{44}NO_6S \cdot Na$	Calbiochem
FM191 (fluorescent)	50–500 μM	$C_{30}H_{42}N_4O_6$	Gift, Dr J Gilmer, TCD
6α-methyl Ursodeoxycholic Acid (6a- MUDCA)	50–1000 μM	$C_{25}H_{42}O_4$	Gift, Prof A. Roda, University of Bologna

Table 2.2: Drugs and Reagents.

Reagent	Abbreviation	Concentration	Source
Carbachol	CCh	100 μM	Sigma Aldrich
Forskolin	FSK	10 μM	Sigma Aldrich
Amphotericin B	Amp B	50 μM	Sigma Aldrich
Nystatin	Nys	100 μg/ml	Sigma Aldrich
Heat stable enterotoxin	ST_a	100 nM	Sigma Aldrich
Cholera toxin	CTX	100 nM	Sigma Aldrich
Bumetanide	Bum	100 μM	Sigma Aldrich
Amiloride	Am	10 μM	Sigma Aldrich
Ouabain	O	100 μM	Sigma Aldrich
Tetra ethyl ammonium chloride	TPeA	100 μM	Sigma Aldrich
Chromanol 293B	C293B	100 μM	Sigma Aldrich
Tetrodotoxin	TTX	100 nM	Sigma Aldrich
Mannitol	Man	25 mM	Sigma Aldrich
Sodium 4- phenyl butyrate	4PBA	5 mM	Sigma Aldrich
Phlorizdin	Phl	1 mM	Sigma Aldrich

Table 2.3: Pharmacological inhibitors used

Drug	Kinase inhibited	Concentration used (μM)
Bisindolylmaleimide	PKC	5 (McKay,2007)
H-89	PKA	5 (Hoque, 2010)
PD98059	ERK1/2 MAPK	5 (Mroz, 2012)
SB203580	p38 MAPK	10 (Keely, 2003)

2.2 Human tissues:

Resected colonic tissue was obtained from adult patients undergoing colorectal surgery and biopsies were from adult patients undergoing colonoscopy in Beaumont Hospital between July 2008 and March 2011. Adult patients undergoing colorectal surgery primarily for colorectal cancer were included. Patients that had received neoadjuvant chemo/radiotherapy, with a background of IBD, or with active inflammation due to diverticulitis or perforation or with concomitant enteric infection such as *Clostridium difficile* at time of resection were excluded for the purposes of this study. Participants agreed to participate by written informed consent explained by the colorectal cancer research nurse or one of the primary clinical investigators (Dr Orlaith Kelly or Professor Frank Murray). This study was approved by Beaumont Hospital Medical Ethics Committee. All surgical specimens were collected on ice from the Pathology Department. Normal colonic mucosa was identified macroscopically and microscopically by individual pathologists. All specimens were taken at least 3 cm clear of tumour margins and at least 3 cm clear of resection margins to avoid injured or cancerous tissue.

Biopsies were also prospectively taken from adult patients who had been treated with oral UDCA for any indication (usual dose 750 mg/ day or 10 kg) who were

undergoing a colonoscopy for any indication. Patients with active inflammation were excluded. Patients with quiescent IBD were included. A healthy control group, i.e. consented asymptomatic patients undergoing colonoscopy for family screening, identified as having a normal appearance at colonoscopy, were also included. These were age and gender matched for the UDCA patients. A total of 6 biopsies per patient were obtained with a large diameter (6 mm) biopsy forceps. Each sample of tissue was transported bathed in ice-cold Ringer's solution to the Molecular Medicine Department. The tissue was then prepared for 1) electrophysiological measurements or 2) crypt isolation for Ca^{2+} imaging and immunofluorescence studies. Patient demographics, including age, gender, indication for resection/endoscopy, area of colon from which sample was taken, relevant findings at time of operation or endoscopy, medications and relevant co-morbidities, were recorded. All patient data were de-identified and patient identifier details were stored separately in a password-protected encrypted database accessible only by the clinical study lead in order to retain patient confidentiality.

2.3 Animals:

Male C57BL6 mice aged 6–12 weeks were used. The animals were maintained in an environmentally-controlled facility on a 12 h light/dark cycle and were given *ad libitum* access to food and water. All experiments were approved by the Royal College of Surgeons in Ireland Ethics Committee. UDCA (10 – 100 mg/ kg) or 6α-MUDCA (10 – 100 mg/ kg), prepared in endotoxin-free phosphate-buffered saline (PBS 0.1 mL), were injected intra-peritoneally and mice were sacrificed 4 h later by cervical dislocation.

2.4 Cell Culture:

T_{84} cells, a human colonic epithelial cell line derived from colon carcinoma, were cultured in DMEM/Hams F12 media (1:1) (Sigma-Aldrich, Gillingham, UK) supplemented with 5% (v/v) new-born calf serum (HyClone, Logan Utah, USA) in an atmosphere of 5% (v/v) CO_2 at 37 ^{0}C, with medium changes every 3–4 days. Cells were split when they reached approximately 90% confluency using trypsin. For electrophysiological studies, 5 x 10^5 cells were seeded onto 12 mm Millicell HA Transwell inserts (Millipore, Bedford MA). For cyclic AMP assays, Western blotting

and surface biotinylation experiments, 1 x 10^6 cells were seeded onto 30 mm Millicell HA Transwells. Cells seeded onto Millicell filters were cultured for 10–15 days prior to use until a trans-epithelial resistance of >1000 ohms.cm^{-2} was achieved. Under these conditions, T_{84} cells develop the polarized phenotype of native colonic epithelial cells and they are widely accepted as being the best available reductionist model for studies of colonic epithelial Cl^- secretion (Barrett, 2000). For Ca^{2+} imaging experiments, cells were seeded on glass-bottomed dishes and were grown to approximately 90 % confluency prior to experimentation. For all experiments carried out on 96-well plates, including ELISAs, cell proliferation, or apoptosis assays, cells were seeded at a density of 1 x 10^5 cells per well.

2.4.1 UDCA treatment:

T_{84} cells were washed in serum-free medium prior to any treatment and allowed to equilibrate for 1 h. Following the equilibration period, cells were treated with UDCA at various concentrations for a period of 15 min, unless otherwise noted in the figure legend.

2.5. Electrophysiological Measurements

Epithelial preparations were placed in Ussing chambers and bathed in physiological solution at 37 ^{0}C and an external current, known as the short-circuit current (I_{sc}), was applied. The spontaneous electric potential difference (PD) was clamped to 0 mV. The electrical parameters measured in these experiments included trans-epithelial resistance (TER) across the epithelial layer, the trans-epithelial PD, and I_{sc}, both under basal conditions and in response to agonist stimulation (see Figure 2.1). The I_{sc} required to maintain the trans-epithelial PD at 0 mV is an indirect measure of vectorial electrogenic ion movement across cell monolayers or tissue preparations (see Figure 2.1 for a diagrammatic representation of the Ussing chamber). After culture for 10–15 days on filter supports, T_{84} cell monolayers were washed in serum-free medium and allowed to equilibrate for 1 h. The formation of a confluent layer was ascertained by trans-epithelial resistance measurements, which in all experiments exceeded 1000 Ω.cm^2. Cells were then mounted in Ussing chambers for measurements of I_{sc}. Conductance measurements were also recorded at intervals throughout the experiments in order to determine any changes in TER which could indicate loss of cell/ tissue viability.

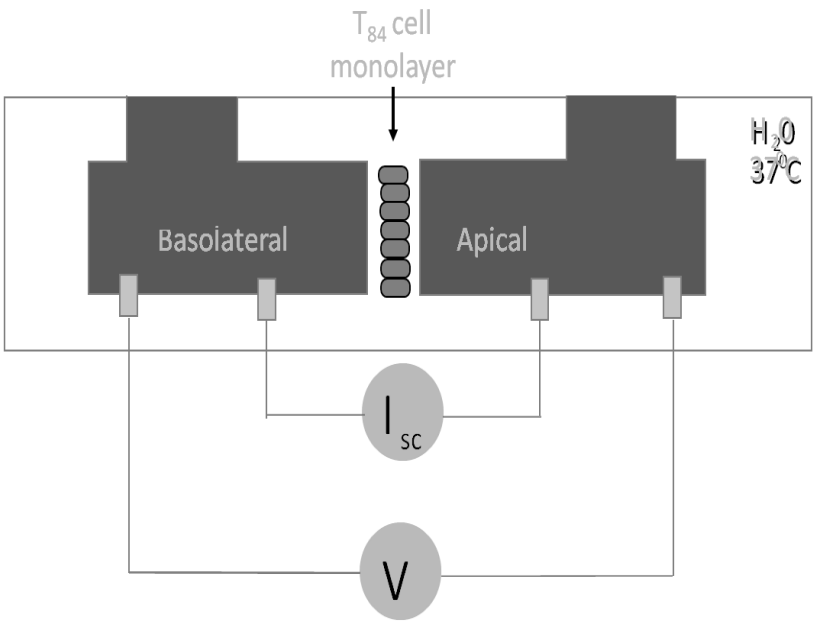


Figure 2.1: Ussing chamber. The epithelial preparation (i.e. epithelial monolayers, human, or mouse tissue) was bathed in Ringer's solution and voltage clamped to 0 mV potential difference by the application of a short circuit current (I_{sc}). TER, changes in I_{sc}, and PD were measured in response to various treatments.

2.5.1. Prototypical agonists of Cl^- secretion.

Carbachol (CCh) is a cholinergic agonist that binds and co-activates nicotinic and muscarinic receptors. It is a non-selective agonist that, in contrast to acetylcholine, is resistant to the action of cholinesterases. Serosal application of CCh to intestinal epithelial monolayers mounted in Ussing chambers causes an immediate increase in short circuit current (I_{sc}) that typically peaks within 5 min and declines rapidly thereafter, although a small increase in I_{sc} may persist for approximately 30 min (Pandol, 1986). Secretory responses to CCh across intestinal epithelial cells are mediated through the G_q protein-coupled M_3 receptor, and subsequent mobilisation of Ca^{2+} from IP_3-sensitive pools. Thus, CCh is routinely employed as a prototypical agonist of the Ca^{2+}-activated secretory pathway in intestinal epithelia (Vaandrager, 1991).

Forskolin (FSK) is a labdane diterpene that is produced by the Indian Coleus plant (*Coleus forskohlii*). FSK is commonly used in experimental settings to raise levels of

intracellular cyclic AMP (cAMP) through activation of adenylyl cyclase. cAMP is an intracellular signalling molecule, important in transducing many cellular responses to hormones and other extracellular signals. Thus, FSK is commonly used as a prototypical agonist for cAMP-induced secretion in intestinal epithelia. I_{sc} responses to agonists such as FSK, are more slow in onset and sustained than those stimulated by Ca^{2+}-dependent agonists (Cuthbert, 1987).

Cholera toxin (CT): CT activates the adenylate cyclase enzyme. The effect is dependent on a specific receptor, monosialosyl ganglioside (GM1 ganglioside) present on the surface of intestinal mucosal cells. The bacterium produces an invasin, neuraminidase, during the colonization stage which degrades gangliosides to the monosialosyl form, which is the specific receptor for the toxin. The net effect of the toxin is to cause cAMP to be produced at an abnormally high rate which stimulates mucosal cells to pump large amounts of Cl^- into the intestinal contents. H_2O, Na^+ and other electrolytes follow due to the osmotic and electrical gradients caused by the loss of Cl^-. The lost H_2O and electrolytes in mucosal cells are replaced from the blood. Thus, the toxin-damaged cells become pumps for water and electrolytes causing the diarrhoea, loss of electrolytes, and dehydration that are characteristic of cholera (Spangler, 1992).

The toxin has been characterized and contains 5 binding (B) subunits of 11,500 Daltons, an active (A1) subunit of 23,500 Daltons, and a bridging piece (A2) of 5,500 Daltons that links A1 to the 5B subunits. Once it has entered the cell, the A1 subunit enzymatically transfers ADP ribose from NAD to a protein (called Gs or Ns), that regulates the adenylate cyclase system which is located on the inside of the plasma membrane of mammalian cells (Bharati, 2011).

Secretory responses of T_{84} cell monolayers to acetylcholine have previously been shown to be greatly potentiated in the presence of CT. CT (0.0001–0.1 µg/ mL (w/v)) applied to the apical domain of T_{84} cell monolayers produces a concentration dependent increase in I_{sc} (Banks, 2004). In our experiments, an optimal concentration of 0.01 µg/ mL (w/v) apically applied was used after a series of concentration experiments.

Heat stable *E. coli* enterotoxin (ST_a): Two families of heat-stable enterotoxins have been identified: ST_a and ST_b, which differ by their physicochemical and biologic characteristics (Burgess, 1978). Although ST_a and ST_b were both discovered in intestinal bacterial strains isolated from humans, ST_b is produced by bacterial strains that preferentially inhabit pigs (Lortie, 1991). Further, ST_a induces diarrhoea through a cyclic nucleotide-dependent mechanism.

ST_a is produced by a variety of enteric pathogenic organisms, including diarrhoeagenic *Escherichia coli* (*E. coli*), *Vibrio cholerae*, *Vibrio mimicus*, *Yersinia enterocolitica*, *Citrobacter freundii*, and *Klebsiella pneumonia.* ST's are translated as precursor peptides which are processed to active peptides (Lin, 2010). Isoforms of ST share a conserved *C*-terminal region of 13 amino acids containing three disulfide bonds responsible for heat stability and biological activity. Investigation of the pathogenesis underlying diarrhoea produced by ST ultimately revealed two intestinal paracrine hormones, guanylin and uroguanylin, and the receptor for these homologous peptides, guanylyl cyclase C (GC-C), encoded by the gene ***GUCY2C*** (Lucas, 2000).

ST_a has been shown to cause Cl^- secretion across T_{84} cell monolayers in a dose-dependent manner when applied to the apical membrane and not when applied to the basolateral surface. This is a cGMP mediated response (Huott, 1988). In these experiments, an optimal concentration of 100 nM was used as determined by a series of optimisation experiments. This is similar to concentrations previously use in Ussing experiments (Trucksis, 2000; Vaandrager 1992).

2.5.2 Experimental Approach.

Confluent T_{84} cell monolayers were mounted in Ussing chambers (aperture = 0.6 cm^2), voltage-clamped to zero potential difference, and monitored for changes in short-circuit current (I_{sc}) using a VCC MC8 voltage clamp (Physiological Instruments, San Diego, CA). Under such conditions, secretagogue-induced changes in I_{sc} across T_{84} monolayers are wholly reflective of changes in electrogenic Cl^- secretion (Cartwright, 1985). I_{sc} measurements were carried out in Ringer's solution gassed with CO_2 (pH 7.4), containing (in mM): 140 Na^+ , 5.2 K^+, 1.2 Ca^{2+}, 0.8 Mg^{2+}, 119.8 Cl^- , 25 HCO^{3-}, 2.4 $H_2PO_4^{2-}$, and 10 Glucose. Results were normalised and expressed

as ΔI_{sc} (μA/ cm^2), or as percentage of control responses (% CR). For preparations of muscle-stripped human or mouse colonic mucosa, Ussing chambers with a window area of either 0.3 cm^2, or in the case of human tissue, 0.5 cm^2 were used.

2.5.3 Measurements apical Cl^- currents, basolateral K^+ currents, and Na^+/K^+ ATPase activity.

Apical Cl^- currents were measured as described by Rochwerger *et al.* (Rochwerger, 1994). T_{84} cell monolayers were mounted in Ussing chambers, and an apical to basolateral Cl^- gradient (119.8 – 4.8 mM) was established by replacing NaCl in the apical solution with equimolar Na-gluconate. Monolayers were basolaterally permeabilized by addition of nystatin (100 μg/ mL), and after a 35 min re-equilibration period, cells were stimulated with forskolin (FSK; 10 μM). Under these conditions, changes in I_{sc} reflect apical Cl^- currents (I_{Cl}).

Basolateral K^+ conductance was analyzed as described by Kirk and Dawson (Kirk, 1983). T_{84} cell monolayers were apically permeabilized with amphotericin B (50 μM). A K^+ gradient (123.2 – 5.2 mM) was created across the basolateral membrane by addition of a high - K^+ (123.2 mM) Ringer's solution, in which NaCl is substituted with K^+ - gluconate, to the apical reservoir. Ouabain (100 μM) was added basolaterally to inhibit Na^+/K^+-ATPase pump activity. Under these conditions, changes in I_{sc} are reflective of changes in basolateral K^+ conductance (I_K).

Na^+/K^+-ATPase activity was measured as described by Lam *et al* (Lam, 2003*).* T_{84} monolayers were bathed bilaterally in low Na^+ (25 mM) Ringer's solution, where NaCl was substituted with equimolar *N*-methyl-D-glucamine (NMDG)-Cl. Apical membranes were permeabilised with amphotericin (50 μM). Under these conditions (*i.e.,* in the absence of ionic gradients across the permeabilised monolayer), changes in I_{sc} are reflective of electrogenic transport through the Na^+/K^+-ATPase pump. Experiments were also performed in the presence of basolateral K^+ current inhibitors, TPEA, chromolyn 2B39, and barium to verify that observed currents were due to Na^+/K^+-ATPase activity alone. These inhibitors were, however, not routinely used in these experiments.

2.5.4. Animal and Human Tissue Experiments

For analysis of their effects on Cl^- secretion in mice, UDCA (10–100 mg/ kg) or 6α-MUDCA (10–100 mg/ kg) were administered by intraperitoneal (IP) injection, with control mice receiving PBS. Mice were then sacrificed by cervical dislocation at 4 h post-treatment, the colons were harvested, and sheets of isolated colonic mucosa were obtained by blunt micro dissection of the overlying muscle layers. These tissues were then divided, mounted in Ussing chambers (aperture 0.3 cm^2), bathed in Ringer's solution, and basal and agonist-stimulated I_{sc} responses were recorded.

For human tissue studies, sections of normal human colonic tissue were collected from surgical resection specimens and transported to the laboratory on ice. Tissues were stripped of their underlying smooth muscle and mounted in Ussing chambers (aperture 0.5 cm^2), as described above.

2.5.5. Measurements of Na^+ absorption through ENaC and SGLT-1.

In order investigate colonic Na^+ absorption through ENaC; the T_{84} cell line was used. However, since these cells do not normally express ENaC, a method described previously, in which expression of ENaC was induced in T_{84} cells by pre-treatment with 4 phenyl-butyrate (5 mM, bilateral, 24 h) prior to experimentation, was employed. Using this approach, an amiloride-sensitive current in T_{84} cells could be reliably produced.

To measure SGLT-1 activity, whole thickness sections of proximal jejunum from mice were used. After mounting in Ussing chambers, the tissues were bathed apically in glucose-free Ringers solution and basolaterally in normal Ringers' solution to create a glucose gradient. To balance osmolarity, 25 mM mannitol was included in the basolateral solution. To stimulate SGLT-1 activity, 25 mM glucose was added apically and changes in I_{sc} were measured. 1mM phlorizin was then added to confirm that increases in I_{sc} were due to SGLT-1 activity (Grubb, 1995).

2.6. Bile Acid Measurements

Caecal contents were collected from treated and control animals and stored in isopropanol at -20 ^{0}C. Caecal bile acid levels were measured by HPLC mass

spectrometry at the University of Bologna as previously described, and results were expressed as % nM (w/v) of the total bile acid pool (Janzen, 2010, Tadano, 2006).

2.7 Crypt Isolation

A standard Ca^{2+} chelation approach for colonic crypt isolation was employed (Schwartz, 1991). Resected normal human colonic tissue was washed with Krebs solution (see Table 2.4) and, using a blunt scalpel, was divided into suitably sized sections (approximately 0.5 cm x 1 cm) and placed in 10 mL of crypt isolation buffer, containing: NaCl, 96 mM: KCl, 1.5 mM: EDTA, 27 mM: sorbitol, 55 mM: HEPES (free acid), 10 mM; Tris base, 10 mM; sucrose, 44 mM; and DTT, 1 mM for 30 min at RT (see

Table **2.**5)**.** After vigorous vortexing for 1 min to dislodge intact crypts, the remaining tissue was discarded. The isolated crypts were then centrifuged (600 G, 8 min). Crypts were washed twice in 10 mL of Krebs solution, containing: NaCl 140 mM, KCl 5 mM, $MgCl_2$ hexahydrate 1 mM, $CaCl_2.2H_2O$ 2 mM, HEPES (free acid) 10 mM, Tris base 10 mM, Glucose 10 mM. Finally, crypts were suspended in an appropriate volume of Krebs solution and treated as required. At the final re-suspension stage, a 50 μL aliquot of solution was also inspected on a glass slide to ensure the presence of intact crypts. For protein extraction, crypts were spun at 1,200 G, immediately placed on ice, and then snap frozen. For mRNA extraction, crypts were suspended in RNAlater (Sigma Aldrich, UK) overnight and then frozen. For Ca^{2+} imaging and confocal imaging, crypts were suspended in physiological perfusion solution and used immediately.

Table 2.4. Krebs Solution

Reagent	Concentration (mM)
NaCl	140
KCl	5
MgCl2 Hexahydrate	1
CaCl2.2H2O	2
HEPES (free acid)	10
Tris base	10
Glucose	10

pH adjusted to 7.4 with HCl. Osmolarity was measured to be 290 ± 5 mOsm

Table 2.5 Crypt Isolation Buffer

Reagents	Concentration (mM)
NaCl	96
KCl	1.5
EDTA	27
Sorbitol	55
Tris (base)	10
Sucrose	44
HEPES (free acid)	10
DTT	1

DTT was added fresh and pH adjusted to 7.with NaOH. Osmolarity was measured to be 380 ± 5 mOsm.

2.8. Confocal Imaging:

In Table 2.6 the antibodies and dilutions used for confocal imaging are outlined.

Table 2.6 Antibodies employed for Confocal Imaging

Primary antibodies	Type	Company	Dilution
Na^+/K^+ ATPase α	Monoclonal mouse antibody	AbCam	1:400
Na^+/K^+ ATPase β	Monoclonal mouse antibody	AbCam	1:400
KCNN4	Monoclonal rabbit antibody	AbCam	1: 1500
AlexaFluor 488-conjugated	Goat anti-mouse	Invitrogen	1:500
AlexaFluor 488-conjugated	Goat anti-rabbit	Invitrogen	1:500
Isotype control	Mouse IgG1	Invitrogen	1:400

Preparation of samples:

Isolated crypts were re-suspended in Krebs solution (c.f Section 2.7 Crypt Isolation) and a 270 μL aliquot was pipetted into each well of a CellTak-coated (BD Biosciences) chamber slide. To minimize crypt damage during treatment, aliquots were pipetted against the well wall. When removing liquid, the slide was tilted and liquid was aspirated from the corner of the well. Crypts were allowed to rest for 30 min prior to further treatment in order to allow adherence.

T_{84} monolayers on inserts and isolated human colonic crypts were treated with UDCA (500 μM, bilateral, 15 min) and were then washed 3 times with ice-cold PBS. Cells or crypts were fixed using 4% (w/v) paraformaldehyde (PFA; 150 μL per well) for 20 min on ice, followed by washing 3 times with ice-cold PBS. This was followed

by permeabilisation (0.4% (v/v) Triton-X-100 , 15 min) and then blocking in 3% (w/v) BSA for 15 min. Cells were incubated with 100 μL of primary antibody (mouse monoclonal anti-Na^+/K^+ ATPase α (1: 400), mouse monoclonal anti-Na^+/K^+ ATPase *β* (1: 400), or rabbit monoclonal anti-KCNN4 (1: 1500) (AbCam) for 2 h. Cells were then incubated with goat anti-mouse or goat anti-rabbit AlexaFluor 488-conjugated secondary antibody (1: 500 dilution) for 1 h in the dark (Invitrogen, Carlsbad, CA, USA). Rhodamine phalloidin was included (1: 2000 dilution) to stain for actin. Control samples were exposed to non-specific isotype control IgG1 (AlexaFluor 488-conjugated). Filters were cut and mounted on glass slides using Vectashield (Hardset) with DAPI. Slides were examined by confocal microscopy using a Zeiss LSM 710 microscope.

2.9. Cell Surface Biotinylation

The protocol used was based on one previously published study (Liu, 2002, Del Castillo, 2005). Following treatment with UDCA, 500 μM for 15 min, monolayers of T_{84} cells were washed with ice-cold PBS. Freshly prepared biotinylation buffer (1 mg/mL Sulfo-NHS-Biotin (Pierce Biotechnology, Rockford, IL, USA) in PBS) was added to the basolateral side. Cells were incubated at 4 ^{0}C for 15 min on a rotating platform after which the buffer was replaced. After a further 15 min, cells were washed twice in ice-cold PBS and incubated with a quenching agent (100 mM glycine in PBS). Cells were again washed twice in ice cold PBS and were subsequently lysed in standard RIPA buffer for 30 min on ice (2.11. Western Blotting). The lysate was centrifuged (10,000 G, 6 min) and the protein concentration of the supernatant was determined and normalised. The samples were then precipitated on a rotator overnight at 4 ^{0}C with 100 μL of streptavidin–agarose beads (Pierce Biotechnology, Rockford, IL, USA). Samples were again centrifuged and washed twice in lysis buffer and then re-suspended in 2x Laemmli buffer (Sigma-Aldrich, UK). Samples were heated to 55 ^{0}C and then subjected to SDS-PAGE analysis. Biotinylated proteins were detected by Western blotting.

2.10. Protein Extraction

After appropriate treatments, cells grown on filters were scraped on ice in 1 mL PBS and placed in 1.5 mL Eppendorf tubes. Samples were then centrifuged at 300 G for 1 min. After removal of supernatant, 100–400 μL of complete lysis buffer (depending

on the size of pellet) were added. Standard lysis buffer consists of: 10 mL NP-40 lysis buffer stock solution, 1x complete mini EDTA-free protease inhibitor tablet, 1 ng/ mL PMSF, and 1 μM sodium vanadate. Samples were vortexed and incubated for 35 min on ice, sonicated (for 30 s), and then centrifuged at 12,000 G for 10 min at 4 ^{0}C. The supernatants were removed and placed in fresh tubes and stored at – 80 ^{0}C until further analysis.

2.11. Western Blotting

After UDCA treatment, T_{84} monolayers were washed with ice-cold PBS and lysed using lysis buffer containing 1% (v/v) Nonidet P-40, 150 mM NaCl, 50 mM Tris Base and supplemented with 1 x complete mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, UK), 0.1 mg/ mL PMSF, and 100nM sodium orthovanadate Na_3VO_4), for 30 min. Lysates were scraped into Eppendorf tubes , sonicated (3 x 10 s pulses), centrifuged (15,300 G; 10 min; 4 °C), and the pellets discarded. Samples were normalized for protein content and 2 x gel loading buffer (50 mM Tris HCl, 100 mM DTT, 40% (v/v) glycerol, and 4% (v/v) SDS) was added to the supernatants. After heating to 55°C for 30 min, samples were loaded on 8% gels and separated by SDS-PAGE (at 120 V for 1 h) and transferred to PVDF membranes permeabilised with 100% (v/v) methanol (transfer was run at 15 V for 60 min). Membranes were then washed with TBST followed by pre-blocking in 5% blocking buffer [w/v; Marvel in Tris buffered saline with 1% (v/v) Tween (TBST)] for 60 min; RT; after which they were probed overnight (16 h) at 4 °C with antibodies against the proteins of interest: Table 2.7 outlines the concentrations used for Western Blotting and biotinylation experiments. After washing (x 4) in TBST, membranes were probed with secondary antibodies conjugated to horseradish peroxidase (HRP) in 5% blocking buffer for 60 min; RT. After further washing (x 4) in TBST, immunoreactive protein bands were visualised by employing enhanced chemoluminescence (Amersham Biosciences, Little Chalfont, U. K.) after exposure to Kodak X-Omat LS Film. Protein levels were quantified by densitometry. Densitometric data were normalized to levels of B-Actin in order to control for differences in protein loading between wells and normalized data were then expressed relative to protein expression in control cells, not treated with UDCA.

Table 2.7 Antibodies employed for Western Blotting

Primary antibodies	Type	Company	Dilution
Na^+/K^+ ATPase α	Monoclonal mouse antibody	AbCam	1:1000 1:500 (biotinylation)
Na^+/K^+ ATPase β	Monoclonal mouse antibody	AbCam	1:1000
KCNN4	Monoclonal rabbit antibody	AbCam	1: 1500
HRP- conjugated Secondary antibodies	Goat anti-mouse	Invitrogen	1:500
	Goat anti-rabbit	Invitrogen	1:500

2.12. cAMP measurements

T_{84} cell monolayers were grown on 30 mm Millicell inserts as described above for 14–21 days. After 24 h in serum-free medium, cells were then acutely treated with UDCA (500 μM, bilateral, 15 min) at 37 ^{0}C in a 5 % CO_2 atmosphere. Cells were then exposed to apical FSK (10 μM) for 10 min and were then lysed on ice using 0.1 M HCl (250 μL for 10 min). Lysates were then centrifuged (600 G for 10 min at 4 ^{0}C) and cAMP levels in the supernatants were measured using a commercially available assay kit (Sigma-Aldrich, Gillingham, UK). Cyclic AMP levels were expressed as picomoles of cAMP/ mg protein.

2.13. Intracellular Ca^{2+} Imaging

Calcium fluorescence measurements in cultured colonic epithelial cells (T_{84}) and isolated human crypts were performed using Fura 2/AM (Molecular Probes, Eugene, OR). The cells were grown on glass coverslips to 80 % confluency, washed in physiological salt solution (PSS; 140 mM NaCl, 5mM KCl, 1mM $CaCl_2$, 10mM D-Glucose and 10 mM HEPES TMA, pH 7.4), and then loaded with 5 μL Fura2/AM

(Molecular Probes, Eugene, OR, USA), dissolved in 0.1% (v/v) pluronic acid F-127 plus 0.1% (v/v) DMSO in PSS at RT for 45 min. For experiments in calcium-free solutions, 1 mM $CaCl_2$ was substituted with 1 mM EGTA (Devor, 1990). In separate series of experiments, cells were pre-treated for 30 min with one of a variety of pharmacological inhibitors of different Ca^{2+} channels. The concentrations used were from previous work in the T_{84} cell model (see Table 2.8 Ca^{2+}- channel blockers employed). Optimisation experiments were also undertaken to ensure reproducibility in the cell passages used (data not shown). Coverslips were then washed in PSS and mounted on a Nikon microscope stage (Nikon, Tokyo, Japan). For control responses, cells were perfused with PSS for 5 min before adding carbachol (CCh 100 μM) to the perfusing solution (Reinlib , 1989). The ratio of Fura-2 fluorescence with excitation at 340 and 380nm ($F_{340/380}$) was measured every 3 s and images were captured using an intensified CCD camera (ICCD 200) and a MetaFluor Imaging System (Molecular Devices Corporation, Sunnyvale, CA, USA). Changes in basal $F_{340/380}$ and CCh-induced responses were also measured after treatment with UDCA (10 – 500 μM). For experiments on human colonic crypts, freshly isolated crypts were used. In order to aid attachment, coverslips pre-coated with poly-D lysine were used. Loading with Fura-2/ AM and experimental treatments were then carried out, as described above.

Table 2.8 Ca^{2+}- channel blockers employed

Pharmacological Inhibitor	Concentration	Channels inhibited
SKF 96365	100 μM	Store operated (Hartford, 2007)
Ruthenium Red	100 μM	TRP channels (N& P-type) Ca^{2+}-ATPase (Irnaten, 2008)
Verapamil	100 μM	L-type channels (van der Meerwe, 2008)

2.14. Cell proliferation and cell viability measurements

To perform cell viability measurements, cells were seeded at a density of 5 x 10^5 cells/ ml and were allowed to attach overnight. Once attached, cells were washed and incubated in serum-free medium for 24 h. Cells were then trypsinised, re-suspended in 2% (v/v) charcoal-stripped foetal bovine serum (FBS)-containing medium, and 100 μL aliquots were seeded into the wells of a 96 well plate. Cells were then treated with 100 μL of twice the desired final concentration of BA in serum-free medium to give a final volume of 200 μL per well. Cells were then left for the required time periods (12–96 h). Wells treated with serum-free or serum-containing medium alone (i.e., without bile acid) were used as controls. A commercially available assay kit was used (Sigma Aldrich, UK) for spectrophotometric measurement of cell viability. The biochemical basis of this kit is the conversion of XTT to a water-soluble coloured formazan derivative by mitochondrial dehydrogenase in viable cells. Absorbance of converted dye was measured at a wavelength of 450 nm after 4 h incubation (Roehm, 1991) (Lee, 2008). Data from bile acid-treated cells were compared to those from controls.

2.15. *In vitro* apoptosis assay

The Caco -2 colon carcinoma cell line was used for these experiments. 1 × 10^4 cells were seeded in 96-well plates. After 24 h incubation at 37°C, cells were then pre-treated with UDCA 100 μM for 24 h. This was followed by treatment with the apoptotic stimulants cycloheximide (5 μg/ mL) and TRAIL (25 ng/ mL). Cells were incubated for a further 24 h at 37 °C. The cells were then harvested, re-suspended in ice cold PBS and incubated with YO-PRO-1, Hoechst 33342 and propridium iodide (Vybrant Apoptosis Assay Kit 7, Invitrogen) according to the manufacturer's protocol. Flow cytometric analyses were carried out using the Cyan ADP analyser (Beckman Coulter) and Summit 4.3 Software. Cells were defined as living, early apoptotic, or late apoptotic/necrotic, according to their relative dye uptake (Tambuwala, 2010).

2.16. Measurements of cytokine levels.

T_{84} cells, grown to 80 % confluency on 96 well plates, were pre-treated with UDCA (250 μM, 30 min) before 12 h stimulation with the TLR-specific ligands, Pam3Cys (10 μg /mL; TLR-2), polyinosinic: polycytidylic acid (Poly I:C 20 μg /mL ; TLR-3),

muramyl dipeptide (MDP 1 μg/mL; NOD2), and lipopolysaccharide (LPS 100 ng /mL; TLR-4). The supernatants were collected and IL-8, IL-6, and RANTES levels were measured by ELISA, as outlined below. For cytokine measurements from human colonic tissue, muscle-stripped resected tissues were mounted in Ussing chambers, as described above. Tissues were pre-treated bilaterally with UDCA (250 μM; 30 min), stimulated with LPS (apical 100 nM), and IL-8 release into the basolateral bathing solution was measured after 6 h. Tissue viability was assessed by monitoring TER over time (Zheng , 2008).

2.16.1. ELISA protocol for cytokine level measurement

ELISA plates were pre-coated with capture antibody (50 μL per well) by incubating at RT overnight. The capture antibody was then aspirated and the plate was washed (x 3) with wash buffer (4L wash buffer: 4L distilled H_2O, 400 mL 10X PBS, 2 mL 0.05% (v/v) PBS Tween) The plate was then blocked using reagent diluent (150 μL/well) (Reagent Diluent composition: 1% (w/v) BSA in sterile PBS) for 1 h at RT followed by aspiration and washing (x 3). Serial dilutions of cytokine standards were prepared. Standards and samples were added to the plate (50 μL) and incubated for 2 h at RT. This was followed by aspiration and washing (x 3). Streptavidin- HRP (50 μL/ well) was then added and the foil-covered plate was incubated with shaking for 20 min at RT. Following further aspiration and washing (x 3), substrate solution was added (50 μL/ well) and the foil-covered plate was incubated with shaking for a further 20 min at RT. To stop the reaction, 50 μL of stop solution (H_2SO_4) was added to each well and the plate was read immediately at 405 nm.

2.17. Statistical Analysis:

Data were graphically expressed as mean ± SEM for a series of *n* experiments. Student's t-tests were used to compare paired data. One-way analysis of variance (ANOVA) with the Student Newman-Keul's post–test was used when 3 or more groups of data were being compared. p values $\leq$ 0.05 were deemed to be statistically significant. All concentration response data is expressed as log concentration and were normalised prior to statistical analysis. Non-linear regression was undertaken and EC50's were calculated. Graph Pad Prism, SPSS and Sigma plot software were used.

Chapter 3

Ursodeoxycholic acid exerts anti-secretory actions on colonic epithelial cells

3.1 Introduction

3.1.1 Regulation of Intestinal Fluid Transport

As discussed, the ability of the intestine to appropriately regulate fluid transport is vitally important. The balance between absorption and secretion is highly dynamic and is tightly regulated by a vast array of neurotransmitters, immunological mediators, and blood-borne factors, such as hormones. These mediators typically act on receptors expressed on, or within, the epithelial cells. Luminal factors, such as bile acids, bacterial toxins, and digestive by-products, such as butyrate and other metabolites, can also profoundly influence epithelial fluid transport in the colon (Keely, 2008). The molecular pathways that regulate epithelial absorptive and secretory processes are described in detail in Chapter 1.

3.1.2 Intestinal Disorders of Fluid Transport

Many intestinal disorders exist can disrupt the finely-tuned balance between absorption and secretion leading to the clinical manifestation of diarrhoea. Diarrhoeal disease is prevalent in the developed world and is a principal feature of many intestinal disorders, including inflammatory bowel disease, infectious diseases, and digestive disorders, such as coeliac disease and lactose intolerance, and irritable bowel syndrome. Diarrhoea can also be common side effect of many drug treatments, particularly chemotherapeutics. A number of different classifications of diarrhoeal disease exist based upon aetiology. These include malabsorptive conditions such as coeliac disease, secretory diarrhoea such as that which occurs in bile acid malabsorption, motility disorders, and infectious or inflammatory diarrhoea. In many conditions, including IBD, some of these aetiologies overlap. Despite major advances in the understanding and treatment of many intestinal diseases, diarrhoea remains a leading cause of global child deaths and a potentially important cause of lifelong morbidity.

3.1.3 Bile Acids as regulators of Fluid transport in the Intestine

BAs have long been recognised as important regulators of epithelial fluid and electrolyte transport. The primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesised in the liver. They are then stored in their conjugated forms in the gallbladder and are released in the duodenum upon ingestion of food. As they

pass through the small intestine, BAs perform their classical functions in facilitating digestion and absorption of lipids and are reabsorbed in the terminal ileum to be recycled back to the liver. Normally, this enterohepatic circulation of BAs is extremely efficient with < 5% of circulating BAs entering the colon after each cycle. Here, they are metabolised by the resident enteric bacteria through deconjugation, dehydroxylation and epimerisation to yield secondary BAs. CA is metabolised to DCA, which is the predominant human colonic BA, while CDCA is metabolised predominantly to ursodeoxycholic acid (UDCA), (Figure 3.1). UDCA is subsequently rapidly metabolized to LCA, and is therefore not present in large quantities in the human colon (Mekjian, 1971, Gordon, 1979).

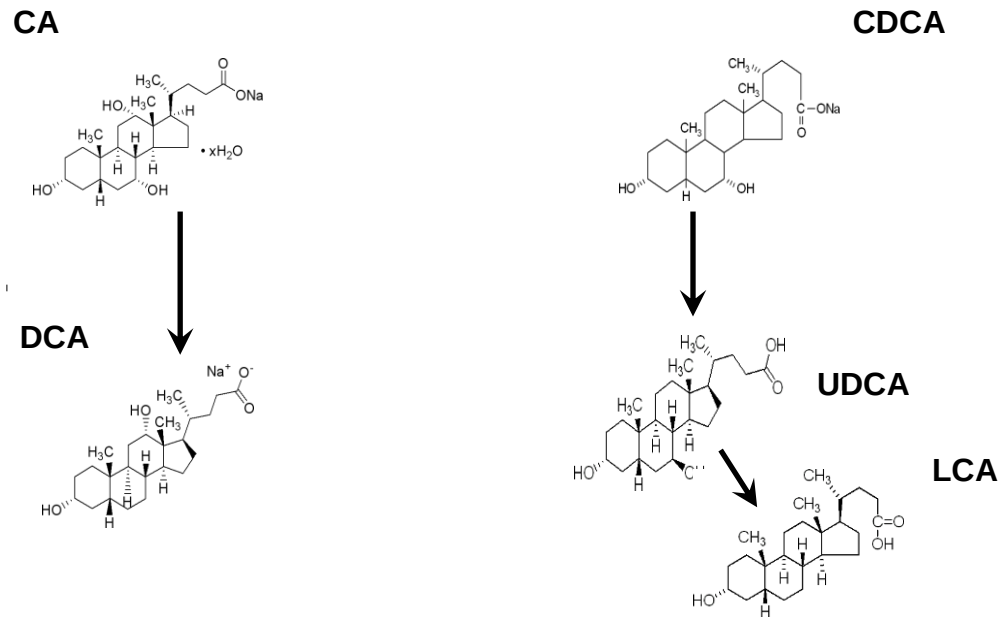


Figure 3.1: Metabolism of bile acids in the colon. The primary BA, cholic acid (CA), is metabolised by the enteric flora to deoxycholic acid (DCA), the predominant faecal BA in humans. Chenodeoxycholic acid (CDCA) is epimerised to ursodeoxycholic acid (UDCA), which subsequently undergoes dehydroxylation to lithocholic acid (LCA).

Studies from this laboratory have recently shown that, when present at physiological concentrations the most common colonic BA, DCA, exerts anti-secretory actions on the colonic epithelium, an effect that has been proposed to promote normal colonic absorptive function (Keating, 2009). However, in conditions of BA malabsorption, increased levels of BAs, in particular DCA and CDCA, are present in the colon, where they are well-known to promote fluid and electrolyte secretion (Mekjian, 1971).

The effects of these pro-secretory BAs have been extensively investigated in a variety of species and cell culture models. While there are considerable interspecies variations in the actions of BAs, it is clear that in humans, most BAs promote secretion only when they are present at pathophysiological concentrations. It is also apparent that strict structure-activity relationships also exist for the pro-secretory actions of BAs, with only dihydroxy BAs being effective. However, UDCA appears to be an exception to this rule (Keely, 2007).

3.1.4 UDCA effects on Colonic Secretion

UDCA is already a well-studied therapeutic agent in its own right. It is primarily used in cholestatic liver disease and has been shown to have choleretic and cytoprotective effects on hepatocytes (Lindor, 1994, Tsagarakis , 2010). In both liver and colonic cell lines, it has been shown to have anti-apoptotic actions (Serfaty, 2010). However, new data emerging on potential detrimental effects of high-dose UDCA in patients with primary sclerosing cholangitis serves to further illustrate the need for a better understanding of the complex actions of this widely used compound (Lindor, 1994, Csutora, 2006, Sinakos, 2010).

UDCA is formed naturally in human colon by bacterial-induced epimerisation of the 7-OH group of CDCA. UDCA is subsequently rapidly metabolized to LCA, and is therefore not normally present in large quantities in the human colon. Previous studies from this laboratory have shown that, unlike other dihydroxy BAs, UDCA is devoid of pro-secretory activity when applied to isolated colonic epithelial cells *in vitro* (Keely, 2007). This supports previous observations that, unlike its parent compound CDCA, UDCA does not stimulate secretory responses across voltage-clamped sections of guinea pig colon in Ussing chambers (Keely, 2007). However, to date a thorough study of the actions of UDCA, or its metabolite LCA, on colonic epithelial secretory function has not been carried out.

3.2 Aims:

In these studies, we aimed to further investigate the role of UDCA in regulation of colonic epithelial fluid and electrolyte transport. In particular, we aimed to:

i) Investigate effects of UDCA on the capacity of epithelial cells to evoke secretory responses.

ii) Investigate the molecular mechanisms underlying UDCA effects on intestinal secretion.

iii) Conduct translational studies to determine the potential for targeting UDCA for treatment of diarrhoea.

3.3 Results

3.3.1 UDCA is predominantly anti-secretory *in vitro*

In contrast to its parent BA, CDCA, which has been shown previously to be pro-secretory (Keely, 2007), addition of UDCA at concentrations up to 1 mM did not alter basal I_{sc} across voltage clamped T_{84} cell monolayers (Figure 3.2). At a concentration of 500 μM, UDCA induced a mean ΔI_{sc} response of 4.8 ± 0.5 μA/cm^2 (n = 6), compared to 40.2 ± 2.7 μA/cm^2 (n = 6) upon CDCA (500 μM) treatment.

However, we found that pre-treatment of the cell monolayers with UDCA (500 μM) did cause a significant attenuation in subsequent responses to the prototypical agonists of Ca^{2+}- and cAMP- induced Cl^- secretion, carbachol (CCh; 100 μM) and forskolin (FSK; 10μM), to 18.9 ± 3.8% and 40.2 ± 7.4% of controls, respectively (n = 18, Figure 3.3).

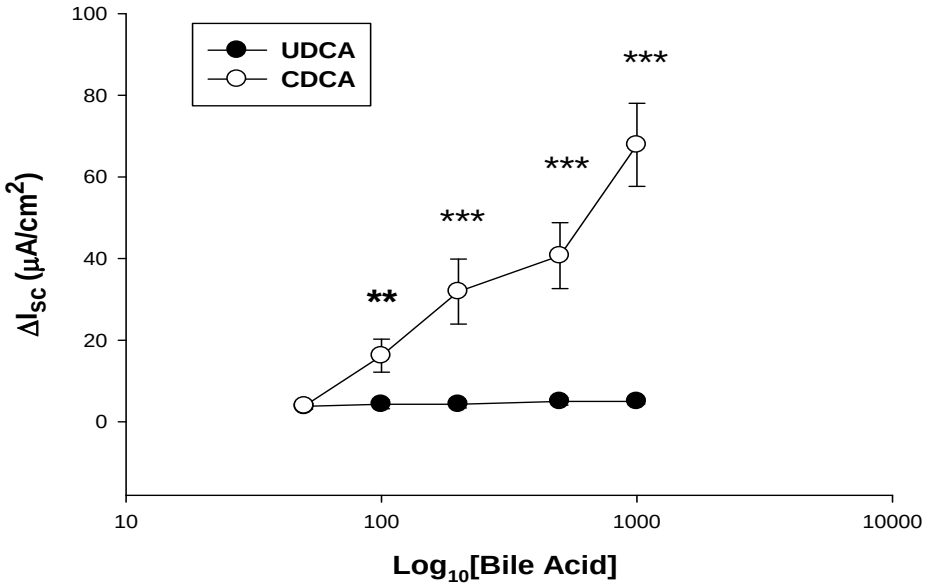


Figure 3.2: UDCA does not alter basal I_{sc} across T_{84} cells monolayers. Monolayers of T_{84} cells grown on permeable supports were mounted in Ussing chambers for measurements of Cl^- secretion. After a 15 min equilibration period, cells were treated with varying concentrations of UDCA (50 μM–1 mM; n = 6) or CDCA (50 μM–1 mM) and ΔI_{sc} was measured **p < 0.01; ***p < 0.001.

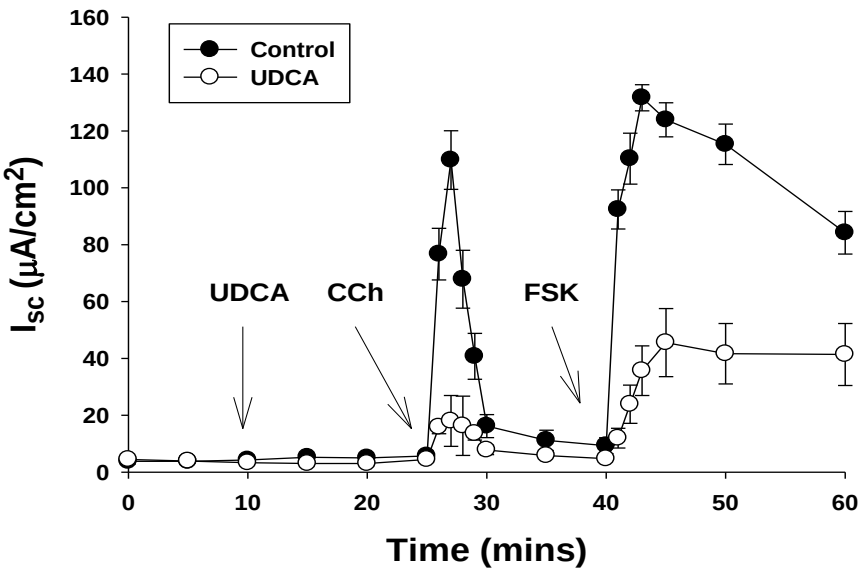


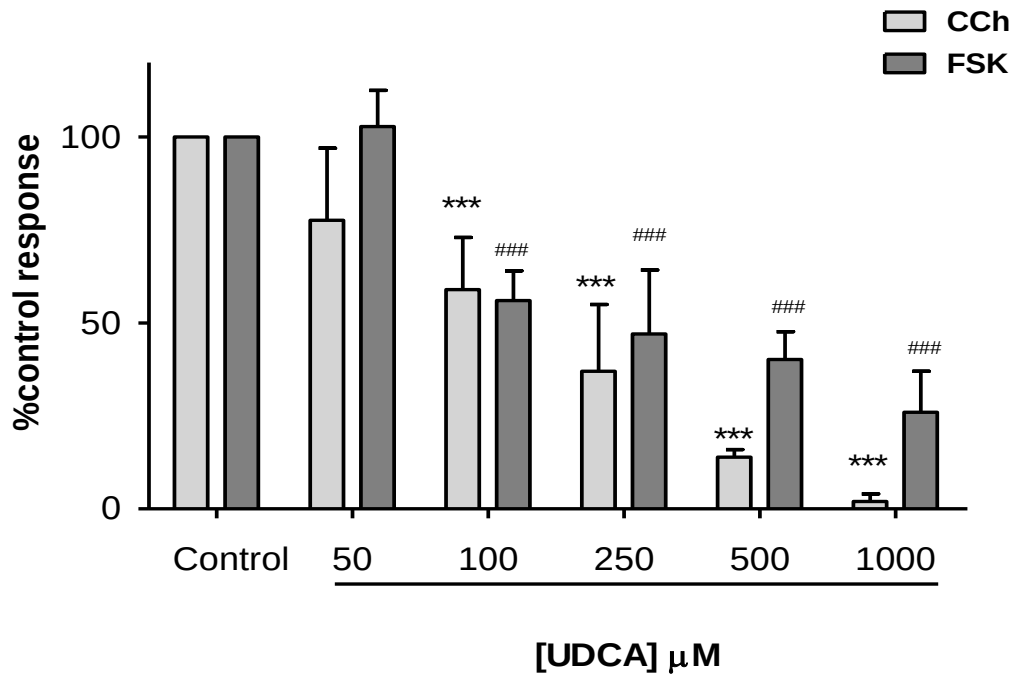
Figure 3.3: UDCA attenuates agonist induced Cl^- secretion across T_{84} cells monolayers. T_{84} cell monolayers grown on permeable supports were mounted in Ussing chambers for measurements of Cl^- secretion. After a 15 min equilibration period, cells were treated with UDCA (500 μM; bilaterally) for a further 15 min. Cells were then stimulated sequentially with CCh (100 μM) and FSK (10 μM) and ΔI_{sc} responses were recorded (n = 18).

The effects of UDCA were concentration dependent, with anti-secretory actions being apparent at 50 μM (Figure 3.4, Panel A) and with an IC_{50} of approximately 200 μM (Figure 3.4, Panel B). Furthermore, UDCA-induced inhibition of Cl^- secretion occurred very rapidly, with responses to CCh being inhibited within 1 min after treatment. Interestingly, anti-secretory effects of UDCA on cAMP-dependent secretion were somewhat slower in onset, becoming apparent only within 15–30 mins after treatment (Figure 3.5).

In further characterising UDCA effects on T_{84} cell monolayers, it was determined that there was no evidence of cellular toxicity. In experiments assessing the effects of UDCA treatment on conductance across confluent T_{84} cell monolayers, it was found that UDCA (50 μM–1 mM; 24 h) had no significant effect, even at the highest concentration tested (n = 6; Figure 3.6).

Furthermore, experiments were carried out in order to determine if the anti-secretory effects of UDCA were reversible. In these experiments, T_{84} monolayers were pre-treated with UDCA (250 μM; 15 min), washed in serum-free medium to remove the bile acid, and then incubated in fresh serum-free medium. I_{sc} responses to CCh or FSK were measured at various time points after the washout period. We found that the anti-secretory effects of UDCA were reversible, with its effects on CCh-induced responses being no longer apparent 120 min after washout (Panel A, Figure 3.7); while its effects on FSK-induced responses were no longer apparent 60 min after washout (Panel B, Figure 3.7).

A



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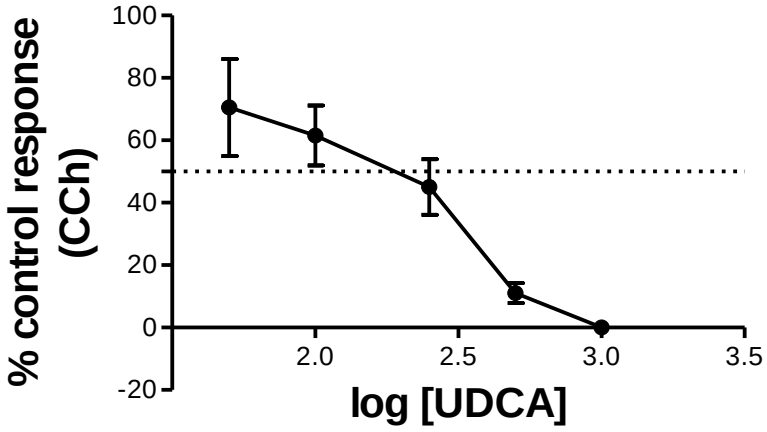


Figure 3.4: Anti-secretory effects of UDCA in T_{84} cell monolayers are concentration-dependent **A)** T_{84} cell monolayers were mounted in Ussing chambers for measurements of Cl^- secretion. After a 15 min equilibration period, cells were exposed to UDCA at differing concentrations (50 μM–1 mM; 15 min) and I_{sc} responses to CCh (100 μM) and FSK (10 μM) were measured. Results were normalised and expressed as a % of control responses to CCh or FSK. Panel **B** shows log [UDCA] (50 μM–1 mM additions; 15 min; bilateral). Data were normalised and expressed as a % of control responses to CCh and were plotted as a linear graph for calculation of the IC_{50} which is 204 μM (n = 6–18, *** $p < 0.001$ compared to control responses to CCh, ### $p < 0.001$ compared to control responses to FSK).

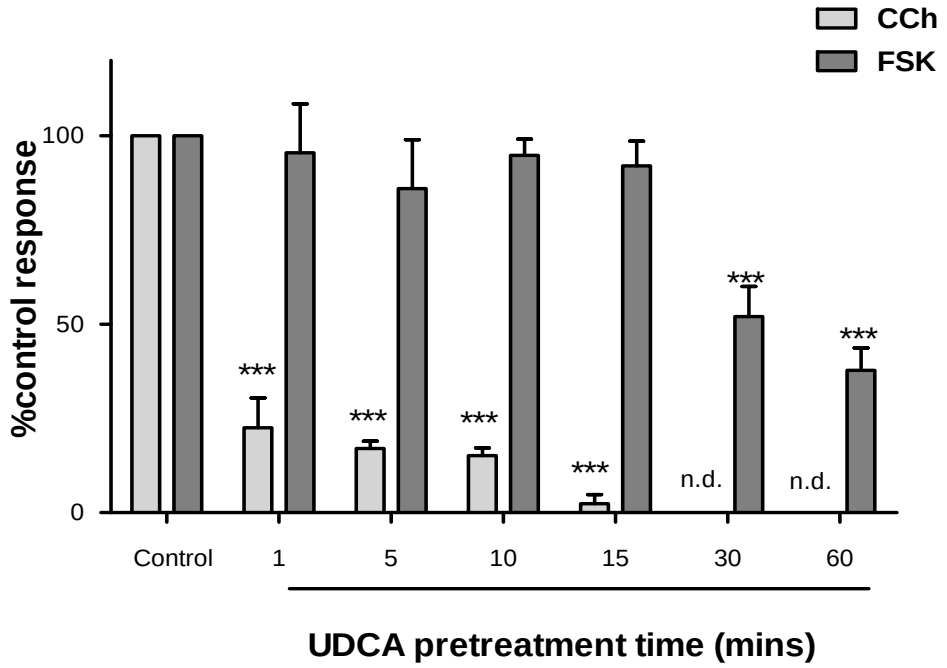


Figure 3.5: Time course of anti-secretory effects of UDCA in T_{84} cell monolayers. T_{84} cell monolayers were mounted in Ussing chambers for measurements of Cl^- secretion. After a 15 min equilibration period, UDCA (500 μM; bilateral) or control (PBS) was added for various time intervals (1–60 min) before measuring secretory responses to CCh (100 μM) and FSK (10 μM). Results were normalised and expressed as a % of control responses to CCh or FSK (n = 4–10, *** $p < 0.001$ compared to control responses to CCh or FSK).

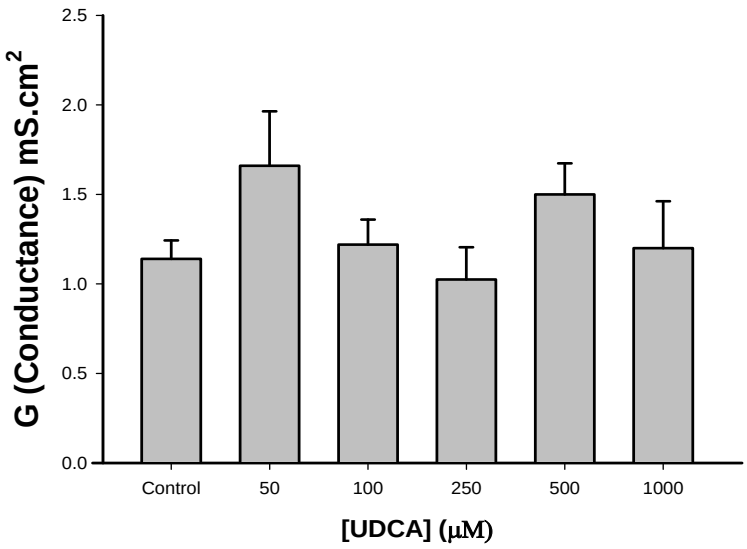


Figure 3.6: UDCA does not significantly alter conductance. T_{84} cell monolayers were mounted in Ussing chambers, allowed to equilibrate and treated with varying concentrations of UDCA (50-1000 μM; 15 min). Trans-epithelial resistance was measured and conductance calculated ($mS.cm^2$) (n = 18).

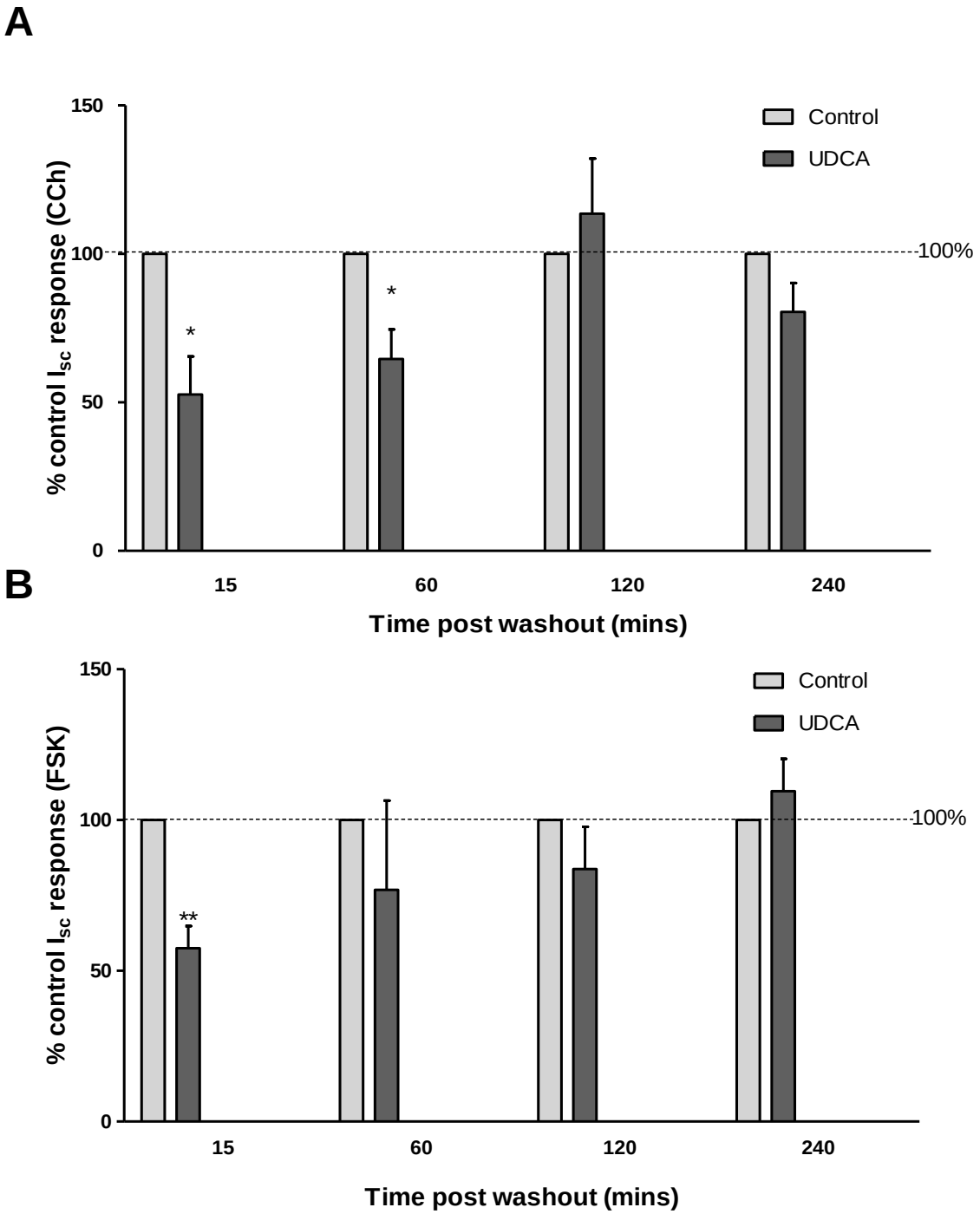


Figure 3.7: Anti-secretory effects of UDCA in T_{84} cells are reversible. T_{84} cell monolayers were pre-treated with UDCA (250 μM; bilateral; 15 min). Cells were then washed in serum-free medium and mounted in Ussing chambers for measurements of Cl^- secretion. Responses to **A)** CCh (100 μM) and **B)** FSK (10 μm) in control or UDCA-pre-treated cells were measured at various time points after washout. Results were normalised and expressed as % of control responses to CCh or FSK (n = 9, * $p < 0.05$, ** $p < 0.01$).

Having demonstrated anti-secretory actions of UDCA on the prototypical Ca^{2+} and cAMP-dependent agonists, CCh and FSK, we next investigated the effects on secretory responses induced by naturally-occurring bacterial toxins normally associated with diarrhoea. Cholera toxin is a potent agonist of colonic Cl^- secretion which acts by stimulating adenylate cyclase and inducing production of cAMP (Van Heyningen, 1976). We found that UDCA treatment (500 μM; 15 min; bilateral) significantly attenuated I_{sc} responses to cholera toxin (0.1 μg/ mL; apical) to 52.1 ± 4.8% of those in control cells ($n = 5$, $p < 0.01$; Figure 3.8, Panel A).

Heat stable *E. coli* enterotoxin (ST_a) is also known to promote Cl^- secretion, but in this case its effects are mediated through via activation of guanylate cyclase and stimulation of cGMP accumulation (Albano, 2005). We found that, similar to its effects on cholera toxin, UDCA significantly attenuated ST_a (100 nM; apical)-induced I_{sc} responses to 32.3 ± 12.0% of those in control cells ($n = 4$, $p < 0.05$; Figure 3.8, Panel B).

Both electrolyte absorption and secretion play important and reciprocal roles in maintaining fluid homeostasis in the colon. Therefore we next examined whether UDCA might also have the capacity to alter intestinal absorptive function. In order to test this, amiloride-sensitive Na^+ channel (ENaC) activity was induced in T_{84} cells using a previously validated method that involves pre-treatment of the cells with 4-phenylbutyrate (4-PBA; 5 mM; 24 h) (Iordache, 2007). Treatment of the cells in this way induced a basal I_{sc} of 7.6 ± 0.9 μA/ cm^2, which was completely abolished by apical treatment with amiloride (10 μM). UDCA (500 μM; 15 min; bilateral) did not significantly alter amiloride-sensitive Na^+ currents in this model ($n = 8$, Figure 3.9).

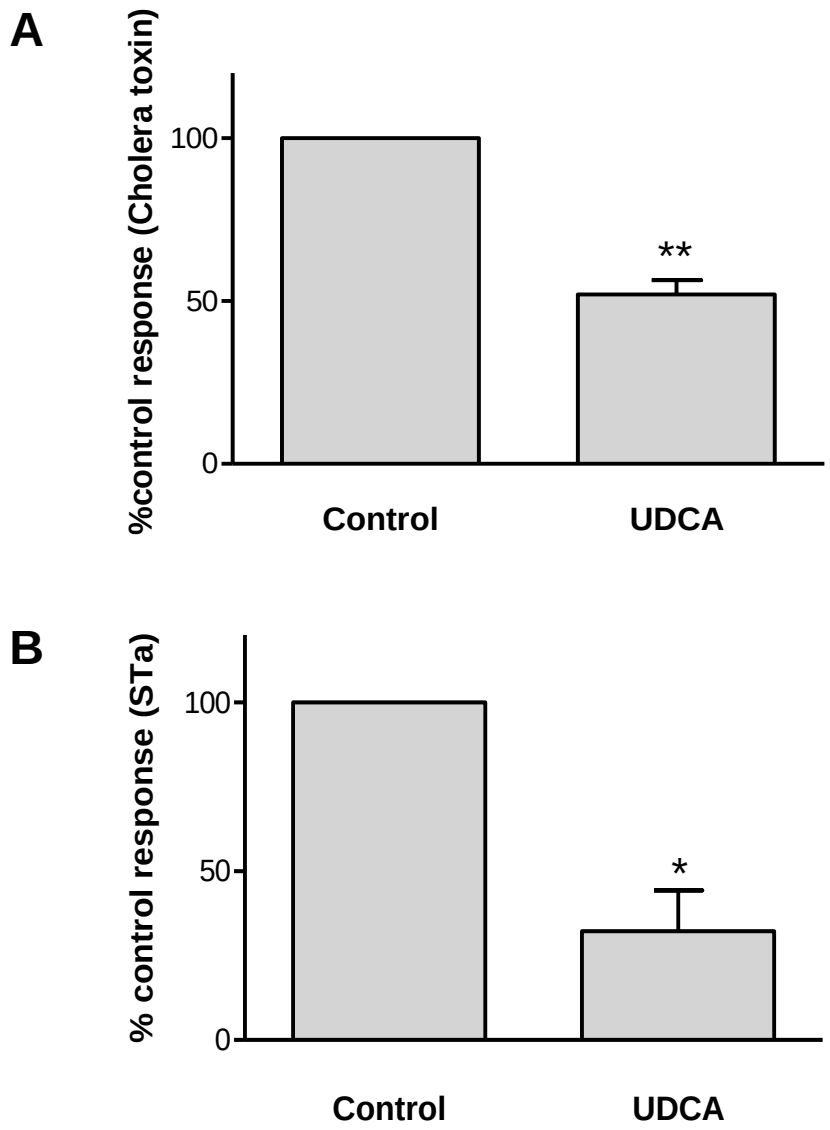


Figure 3.8: UDCA significantly attenuates cholera toxin-induced and heat stable *E. coli* enterotoxin-induced secretory responses across T_{84} colonic epithelial cells. T_{84} cell monolayers were mounted in Ussing chambers for measurements of Cl^- secretion. After a 15 min equilibration period, UDCA (500 μM; 15 min; bilateral) was added before measuring secretory responses to (A) cholera toxin (100 nM; apical) and (B) ST_a (100 nM; apical). Results were normalised and expressed as a % of control responses to cholera toxin or ST_a (n = 5, * $p < 0.05$, ** $p < 0.01$).

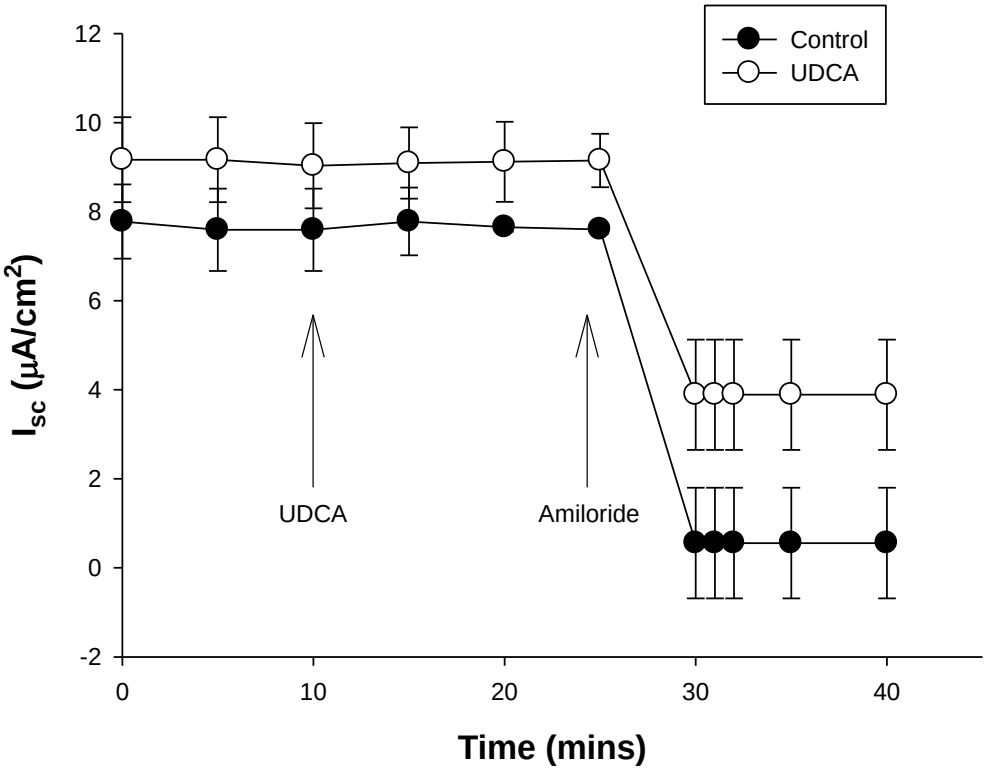


Figure 3.9: UDCA does not alter amiloride-sensitive Na^+ currents in cultured epithelial cells. Monolayers of T_{84} cells were pre-treated with 4-phenylbutyrate (4-PBA; 5mM) for 24 h to induce amiloride-sensitive Na^+ channel (ENac) activity, as previously described (Iordache, 2007). Cells were then mounted in Ussing chambers for measurements of I_{sc} and were sequentially treated with UDCA (500 μM; bilateral) and amiloride (10 μM; apical; n = 8).

3.3.2 UDCA potentiates secretory function *in vivo.*

It has been previously determined that concentrations of UDCA in human colonic fluid are normally in the region of 10 μM (Mekjian, 1971, Edenharder, 1981, Gordon , 1979). However, levels of the BA in patients on UDCA therapy are likely to be much higher. Clinically, UDCA therapy can cause diarrhoea as a side effect (Caspary, 1980). Here, however we, demonstrate that it has an anti-secretory effect when directly applied to colonic epithelium. To further investigate this paradox, we analysed the effects of UDCA on colonic secretory function after administration to mice. Male C57B6 mice were administered UDCA (25 mg/ kg in 0.1 ml PBS (w/v)) by intra-peritoneal (IP) injection and after 4 h, agonist-induced secretory responses across *ex vivo* colonic tissue were measured in Ussing chambers. Interestingly, in contrast to its anti-secretory actions in cultured colonic epithelial cells, in UDCA-pre-treated mice responses to CCh were potentiated to 284 ± 42% of those in control mice while FSK–induced responses were potentiated to 195 ± 50% of controls (n = 4, $p < 0.05$; Figure 3.10). Furthermore, baseline trans-mucosal conductance in mice treated with UDCA was also increased to 14.7 ± 2.0 $mS.cm^2$ compared to 8.3 ± 0.9 $mS.cm^2$ in control mice (n = 5, $p < 0.05$). Baseline I_{sc} in UDCA treated mice was also increased to 41± 5.6 compared with 23.6 ± 0.6 $\mu A/ cm^2$ in controls (n = 5, $p < 0.05$).

We hypothesised that such different effects of UDCA *in vitro* and *in vivo* may be due to its conversion by colonic bacteria to LCA (Fromm, 1983, Edenharder , 1981). To test this, in collaboration with Professor Aldo Roda (University of Bologna), we analysed caecal BA levels in control and UDCA-treated mice. Using HPLC mass spectrometry, we found that the total BA concentration in the caecal contents was 206 nmol /g in control mice and 215 nmol/ g in UDCA-treated mice, with no significant difference between the groups. However, a significant difference was noted in the qualitative composition of individual BAs. LCA concentrations in control mice were a mean of 3.4 nmol/ g (4 % of total BAs), compared to 9.7 nmol /g (16% of total BA) in UDCA-treated mice. Thus, caecal LCA levels in UDCA-treated mice were approximately 4-fold higher than those of controls (n = 4, $p < 0.001$;

Figure **3.11**), supporting our hypothesis. Figure 3.12 shows a more complete analysis of the % composition of individual BAs in control and UDCA-treated groups.

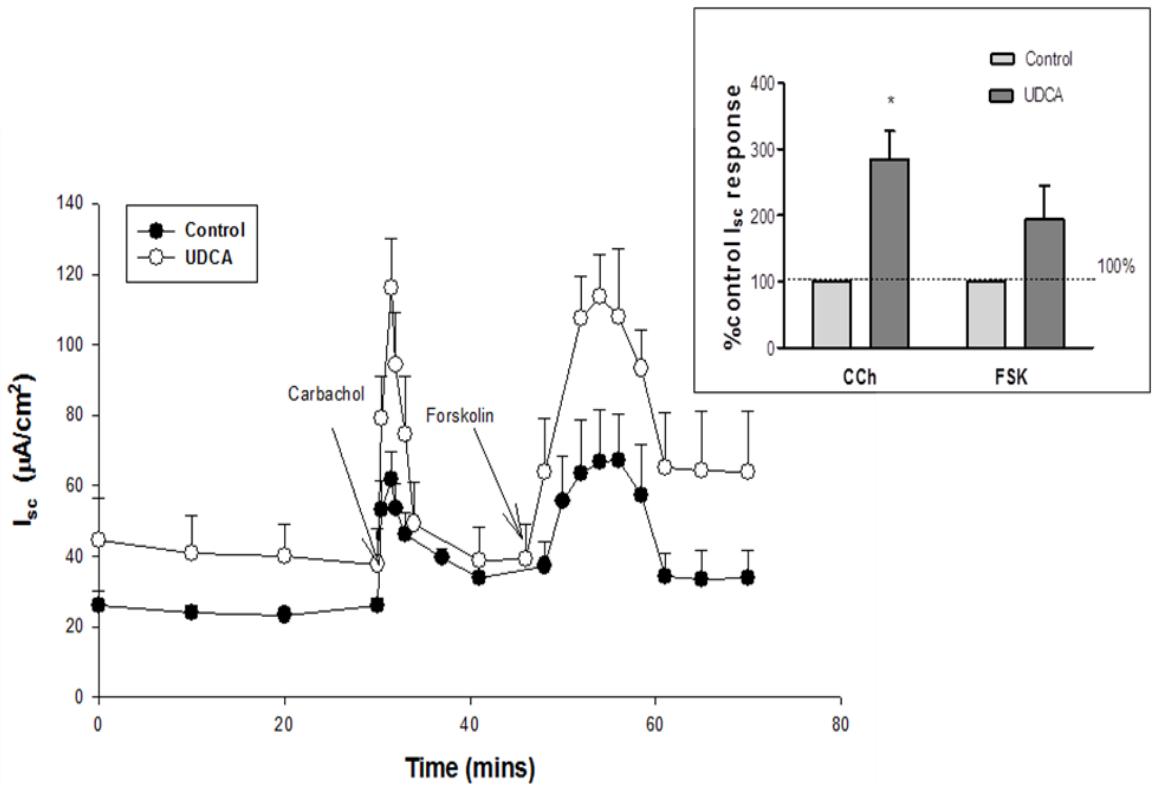


Figure 3.10: UDCA administration *in vivo* potentiates agonist-induced Cl^- secretory responses across *ex vivo* colonic tissues. Male C57B6 mice were intraperitoneally injected with either UDCA (25 mg/ kg) or vehicle control (PBS), and after 4 h were sacrificed. Muscle-stripped colonic mucosa was then mounted in Ussing chambers for measurements of ΔI_{sc}. After a 30 min stabilisation period, tissues were sequentially treated with CCh and FSK and I_{sc} responses were measured. The inset shows CCh and FSK-induced changes in I_{sc} in UDCA-pre-treated cells as a % of control responses (n = 5, * $p < 0.05$).

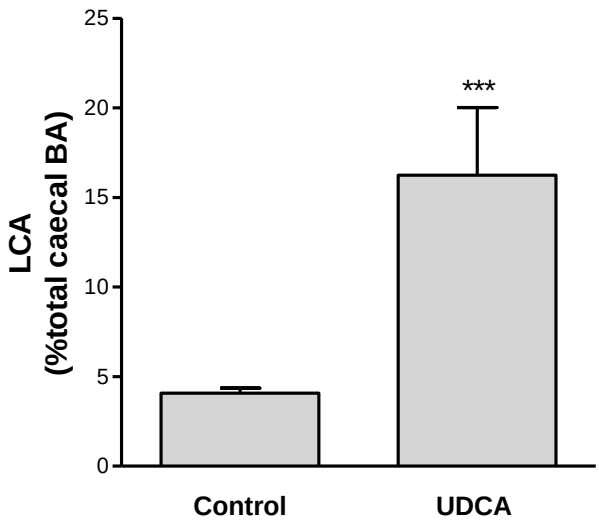


Figure 3.11: UDCA is metabolised to LCA *in vivo*. Male C57B6 mice were intraperitoneally injected with either UDCA (25 mg/ kg) or vehicle control (PBS), and after 4 h were sacrificed. The caecum was removed and LCA levels in the caecal contents were measured by HPLC mass spectrometry. Data are expressed as % of total BA pool (n = 4, *** $p < 0.001$)

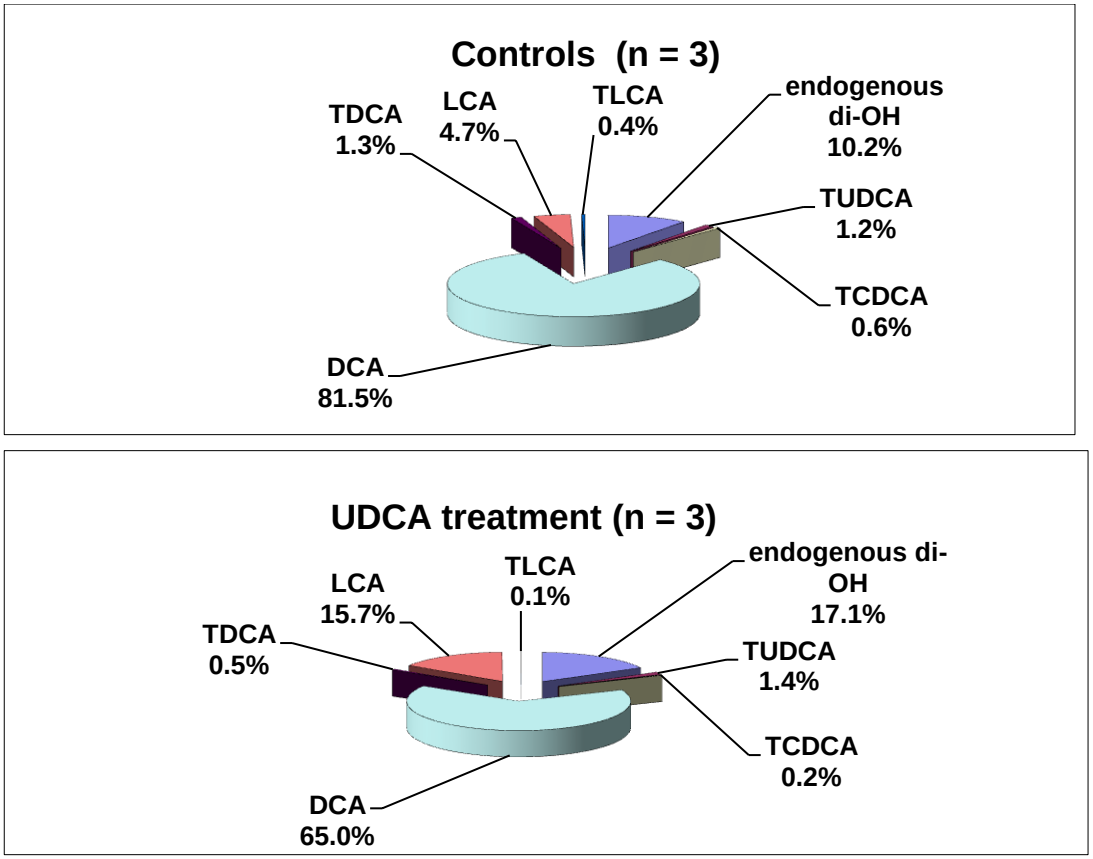


Figure 3.12: Composition of the bile acid pool in the caecal content of control and UDCA-treated mice. Male C57B6 mice were intra-peritoneally injected with either UDCA (25 mg/ kg) or vehicle control (PBS), and after 4 h were sacrificed. Bile acid levels in the caecal contents of UDCA-treated and control mice were measured by HPLC mass spectrometry. Data are expressed as % of total BA pool. Endogenous- (OH) is an abbreviation for endogenous dihydroxy BAs the majority component of which is UDCA and excludes other di-(OH) BAs listed (i.e. DCA).

3.3.3 LCA potentiates agonist-induced secretory responses across human colonic epithelium

Next, we examined the effects of LCA on agonist-induced secretory responses across cultured colonic epithelial cells. Similar to UDCA, pre-treatment of T_{84} cells with LCA (50–250 µM; 15 min) had no effect on basal I_{sc}. However, in contrast to UDCA, LCA treatment significantly potentiated subsequent responses to CCh (n = 9, $p < 0.001$;

Figure **3.13**, Panel A). LCA also caused increase in trans-epithelial conductance of 1.5 ± 0.3 mS.cm^2 compared to controls (n = 9, $p < 0.05$). Similar effects of LCA were found in human colonic mucosa mounted in Ussing chambers. LCA (50 µM) enhanced CCh-induced I_{sc} responses in tissues to 130.3 ± 9.9% of controls (n = 5, $p < 0.05$;

Figure **3.13**, Panel B). Increase in conductance of 4.5 ± 1.3 mS.cm^2 was also noted, but was not statistically significant.

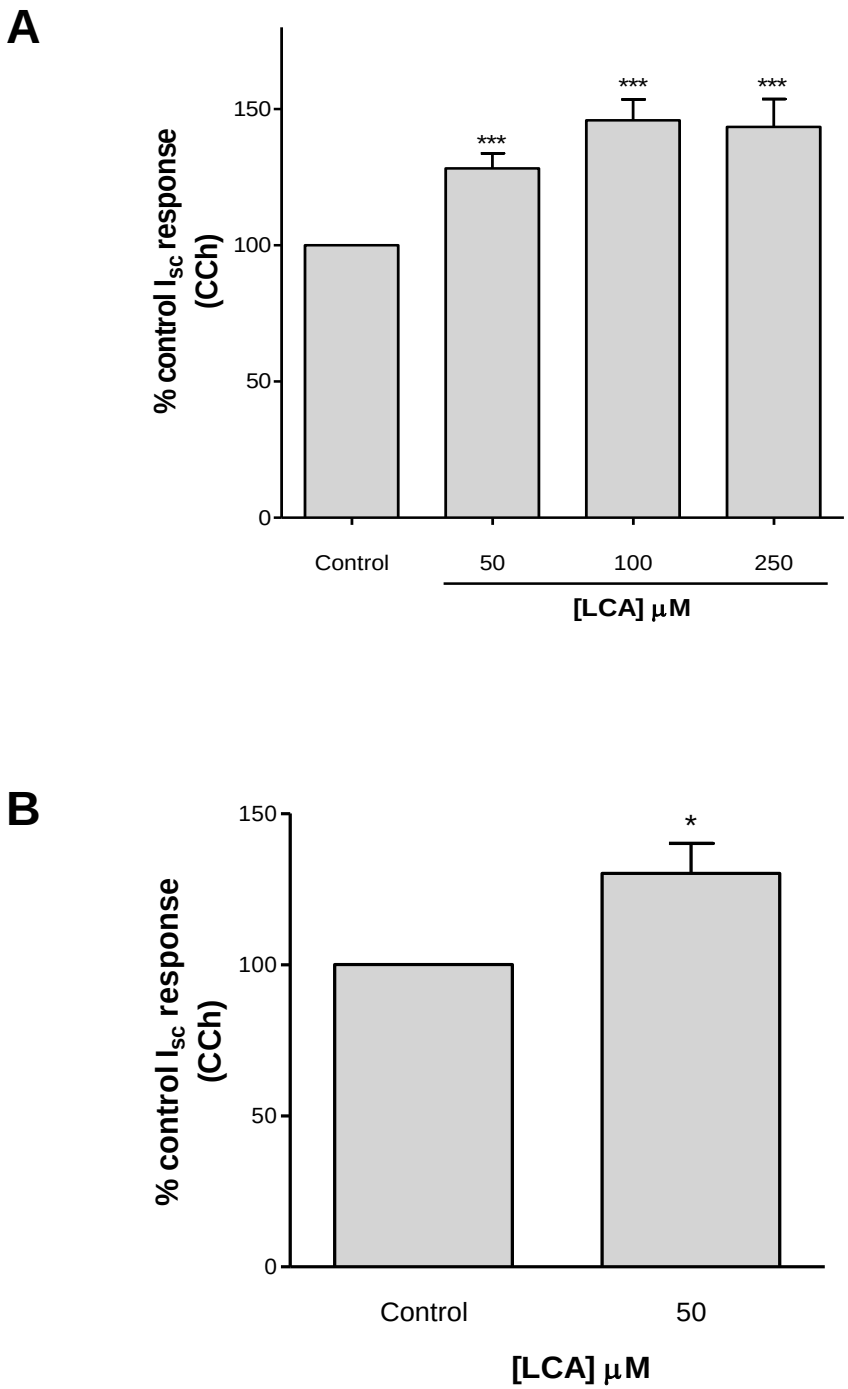


Figure 3.13: LCA potentiates agonist-induced Cl^- secretion across human colonic epithelium. A) T_{84} cell monolayers were mounted in Ussing chambers for measurements of Cl^- secretion. After a 15 min equilibration period, cells were treated with LCA (50 – 250 μM; 15 min) before measuring secretory responses to CCh (n = 9). In LCA-pre-treated cells (250 μM; 15 min), responses to CCh were enhanced to 143 ± 10.3% of those in control cells**. B)** Muscle-stripped sections of human colonic mucosa were mounted in Ussing chambers and treated with LCA (50 μM;

15 min). LCA enhanced I_{sc} responses to CCh to 130.3 ± 9.9% of those in controls (n = 5). Data are expressed as a % of control responses to CCh. *p < 0.05; ***p < 0.001).

3.3.4 6α-MUDCA, a dehydroxylation-resistant analogue of UDCA, inhibits Cl^- secretion both *in vitro* and *in vivo*.

To further investigate our hypothesis that metabolism to LCA underlies the lack of anti-secretory actions of UDCA *in vivo*, we employed a non-metabolisable analogue of the BA, provided to us by Prof. Aldo Roda (University of Bologna). It has been previously shown that 6α-MUDCA, unlike UDCA, cannot be dehydroxylated to LCA by colonic bacteria *in vitro* (Roda, 1994). To confirm this *in vivo*, we examined caecal LCA levels in mice treated with 6α-MUDCA and found them to be similar to those in control PBS-treated mice (Figure 3.14).

We next tested whether 6α-MUDCA shared the same anti-secretory actions as its parent BA, UDCA, or whether the addition of a methyl group at the 6-OH position altered its biological activity. However, we found that apical pre-treatment of T_{84} cells with 6α-MUDCA (500 μM; 15 min) resulted in a similar attenuation of agonist-induced secretory responses to that evoked by UDCA. Treatment with 6α-MUDCA (500 μM; 15 min) attenuated CCh- and FSK-induced responses to 37.1 ± 4.3% and 48.8 ± 9.1% of those in control cells, respectively (n = 7; Figure 3.15).

Having shown that 6α-MUDCA is not metabolised to LCA but retains the anti-secretory activity of UDCA, we next went on to investigate its effects on agonist-induced secretory responses across *ex vivo* colonic tissue from mice. Mice were administered 6α– MUDCA (25 mg/ kg) by intra-peritoneal injection, and after 4 h, colonic secretory responses were measured *ex vivo*. Interestingly, we found that, in contrast to the potentiating effects of UDCA on agonist-induced secretory responses, 6α– MUDCA inhibited CCh- and FSK-induced responses to 52.5 ± 14.7% and 36.69 ± 8.51 % of those in control mice, respectively (Figure 3.16).

Furthermore, it was interesting to note that, in contrast to mice treated with UDCA, in those treated with 6α-MUDCA, tissue conductances were unchanged compared to controls. 6α-MUDCA was also without effect on basal I_{sc}. Next, to determine the specificity of 6α–MUDCA in regulation of epithelial transport processes *in vivo*, we

examined its actions on Na^+-dependent glucose transport, an important pathway for fluid absorption in the small intestine. Mice were treated with 6α-MUDCA (25mg/ kg; IP), or sterile PBS for controls. After 4 h, un-stripped sections of proximal jejunum were mounted in Ussing chambers. Na^+-dependent glucose transport was measured as changes in phlorizdin-sensitive I_{sc} induced by addition of 10 mM glucose to the apical reservoir (Tavakkolizadeh, 2001). In these experiments, we found that glucose-induced changes in I_{sc} were similar in control animals (36.3 ± 15.1 μA/ cm^2) to those in 6α-MUDCA pre-treated mice (37.1 ± 10.8 μA/ cm^2; n = 7, Figure 3.16). Thus, 6α-MUDCA does not appear to alter jejunal Na^+-dependent glucose transport in mice.

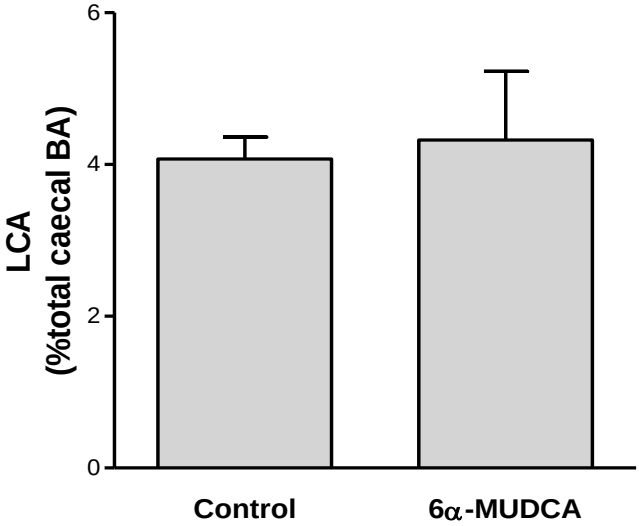


Figure 3.14: 6α–MUDCA is a stable analogue of UDCA and is not metabolised to LCA *in vivo.* Male C57B6 mice were intra-peritoneally injected with either 6α-MUDCA (25 mg/kg) or vehicle control (PBS), and after 4 h were sacrificed. The caecum was removed and LCA levels in the caecal contents were measured by HPLC mass spectrometry. Data are expressed as % of total BA pool (n = 4)

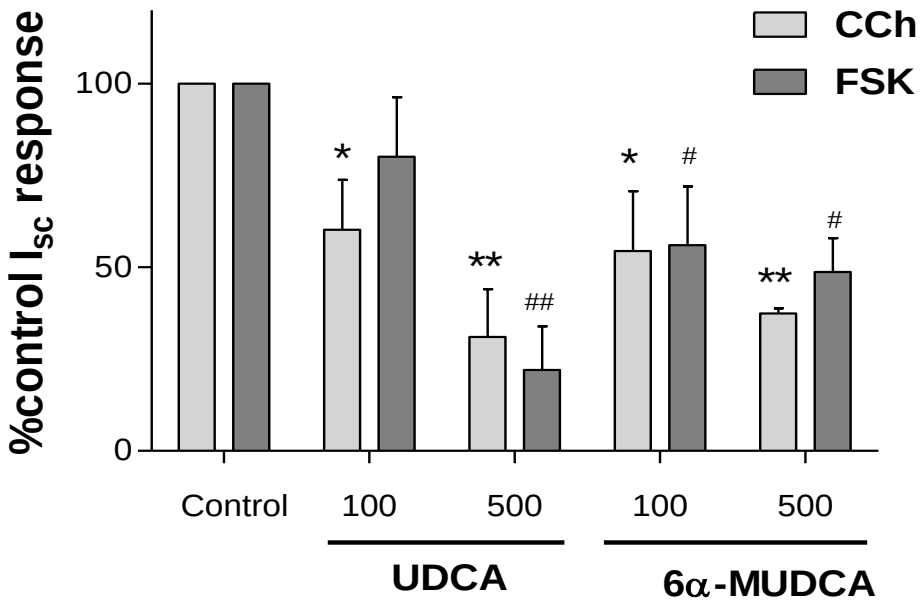


Figure 3.15: 6α-MUDCA has equivalent anti-secretory actions to UDCA *in vitro*. T_{84} cell monolayers were mounted in Ussing chambers for measurements of Cl^- secretion. After a 15 min equilibration period, cells were pre-treated with 6α-MUDCA (100 μM, 500 μM; 15 min, apically) or UDCA (100 μM, 500 μM; 15 min, apically) and secretory responses to CCh (100 μM) and FSK (10 μM) were measured. Data are expressed as a % of control responses to CCh or FSK (n = 7, * $p < 0.05$, ** $p < 0.01$).

Table 3.1: Conductance (G) and I_{sc} across *ex vivo* colonic tissues from mice treated with UDCA or 6α–MUDCA (n = 8; * $p < 0.05$)

Treatment(μM)	**I_{sc}(μA/cm^2)**	**G(mS.cm^2)**
Control	62.1 ± 4.0	7.9 ± 0.5
UDCA	95.4 ± 22.7	14.7 ± 2.1 *
6α-MUDCA	64.4 ± 14.9	8.1 ± 0.61

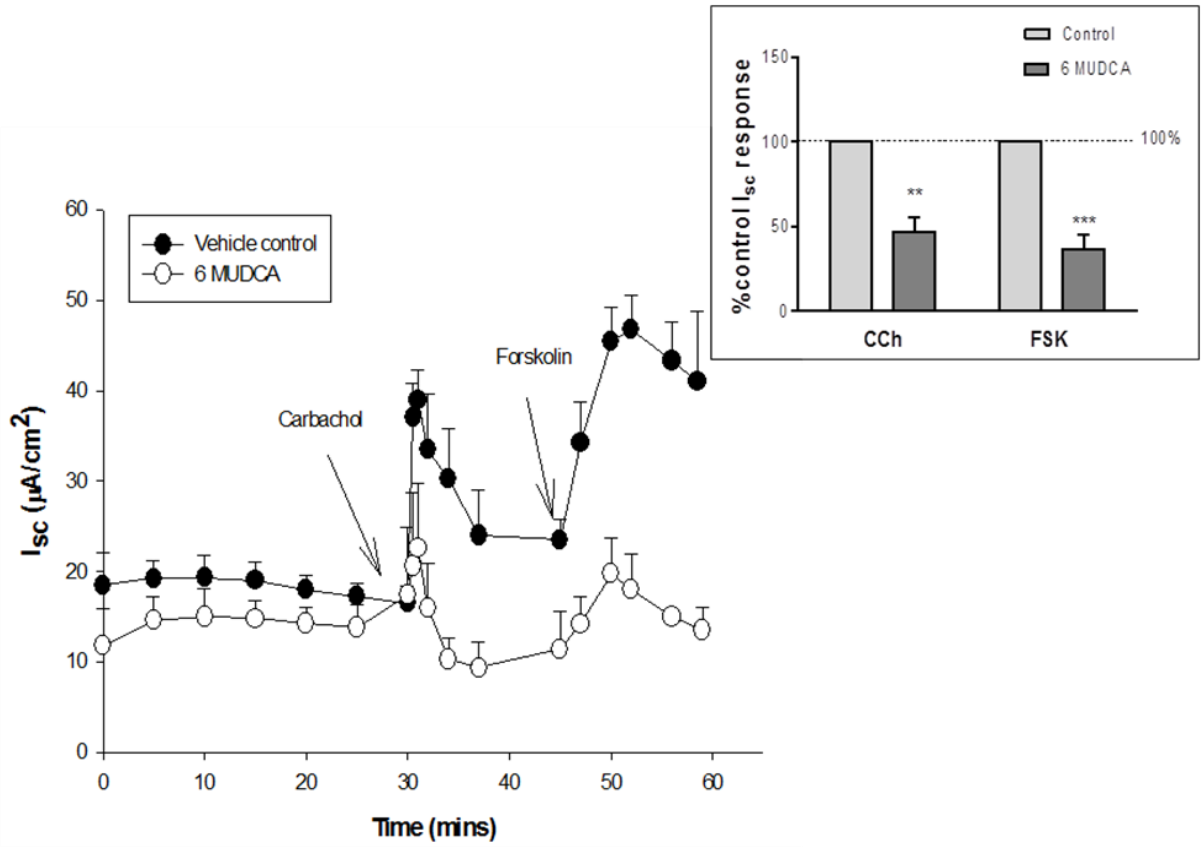


Figure 3.16: *In vivo* administration of 6α-MUDCA inhibits colonic secretory function. Mice were intra-peritoneally injected with 6α–MUDCA (25 mg/ kg) or vehicle control (PBS) and then sacrificed after 4 h. Muscle-stripped colonic mucosa was mounted in Ussing chambers and changes in I_{sc} in response to CCh (100 μM) and FSK (10 μM) were measured. Agonist induced responses were significantly reduced in animals pre-treated with 6α- MUDCA compared to controls. The trace shows the changes in I_{sc} over time while the inset shows data expressed as a % of control responses to CCh or FSK (n = 5; ** $p < 0.01$, *** $p < 0.001$).

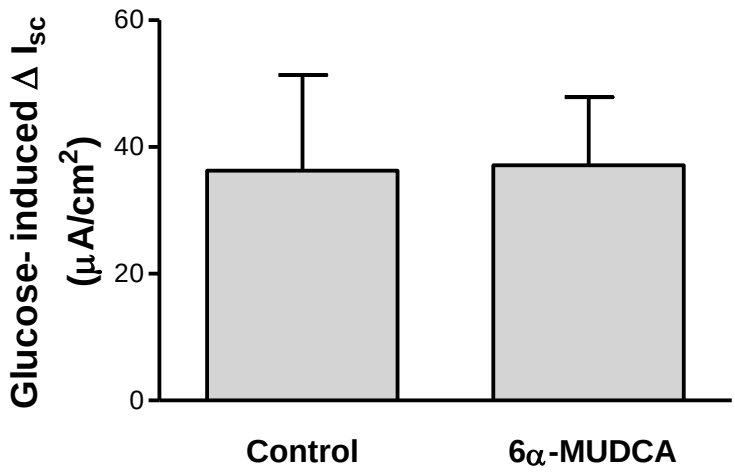


Figure 3.16: 6α-MUDCA does not alter SGLT activity across mouse jejunum. Male C57B6 mice were intra-peritoneally injected with 6α-MUDCA (25 mg/ kg) or vehicle control (PBS) and after 4 h were sacrificed and the proximal jejunum was removed and mounted in Ussing chambers. After an equilibration period of 15 min, changes in I_{sc} in response to addition of glucose (25 mM, apically) were measured (n = 7)

3.3.5 Sidedness of the anti-secretory effects of UDCA

To further characterise the anti-secretory effects of UDCA, we next examined if there is a sidedness, or polarity, to its actions. These experiments were carried out by assessing the effects of UDCA, or its membrane-impermeable taurine conjugate, TUDCA, when added apically or basolaterally to T_{84} cell monolayers mounted in Ussing chambers. As shown in Figure 3.17, Panel A, these studies revealed that attenuation of CCh-induced responses by UDCA was most prominent when it was added bilaterally (15.8 ± 4.0% of control response, $n = 13$, $p < 0.001$) or basolaterally (29.8 ± 5.2% of controls, $n = 13$, $p < 0.001$). Apical UDCA addition also inhibited CCh-induced responses but to a lesser extent (77.0 ± 9.4% of controls, $n = 13$, $p < 0.05$). UDCA, and other unconjugated C24 BAs, are membrane-permeable and thus can be absorbed passively from the intestinal lumen (Hofmann, 2008). However, TUDCA is more hydrophilic and cannot permeate cell membranes, unless a transporter is present. Basolateral and bilateral addition of TUDCA (500 μM) attenuated CCh-induced Cl^- secretion to a similar degree as UDCA with bilateral addition, reducing responses to 39 ± 7.1 % ($n = 10$, $p < 0.01$) of controls and basolateral addition to 30.9 ± 7.8% of controls ($n = 10$, $p < 0.001$). In contrast, apical addition of TUDCA did not significantly alter CCh-induced secretory responses, which were 108 ± 11.9% of those in control cells ($n = 10$). Both UDCA (500 μM) and TUDCA were less effective in inhibition of FSK and the pattern was similar (Figure 3.17, panel B).

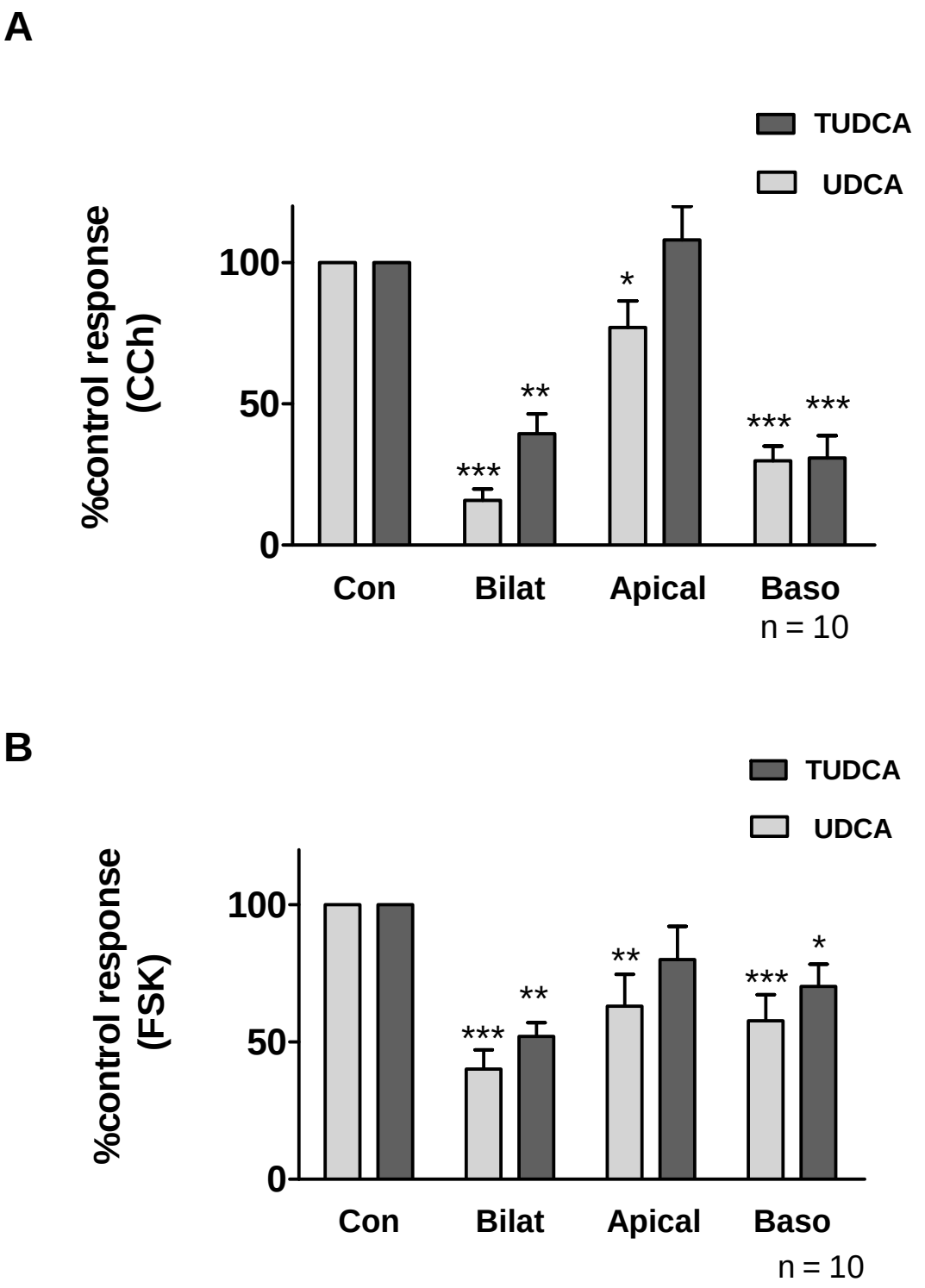


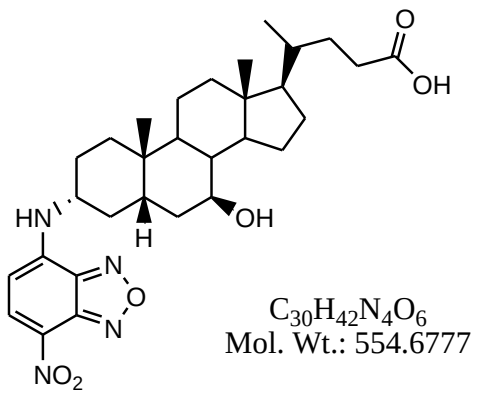
Figure 3.17: **Sidedness of UDCA effects on agonist induced secretory responses in T_{84} cells.** T_{84} cell monolayers were mounted in Ussing chambers for measurements of Cl^- secretion. After a 15 min equilibration period, cells were treated with UDCA (500 μM) or TUDCA (500 μM), added bilaterally, apically or basolaterally and I_{sc} responses to CCh (100 μM) and FSK (10 μM) were measured. Apical addition of TUDCA did not inhibit responses significantly in contrast to UDCA, suggesting that UDCA enters the cell to induce attenuation of secretion. Data are expressed as % of control responses (n = 10, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3.6 Distribution of FM191, a fluorescent derivative of UDCA, in T_{84} cell

In further experiments in collaboration with Dr. John Gilmer (Trinity College Dublin), we sought to further examine how UDCA may act on epithelial cells. We employed a novel fluorescent derivative of UDCA, known as FM191, in order to directly visualise localisation of the bile acid after treatment of T_{84} monolayers. FM191 is a synthetic derivative of UDCA containing a fluorophore attached to the side chain. The fluorescent moiety consists of a carbon ring with 2 amide bonds (See Panel A, Figure 3.18) and the compound emits fluorescence between 530 and 540 nm. The absorption maximum of the compound was found to be between 460 and 470 nm.

Firstly, the biological efficacy of FM191 was assessed in voltage-clamped T_{84} cell monolayers. In these experiments, FM191 (apical, 250 μM) was found to exert similar anti-secretory effects to UDCA (apical, 500μM) (Figure 3.18, Panel B). Next, T_{84} cell monolayers were treated with FM191 (250 μM, 15 min), and were then fixed and stained for confocal microscopy. Nuclear staining was performed with DAPI. Staining was also performed for the cytoskeletal protein actin. We found that FM191 appears to mainly associate with the membrane, with a small amount distributed in cytoplasm (Figure 3.20).

A



The molecular structure of FM191.

B

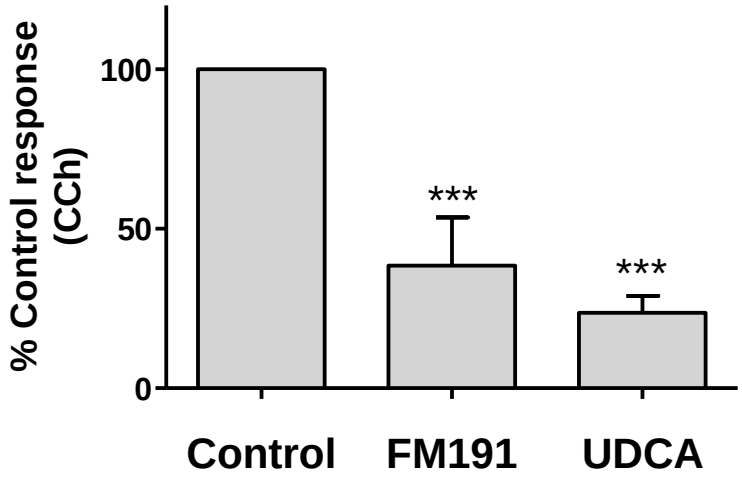
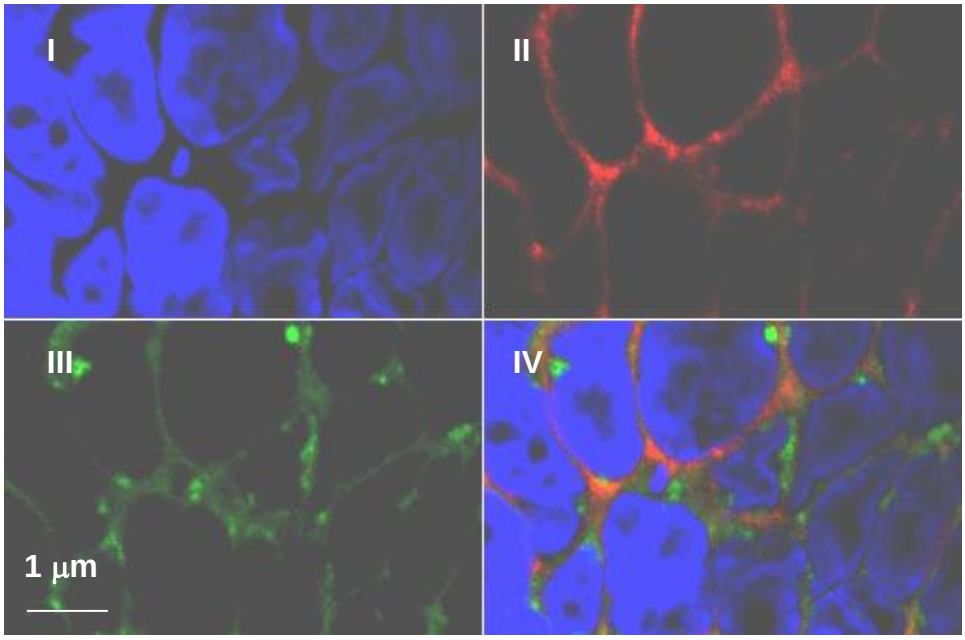


Figure 3.18: FM191 exerts similar anti-secretory actions to UDCA. A) The structure of FM191**. B)** T_{84} cell monolayers were mounted in Ussing chambers for measurements of Cl^- secretion. After a 15 min equilibration period, cells were treated FM191 (250 μM; apical) or UDCA (500 μM; apical) for 15 min, after which I_{sc} responses to CCh (100 μM) were measured. Data are expressed as % of control responses to CCh (n = 6; *** $p < 0.001$).

A



B

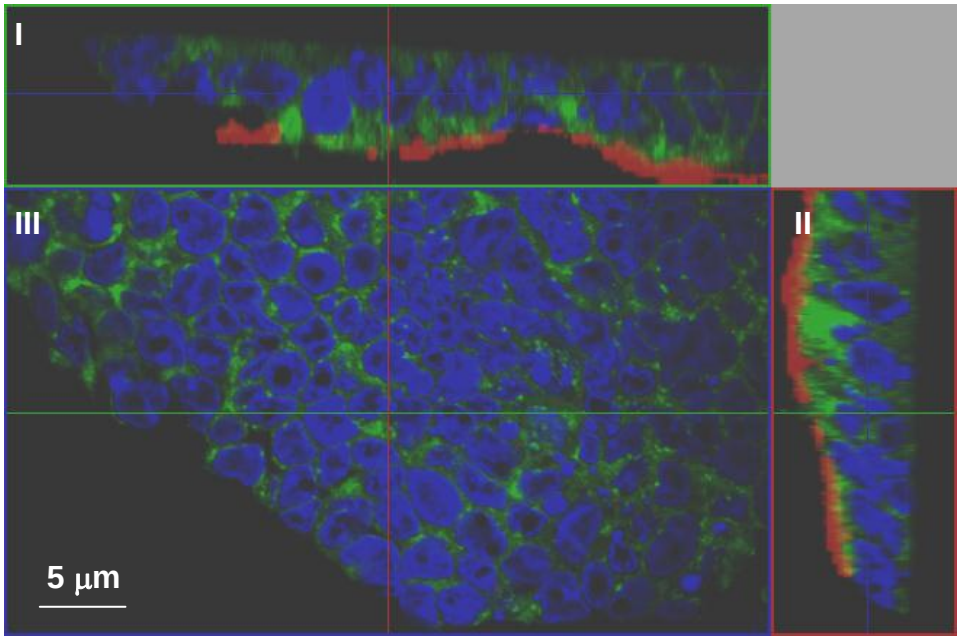


Figure 3.20: Confocal imaging following apical addition of FM191 A) T_{84} cells were grown to confluency on transparent inserts and treated apically with FM191 (250 μM; 15 min), after which they were prepared for confocal microscopy. This is a representative confocal image. Panel I shows nuclear staining with DAPI (blue), panel II shows staining for the cytoskeletal protein, actin (red), and panel III shows the fluorescent UDCA derivative, FM191 (green). Note, that the derivative is primarily associated with the cell membrane. Panel IV is a composite image showing all 3 stains (n = 6). **B)** Z- series stack images were captured of 0.5 μm- thick optical sections encompassing the entire T_{84} cell monolayer. This is a representative cross-sectional image which demonstrates that FM191 (green) is present both apically and basolaterally within the monolayer, suggesting that it is capable of diffusing into the cell in order to induce its effects on secretion. Panel I shows the apical side of the cells at the top of the image in panel II the apical aspect is to the right and III shows the image from above.

3.3.6 UDCA inhibits Na^+/ K^+ ATPase pump activity and basolateral K^+ channel currents in T_{84} cells.

Having shown that UDCA, and its analogues, exert anti-secretory actions on colonic epithelial cells, we next set out to investigate the potential mechanisms involved. Since the effects of UDCA occur very rapidly (i.e. within seconds to minutes), we hypothesised that it is likely to act either by modulating transport protein activity or through altered expression of transport proteins at the cell surface.

First, we examined the effects of UDCA on the activity of key transport proteins that comprise the Cl^- secretory pathway, i.e., apical Cl^- channels, basolateral K^+ channels, and Na^+/K^+ ATPase pumps. Experimental conditions employed to isolate the activities of each of these transport proteins are described in Chapter 2. In these experiments, UDCA (500 μM) was found to inhibit CCh (100 μM)-stimulated Na^+/K^+ ATPase pump activity to 16.2 ± 3.9% of that in control cells (Figure 3.19; $n = 6$, $p < 0.001$). Similarly, under conditions to isolate basolateral K^+ conductances, UDCA pre-treatment attenuated I_{sc} responses to 13.7 ± 4.1% of those in control cells (Figure 3.20; $n = 7$, $p < 0.001$). However, we found that UDCA pre-treatment did not significantly alter apical Cl^- conductances (Figure 3.21, $n = 8$).

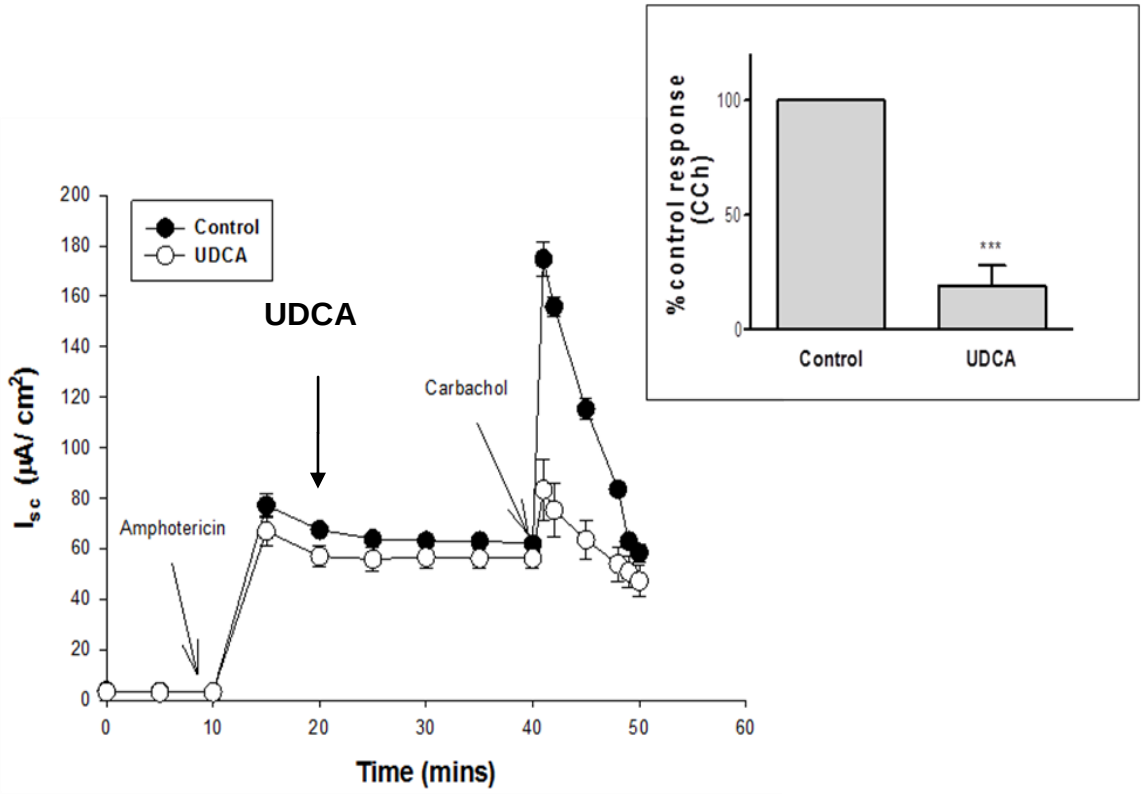


Figure 3.19: UDCA inhibits Na^+/K^+ ATPase pump activity in T_{84} cells. T_{84} cell monolayers were mounted in Ussing chambers in low Na^+ Ringers solution and after a 15 min equilibration period, apical membranes were permeabilised with amphotericin B (50 µM). After 15 min, UDCA (500 µM, bilateral) was added and after a further 15 min, I_{sc} responses to CCh (100 µM) were measured. Under these conditions (i.e., in the absence of ionic gradients across the permeabilised monolayer), changes in I_{sc} are reflective of electrogenic transport through the Na^+/K^+-ATPase pump. The inset shows maximal changes in I_{sc} in UDCA-treated cells expressed as a % of control cells (n = 6, *** $p < 0.001$).

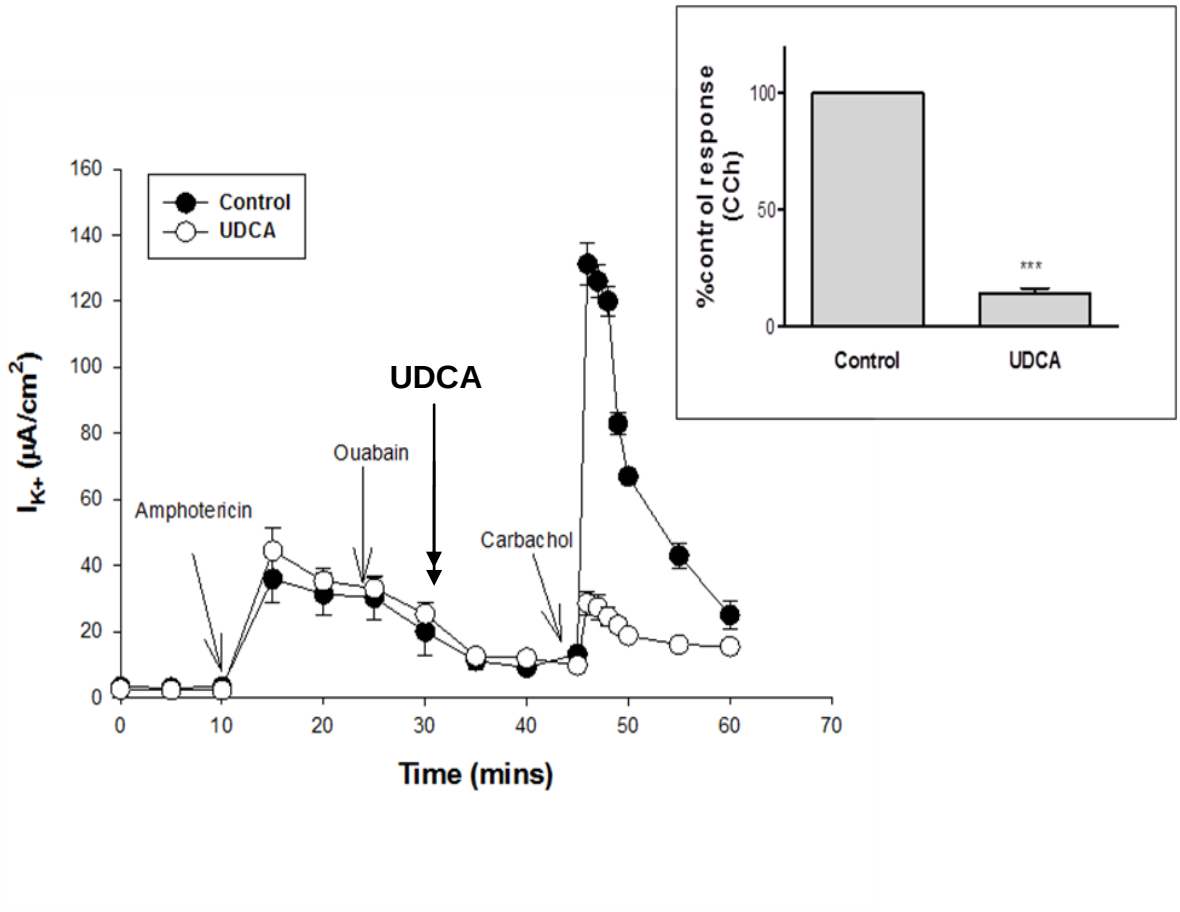


Figure 3.20: UDCA inhibits basolateral K^+ channel currents in T_{84} cells. T_{84} cell monolayers were apically permeabilized with amphotericin B (50 µM). A K^+ gradient (123.2–5.2 mM) was created across the basolateral membrane by addition of a high K^+ (123.2 mM) Ringer's solution, in which NaCl is substituted with K^+ - gluconate, to the apical reservoir. Ouabain (100 µM) was added basolaterally to inhibit Na^+/K^+-ATPase pump activity. Under these conditions, changes in I_{sc} are reflective of changes in basolateral K^+ conductance (I_K). The inset shows maximal changes in I_K in UDCA-treated cells expressed as a % of control cells (In conditions, as described, to isolate basolateral K^+ current, UDCA (500 µM, 10 min, bilateral) attenuated I_{sc} to 13.7 ± 4.1 (n = 7, *** $p < 0.001$) of controls.

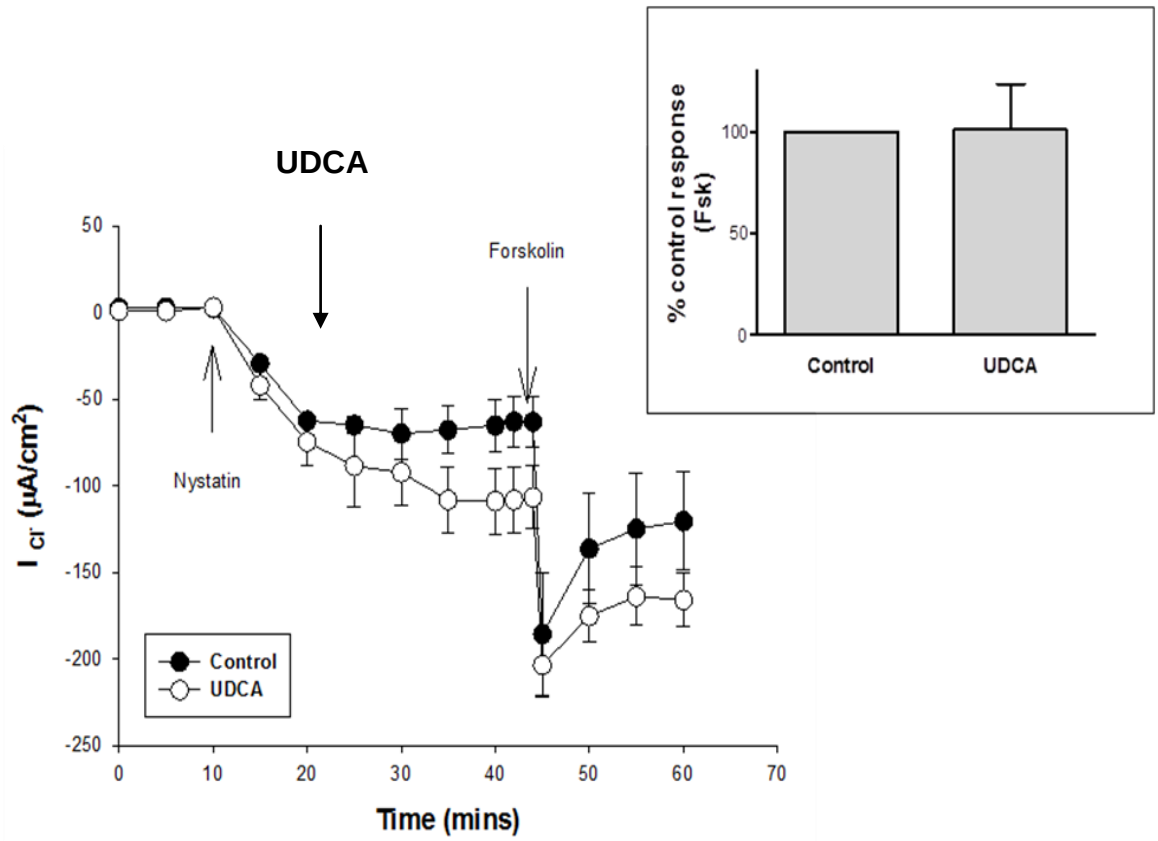


Figure 3.21: UDCA does not alter apical Cl^- conductances. T_{84} cell monolayers were mounted in Ussing chambers and an apical to basolateral Cl^- gradient (119.8 – 4.8 mM) was established by replacing NaCl in the apical solution with equimolar Na-gluconate. Monolayers were basolaterally permeabilized by addition of nystatin (100 µg/ mL), and after a 35 -min re-equilibration period, cells were stimulated with forskolin (FSK; 10 µM). Under these conditions, changes in I_{sc} reflect apical Cl^- currents (I_{Cl}). The inset shows maximal changes in I_{Cl} in UDCA-treated cells expressed as a % of control cells (n = 8).

3.3.7 Effects of UDCA on cellular localisation of Na^+/K^+ ATPase pumps and KCNN4.

Since UDCA inhibits the activity of Na^+/K^+ ATPase pumps and K^+ channels, we next went on to determine if UDCA might be altering the cell surface expression of these proteins. Firstly, using an immunohistochemical approach, the effects of UDCA on localisation of these proteins was assessed in both T_{84} cells and isolated human colonic crypts. However, in these experiments we found no differences in the localisation of Na^+/K^+ ATPase α subunit in T_{84} cells, or KCNN4 in T_{84} cells or human colonic crypts, were apparent between UDCA pre-treated cells or crypts and untreated cells or crypts. Figure 3.24 shows representative stains for Na^+/K^+ ATPase α and KCNN4 from control and UDCA treated monolayers of T_{84} cells (Kelly, 2012) and Figure 3.25 shows image panels stained with actin/ DAPI and anti KCNN4 from UDCA treated and control isolated human colonic crypts (Control pre-treatment was with PBS).

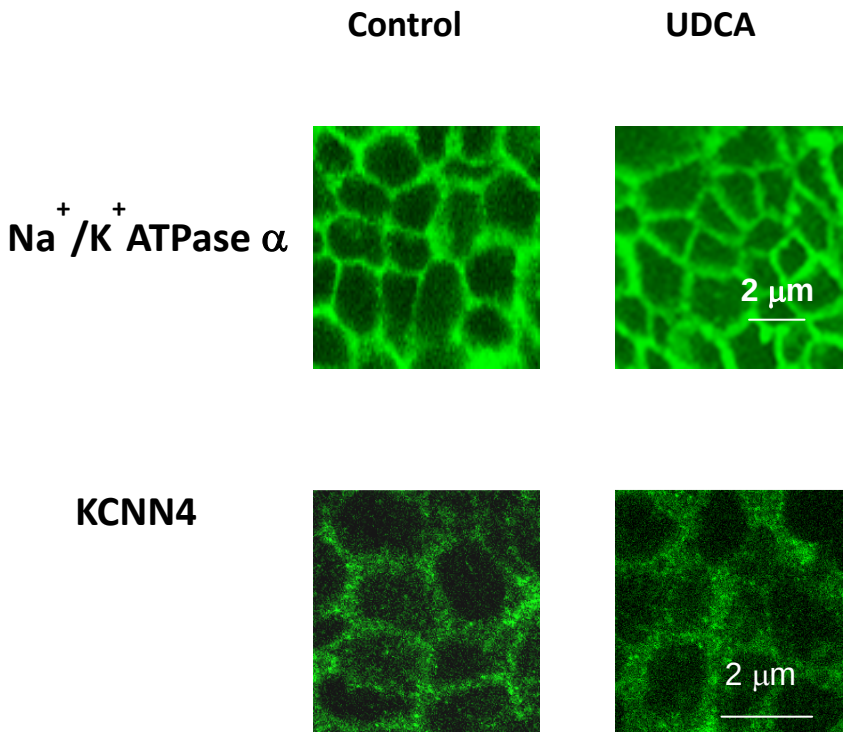


Figure 3.24: UDCA does not alter localisation of the Na^+/K^+ ATPase-α subunit or KCNN4 inT_{84} cells. T_{84} cells were grown to confluency on transparent filters. Cells were treated apically with UDCA (500 μM, 15 min), after which localization of Na^+/K^+ ATPase-α subunits and KCNN4 was investigated by confocal microscopy in two separate series of experiments. The image shows a 4 panel split planar image from control and UDCA-treated cells stained for Na^+/K^+ ATPase-α or KCNN4. There was no significant difference noted in localisation of either antibody (n= 4-12).

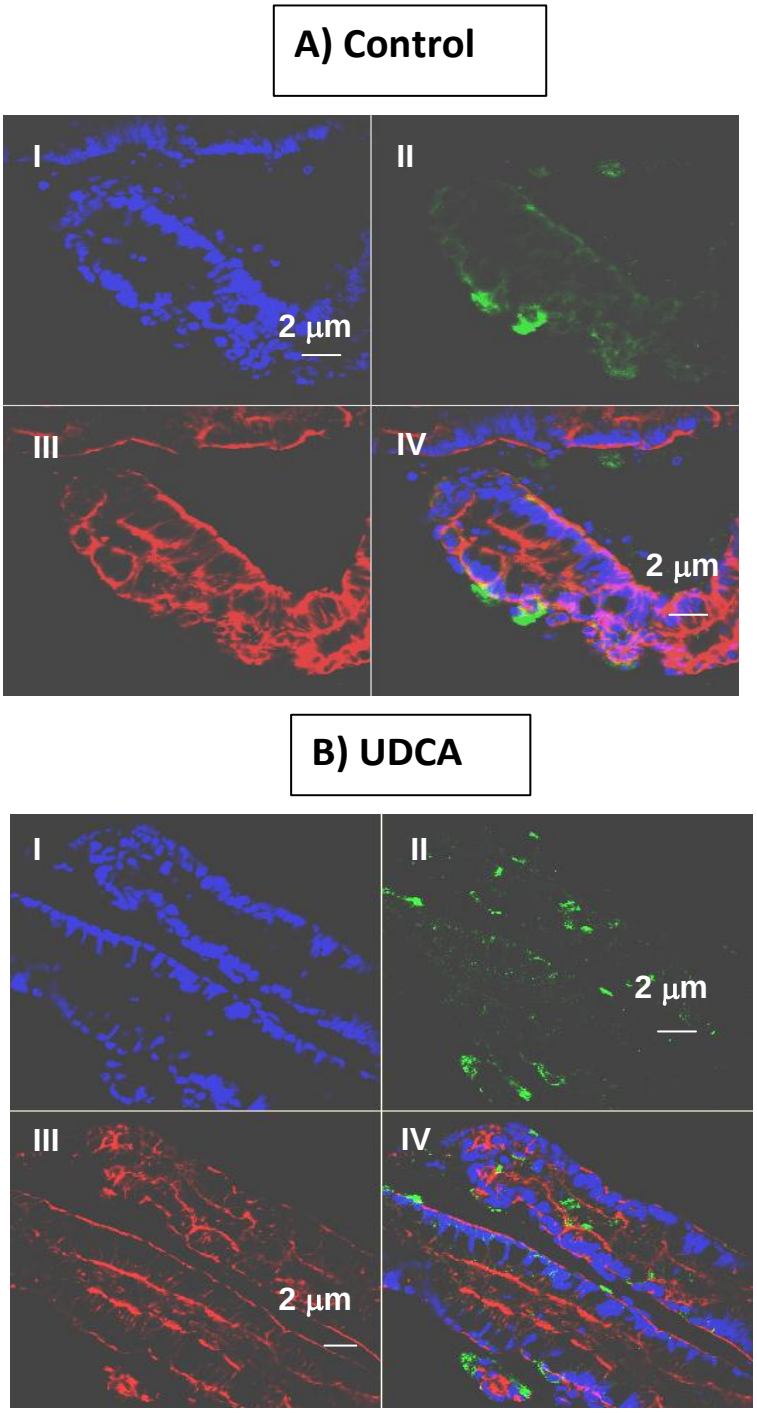


Figure 3.23: UDCA does not affect KCNN4 localisation in human crypts. Human colonic crypts were isolated and treated with UDCA (500 μM, 15 min). Crypts were then fixed and stained for confocal imaging. The image is representative of 3 similar experiments and shows a 4 panel split planar image from **A)** control and **B)** UDCA-treated colonic crypts. I Blue staining represents nuclear staining with DAPI II green staining represents KCNN4, III red represents the cytoskeletal protein actin and IV shows a composite image with all stains present.No significant difference was noted in localisation of KCNN4 after UDCA treatment in isolated human crypts (n = 3).

In further experiments surface expression of both the Na^+/K^+-ATPase pumps and KCNN4 in UDCA-treated T_{84} cell monolayers was determined quantitatively by cell surface biotinylation. This confirmed that there was no significant alteration in either the cellular or surface expression of Na^+/K^+-ATPase α subunits or KCNN4 after treatment with UDCA (Figure 3.24).

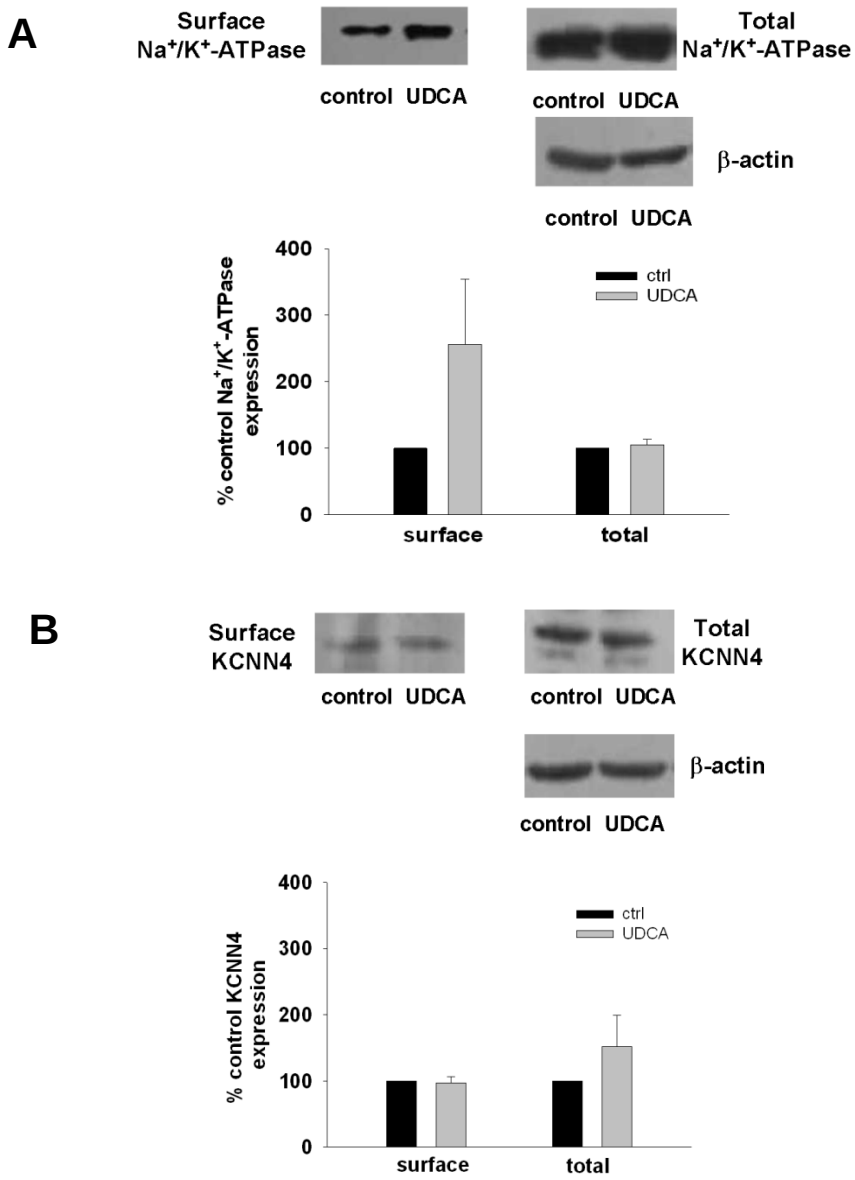


Figure 3.24: UDCA does not alter cell surface expression of Na^+/K^+ ATPase α or KCNN4. A) After treatment with UDCA (500 µM; 15 min), total cellular and basolateral surface expression of the Na^+/K^+-ATPase α subunit was analysed by western blotting or cell surface biotinylation, respectively. The bar chart represents densitometric analysis of 8 similar experiments. **B)** After treatment with UDCA (500 µM; 15 min) total cellular and basolateral surface expression of KCNN4 was analysed. The lower panel shows densitometric analysis of 3 similar experiments.

3.4 Discussion:

Previously published studies from this, and other, groups have suggested that acute exposure to UDCA does not promote Cl^- secretion across colonic epithelial cells (Keely, 2007). In this chapter, we confirm these previous findings, but more importantly, we go on to further demonstrate that UDCA exerts potent anti-secretory effects on agonist-induced secretory responses. Of note, we show that UDCA did not alter TER, suggesting the effects of the BA are not due to a loss of cell viability, but are rather due to specific actions on the colonic Cl^- secretory pathway. The anti-secretory actions of UDCA are intriguing compared to other dihydroxy BAs, such as DCA and CDCA, which are known to be pro-secretory at pathophysiological concentrations and which are thought to cause diarrhoea in conditions of bile acid malabsorption (Hofmann, 2008). The studies described here also underline a marked chemical specificity which exists for BA-induced Cl^- secretion and suggest that modification of BA structure by colonic bacteria is likely to significantly modulate their functional properties.

Anti-secretory effects of UDCA are very rapid in onset, becoming apparent as early as 1 min after UDCA treatment. Such a rapid onset of action suggests that the effects of UDCA are mediated by post-transcriptional mechanisms, such as those involving membrane perturbations, modifications of key membrane transport proteins, or alterations in the generation of intracellular 2nd messengers. Interestingly, the effects of UDCA treatment on cAMP-induced secretory responses are slower in onset than its effects on Ca^{2+}-mediated agonists, with significant effects only becoming apparent between 15–30 min after treatment. Such temporal differences in the effects of UDCA on Ca^{2+}- and cAMP-dependent secretory responses suggest the involvement of multiple signalling pathways, and these issues are further explored in Chapter 4.

When assessing the specific cellular compartments upon which UDCA acts, factors such as membrane permeability and diffusion capacity must be taken into account. It has been previously demonstrated that the most common mechanism by which unconjugated Bas enter into colonic epithelial cells is by passive diffusion (Hofmann, 2008). Thus, unconjugated BAs can exert their effects either at the cell surface or in

the cytosol. In an effort to determine whether UDCA must enter colonic epithelial cells to exert its effects on Cl^- secretion, we adopted a number of approaches. Initial sidedness studies revealed that UDCA has greater efficacy when added basolaterally. These data are interesting in light of previous studies that show that 3 basolateral transporters, Na^+-taurocholate co-transporting polypeptide (NTCP, SLC10A1), organic anion transporting polypeptide (OATP) 1B1 (OATP-C) and OATP1B3 (OATP8) mediate the uptake of UDCA, GUDCA, and TUDCA by human hepatocytes. Thus, a basolateral transporter may also mediate UDCA uptake in colonic epithelial cells. Previous work has noted an enhanced expression of the basolateral human multidrug resistance protein 3 (MRP3) in the presence of another BA, CDCA (Maeda, 2006, Inokuchi, 2001). To test this hypothesis, we employed the taurine conjugate of UDCA, TUDCA, which due to its increased hydrophobicity, cannot cross cell membranes unless a transport protein is present. These experiments showed that similar to UDCA, TUDCA inhibited agonist-induced secretory responses when added to the basolateral side of T_{84} cells. However, in contrast to UDCA, its effects on CCh were less notable when applied apically. It is worth noting that apical addition of UDCA is also less potent than basolateral addition. Interestingly, responses to FSK were similar to those of UDCA apically. Thus, our studies support the idea that there is likely to be a transport protein for UDCA, and its conjugated derivatives, on the basolateral membrane and that apical entry of the BA occurs to a lesser extent, and by passive diffusion. This is supported by previous findings from this group which showed that basolateral UDCA entry into T_{84} cells is approximately 6-fold more efficient than apical entry (Keely, 2007). Interestingly, immune-histochemical studies after apical addition of a fluorescent UDCA analogue, FM191, showed preferential localisation of the compound to the cell membrane but also that there was diffusion into the cytosol. This supports the idea that apical UDCA can enter the cytosol and then subsequently acts on basolateral transport proteins to inhibit secretion. Finally, the semi-synthetic derivative of UDCA, 6α-MUDCA, exerted similarly potent anti-secretory effects when added apically, suggesting that 6-methylation does not alter the diffusion capacity of the compound negatively, as described previously (Roda, 1994).This similar permeability of 6α-MUDCA across the apical membrane of colonic epithelia would be

favourable when considering the potential use of the compound as an orally-administered therapeutic.

In further experiments, possible mechanisms by which UDCA inhibits Cl^- secretion across colonic epithelial cells were explored. We turned our attention to a number of downstream targets by investigating the effects of UDCA on the activities of specific transport proteins that comprise the Cl^- secretory pathway. We first investigated the effects of UDCA on Na^+/K^+-ATPase pump activity using a well-established assay (Lam, 2003). In these experiments, we found that UDCA treatment attenuated Na^+/K^+-ATPase activity to a similar degree to that by which it inhibited agonist-induced trans-epithelial Cl^- secretory responses. However, in further experiments we found that UDCA did not alter cellular abundance of the Na^+/K^+-ATPase α subunit, suggesting that it does not alter pump expression. This is hardly surprising given the rapidity by which UDCA exerts its anti-secretory effects. Several studies suggest that Na^+/K^+-ATPase activity can also be regulated through altered trafficking of the α subunit to the cell membrane (Chow, 1995, Bystriansky, 2007). However, when we examined localisation of the protein by confocal microscopy or cell surface biotinylation, we found this to be also unaltered by UDCA treatment. Thus, UDCA appears to rapidly inhibit Na^+/K^+-ATPase activity through a mechanism that does not involve its altered expression or cell surface localisation. Other possible mechanisms that could be involved include reductions in mitochondrial ATP production and availability (Papandreou, 2006), direct effects on the pump, or effects on regulatory proteins, such as FXYD proteins (Papandreou, 2006).

By maintaining membrane hyperpolarisation, K^+ efflux across the basolateral membrane is a rate-limiting step for Ca^{2+}-dependent epithelial secretory responses (Barrett, 2000). We therefore examined whether UDCA exerts its anti-secretory actions through modulation of the Ca^{2+}-dependent basolateral K^+ channel conductance, KCNN4 (Flores, 2007). Employing a well-established technique for measuring basolateral K^+ currents (Dawson, 1983), we found that, similar to its actions on trans-epithelial Cl^- secretion, UDCA treatment also inhibited CCh-induced K^+ currents. This suggests that UDCA limits Ca^{2+}-dependent Cl^- secretion by inhibiting K^+ recycling through basolateral KCNN4 channels. Although there is little

information in the literature regarding regulation of K^+ channels by UDCA, it is worth noting that previous work has shown that the pro-secretory BA, TDCA, activates Ca^{2+}-dependent basolateral K^+ channels, thereby providing the driving force for apical exit of Cl^- (Moschetta, 2003). In further experiments, we employed confocal microscopy to determine whether UDCA inhibits K^+ conductances by altering the localisation or abundance of KCNN4 in the basolateral membrane. However, we observed no apparent alterations in the localisation or abundance of KCNN4, suggesting UDCA acts by an alternative mechanism that regulates channel activity, rather than membrane expression. A possible mechanism could be indirect modulation of one or more of the basolateral K^+ channels via activation of protein kinases and subsequent channel phosphorylation which could subsequently lead to reduced channel activity and thus reduction in Cl^- secretion. In support of this suggested hypothesis, previous work from our laboratory has shown that oestradiol, which has inhibitory effects on Cl^- secretion, activates PKCδ and PKACI, which in turn associate with the KCNQ1 channel resulting in its phosphorylation (O'Mahony, 2007). It has also been observed that the PKC agonist phorbol ester may block basolateral K^+ currents in T_{84} cells and reduced Cl^- secretion (Reenstra, 1993).

While the anti-secretory effects of UDCA demonstrated in these studies suggest that the BA may be useful as a new drug to treat diarrhoea, this idea is not borne out in clinical practice. In fact, when used in the clinic, UDCA is an exceptionally safe drug, with the only notable side effect being diarrhoea. Interestingly, in the current studies we found that, in contrast to its anti-secretory effects on cultured epithelial cells, administration of UDCA to mice *in vivo*, led to an enhancement of subsequent secretory responses across *ex vivo* colonic tissues. We hypothesised that the pro-secretory effect of UDCA that occurs *in vivo* could be explained by the fact that the bile acid is rapidly dehydroxylated by enteric bacteria to yield LCA (Fromm, 1983). In support of this hypothesis, we found that there were significantly greater amounts of LCA in the caecal contents of UDCA-treated animals compared to controls. Furthermore, in direct contrast to the anti-secretory actions of UDCA, LCA enhanced agonist-induced secretory responses, both in cultured colonic epithelia and in resected human tissues.

In light of our findings that administration of UDCA to mice increases colonic LCA levels and enhances agonist-induced secretory responses, we hypothesised that a non-metabolisable analogue of UDCA might retain anti-secretory actions *in vivo*. To test this hypothesis we employed 6α-MUDCA, a dehydroxylation-resistant analogue of UDCA. In contrast to UDCA, 6α-MUDCA is stable against 7-dehydroxylation when incubated with human stool in anaerobic conditions, suggesting the compound would not be metabolised in the colon (Roda, 1994).This was confirmed in our *in vivo* mouse studies where LCA levels in 6α-MUDCA-treated animals were comparable to those in controls. Importantly, we also demonstrated that addition of a hydroxyl group to the 6-carbon position of UDCA did not alter its anti-secretory actions *in vitro*. Furthermore, we found that, in contrast to UDCA, 6α-MUDCA exerts anti-secretory actions *in vivo*, supporting the idea that metabolism to LCA prevents the anti-secretory activity of UDCA from being apparent *in vivo*.

While our data suggest that stable analogues of UDCA may be useful for treatment of diarrhoeal diseases, it is widely accepted that, in health at least, absorption of fluid and electrolytes predominates throughout the intestine (Geibel, 2005). Thus, for appropriate fluid homeostasis to occur there must be reciprocal regulation of Cl^- secretion and Na^+ absorption. Therefore, experiments were also conducted to examine the effects of UDCA on intestinal epithelial absorptive function. To measure UDCA effects on ENaC function, expression of the channel was induced in T_{84} cell monolayers using an established experimental approach (Iordache, 2007), while in order to assess its effects on SGLT-1 activity, proximal jejunum from 6α-MUDCA-treated mice was used. In these experimental settings, it was found that neither UDCA nor 6α-MUDCA had significant effects on absorptive processes. Thus, while potential effects of UDCA on Na^+/H^+ exchange remain to be investigated, our data suggest that UDCA, and its analogues, specifically inhibit secretory processes and that should therefore be useful in treating intestinal disorders associated with secretory diarrhoea.

One important question that arises from these studies relates to how UDCA can specifically inhibit secretory processes through inhibition of Na^+/K^+ ATPase activity, but at the same time does affect absorption. One possibility could be that UDCA

alters interactions between the Na^+/K^+ ATPase pump and regulatory proteins in a cell-specific manner. As already mentioned, several proteins are known to bind to and regulate Na^+/K^+ ATPase activity, including FXYD proteins (Lindzen, 2006), translationally-controlled tumour protein (Jung, 2004), and modulator of Na^+/K^+ ATPase (Mao, 2005). Indeed, such regulatory proteins provide an attractive mechanism for inhibition of the Na^+/K^+ ATPase pump, since many are expressed in a cell-specific manner and could explain why UDCA specifically inhibits Cl^- secretory responses, while Na^+ absorptive processes, including electrogenic Na^+ absorption through ENaC and SGLT-1 activity, remain unaltered. Similarly, proteins that regulate the activity of KCNN4, the Ca^{2+}-dependent K^+ channel, are known to exist and could represent targets for UDCA action (Joiner, 2001). Future studies will focus on elucidating the molecular mechanisms by which UDCA inhibits the activities of these transport proteins.

In summary, we present data in this chapter to suggest that, unlike other dihydroxy BAs, UDCA exerts anti-secretory actions on colonic epithelial cells. We also present data to suggest that such anti-secretory actions are not normally apparent *in vivo* since UDCA is rapidly metabolised to LCA by bacteria in the colon. Anti-secretory effects of UDCA, and its derivatives, appear to be mediated by inhibition of multiple components of the Cl^- secretory pathway, including Na^+/K^+ ATPase pumps and basolateral K^+ channels. While mechanisms by which UDCA inhibits transport protein activity remain to be elucidated, they do not appear to involve altered protein expression or localisation. In conclusion, our data suggest that non-metabolisable analogues of UDCA may represent a novel class of anti-diarrhoeal drug that acts through directly targeting epithelial secretory function.

Chapter 4

Signalling mechanisms underlying the anti-secretory actions of UDCA in colonic epithelial cells

4.1. Introduction

In Chapter 3, novel effects of UDCA in attenuating colonic epithelial secretory function were described and the non-metabolisable analogue of UDCA, 6α-MUDCA, was shown to have potent anti-secretory effects *in vivo*. However, before we can further target UDCA, or its derivatives, as potential therapeutic agents for diarrhoeal disease, it is important to further elucidate the mechanisms by which they exert their anti-secretory effects.

As a basis for the studies described in this Chapter, it is first necessary to consider what is already known of the signalling mechanisms that regulate fluid and electrolyte secretion in the colon. Such mechanisms can involve modulation of the activity, abundance, or localisation of the specific transport proteins involved. However, given that the anti`-secretory effects of UDCA occur very rapidly (within minutes) and are associated with alterations in the activity, but not surface expression, of key transport proteins, it is most likely that UDCA modulates signalling processes that are involved in acute regulation of the secretory pathway. Such mechanisms could include i) direct interaction with transport proteins, ii) alterations in production of pro-secretory 2nd messengers, or iii) interaction with downstream effector enzymes. To set these studies in context, in the following sections an introduction to the main 2nd messengers and effector enzymes involved in regulation of epithelial secretory responses is provided.

4.1.1. Intracellular Ca^{2+}

Ca^{2+} is an important intracellular 2nd messenger, that controls a diverse range of cellular processes, including enzyme activities, cell attachment, motility, morphology, metabolic processes, cell-cycle progression, signal-transduction, replication, gene expression and, of particular relevance to the current studies, electrochemical responses (Berridge, 2000, Hofer, 2005). As a consequence of its critical roles in regulating cellular physiology, extracellular Ca^{2+} concentrations are tightly regulated at the levels of intestinal Ca^{2+} absorption, exchange of Ca^{2+} to and from bone, and renal Ca^{2+} reabsorption (Barrow, 2006). Resting intracellular concentrations of Ca^{2+} are normally maintained in the range of 10 –100 nM, typically 4 orders of magnitude lower than external concentrations. To maintain this low intracellular concentration,

Ca^{2+} is actively pumped from the cytosol to the extracellular space, or into the endoplasmic reticulum (ER), and to a lesser extent, the mitochondria. Upon appropriate stimulation, Ca^{2+} levels in the cytoplasm can increase, either as a consequence of its release from intracellular stores or its entry across the plasma membrane (Clapham, 2007).

Ca^{2+} release from intracellular stores

When Ca^{2+} signalling is stimulated in a cell, Ca^{2+} enters the cytoplasm from one of two general sources: it is released from intracellular stores, or it enters the cell across the plasma membrane. In Ca^{2+}-storing organelles, such as the ER and mitochondria, Ca^{2+} ions bind to specialized Ca^{2+}-binding proteins, such as calsequestrin (Katz, 2005). In the cytosol, there are also Ca^{2+} buffers; the calbindins, calmodulin, and S-100 protein families, which serve to redistribute Ca^{2+} within the cell (Gackiere, 2006, Sharma, 2006).

The most common signalling pathway triggering release of Ca^{2+} from the intracellular stores involves activation of PLC and generation of IP_3 (inositol triphosphate) and DAG from PIP_2 (phosphatidylinositol (4, 5)-bisphosphate). PLC can be differentially activated by a wide variety of cell surface receptors, including those for growth factors (Hyun, 2011); cytokines (Chung, 2012); G-protein coupled receptors (GPCRs) (Yan, 2003; Okamoto, 2004; O'Mullane, 2009), and integrins (Hyduk, 2007). PLC hydrolyses the membrane phospholipid, PIP_2 to form the two classical intracellular 2nd messengers IP_3 and DAG. DAG activates protein kinase C (PKC), while IP_3 diffuses to the endoplasmic reticulum (ER), binds to the IP_3 receptor, and thus releases Ca^{2+} (Kachintorn,1993) from the ER (Gackiere, 2006).

Cellular Ca^{2+} entry: The most common mechanism of regulated Ca^{2+} entry into epithelial cells is however through store-operated Ca^{2+} entry (SOCE), whereby depletion of intracellular stores due to the actions of IP_3, or other Ca^{2+}-releasing signals, activates a signalling pathway that leads to the opening of plasma membrane Ca^{2+} channels, known as store-operated channels (SOCs) (Putney, 2001). The resulting current is referred to as a Ca^{2+}-release-activated Ca^{2+} current (I_{CRAC}). Mechanisms through which I_{CRAC} occurs are still under investigation, but recent studies suggest that phospholipase A_2, nicotinic acid adenine dinucleotide

phosphate (NAADP) (Moccia, 2003), and STIM-1 (Baba, 2006) may also be involved.

In excitable cells, Ca^{2+} influx may also occur through highly selective voltage-gated Ca^{2+} channels (VGCCs). VGCCs are expressed in colonic epithelia and T_{84} cells (Wang, 2000). L-type Ca^{2+} channels have been shown to play a key role in the mediation of oscillation at the neuro-epithelial junctions of guinea pig colon where Ba^{2+}, a K^{+} channel inhibitor, evokes Ca^{2+}-dependent oscillatory Cl^{-} secretion via activation of sub-mucosal cholinergic neurons in guinea pig distal colon (Nishikitani, 2007).

Transient receptor potential (TRP) channels are non-specific cation channels, which are subdivided into 3 groups; TRPC, TRPV and TRPM. TRPC channels respond indirectly to hormones and transmitters through PLC activation and via 2nd messengers, such as DAG (Gackiere, 2006, Nowycky, 2002).TRPA-1 channels are expressed in human and rat colon (Kaji et al., 2011). TRP channels are often functionally associated with GPCRs and linked with Ca^{2+} signalling and are involved in the motility of the small intestine (Nozawa, 2009).

Regulation of colonic secretion by Intracellular Ca^{2+}:

There is a significant body of evidence to suggest that alterations in intracellular Ca^{2+} play a significant role in the regulation of intestinal fluid and electrolyte transport. In early studies, Donowitz et al. used 3,4,5 -trimethoxybenzoate 8-(N,N-diethyl amino) octyl ester (TMB8), an inhibitor of intracellular Ca^{2+} release, to assess the role of Ca^{2+} in electrolyte transport in rabbit ileum, and found that the effects of the muscarinic agonist, CCh, but not serotonin, were inhibited. This suggests specific roles for different pools of intracellular Ca^{2+} in mediating transport responses to different neuroimmune agonists (Donowitz, 1986). The same group later went on to show that increases in intracellular Ca^{2+} act through PKC to regulate NaCl absorption in the rabbit ileum (Donowitz, 1989). Increases in intracellular Ca^{2+} have also been shown to stimulate both Cl^{-} and HCO_3^{-} secretion in some intestinal cell lines and to inhibit electroneutral NaCl absorption and electrogenic Na^{+} absorption (Barrett, 2006).

Agonist-induced elevations in intracellular Ca^{2+} have been shown to regulate epithelial Cl^- secretion by several mechanisms. Barrett et al., have shown that elevations in $[Ca^{2+}]_i$ alone are a sufficient signal to induce Cl^- secretion in colonic epithelial cells (Kachintorn, 1993) . Indeed, intracellular Ca^{2+} directly regulates the activity of key transport proteins involved in epithelial secretion. K^+ efflux across the basolateral membrane through the Ca^{2+}-dependent channel, KCNN4, is an important, rate-limiting, step in the Ca^{2+}-dependent epithelial secretory responses. This process appears to involve calmodulin (Fogg, 1994).This is very relevant to the effects of UDCA on colonic secretion given that we have already demonstrated that UDCA inhibits basolateral K^+ channel activity.

Furthermore, the molecular identification of TMEM-16A, an apical Ca^{2+}-activated Cl^- channel, has provided new insights into how elevations in intracellular Ca^{2+} promote Cl^- secretion. However, the role of this channel in mediating Ca^{2+}-dependent secretory responses in the intestine is still unclear (Ousingsawat, 2009).

Although release of Ca^{2+} from intracellular stores exerts pro-secretory effects on colonic epithelial cells, there is also some evidence to suggest that increases in intracellular Ca^{2+} can also activate signalling pathways that negatively regulate Cl^- secretion. For example, at the same time that they induce Cl^- secretion, Ca^{2+}-dependent agonists also activate a MAPK-mediated anti-secretory mechanism, which may serve to limit the extent and duration of secretory responses to such agonists (Keely, 2003). Furthermore, previous studies have shown that while release of Ca^{2+} from intracellular stores is pro-secretory in colonic epithelial cells, the subsequent influx of extracellular Ca^{2+} across the plasma membrane is anti-secretory (Vajanaphanich, 1995). The existence of such anti-secretory mechanisms likely underlies the observations that there is a relatively poor correlation between the magnitude and duration of increases in intracellular Ca^{2+} and associated Cl^- secretory responses (Dharmsathaphorn, 1989a). In general, Ca^{2+}- evoked secretory responses are transient and terminate even if the Ca^{2+} signal persists (Keely, 2000).

4.1.2. Cyclic nucleotides

Levels of cAMP within epithelial cells are regulated by a family of adenylate cyclases (AC) that respond to neuroimmune agonists of GPCRs. Consequently, intracellular

levels of cAMP can be up- or down-regulated, depending upon which type of GPCR is activated. While G_sPCRs are positively coupled to AC, G_iPCRs inhibit activity of the enzyme (Hall, 2012). Several neural, immune and hormonal regulators of cAMP levels exist, including VIP and prostaglandins, which stimulate AC activity, and somatostatin, a G_iPCR agonist, which is inhibitory (Kimberg, 1971). Furthermore, exogenous substances, such as *Vibrio cholera* enterotoxin, can over-activate the cAMP-dependent signalling pathway through irreversible activation of G_s proteins by ADP ribosylation and prolonged adenyl cyclase activation.

cGMP is another important cyclic nucleotide which acts as a 2nd messenger in epithelial transport. Association of cGMP agonists with the extracellular domain of guanylyl cyclase receptor (GC-R) activates the intracellular catalytic domain that then converts GTP into cGMP (Parkinson, 1997). cGMP, in turn, activates cGMP-dependent protein kinase (PKG) II, the conventional downstream effector for this cyclic nucleotide, resulting in secretory diarrhoea (Parkinson, 1997, Zhang, 1999). Heat stable enterotoxin (ST_a) is a cGMP agonist and binds to the GCR, thus increasing cGMP levels and producing a pro-secretory effect as described above and thus this relationship forms part of the crosstalk between enteric pathogens and the intestine (Fasano, 2000) . The endogenous ligand for this receptor is guanylin. This family of peptides play an important role in intestinal fluid and electrolyte homeostasis and also in linking the mechanisms of the kidney and the intestine.

With respect to intestinal fluid and electrolyte transport, increases in either cAMP, or cGMP, have been shown to stimulate Cl^- secretion and to inhibit Na^+ absorption (Donowitz, 1986). cAMP regulates each of the transport proteins involved in the Cl^- secretory pathway, including CFTR , Na^+/K^+ ATPase pumps, NKCC, and other K^+ channels (Reynolds., 2007, Bargon, 1998, Alzamora, 2011, Schulzke, 2011, Ward, 2011). Cellular responses to agonists that elevate levels of cAMP are terminated by the activity of phosphodiesterases (PDE), which hydrolyses cAMP to 5'-AMP, and also by complex signalling mechanisms that lead to receptor desensitisation and internalisation (Kunzelmann, 2002).

Importantly, it has been shown that the Ca^{2+} and cAMP-dependent pro-secretory pathways do not function independently of one another but that crosstalk exists

between them. For example, in the rat colon, agonist-induced activation of adenylayte cyclase (AC) is positively affected by the presence of Ca^{2+} (Calderaro, 1993). Conversely, regulation of Ca^{2+} transients by VIP occurs via activation of AC and increased synthesis of cAMP (Hagen, 2006). Important crosstalk exists between these two pathways and in fact synergistic Cl^- secretion may occur when both types of agonist are present in T_{84} cells (Vajanaphanich, 1995).

4.1.3. Protein Kinases

The human genome contains about 500 genes encoding kinases (Manning, 2002), many of which are involved in mediating downstream effects of intracellular Ca^{2+} and cAMP in regulating epithelial transport responses. Kinases that have received particular interest with respect to regulation of epithelial transport include the mitogen-activated protein kinases PKC, PKA and ERK-MAP kinases.

The PKC family of kinases:

PKC isoforms are divided into 3 groups, conventional (cPKC), novel (nPKC) and atypical PKCs (aPKC) as demonstrated in Figure 4.1, cPKCs are known to be Ca^{2+}-activated, phospholipid-dependent and sensitive to phorbol esters. nPKCs are activated by phospholipids and similar to cPKC isoforms, are also activated by phorbol esters. However, they do not require Ca^{2+} for catalytic activity. The aPKC group is not activated by either phospholipids or phorbol esters. Each member of the PKC family has regulatory and catalytic domains. The catalytic domains are highly homologous within the family and have 3 phosphorylation sites. One is a threonine residue located in the catalytic domain and the others are Ser and Thr residues located within the carboxyl-terminal region of the catalytic domain (Yamamoto, 2006). In rabbit distal colon PKCα activation appears to be necessary for BA stimulation of Cl^- secretion (Kanchanapoo, 2007).

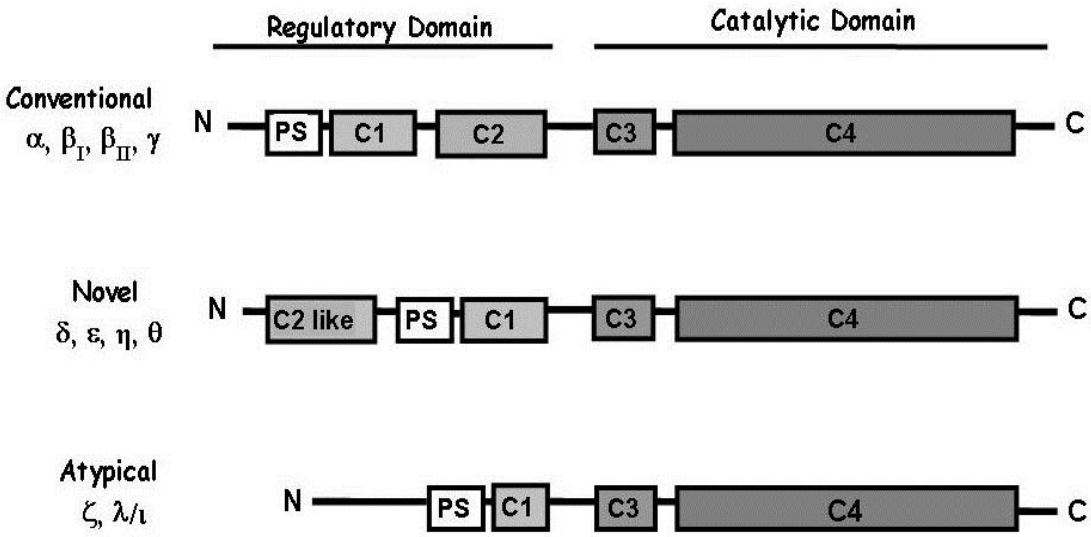


Figure 4.1: The PKC super family (Byrne , 2010). Each isoform is composed of a regulatory and catalytic domain and the family is divided into 3 subgroups based on their structures: the conventional, novel, and atypical PKCs.

In relation to colonic epithelial secretion and of interest to our findings in relation to the anti-secretory effects of UDCA, inhibitory signalling in response to PKC activation has been shown to play a role. PKC activation is thought to occur downstream or in parallel with EGFr transactivation in the negative regulation of chloride secretion in the colonic epithelium (Reynolds, 2007). Direct activation of PKC is thought to inhibit Cl^- secretion by preventing activation of the secretory machinery of the cell through subsequent elevation in intracellular Ca^{2+} (Kachintorn, 1993) or cAMP (Matthews, 1993).

PKA: (Protein Kinase A) refers to a family of enzymes whose activity is dependent on cellular levels of cAMP. PKA is also known as cAMP-dependent protein kinase. When the concentration of cAMP rises (e.g. through activation of ACs by GPCRs), cAMP binds to the two binding sites on the regulatory subunits, which leads to the release of the catalytic subunits. They then catalyse the transfer of ATP terminal phosphates to protein substrates. This phosphorylation usually results in a change in activity of the substrate. PKA and cAMP are involved in many cellular processes including colonic epithelial fluid secretion where PKA is involved with acute regulation of Cl- exit apically through cAMP-induced secretion. PKA has been shown to directly interact with CFTR through direct phosphorylation of the channel (Berger, 1993). NKCC1 is also phosphorylated by PKA. cAMP is also responsible for gating

KCNQ1/KCNE3 K^+ channels. The kinases which are involved with the Ca2+ signalling pathway are less well understood, but it has been demonstrated that PKA can activate the Ca^{2+} sensitive K^+ channel but that PKC does not (Cole, 1996).

Mitogen-Activated Protein Kinases: The MAPKs are a group of enzymes responsible for phosphorylating serine and threonine amino acids in many effector proteins. There are 7 families of MAPKs, the best characterised of which are extracellular regulated kinase 1/2 (ERK 1/2), p38 MAP kinase, and the c-Jun N-terminal kinase (JNK) (Coulombe, 2007). GPCRs recruit these pathways through a number of methods, including transactivation of the EGFr or activation of the Src family kinases (Keely, 2003). Once activated, MAPKs may stimulate other effector proteins or translocate to the nucleus and activate gene transcription. The activation of ERKMAPKs has been extensively studied (Broom, 2009) and activation may occur with many GPCR agonists including VIP and CCh (Bertelsen , 2004a). This leads to a signalling cascade involving Ras, Raf (MAPKKK), and mitogen extracellular kinase (MEK) (MAPKK).

MAP kinases are an important regulatory mechanism in epithelial ion transport. Interestingly, Barrett et al., have demonstrated that Ca^{2+}-mediated transactivation of EGFR/MAP kinase pathways in T_{84} cells play a central role in the negative regulation of Ca^{2+}-mediated Cl^- secretion (Keely, 2003, Keely, 1998). These studies established the M_3AChR–Ca^{2+}–EGFR transactivation pathway and the contribution of downstream MAP kinase signalling to negative regulation of Ca^{2+}-dependent fluid secretion in the T_{84} cell model.

4.1.4. BA effects on 2nd messengers and kinase activity

There are a number of studies which show that UDCA and other BAs regulate 2nd messengers and kinases that are involved in regulation of epithelial transport function. For example, it has been shown that pro-secretory BAs increase colonic epithelial Cl^- secretion through elevating intracellular levels of Ca^{2+} (Dharmsathaphorn, 1989b). Similarly, Moschetta et al., have demonstrated that activation of Ca^{2+}-dependent basolateral K^+ currents, through TDC-induced apical Ca^{2+} influx, provides the driving force for epithelial secretion of Cl^- ions (Moschetta,

2003). Effects of BAs on colonic epithelial intracellular Ca^{2+} levels have been previously reported with DCA causing a rise while UDCA was not deemed to have an effect in the ACL-15 colonic cancer cell line however its effects on intracellular Ca^{2+} have not been previously demonstrated in either a secretory cell line or in isolated human crypts as shown here (Momen, 2002) . It has also been demonstrated that amylase secretion from rat pancreatic acini in response to the BA are abolished by removal of extracellular Ca^{2+}, implying the involvement of Ca^{2+} influx (Shinozaki, 1995). Furthermore, UDCA and TUDCA induce sustained elevations in Ca^{2+} in hepatocytes (Beuers, 1993, Bouscarel, 1993) and activate membrane-bound PKCs (Stravitz , 1996), while cytosolic free Ca^{2+} is required for TUDCA-induced exocytosis and insertion of transport/carrier proteins into the apical membrane (Beuers , 1993).

In relation to BA effects on cAMP-induced secretion, it has been demonstrated previously that DCA affects the transport properties of the distal colon through modulation of both the cAMP- and Ca^{2+}-dependent signalling pathways (Mauricio, 2000). In contrast, in isolated crypts DCA activated a Ca^{2+}-regulated K^{+} conductance but had no effect on cAMP (Mauricio, 2000). There is little known of the role of UDCA in regulating cAMP levels in colonic epithelial cells, but Bouscarel et al., have demonstrated that the BA inhibits glucagon-induced cAMP formation in hepatocytes (Bouscarel, 1995). In contrast, UDCA has also been shown not to affect basal cAMP levels in rabbit intestinal loops but in this model it was also without effects on net secretion (Rahban, 1980).

In addition to regulating intracellular levels of pro-secretory 2nd messengers, BAs also signal to a number of kinases that can be involved in regulating epithelial transport function. For example, BAs have been shown to modulate the Golgi membrane fission process via a protein kinase C and protein kinase D-dependent pathway in colonic epithelial cells (Byrne, 2010). In hepatocytes, inhibition of bile salt-induced apoptosis by cAMP involves a PKA-dependent Ser/Thr phosphorylation of the CD95 (Reinehr, 2004). Interestingly, inhibition of the MAPK and PI3K pathways enhances UDCA-induced apoptosis in primary rodent hepatocytes (Qiao, 2002). In relation to effects on colonic secretion it has been found that physiological concentrations of DCA (50 μM) can result in rapid phosphorylation of both ERK and

p38 MAP kinases implicating them in its effects on down regulation of colonic Cl^- secretion at these concentrations (Keating, 2009).

4.2. Aim:

While, BAs have the capacity to modulate 2nd messengers and signalling pathways that are known to be involved in regulating intestinal epithelial transport function, how UDCA exerts its actions in colonic epithelia are still poorly understood. The primary goal of this chapter was to investigate signalling mechanisms underlying the anti-secretory effects of UDCA on colonic epithelial cells. In particular, we aimed to:

- Examine the effects of UDCA on intracellular levels of the pro-secretory 2nd messengers, Ca^{2+} and cAMP, in colonic epithelial cells.
- Examine the role of PKA, PKC, and ERK-dependent signalling mechanisms in mediating anti-secretory actions of UDCA in colonic epithelial cells.

4.3. Results:

4.3.1. UDCA elevates intracellular levels of Ca^{2+} in colonic epithelial cells

Previous studies have shown that agonists of G_qPCRs, typified by CCh, induce biphasic increases in cytosolic [Ca^{2+}] in colonic epithelial cells: an initial release from intracellular stores, followed by influx from the extracellular milieu that is required for sustained, oscillatory signalling (Kachintorn, 1993). It has also been shown that despite prolonged elevations in $[Ca^{2+}]_i$, Cl^- secretory responses to CCh are transient in nature (Kachintorn, 1993), suggesting that there is a complex relationship between intracellular Ca^{2+} levels and Cl^- secretory responses. Thus, having demonstrated anti-secretory effects of UDCA on Ca^{2+}-dependent secretory responses in Chapter 3 of this Thesis, we became interested in investigating how the BA might regulate levels of intracellular Ca^{2+}.

Experiments were first carried out to assess the effects of CCh on intracellular Ca^{2+} in our T_{84} cells. A standard approach for measuring intracellular Ca^{2+} levels, using Fura-2/AM-loaded T_{84} cells and assessing changes in fluorescence ratio upon addition of CCh (100 μM), was employed. We found that CCh stimulated an immediate elevation of intracellular Ca^{2+}, corresponding to Ca^{2+} release from intracellular stores, followed by a plateau phase, corresponding to Ca^{2+} entry (n = 12;

Figure 4.2: Panel A). Next, the effects of UDCA on intracellular Ca^{2+} levels were assessed. UDCA (250 μM) also increased intracellular levels of Ca^{2+} (n = 12; Figure 4.2: Panel B). Responses to UDCA occurred immediately and were concentration-dependent, with effects being observed at concentrations from 10−500 μM (Figure 4.3). In subsequent studies, UDCA was employed at a concentration of 250 μM, since this concentration elevates intracellular Ca^{2+} and effectively inhibits agonist-induced Cl^- secretion (IC_{50} ~ 200 μM).

Further investigation revealed that UDCA (250 μM) also inhibited subsequent CCh-induced Ca^{2+} mobilization by 63 ± 0.6% compared to controls ($n = 12$, $p < 0.05$; Figure 4.4, Panel A). Similarly, pre-treatment with CCh (100 μM) ameliorated UDCA-induced elevations in intracellular Ca^{2+} ($n = 12$, $p < 0.01$, Figure 4.4; Panel B).

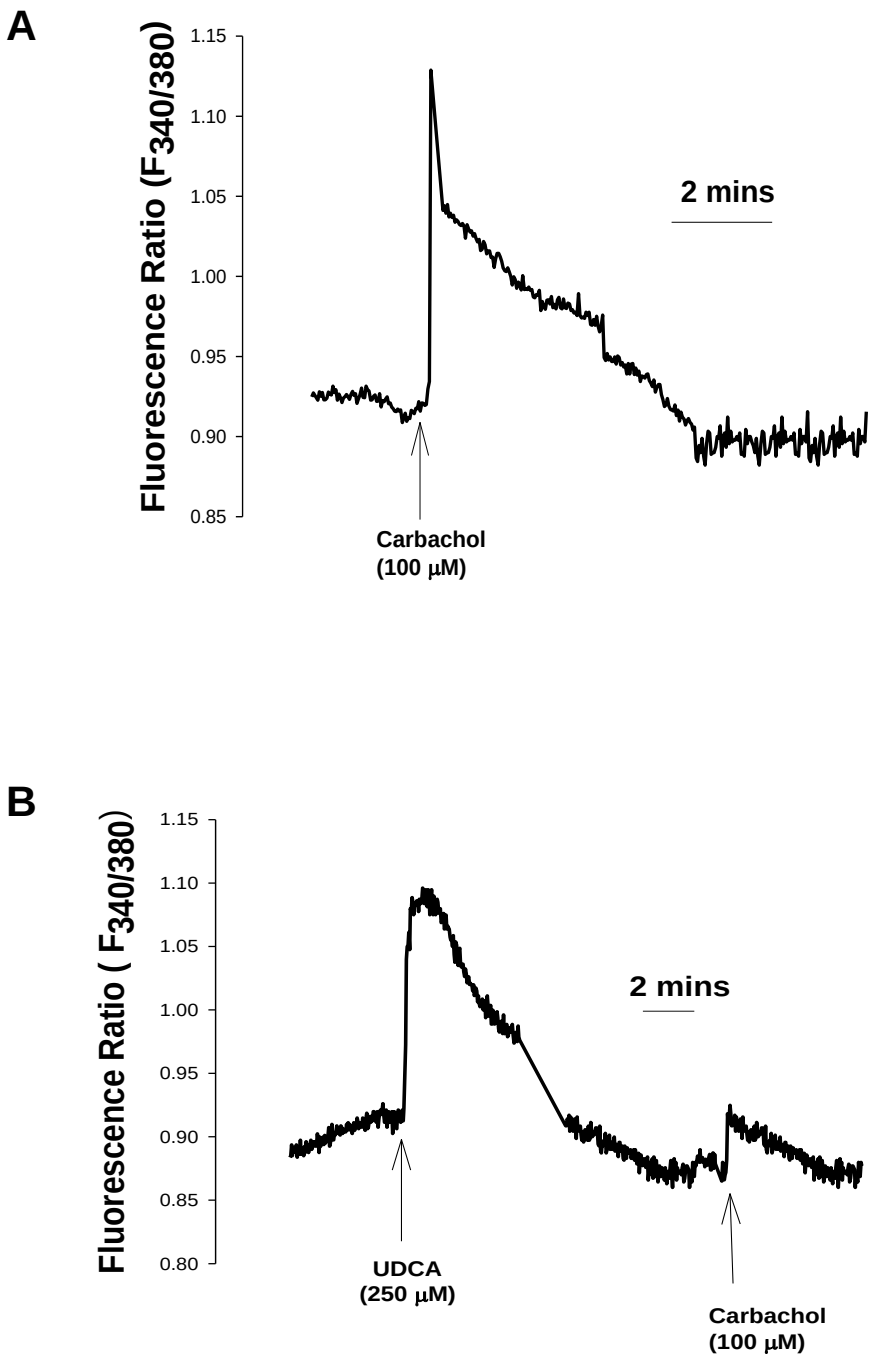


Figure 4.2: UDCA elevates intracellular Ca^{2+} in T_{84} cells. T_{84} cells were grown to 70% confluency on glass coverslips and loaded with Fura/2AM for 45 min in serum-free medium. Cells were perfused with physiological perfusion solution, mounted on the stage of a Nikon microscope and after equilibration for 15 min were stimulated with A) CCh (100 μM) or B) UDCA (250 μM) followed by CCh (100 μM). Changes in intracellular Ca^{2+} were measured by determining the $F_{340/380}$ ratio in 15-20 regions of interest (ROI). Traces shown are representative of n = 12 individual experiments.

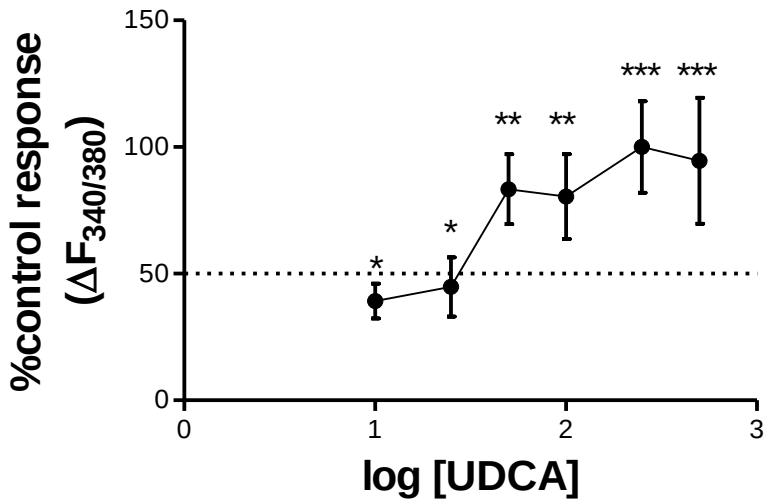


Figure 4.3: UDCA induces elevations in intracellular Ca^{2+} in a concentration-dependent manner. T_{84} cells were grown to 70% confluency on glass coverslips and loaded with Fura/2AM for 45 min in serum-free medium. Cells were then mounted on the stage of a Nikon microscope and after equilibration for 15 min, UDCA was added and changes in fluorescence recorded ($\Delta F_{340/380}$). UDCA (10 - 500 μM) induced significant increases in intracellular Ca^{2+} compared to control even at low doses with maximal responses noted at concentrations ≥ 250 μM. Graph represents the log concentration dose response. Calculated EC_{50} was 29 μM (n = 6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

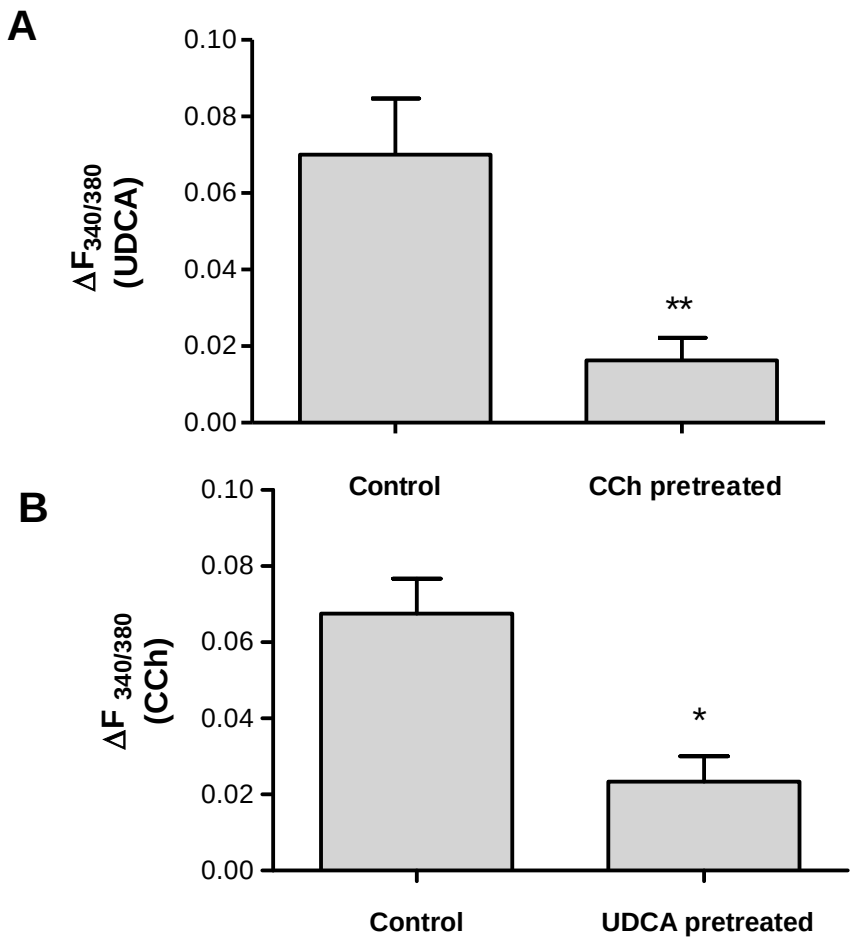


Figure 4.4: UDCA pre-treatment ameliorates subsequent Ca^{2+} mobilisation by CCh. UDCA (250 μM; apical) was added to Fura 2AM loaded T_{84} cells and $\Delta F_{340/380}$ was measured. This was followed by addition of CCh (100 μM) **A)** UDCA (250 μM) inhibited subsequent Ca^{2+} mobilization responses to CCh by 63 ± 0.57% of control cells in Fura/ 2AM loaded T_{84} cells (n = 12, *p < 0.05). **B)** Similarly, pre-treatment with CCh (100 μM) ameliorated subsequent Ca^{2+} mobilization responses to UDCA (250 μM), (n = 12, **p < 0.01).

4.3.2. UDCA induces Ca^{2+} influx and store-operated Ca^{2+} entry in T_{84} cells.

To determine whether UDCA-induced elevations of intracellular Ca^{2+} were due to influx of extracellular Ca^{2+} or release from intracellular stores, extracellular Ca^{2+} was removed from the perfusion solution and replaced with the Ca^{2+} chelator, EGTA (ethylene glycol-bis(2- aminoethylether)- *N, N, I', N'*- tetraacetic acid; 1mM). Under these conditions, the CCh-induced peak in intracellular Ca^{2+}, which is due to release from intracellular stores, was not significantly altered, with the $\Delta F_{340/380}$ value being 0.099 ± 0.05 in Ca^{2+}-free PSS compared to 0.12 ± 0.005 in controls (Figure 4.5, Panel A, n = 6). In contrast, the mean difference between baseline and average plateau phase values was reduced to approximately 30% of controls in cells in Ca^{2+}-free medium ($\Delta F_{340/380}$ = 0.03 ± 0.004 compared to 0.1 ± 0.002 in controls (n = 6; $p < 0.01$)). This finding would be expected because sustained Ca^{2+} signalling requires influx from the extracellular milieu. Interestingly, removal of Ca^{2+} from the extracellular bathing solution abolished UDCA-induced Ca^{2+} elevations. $\Delta F_{340/380}$ values were 0.12 ± 0.007 in control cells compared to 0.02 ± 0.009 in Ca^{2+}-free bathing solution (Figure 4.5, Panel B, n = 6, ** $p < 0.01$).

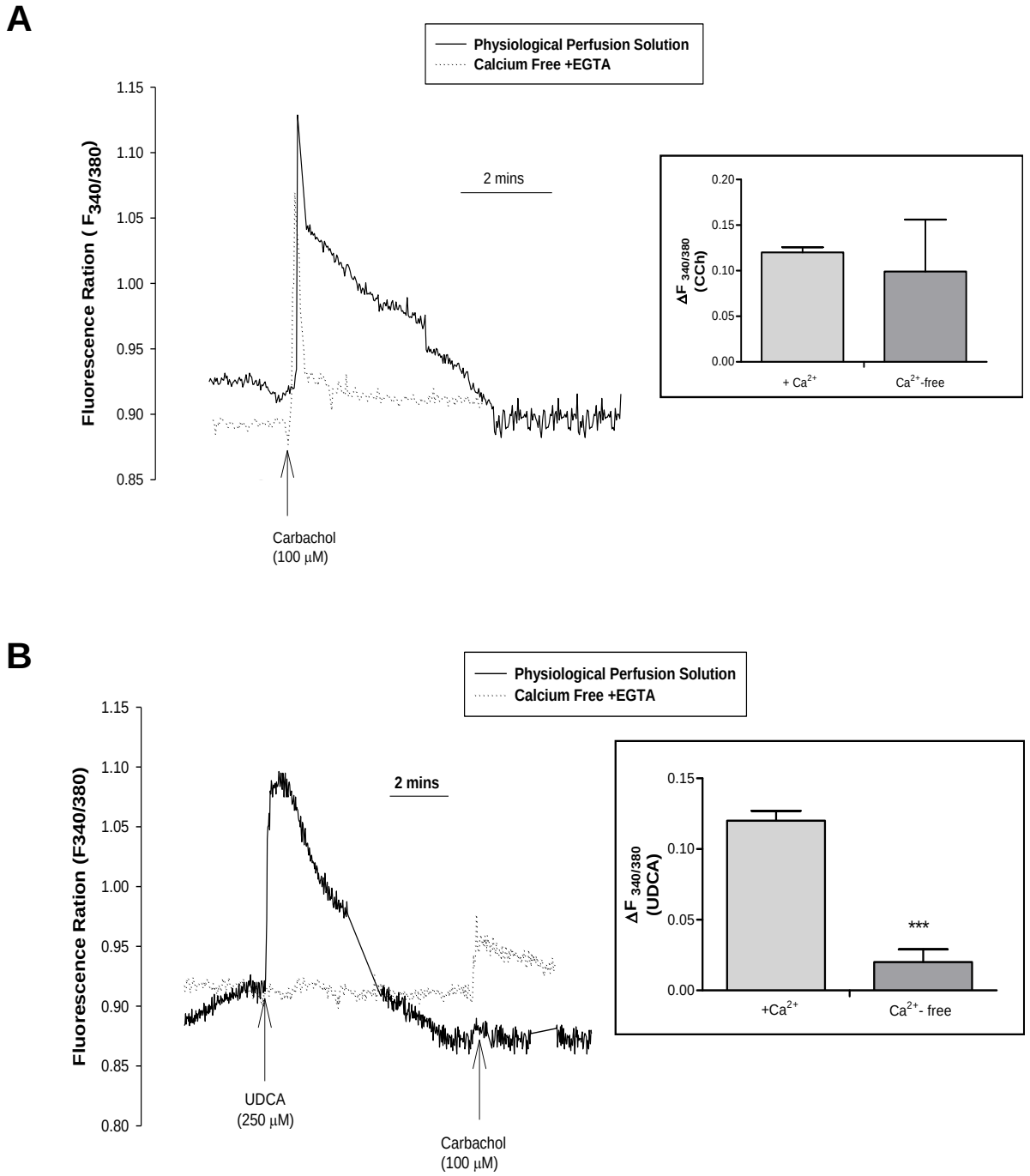


Figure 4.5: UDCA-induced elevations in intracellular Ca0.1^{+} are due to influx of extracellular Ca^{2+}. T_{84} cells were grown to 70% confluency on glass coverslips and loaded with Fura/2AM for 45 min in serum-free medium. Cells were perfused with physiological perfusion solution, mounted on the stage of a Nikon microscope and after equilibration for 15 min were stimulated with UDCA or CCh in the presence or absence of Ca^{2+} in the medium. **A)** CCh-induced peak Ca^{2+} levels were not significantly affected by removal of extracellular Ca^{2+}, as can be seen on the trace and the bar-chart insert showing mean $\Delta F_{340/380}$, however the plateau phase was reduced with a marked dissipation occurring within seconds of the spike in contrast to the longer plateau phase when Ca^{2+} is present (n = 6, **p < 0.01). **B)** Removal of Ca^{2+} from the extracellular bathing solution abolished UDCA-induced Ca^{2+} elevations. Both trace and insert demonstrate little change in baseline intracellular Ca^{2+} when UDCA is added in the absence of extracellular Ca^{2+}(n = 6, ** p < 0.01).

Having shown that influx of extracellular Ca^{2+} was necessary for UDCA to elevate intracellular Ca^{2+}, we next employed SKF96365 (SKF; 100 µM), an inhibitor of store-operated Ca^{2+} entry (SOCE) (Merritt, 1990) to further investigate the role of Ca^{2+} influx. Firstly, responses to CCh were analyzed in the presence of extracellular Ca^{2+}. CCh induced an initial peak of $[Ca^{2+}]_i$, corresponding to release from intracellular stores, followed by a plateau phase which was inhibited to 70 ± 11% of controls in the presence of SKF96365 (n = 6; *p < 0.05). Pre-treatment with SKF96365 (100 µM; 30 min) did not significantly alter the initial peak in intracellular Ca^{2+} induced by CCh (79.8 ± 12.3% of controls, n = 6, Figure 4.7 4.6).

Pre-treatment with SKF96365 (100 µM; 30 min) (Hartford, 2008) ameliorated the UDCA-induced rise in intracellular Ca^{2+} to a $\Delta F_{340/380}$ value of 0.0126 ± 0.008 (Panel A, Figure 4.7.7), representing a reduction to 5.2 ± 3.2 % of control responses (**p < 0.01, n = 6, Panel B, Figure 4.7). Pre-treatment with SKF96365 also partially reversed the inhibitory effects of UDCA on subsequent Ca^{2+} mobilization by CCh (100 µM). These data suggest that UDCA-induced elevations in intracellular Ca^{2+} are dependent on SOCE and that by inhibiting SOCE, subsequent CCh-induced Ca^{2+} mobilization can be restored.

Concurrent experiments were carried out to assess how SOCE inhibition altered effects of UDCA on Cl^- secretion. T_{84} cell monolayers were pre-treated with SKF96365 (100 µM; apical; 30 min) and placed in Ussing chambers for measurements of CCh-induced I_{sc}. ΔI_{sc} responses to CCh in cells pre-treated with UDCA (500 µM; bilateral; 15 min) in the presence of SKF96365 (100 µM; 30 min pre-treatment) were 50.3 ± 18.0% of controls (*p < 0.05, n = 6). This was approximately 5-fold greater than responses in cells exposed to UDCA alone (9.7 ± 3.6% of controls, ***p < 0.001, n = 6, Figure 4.8), suggesting the involvement of SOCE in mediating the anti-secretory effects of UDCA.

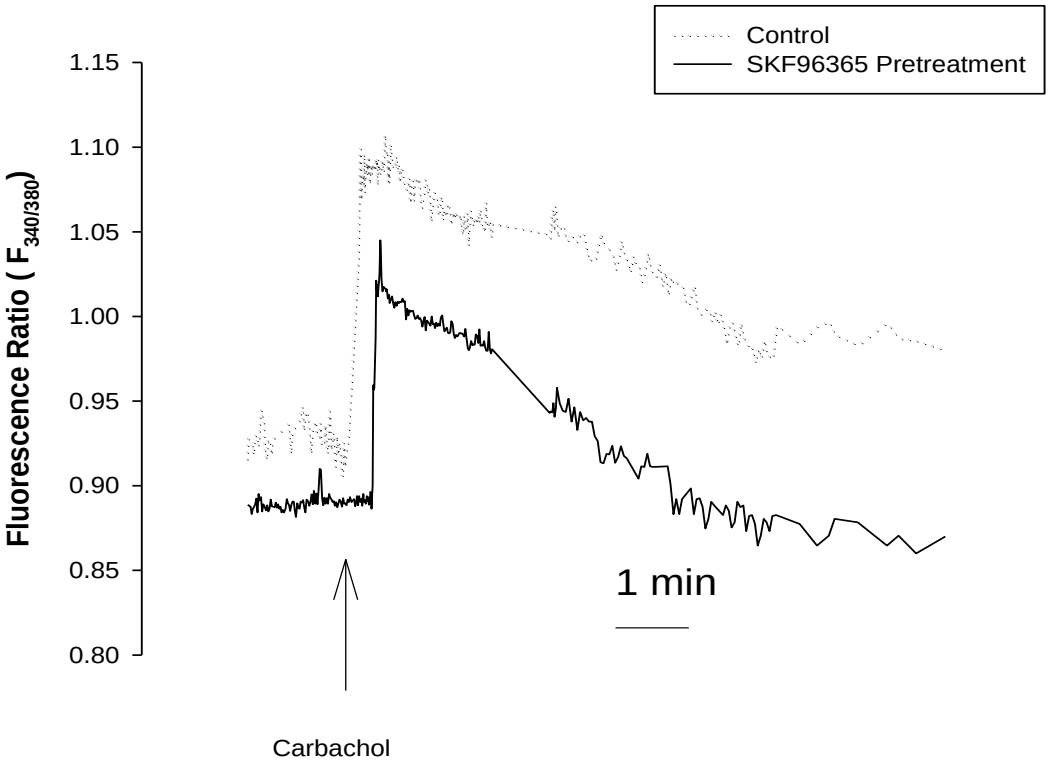
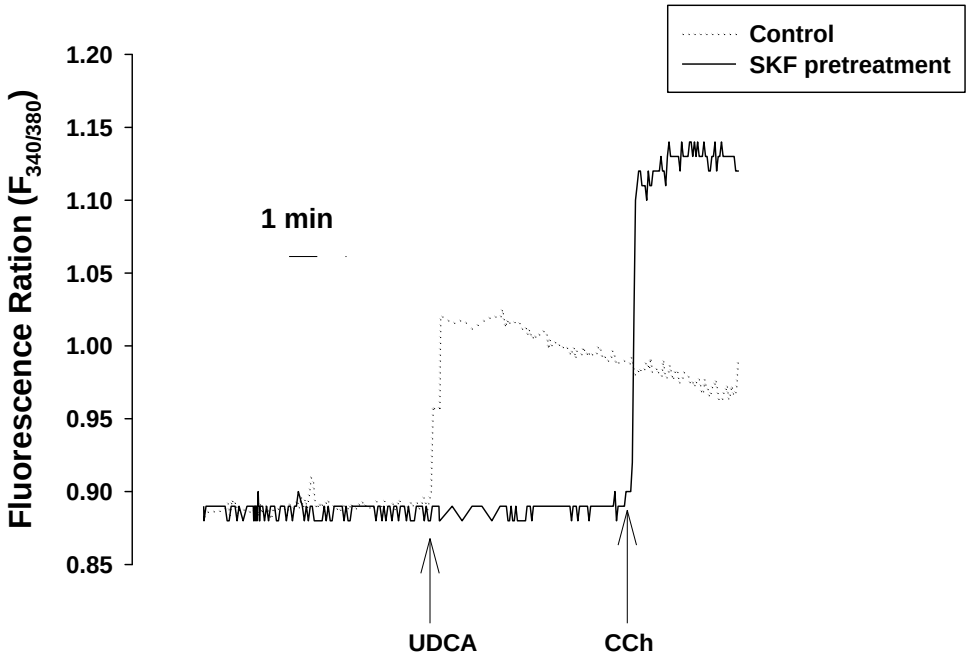


Figure 4.6:SKF96365 inhibits store-operated Ca^{2+} entry in T_{84} cells. T_{84} cells, grown to 70% confluency on coverslips, were loaded with Fura-2/AM. In controls, CCh (100 µM) induced an initial peak of $[Ca^{2+}]_i$, corresponding to release from intracellular stores, followed by a plateau phase. This plateau phase in particular, was inhibited significantly in the presence of SKF96365 (100 µM; 30 min pre-treatment; n = 6; $p < 0.05$). SKF96365 did not however significantly alter the peak elevation in intracellular Ca^{2+} induced by CCh (n = 6).

A



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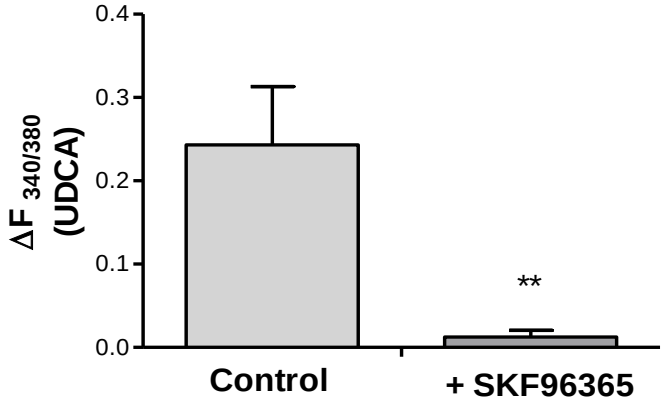


Figure 4.7: UDCA-induced elevations in intracellular Ca^{2+} are mediated by store operated Ca^{2+} entry. A) T_{84} cells loaded with Fura-2/AM were pre-treated with SKF96365 (100µM; 30 min) and response to UDCA and CCh (Δ $F_{340/380}$) was measured (n = 6). Panel **B)** demonstrates a reduction in UDCA response in the presence of SKF96365 to 5.18 ± 3.2 % of control UDCA response (n = 6, ** $p < 0.01$).

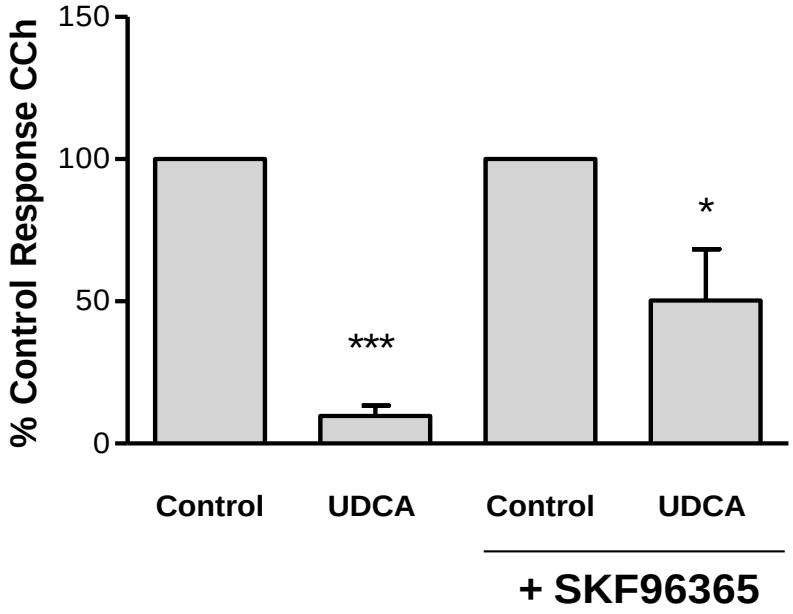


Figure 4.8: SKF96365 partially reverses the anti-secretory effect of UDCA on CCh responses. T_{84} cells pre-treated with SKF96365 (100 µM; 30 min) were placed in Ussing chambers and I_{sc} responses to secretory agonists were measured. Δ I_{sc} in response to CCh in cells pretreated with UDCA (500 µM; 15 min) and SKF96365 was 50.3 ± 18% of controls (*$p < 0.05$, n = 6), 5 -fold higher than CCh -induced I_{sc} responses in cells pre-treated with UDCA alone which was 9.7 ± 3.6% of controls (n = 6, *** $p < 0.001$).

4.3.3. The effects of Ca^{2+} channel inhibitors on UDCA-induced intracellular Ca^{2+} responses

It has been previously demonstrated in T_{84} cells that the initial increase in $[Ca^{2+}]i$ in response to CCh is due to the release of Ca^{2+} from internal stores, whereas the contribution of extracellular Ca^{2+} occurs later and, at least partially, involves verapamil-sensitive channels (Reinlib, 1989). Therefore, we wished to assess role of L-type Ca^{2+} channels in mediating responses to UDCA. T_{84} cells were pre-treated with verapamil, a selective inhibitor of L-type Ca^{2+} channels (100 µM; 30 min, optimised through series of experiments, not shown) and cells were subsequently exposed to UDCA (250 µM) and CCh (100 µM). Verapamil pre-treatment led to significant attenuation of UDCA-induced increases in intracellular Ca^{2+} to 25.5% ± 0.5% of those in control cells (n = 4, $p < 0.05$, Figure 4.9, Panels A and B).

Interestingly, we found that pre-treatment with verapamil also partially reversed the effects of UDCA on subsequent CCh-induced Ca^{2+} mobilization (Panel A).

Two members of the transient receptor potential (TRP) super-family, TRP vanilloid (TRPV5 and TRPV6), are specialised epithelial Ca^{2+} channels responsible for Ca^{2+} entry in the intestine and kidney, respectively (Nijenhuis et al., 2005). Ruthenium red (RR) is a non-selective TRP channel inhibitor and also a modulator of Ca^{2+}-ATPase pumps. To determine the involvement of these proteins in mediating responses to UDCA, T_{84} cells were pre-treated with RR (100 μM; 30 min, optimized and previously published, Nijenhuis, 2005) and subsequently exposed to UDCA (250 μM) and CCh (100 μM) and changes in intracellular Ca^{2+} noted. It was found that, in contrast to inhibitors of SOCE or L-type channels, RR did not significantly alter UDCA-induced Ca^{2+} mobilization (Figure 4.10, n = 4, Panels A and B).

In summary, our findings from these studies suggest that UDCA induces large and transient increases in intracellular Ca^{2+} through activation of store-operated and voltage-gated channels in colonic epithelial cells. Furthermore, this influx of Ca^{2+} appears to, at least partially; mediate anti-secretory actions of UDCA. In our next series of experiments we set out to more fully elucidate the molecular mechanisms involved by investigating the potential roles of ERK-MAP kinase and PKC in UDCA-induced responses.

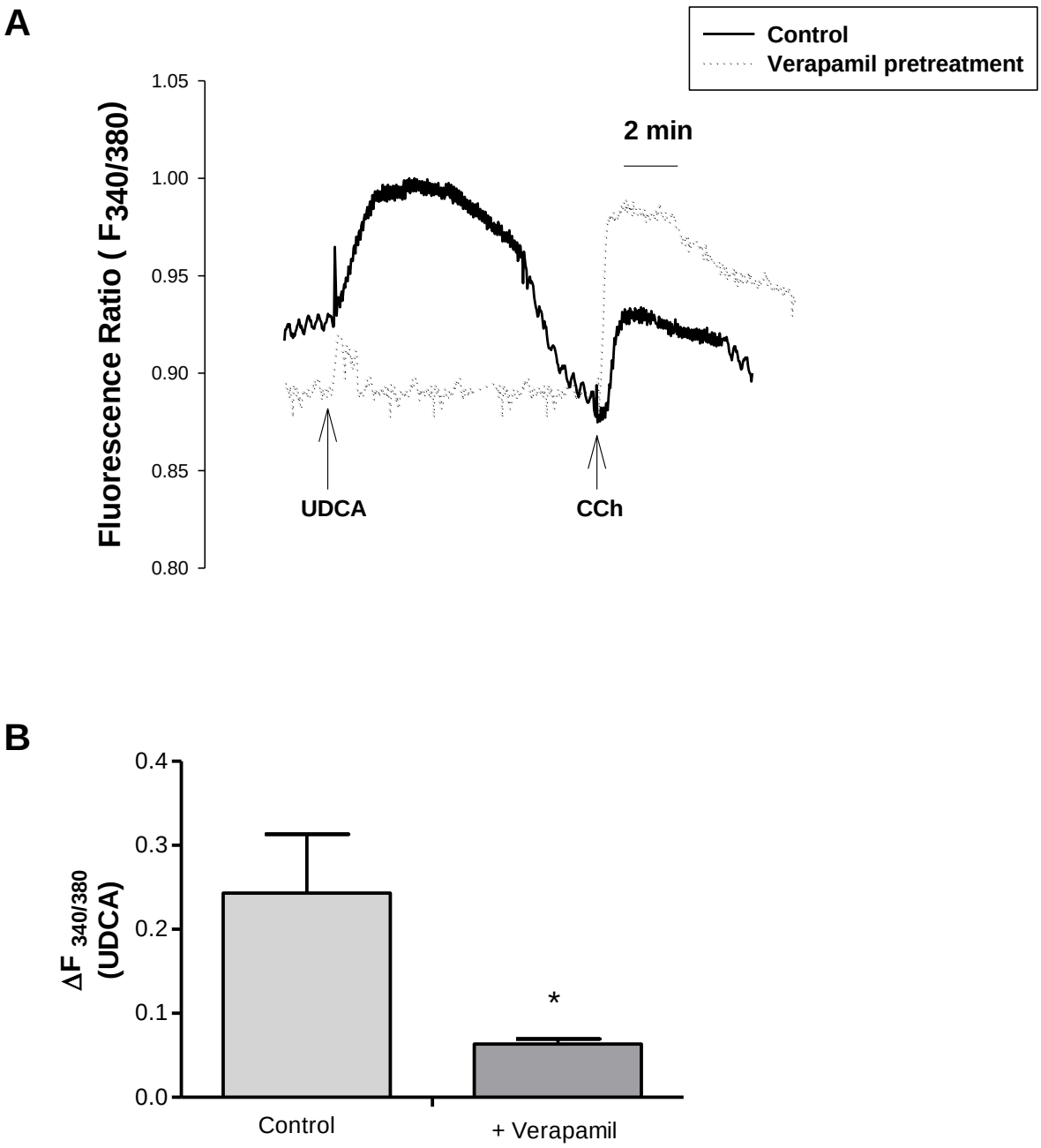
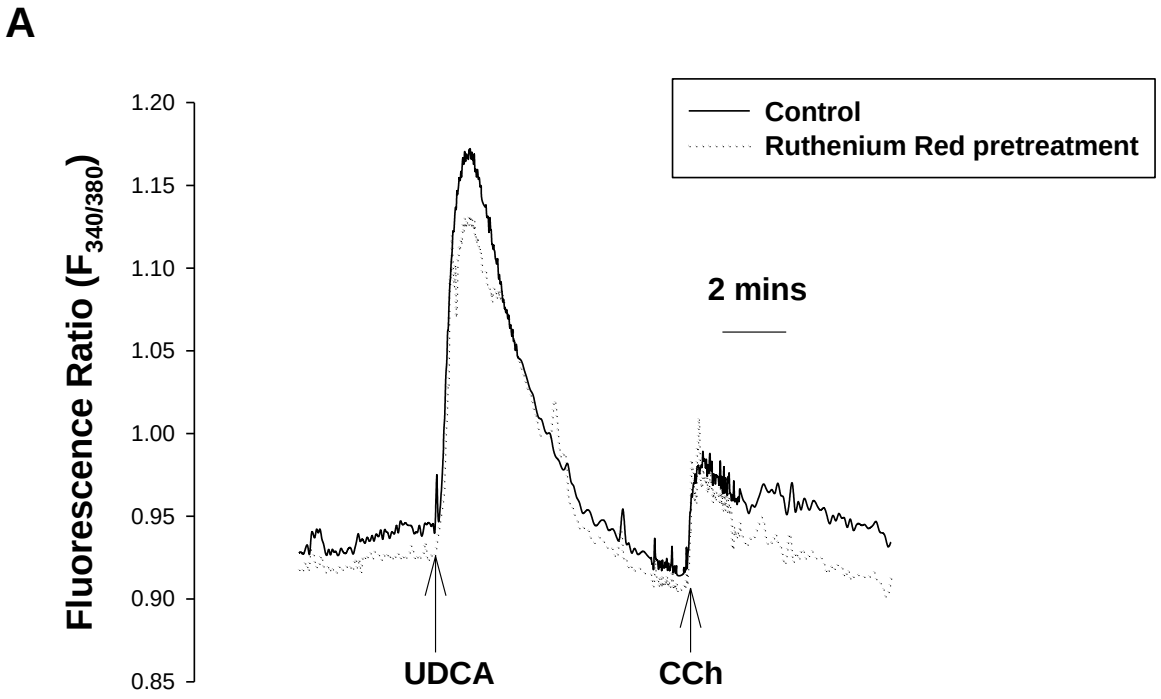


Figure 4.9: Verapamil inhibits UDCA-induced intracellular Ca^{2+} responses in T_{84} cells. T_{84} cells were grown to 70% confluency on glass coverslips and loaded with Fura/2AM for 45 min in serum-free medium. Cells were perfused with physiological perfusion solution, mounted on the stage of a Nikon microscope and after equilibration for 15 min were stimulated and $\Delta F_{340/380}$ measured. **A)** Cells pre-treated with verapamil (100 μM; 30 min) were subsequently treated with UDCA (500 μM) and CCh (100 μM) and Δ $F_{340/380}$ measured. **B)** Verapamil pre-treatment attenuated UDCA-induced Ca^{2+} mobilization (n = 4, *p < 0.05).



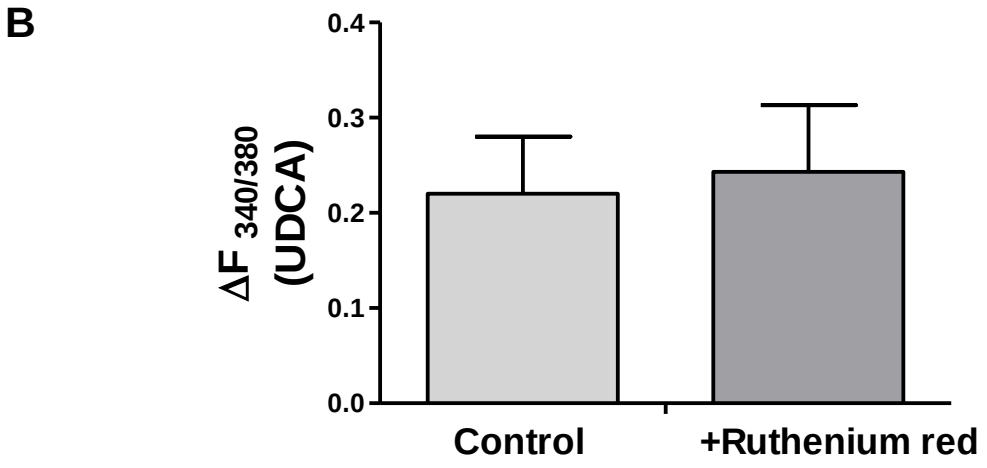
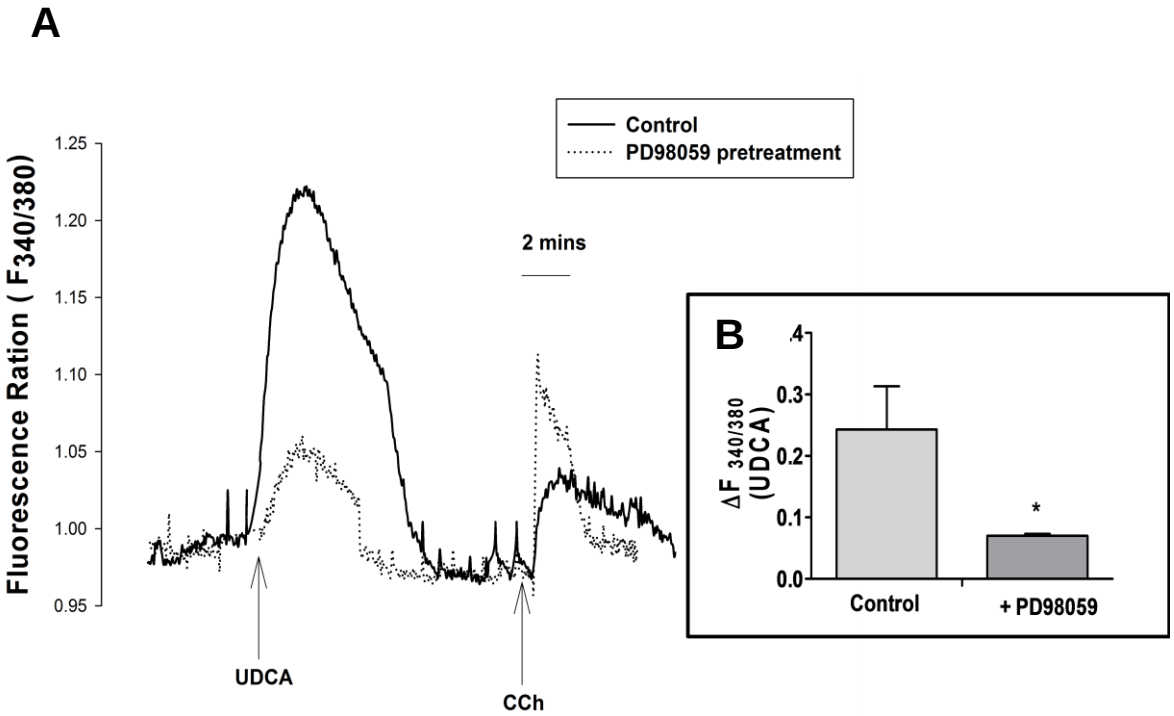


Figure 4.10: Ruthenium Red does not alter UDCA-induced intracellular Ca^{2+} responses. T_{84} cells were grown to 70% confluency on glass coverslips and loaded with Fura/2AM for 45 min in serum-free medium. Cells were perfused with physiological perfusion solution, mounted on the stage of a Nikon microscope and after equilibration for 15 mins were stimulated andΔ $F_{340/380}$ measured. **A)** Cells pre-treated with Ruthenium Red (100 μM; 30 min) were subsequently treated with UDCA (500 μM) and CCh (100 μM) and Δ $F_{340/380}$ measured. **B)** Pre-treatment with Ruthenium Red did not significantly alter UDCA-induced Ca^{2+} mobilization (n = 4).

4.3.4. The effects of ERK MAPK and PKC inhibitors on the antisecretory effects of UDCA in T_{84} cells

Previous work has shown that G_qPCR-induced transactivation of the EGFr with subsequent activation of ERK MAP kinase constitutes a signalling pathway limiting secretory responses to CCh (Keely, 1998). Furthermore, Vajanaphanich et al., have shown that inhibition of Ca^{2+} influx, in contrast to release of Ca^{2+} from intracellular stores may underlie an anti-secretory mechanism in colonic epithelia (Vajanaphanich , 1995). Given this knowledge, and that our data suggests that anti-secretory effects of UDCA require Ca^{2+} influx, we decided to examine the potential involvement of ERK -MAP kinase in UDCA-induced elevations in intracellular Ca^{2+} inhibition of ERK MAP kinase with the inhibitor PD98059 (10 μM; 30 min, conditions optimised for all kinase inhibitors used in T_{84} cells, Mroz, 2012), reduced UDCA-induced elevations in intracellular Ca^{2+} to 28.8 ± 0.4% of those in control cells (n = 3, ** $p < 0.01$, Figure 4.11, Panels A and B). Furthermore, in cells pre-treated with PD98059, UDCA (250 μM) did not inhibit subsequent CCh–induced elevations in intracellular Ca^{2+}. In fact, spike responses to CCh in UDCA + PD98059-treated cells were comparable to those in control cells treated with CCh alone ($\Delta F_{340/380}$ = 0.12 ± 0.06; n = 6, Figure 4.11, Panel C). These data suggest that activation of ERK MAPK is involved in mediating the effects of UDCA on influx of extracellular Ca^{2+}.

Having demonstrated a role for ERK-MAPK in mediating the effects of UDCA on Ca^{2+} influx, we assessed its involvement in mediating anti-secretory actions of the BA. T_{84} cell monolayers pre-treated with PD98059 (10 μM; 30 min) were placed in Ussing chambers and I_{sc} responses to secretagogues were measured in cells exposed to UDCA (500 μM; 15 min). Responses to CCh in cells treated with UDCA were 15.0 ± 3.2% of those in controls ($p < 0.001$, n = 6) compared to 60.0 ± 22.5 % in cells treated with UDCA + PD98059 (Figure 4.12), implying partial reversal of effects with ERK-MAPK inhibition.



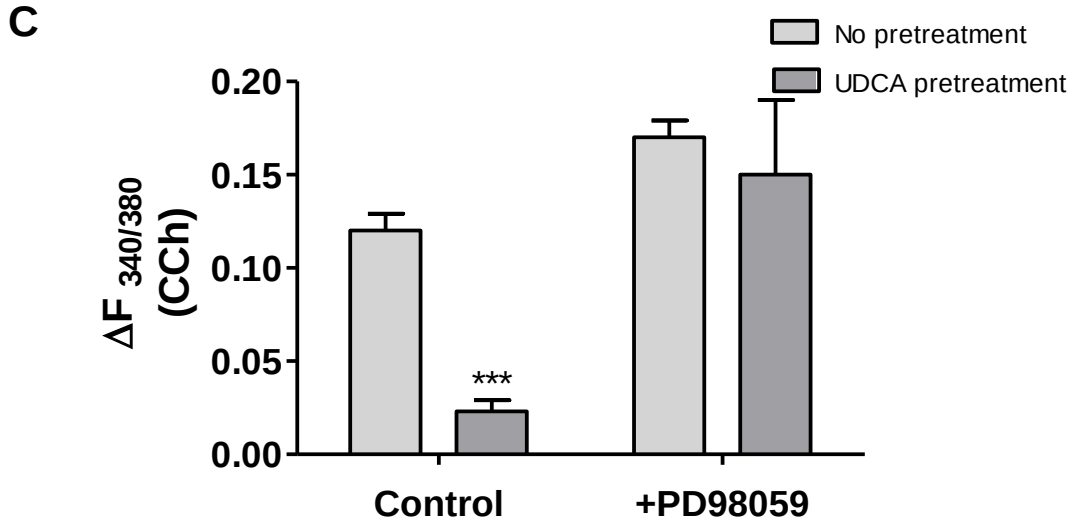


Figure 4.11: ERK-MAPK inhibition with PD98059 reduces UDCA–induced elevations in intracellular Ca^{2+} and restores subsequent CCh-induced Ca^{2+} mobilization. T_{84} cells were grown to 70% confluency on glass coverslips and loaded with Fura/2AM for 45 min in serum-free medium. Cells were perfused with physiological perfusion solution, mounted on the stage of a Nikon microscope and after equilibration for 15 min were stimulated andΔ $F_{340/380}$ measured. **A)** Cells pre-treated with PD98059 (10 μM; 30 min) were exposed to UDCA (250 μM; 15 min) and CCh (100 μM) and $\Delta F_{340/380}$ measured. Pre-treatment with PD98059 reduced UDCA-induced intracellular Ca^{2+} elevation (n = 3, *$p < 0.05$, See Inset).**B)** PD98059 pre-treatment abolished the inhibitory effect of UDCA on subsequent CCh responses (n= 6, ***$p < 0.001$).

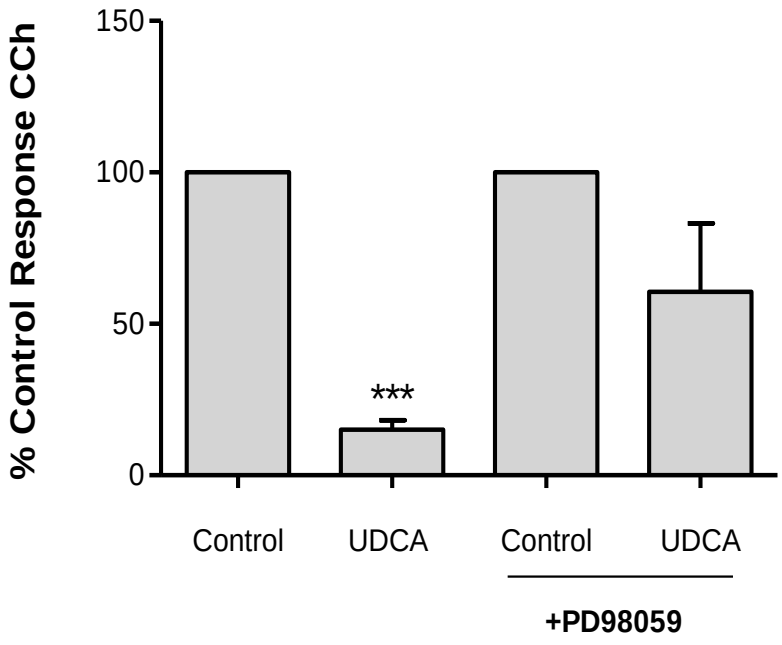


Figure 4.12: ERK-MAP kinase inhibition partially reverses the anti-secretory effects of UDCA on CCh responses. T_{84} cells seeded onto Millicell filters were cultured for 10–15 days prior to use until a trans-epithelial resistance of >1000 ohms.cm^{-2} was achieved. T_{84} monolayers placed in Ussing chambers were pre-treated with PD98059 (10 μM; 30 min) followed by UDCA (500 μM, 15 min) and I_{sc} responses to CCh were measured. CCh responses in cells treated with both UDCA and PD98059 were partially ameliorated, compared to cells treated with UDCA (n = 6, *** $p < 0.001$).

Previous studies have shown that PKC activation can occur upstream or downstream of ERK MAPK and that it can also exert negative effects on colonic epithelial secretory responses (Fiorotto, 2007). Thus, in order to investigate a potential role for PKC in mediating the anti-secretory actions of UDCA, a general PKC inhibitor, bisindolylmaleimide I (Bis I), was used. However, we found that PKC inhibition did not alter the anti-secretory effects of UDCA, with responses to CCh in cells pre-treated with both Bis I (5 μM; 30 min) and UDCA (500 μM; 15 min) being 13.2 ± 4.1 % of controls, compared to responses that were 10.7 ±.4.2 % of controls in cells treated with UDCA alone (n = 6, Figure 4.13).

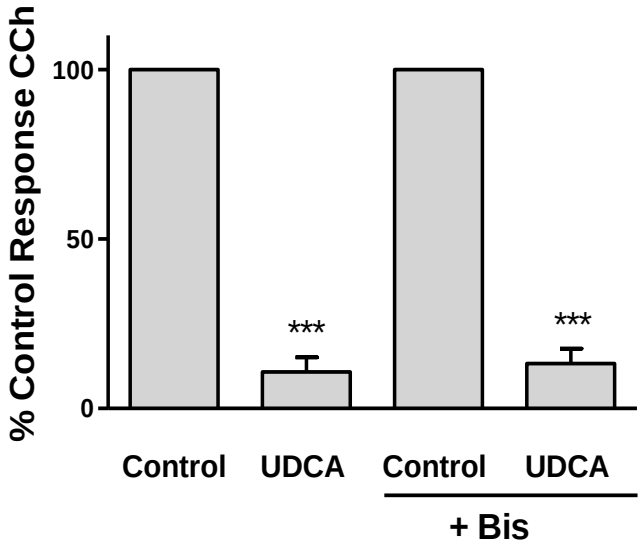


Figure 4.13: PKC inhibition does not alter UDCA inhibition of CCh- induced Cl^- secretion. T_{84} cells seeded onto Millicell filters were cultured for 10–15 days prior to use until a trans-epithelial resistance of >1000 ohms.cm^{-2} was achieved. T_{84} monolayers placed in Ussing chambers were then pre-treated with bisindolylmaleimide I (Bis; 5 μM; 30 min) followed by UDCA (500 μM; 15 min). UDCA reduced I_{sc} responses to CCh (100 μM) to a similar extent both in cells pre-treated or not pre-treated with Bis (n = 6, *** $p < 0.001$).

4.3.5. The effects of UDCA on cAMP-induced secretory responses

In Chapter III, it was noted that UDCA not only exerts anti-secretory effects on Ca^{2+}-dependent responses, but that it also downregulates cAMP-dependent responses in colonic epithelial cells. Having investigated the role of Ca^{2+} influx in mediating anti-secretory actions of UDCA on Ca^{2+}-dependent secretagogues, we next went on to elucidate potential mechanisms involved in its effects on cAMP-induced secretion. Given that previous work has suggested crosstalk between Ca^{2+}- and cAMP-dependent signalling pathways, we investigated the effects of inhibiting SOCE and ERK MAPK on the inhibitory effects of UDCA on cAMP-induced secretory responses in T_{84} cells. First, we found that the SOCE inhibitor, SKF96365 (100 μM; 30 min), did not significantly alter the inhibitory effect of UDCA (500 μM; 30 min) on FSK-induced responses. In untreated cells, UDCA reduced FSK-induced response to 23 ± 5% of those in control cells , while in SKF-pre-treated cells, UDCA attenuated FSK responses to 54 ± 9% of controls ($p < 0.05$, n = 5; Figure 4.14, Panel A). We also assessed the effects of ERK-MAPK inhibition on FSK-induced responses. Interestingly, it was noted that PD98059 pre-treatment (10 μM; 30 min), partially

reversed attenuation of FSK-induced responses by UDCA with responses being restored to 74 ± 16 % of controls (n = 5, Figure 4.14, Panel B).

Next, experiments were carried out to determine if UDCA has the capacity to alter basal or agonist-stimulated levels of cytosolic cAMP. T_{84} cell monolayers were grown on permeable supports and after 24 h in serum-free medium, cells were treated with UDCA (500 μM, bilateral) for 30 min at 37 ^{0}C in a 5% CO_2 atmosphere. Cells were then exposed to apical FSK (10 μM) for 10 min, after which they were lysed on ice using 0.1M HCl. Lysates were then centrifuged and cAMP levels in the supernatants were measured using a commercially available assay. In these experiments we found that UDCA treatment did not significantly alter basal or FSK-stimulated levels of cAMP (Figure 4.15, n = 6).

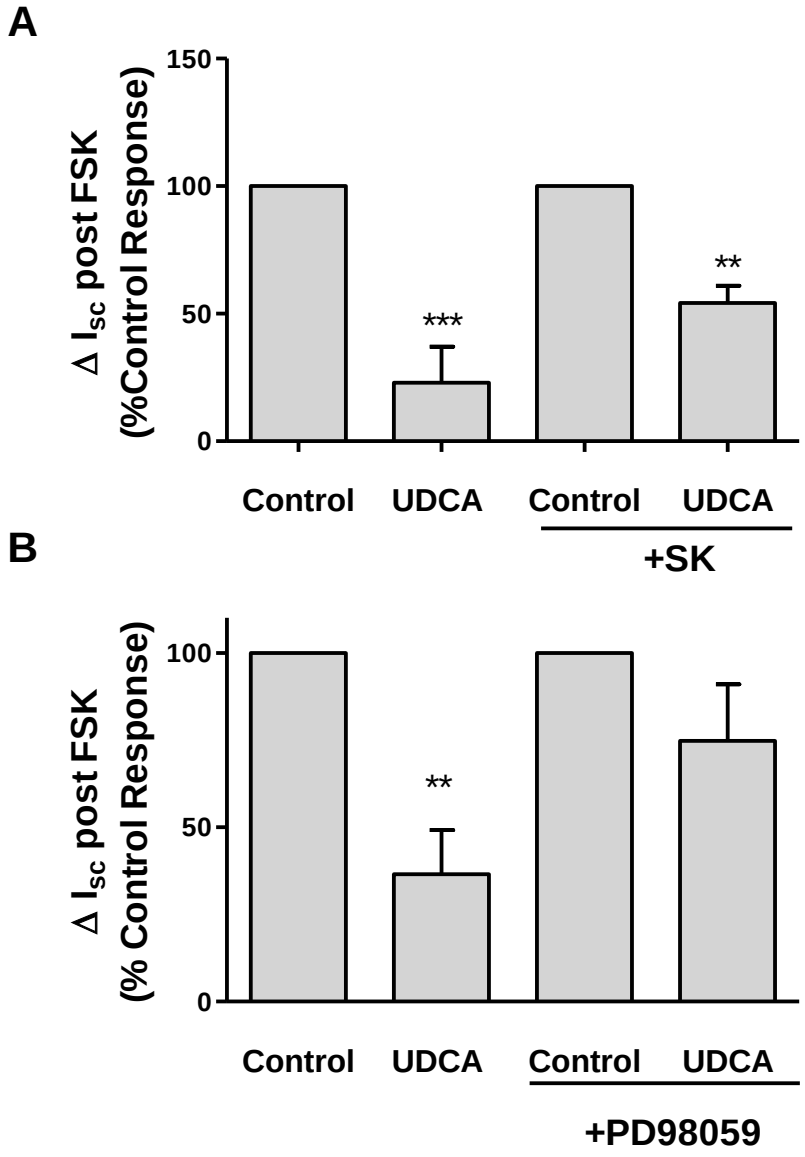


Figure 4.14: Effects of SKF96365 and PD98059 on UDCA inhibition of forskolin-induced cAMP-mediated secretory responses in T_{84} cells. T_{84} cell monolayers seeded onto Millicell filters were cultured for 10–15 days prior to use until a trans-epithelial resistance of >1000 ohms.cm^{-2} was achieved. Monolayers were then placed in Ussing chambers and pre-treated with **A)** SKF96365 (SK; 100 μM; 30 min) or **B)** PD98059 (10 μM; 30 min) and then treated with UDCA (500 μM; 15 min). Secretory responses to FSK (100 μM) were measured as ΔI_{sc} and are presented as % control responses (n = 5, ** $p < 0.01$, *** $p < 0.001$).

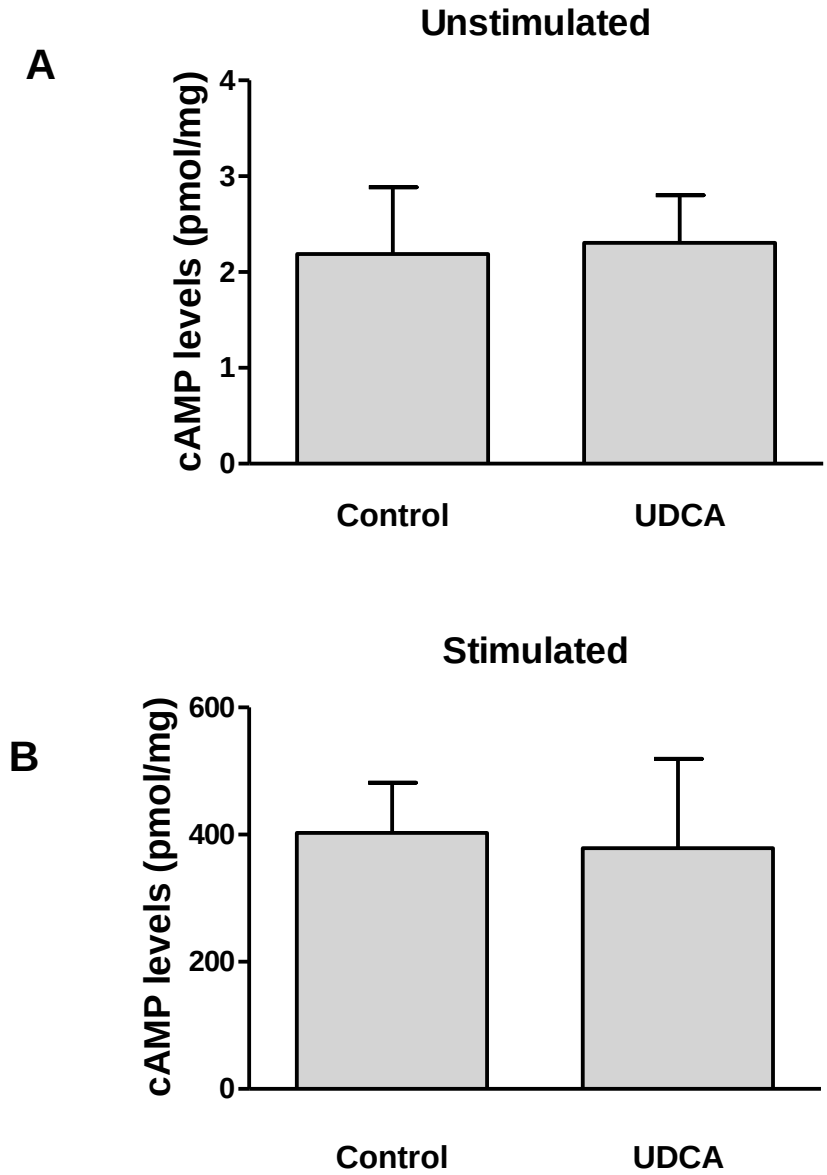


Figure 4.15: UDCA does not alter basal or FSK-induced elevations in intracellular cAMP. T_{84} cells seeded onto Millicell filters were cultured for 10–15 days prior to use until a transepithelial resistance of >1000 ohms.cm^{-2} was achieved. Monolayers were then acutely treated with UDCA (500 μM; 30 min), stimulated with FSK (10 μM; 10 min), and then lysed. cAMP levels in the lysates were measured using a commercially-available assay kit and results were expressed as pmol/ mg of protein. UDCA did not significantly alter **(A)** basal or **(B)** FSK-stimulated cAMP levels (n = 6).

Since our data suggested that UDCA inhibits cAMP-induced secretory responses without altering cAMP levels, experiments were next carried out to determine if a more downstream target is involved. In these experiments, T_{84} cell monolayers were pre-treated with the PKA inhibitor, H-89 (5 μM, 30 min), and subsequent I_{sc} responses to FSK (10 μM) were recorded. We found that in cells pre-treated with UDCA (500 μM), PKA inhibition reversed the anti-secretory actions of UDCA (n = 3, Figure 4.16).

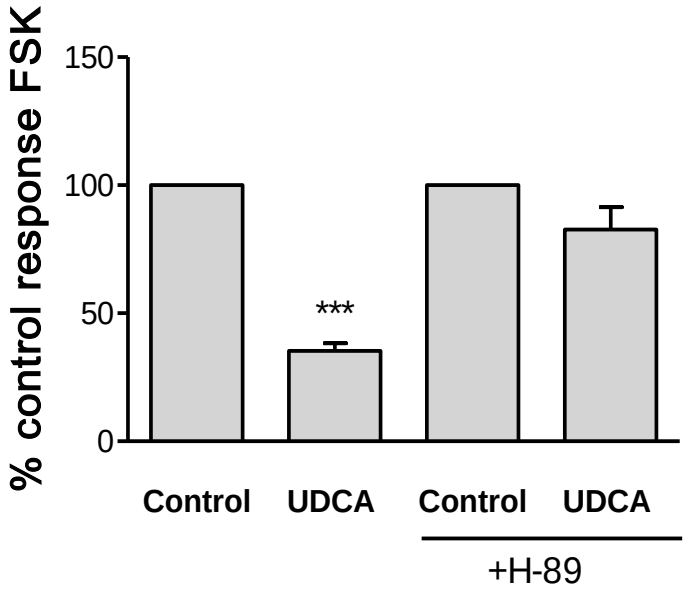


Figure 4.16: PKA inhibition reverses UDCA effects on forskolin- induced Cl^- secretion. Cells pre-treated with H-89 were then treated with UDCA (500 μM; 15 min) and subsequent FSK responses were recorded. In control cells, UDCA reduced FSK response to 35.05 ± 3.02% of controls, while in H-89 pre-treated cells, there was no significant difference between FSK responses between control and UDCA treated cells (n = 3, *** $p < 0.001$).

4.4 Discussion

In Chapter 3, we found that UDCA inhibits both Ca^{2+}- and cAMP-dependent Cl^- secretion across colonic epithelial cells through inhibition of basolateral Ca^{2+}-dependent K^+ channels and Na^+/ K^+ ATPase activity. In the studies described in this Chapter, we set out to determine signalling mechanisms involved by investigating the effects of UDCA on levels of 2nd messengers and protein kinases known to be involved in regulating colonic Cl^- secretion. Intriguingly, our experiments revealed that UDCA rapidly induces elevations in intracellular Ca^{2+} in T_{84} cells. Furthermore, even though increases in intracellular Ca^{2+} are normally associated with pro-secretory actions in epithelial cells (Kachintorn, 1993), in the case of UDCA, they appear to mediate the anti-secretory effects of the BA.

Removal of extracellular Ca^{2+} abolished UDCA-induced elevations in intracellular Ca^{2+}. Thus, the rise in intracellular Ca^{2+} with UDCA is likely to be mediated by influx of Ca^{2+} from the extracellular space, rather than release from intracellular stores. BAs have been shown in multiple studies to stimulate increases in intracellular Ca^{2+}. TDCA, at high pro-secretory concentrations causes release of intracellular Ca^{2+} and stimulates Cl^- secretion in T_{84} cells (Devor, 1993). This, in direct contrast to the effects we have found with UDCA, appears to occur through activation of K^+ and Cl^- conductances via an IP_3-mediated release of Ca^{2+} from intracellular stores. Increases in free cytosolic Ca^{2+} derived from extracellular sources, with subsequent increases in Cl^- secretion have also been previously described in response to high concentrations of other BAs in T_{84} cells (Dharmsathaphorn, 1989b). Interestingly, we found that, although UDCA induces similar elevations in intracellular Ca^{2+} it results in an anti-secretory effect. These data suggest that different pools of Ca^{2+} may exert opposing effects on colonic Cl^- secretion.

This idea is supported by the previous work by Vajanaphanich et al., demonstrating that G_qPCR agonists stimulate biphasic increases in intracellular Ca^{2+} with the initial spike in intracellular Ca^{2+} due to release from intracellular stores and the later influx of extracellular Ca^{2+} resulting in an anti-secretory effect (Vajanaphanich, 1995).

Furthermore, previous data presented from this laboratory has demonstrated that ERK-mediated Ca^{2+} influx across the apical membrane of T_{84} cells constitutes an anti-secretory signalling mechanism that limits Ca^{2+}-dependent Cl^- secretory responses in colonic epithelial cells (Hartford, 2007). *In vivo*, such a pathway would serve to limit the extent of fluid secretion occurring when mucosal levels of Ca^{2+}-dependent neuro-immune agonists are elevated. Thus, the data from the current studies suggest that UDCA exerts anti-secretory actions because it only stimulates Ca^{2+} influx, without inducing a pro-secretory spike in Ca^{2+} release from intracellular stores.

One of the crucial findings of this work is the apparent modulation of store- operated Ca^{2+} entry by UDCA resulting in inhibition of Cl^- secretion. Having shown that elevations in intracellular Ca^{2+} in response to UDCA are due to Ca^{2+} influx, we then sought to determine how Ca^{2+} influx was occurring. Previous studies investigating inhibition of Ca^{2+}-dependent Cl^- secretion across canine colonic epithelium by prostanoids has suggested a locus for the inhibitory effects at the level of store operated Ca^{2+} entry (Larsen, 2002). Interestingly, we found that in the presence of a selective inhibitor of SOCCs, SKF96365, UDCA-induced elevations in intracellular Ca^{2+} were significantly reduced. Furthermore, SKF96365 was found to reverse the anti-secretory effects of UDCA on CCh-induced responses in Ussing chambers. Thus, it appears that Ca^{2+} influx through SOCCs, which are known to be expressed in colonic epithelium (Koslowski, 2008, Sun, 2012), mediate the anti-secretory actions of UDCA. Although, the mechanism by which this occurs remains speculative, one candidate for store-operated Ca^{2+} entry is MS4A12, a novel colon-specific store-operated Ca^{2+} channel. Koslowski et al., identified MS4A12, a sequence homologue of CD20, to be a colonic epithelial cell lineage gene confined to the apical membrane of colonocytes with strict transcriptional repression in all other normal tissue types. Ca^{2+} flux analyses in that study identified MS4A12 as a novel component of store-operated Ca^{2+} entry in intestinal cells (Koslowski, 2008). In future work, it would be interesting to assess the effects of UDCA on the activity of these channels.

In addition to SOCCs, we also investigated the potential for voltage-gated Ca^{2+} channels, Ca^{2+} ATPase activity, and TRP channels in mediating responses to

UDCA. L-type Ca^{2+} channels are expressed in colonic epithelial cells where they have a predominantly membrane distribution and they have been shown to have increased expression in colon cancers (Wang, 2000). Similarly, Ca^{2+} ATPase pumps are expressed in normal colonic mucosa as well as in reduced quantities in colon cancer specimens (Ruschoff , 2012), while TRPA1 has been shown to be expressed in isolated normal rat colonic mucosa (Kaji , 2011) while TRPV6 is expressed in the colon and is likely to have a protective effect in colon cancer (Lehen'kyi , 2012). Our studies suggest that UDCA effects are selective for SOCCs and voltage-gated entry, but that the BA does not affect TRP channels or Ca^{2+} ATPase activity. This is based on the observations that ruthenium red, which blocks both TRP channels and Ca^{2+}ATPase pumps, did not alter UDCA-induced elevations in intracellular Ca^{2+}, while verapamil, an L-type Ca^{2+} channel blocker, similar to SKF96365, attenuated UDCA-induced Ca^{2+} responses.

Interestingly, in addition to Ca^{2+} influx, our studies also implicate ERK MAP kinase in mediating the anti-secretory effects of UDCA. Of relevance to these findings, previous studies have also implicated MAPKs in anti-secretory signalling mechanisms stimulated by Ca^{2+}-dependent agonists in colonic epithelial cells. For example, the muscarinic receptor agonist, CCh, stimulates ERK MAPK activation in T_{84} cells via a mechanism involving transactivation of EGFR, thus limiting agonist-induced secretory responses (Keely, 1998). Subsequent studies also demonstrated that G_qPCR-induced p38 MAPK activation constituted a similar, but distinct, anti-secretory signalling pathway to that of ERK, while JNK MAPK has also been found to be involved in limiting Cl^- secretory responses by inhibition of K^+ channel currents (Donnellan, 2010). The effects of G_qPCR agonists on activation of MAPKs were shown to be mimicked by thapsigargin, an agent that specifically elevates intracellular Ca^{2+}, and were abolished by the Ca^{2+} chelator BAPTA-AM, implying a role for intracellular Ca^{2+} in mediating these responses (Keely, 2003).

In examining a possible role for ERK MAPK in mediating UDCA-induced Ca^{2+} influx in colonic epithelial cells, we found that PD98059, a highly specific ERK MAP kinase inhibitor (Alessi, 1995), reduced UDCA-induced elevations in intracellular Ca^{2+} and partially reversed the anti-secretory effect of the BA. Thus, our data support the idea that activation of ERK-MAPK is likely to be involved in mediating effects of UDCA on

influx of extracellular Ca^{2+} through SOCCs. In support of this idea, there have been previous reports in the literature that UDCA can induce activation of ERK MAPK in intestinal epithelial cells and hepatocytes (Schoemaker, 2004; Marzioni,2006; Krishna-Subramanian, 2012). Furthermore, ERK activation has also been shown to play an important role in agonist-induced elevations in intracellular Ca^{2+} through activation of SOCCs in T_{84} cells (Hartford, 2007) and also in other cell types (Selway, 2012).

Previous work suggests that PKC plays an important role in mediating the effects of UDCA in some systems (McMillan, 2003, Marzioni, 2006). Furthermore, various isoforms of PKC play important roles in regulating colonic epithelial secretion (van den Berghe, 1992, Chow, 2000, Harvey, 2002), and PKC has also been shown to be activated both upstream and downstream of ERK-MAPK in various systems (Palma-Nicolas, 2008, Moon, 2010). Interestingly, PKC has also been shown to mediate both proliferative and apoptotic responses to UDCA in colonic epithelial cells (Byrne, 2010, Looby, 2005). Thus, we also carried out preliminary experiments to investigate a potential role for PKC in mediating the anti-secretory effects of UDCA. However, using Bis I as a general inhibitor of PKC, we found it to have no significant effect on the anti-secretory activity of UDCA, even though the inhibitor has been previously shown to be effective in T_{84} cells at the concentration employed (McKay, 2007). Thus, our current studies do not support a role for PKC in mediating UDCA actions on colonic secretion. However, it must be acknowledged that these studies are quite preliminary and further work assessing the effects of UDCA on activation of different PKC isoforms in colonic epithelial cells should be conducted.

While elevation in intracellular Ca^{2+} is one of the major pathways for promoting intestinal epithelial Cl^- secretion, the other predominant pathway is that mediated by cAMP. Thus, we also investigated UDCA actions on basal and FSK-stimulated levels of cAMP in colonic epithelial cells. However, we found UDCA to be without effect, which is in keeping with previous findings where UDCA was shown to be without effect on mucosal cAMP levels in a rabbit intestinal loop model (Rahban, 1980). Similarly, Bouscarel et al found no alterations in basal cAMP levels in hepatocytes in response to UDCA (Bouscarel, 1995). Previous studies have shown that in intestinal epithelial cells, as in most cell types, cAMP-dependent signalling occurs through

activation of PKA (Rudolph, 2007). Future work could assess the role of PKA in mediating the anti-secretory effects of UDCA on cAMP-dependent responses.

In summary, in the current Chapter, we sought to identify signalling mechanisms underlying the anti-secretory actions of UDCA on colonic epithelial cells. We identified that UDCA stimulates an acute and transient rise in intracellular Ca^{2+} which appears to be due to an influx of extracellular Ca^{2+} through membrane SOCCs and L-type Ca^{2+} channels. In turn, influx of Ca^{2+} appears to attenuate subsequent secretagogue-induced release of Ca^{2+} from intracellular stores. This mechanism appears to involve activation of ERK MAP kinase but seems to be independent of PKC. This pathway appears to be a key mechanism by which UDCA inhibits Ca^{2+}-dependent secretion and might explain why agonist-induced KCNN4 and Na^{+}/K^{+} ATPase activity become attenuated after UDCA treatment (cf. Chapter III, Figures 3.21-3.27). In contrast, although UDCA also attenuates cAMP-induced Cl^{-} secretory responses, it does so without altering intracellular levels of this 2nd messenger.

In conclusion, UDCA exerts complex actions on both Ca^{2+}- and cAMP-dependent Cl^{-} secretory pathways in colonic epithelial cells, effects which *in vivo* would be expected to be associated with reduced luminal fluid and electrolyte secretion in response to neuro-immune agonists (Figure 4.17). Further elucidation of the mechanisms involved may lead to the development of new ways in which to target epithelial transport processes for treatment of intestinal diseases.

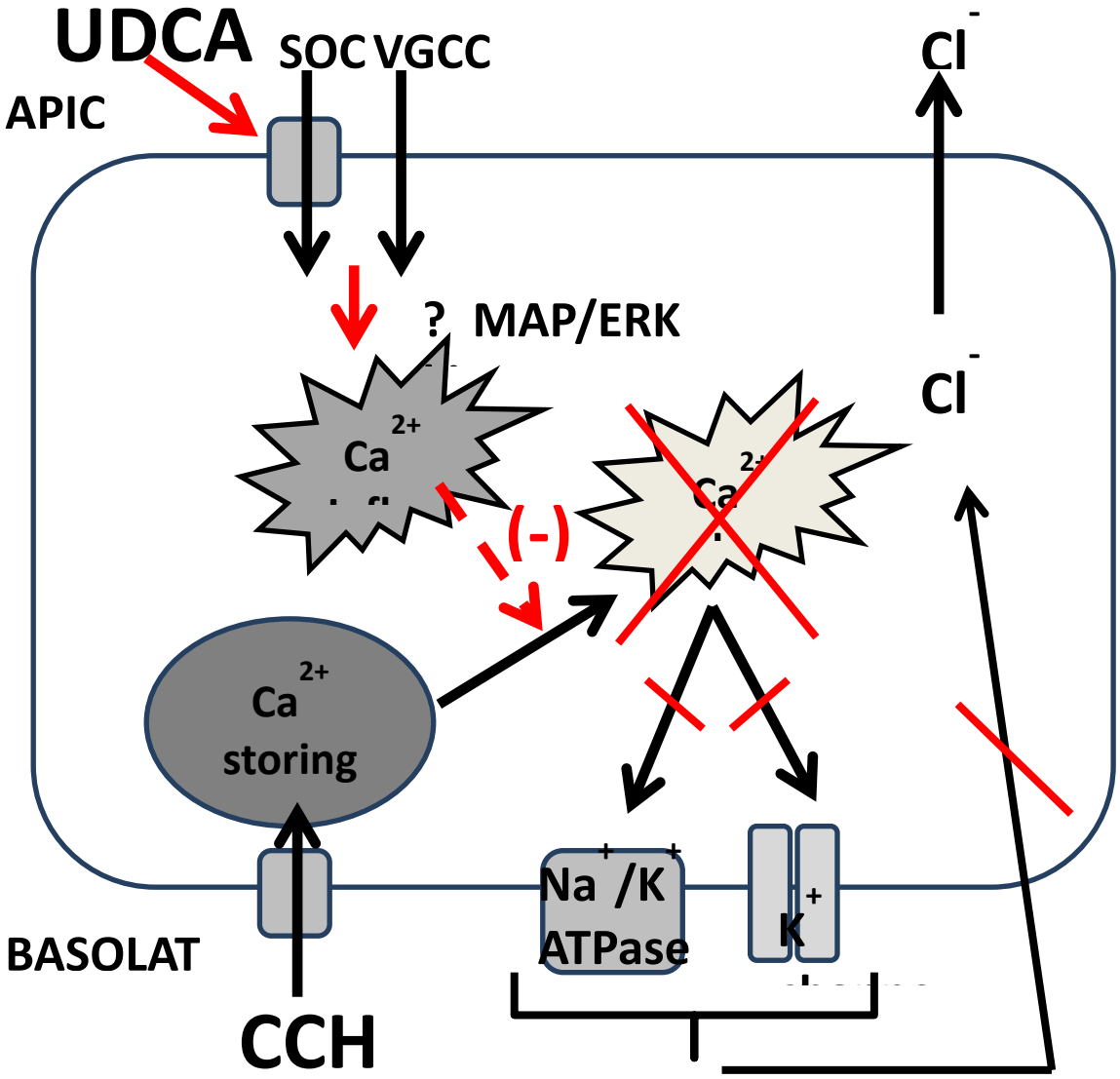


Figure 4.17: Schematic representation of UDCA effects on Ca^{2+} signalling and Cl^- secretory responses in the colonic epithelium. Pro-secretory agonists such as CCh, stimulate release of Ca^{2+} from intracellular stores, which promotes Cl^- secretion (black arrows). UDCA stimulates increases in cytosolic Ca^{2+} through activation of voltage-gated Ca^{2+} channels and store-operated Ca^{2+} channels in an ERK MAPK-dependent fashion (red arrows). By a mechanism that has yet to be defined, Ca^{2+} influx attenuates subsequent Cl^- secretory responses by inhibiting the activity of basolateral K^+ channel currents and Na^+/ K^+ ATPase activity.

Chapter 5

The effects of UDCA on Cl^- secretion in rat and human colonic mucosa

5.1 Introduction

Studies described in Chapter III demonstrated a novel anti-secretory action of UDCA in isolated colonic epithelial cells. However, while cultured epithelial cells are very useful as reductionist models for studies of epithelial transport, transport function *in vivo* is typically regulated by nerves, entero-endocrine cells, and immune cells, which upon activation release a diverse group of mediators and neurotransmitters. Thus, in order to assess its effects under more physiological conditions, we carried out studies to examine the role that UDCA plays in regulating secretory function across intact colonic mucosal tissues from rats and humans *in vitro*.

5.1.1 Neuroimmune regulation of colonic secretion

As discussed in the general Introduction, It is well known that the enteric nervous system (ENS) plays an important role in regulating intestinal fluid transport (Hubel, 1985). Signals from the ENS serve to control intestinal blood flow, motility and fluid transport processes (Hubel, 1985). Among the most predominant neurotransmitters involved in regulating fluid and electrolyte secretion in the colon are acetylcholine (ACh), which acts via increases in intracellular Ca^{2+}, and vasoactive intestinal polypeptide (VIP), which elevates intracellular cAMP (Kunzelmann, 2002). However, there are a myriad of other neurotransmitters which exert varying levels of control on secretory responses in the colon (see Table 1.1, Chapter 1). Furthermore, not all neurotransmitters are pro-secretory, for example, neuropeptide Y inhibits colonic epithelial secretion, and is commonly found in interneurons, motor neurons or secreto-motor neurons. Its analogue, peptide YY is located in neuroendocrine L-cells which predominate in the colorectal mucosa (Cox, 2010), while pancreatic polypeptide is found in only a few scattered neurons. Three Y receptor types are functionally significant in human colon (Y1, 2 and 4) and their activation results in anti-secretory or pro-absorptive responses (Cox, 2007, Christofi, 2004, Mannon 1999).

Cells of the mucosal immune system are densely packed into the lamina propria of the intestine and many mediators released from these cells have important actions in regulating Cl^- secretion (Keely, 2009). These cells can be activated in response to luminal pathogens, and a wide array of other stimuli, and it is thought the pro-

secretory actions of their mediators serves to wash away luminal toxins. Such responses mainly involve mast cells and neutrophils, which release many different secretagogues including, prostaglandins, leukotrienes, adenosine and reactive oxygen species (Barrett, 2000).

5.1.2 Bile acids in the intact colon

It has been well documented in humans, that when levels of colonic BAs enter the pathophysiological range they exert pro-secretory and anti-absorptive actions (Mekjian , 1971). These effects are thought to underlie the diarrhoea that commonly occurs in many conditions of BA malabsorption, such as in terminal ileitis, right colonic Crohn's Disease, or post-resection of the right hemicolon or terminal ileum. There is also evidence to suggest that chronic diarrhoea induced by BAs can occur as a consequence of excessive BA synthesis, rather than malabsorption (Hofmann, 2009).Though the effects of BAs on mucosal permeability and bacterial uptake have been studied in human biopsies , their effects on human colonic Cl^- secretion have not been fully elucidated (Munch, 2007).

Both conjugated and unconjugated BAs have been shown to rapidly induce Cl^- secretion in a variety of experimental models *in vitro* and *in vivo* (Chadwick, 1979, Dharmsathaphorn, 1989b, Binder, 1975); (Mauricio , 2000); (Venkatasubramanian, 2001, Gelbmann , 1995). In these models, BAs can exert these pro-secretory effects either by acting directly on the epithelial cells themselves, or indirectly, through stimulation of nerves and other regulatory cells within the mucosa (Gelbmann et al., 1995).There is however a strict structure-activity relationship for luminal BAs in promoting secretion, with only dihydroxy BAs, such as DCA and CDCA, being effective (Chadwick,1979, Gordon, 1979).There have, to date, been very few studies of the effects of UDCA on secretory function in intact colonic tissues, with those that have been carried out being limited to animal models (Caspary, 1980, Hardcastle, 1987).

5.2 Aims:

The primary aims of this chapter were to:

- Examine the effects of UDCA on Cl^- secretion across intact colonic mucosal tissues from rats and humans.
- Determine the role of the ENS in mediating responses to UDCA.
- Examine the effects of long-term treatment with UDCA on colonic epithelial function.

5.3 Results

5.3.1 Tissue collection and patient selection

Resected human colonic tissue was collected from consented patients undergoing elective colorectal surgery in our institution between Jan 2009 and March 2011 (See Materials and Methods for detail on collection and selection process). The patient data were anonymised and relevant demographic information, indication for surgery and site of sample were recorded. Those with rectal cancers who had received standard of care neoadjuvant chemo/radiotherapy were excluded from this study on the grounds of the potential presence of inflammation and fibrosis after treatment. Likewise, patients undergoing emergency surgery for obstructive symptoms were excluded, given the likely presence of acute inflammation. Patient medications were examined and record made of any patients taking BA sequestrants or UDCA.

64 subjects undergoing colonic resection were included in the initial studies described in this chapter, none of which were on UDCA or BA sequestrant therapy at the time of tissue collection. The majority of patients were male (64%) and the median age was 68. The majority of patients underwent surgery for colorectal tumour excision. Below is a table summarising the patient demographics (Table 5.2). A different subgroup of patients and controls from which endoscopic biopsies were obtained are described later (See Table 5.3).

Table 5.2 Patient Demographics

Patients (n)	Male: Female ratio	Median Age (interquartile range and range)	Region of colon (%)	Indication for surgery (%)
64	1.8:1	68 (60–74) (38-85)	65% left 35% right	96.8% tumour resection 3.2% high grade dysplastic polyp resection

5.3.2 Bilateral UDCA stimulates basal ion transport across human and rat colonic mucosa.

We first set out to determine what effect, if any, UDCA (500 μM; bilateral) had on basal I_{sc} across muscle-stripped human colonic mucosal tissues mounted in Ussing chambers. We found that at this concentration, bilateral addition of UDCA caused an immediate, although transient, increase in basal I_{sc} when applied bilaterally. In UDCA-treated tissues I_{sc} increased by 118.5 ± 37.8% compared to control (i.e., to 156.8 ± 27.4 μA/ cm^2 compared to a basal I_{sc} of 78.7 ± 17.8 μA/ cm^2 in untreated controls (mean ΔI_{sc} = 77.3 ± 10.8 μA/ cm^2; n = 4; $p < 0.05$, Figure 5.1). These findings were in marked contrast to the lack of effect of UDCA on basal I_{sc} in T_{84} cells (cf. Chapter III, Figure 3.3).

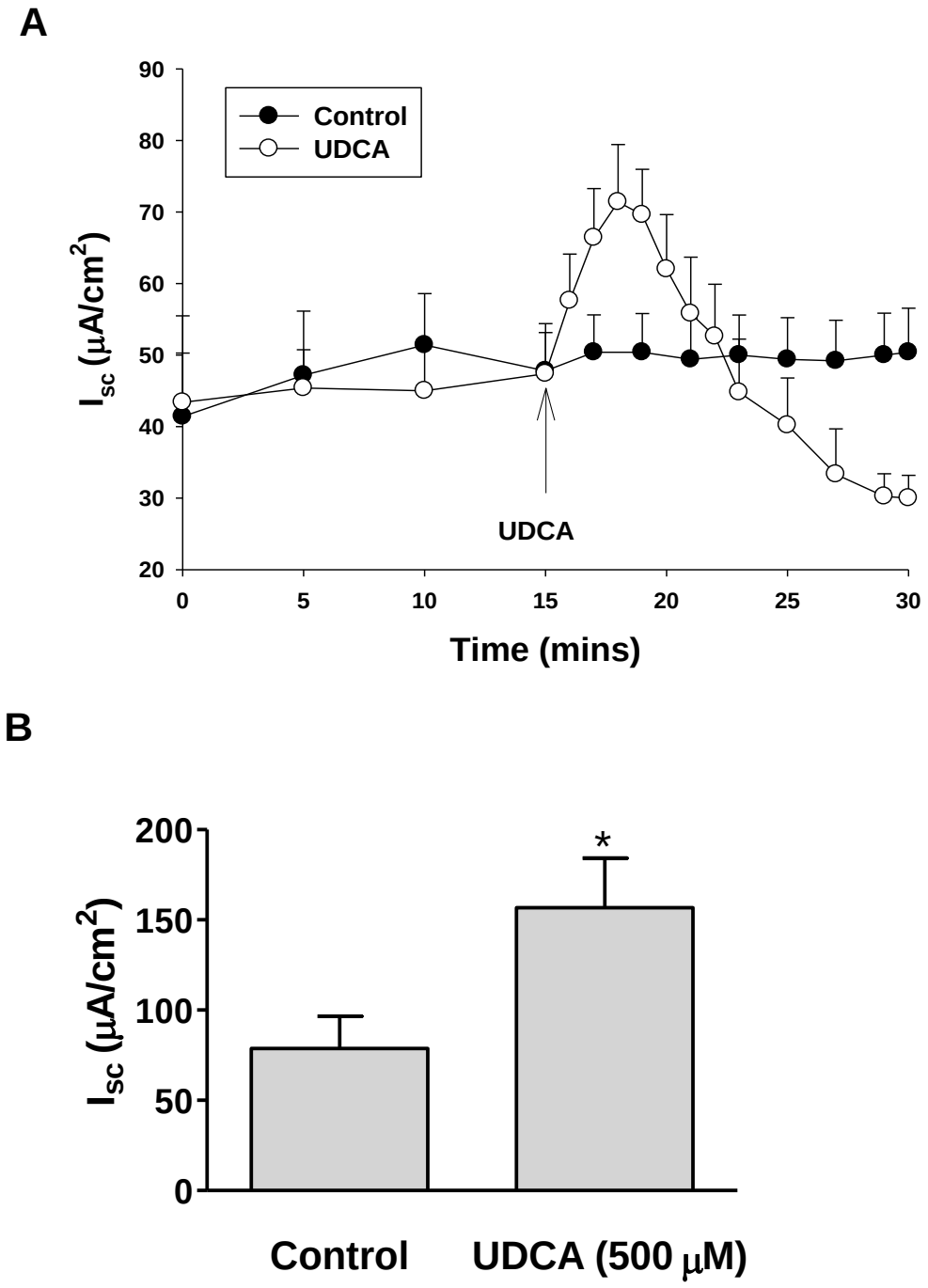


Figure 5.1: Bilateral UDCA stimulates a transient increase in I_{sc} across human colon. Muscle-stripped human colonic mucosa was mounted in Ussing chambers and allowed to equilibrate. UDCA (500 μM; bilateral addition) was then added and changes in I_{sc} were measured). Panel **A** demonstrates the time course of I_{sc} responses to UDCA (added at t = 0), while Panel **B** demonstrates the mean peak I_{sc} in UDCA treated–tissues, compared to paired controls (n = 4, *p < 0.05).

Next, the concentration-dependence of responses to UDCA was examined, with concentrations ranging from 100 μM–1 mM. These experiments revealed the pro-secretory actions of UDCA to be concentration-dependent with a maximal effect occurring at 500 μM (n = 4, Figure 5.2).

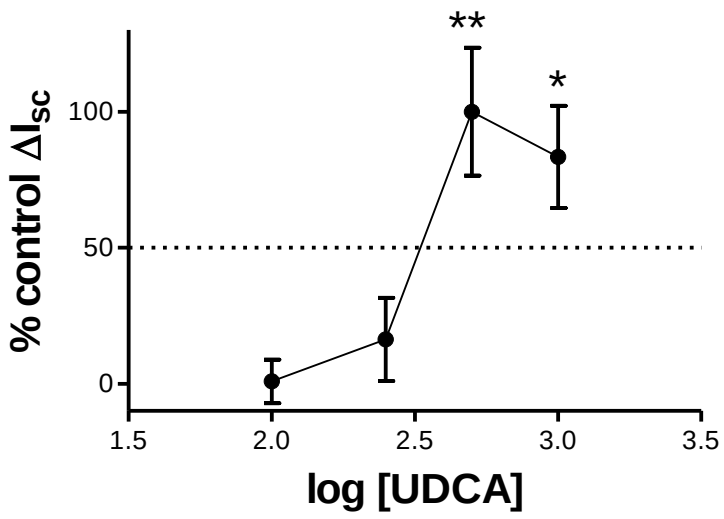


Figure 5.2: Effects of UDCA on human colonic electrolyte transport are concentration-dependent. UDCA (100 μM–1 mM, bilateral) was added to voltage-clamped sections of muscle-stripped human colonic mucosa mounted in Ussing chambers. I_{sc} was measured over time and compared to untreated controls. Maximal increases in I_{sc} were noted at concentrations of 500 μM. Mean basal I_{sc} was 40.2 ± 12.1 μA/ cm^2 before treatment, with UDCA (500 μM) inducing a maximal ΔI_{sc} response of 116 ± 7 μA/cm^2 (n = 4, $p < 0.01$). UDCA at concentrations of 100 μM, 250 μM, and 1 mM resulted in increased mean ΔI_{sc} of 1.2 ± 5 μA/cm^2, 19.2 ± 7 μA/ cm^2, and 97 ± 21 μA/ cm^2, respectively (n = 5, *$p < 0.05$, **$p < 0.01$). Graph is representative of the log concentration dose response and calculated EC_{50} was 339 μM.

In the next series of experiments, we set out to determine the sidedness of responses to UDCA in human colon. UDCA was added either apically or basolaterally and ΔI_{sc} responses were compared to untreated control samples. These studies revealed that pro-secretory actions of UDCA were only apparent upon basolateral addition. Basolateral addition of UDCA (500 μM) stimulated an I_{sc} response of 50 ± 6.3 μA/ cm^2 (n = 7, $p < 0.001$). In contrast, apical addition of UDCA (500 μM) did not significantly alter I_{sc} when compared to the spontaneous increases in basal I_{sc} observed in untreated controls (n = 7; Figure 5.3).

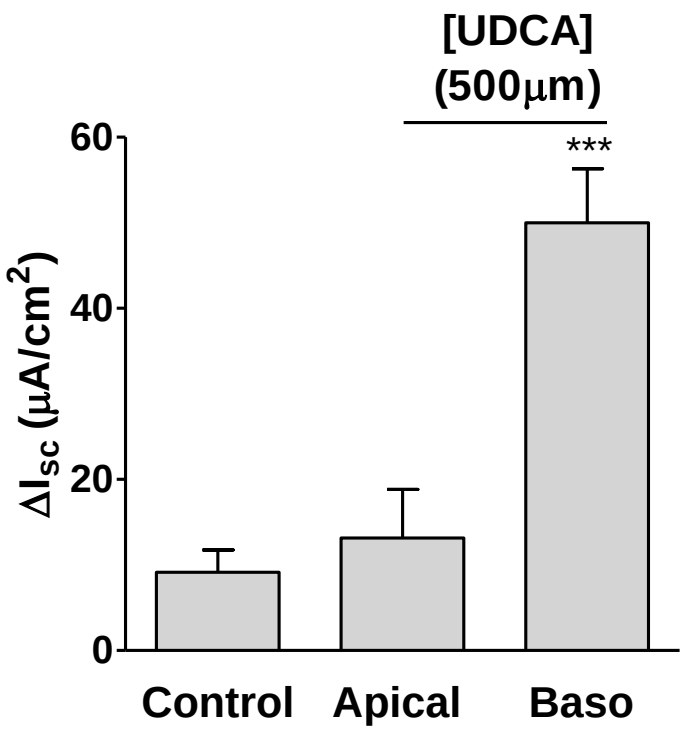


Figure 5.3: I_{sc} responses to UDCA in human colon are apparent only upon basolateral addition. UDCA (500 μM) was added to voltage-clamped sections of human colon either apically or basolaterally and ΔI_{sc} was measured and compared to untreated controls (n = 7; *** $p < 0.001$).

In parallel experiments we also studied the effects of UDCA on muscle-stripped segments of rat colon. Male Sprague-Dawley rats aged 6–12 weeks were sacrificed, their colons removed, stripped of underlying smooth muscle, and mounted in Ussing chambers (0.3 cm^2 inserts) for measurements of ΔI_{sc}. Similar to its effects in human tissue, UDCA (500 μM) stimulated transient increases in I_{sc} in rat colon. Effects of UDCA were significant only at the higher concentrations tested, with mean ΔI_{sc} responses of 29 ± 6.25 μA/ cm^2 and 20 ± 2.94 μA/ cm^2, occurring at 500 μM and 1mM UDCA, respectively (n = 4, $p < 0.05$, Figure 5.4

Figure 5.4). Also similar to findings in human tissue, increases in I_{sc} due to UDCA were only apparent upon basolateral addition of the bile acid. Basolateral addition of UDCA (500 μM) resulted in a ΔI_{sc} of 35.5 ± 11.4 μA/cm^2 (n = 3, $p < 0.05$), while apical addition did not significantly alter basal I_{sc} (Figure 5.5, n = 3).

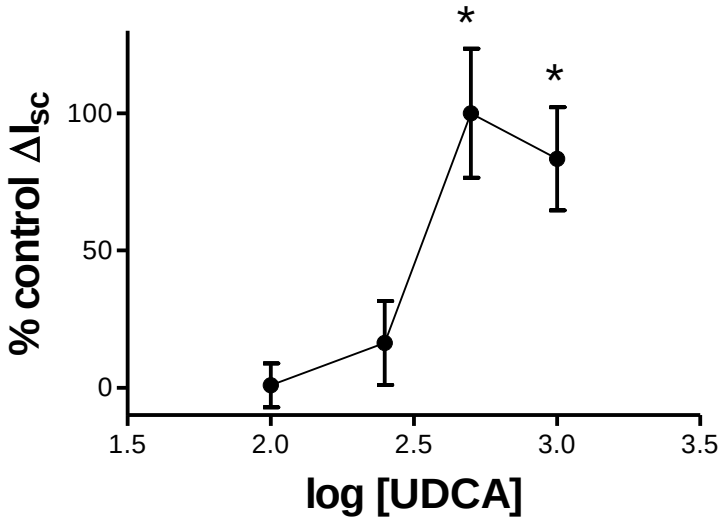


Figure 5.4: UDCA stimulates I_{sc} responses in a concentration-dependent manner in rat colon. UDCA (50 μM–1 mM; bilateral) was added to muscle-stripped sections of rat colon in Ussing chambers and changes in I_{sc} were measured. Graph represents the log concentration dose response. Calculated EC_{50} was 349 μM (n = 4; *p < 0.05 for 500 and 1000 μM additions)

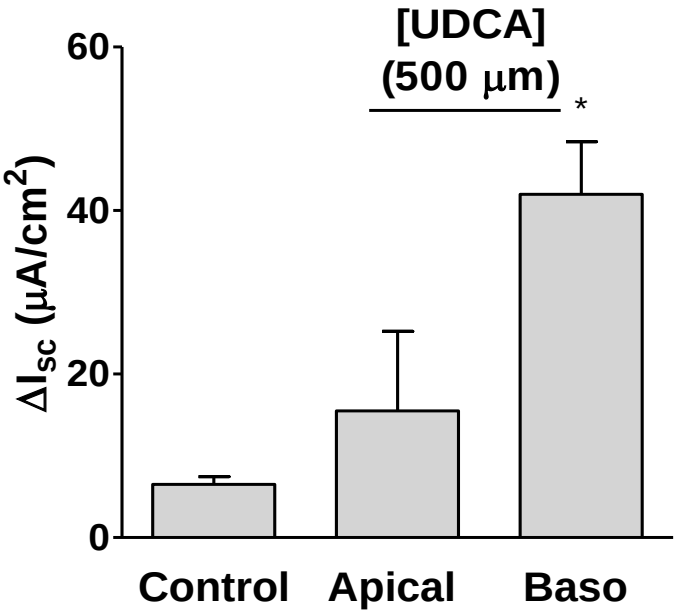


Figure 5.5: I_{sc} responses to UDCA are only apparent upon basolateral addition to rat colonic mucosa. UDCA (500 μM) was added to voltage-clamped sections of rat colon either apically or basolaterally and ΔI_{sc} was measured and compared to untreated controls (n = 3; *p < 0.05).

Thus, our findings indicate that, in contrast to its anti-secretory effects in cultured epithelial cells, UDCA stimulates I_{sc} responses in intact colonic tissue. Although in the current studies, we did not carry out a characterization of the charge carrying ion involved, such responses are most likely to be due to increased Cl^- secretion, as has been shown for UDCA in the small intestine (Hardcastle, 2001). Since responses to UDCA are only observed in the setting of intact colonic tissue, we hypothesized that its actions are likely to be indirect and mediated by activation of enteric nerves.

5.3.3 UDCA-induced I_{sc} responses in human colon are mediated by enteric nerves.

To investigate the potential involvement of the ENS in mediating responses to UDCA in human colon, a potent neurotoxin, tetrodotoxin (TTX), was used. In tissues pre-treated with TTX (0.1 μM), we found that UDCA-induced I_{sc} responses were significantly reduced, suggesting that they are neuronally-mediated. Basolateral addition of UDCA (500 μM) stimulated increase in I_{sc} in untreated tissues compared to TTX-pre-treated tissues ($n = 7$, $p < 0.05$, Figure 5.6). ΔI_{sc} in TTX-pre-treated tissues after UDCA treatment was similar to that seen in control tissues, treated with TTX alone (Figure 5.6). In order to further characterise neuronally-mediated responses to UDCA, we used atropine (1μM; 30 min), a competitive antagonist of muscarinic acetylcholine receptors. In these experiments, ΔI_{sc} responses to UDCA (500 μM) were significantly attenuated in the presence of atropine from 38.3 ± 6.8 to 15.3 ± 2.2 $\mu A/cm^2$ ($n = 3$, $p < 0.05$), suggesting that they are mediated by activation of muscarinic secreto-motor neurons (Figure 5.7).Thus, taken together, these data indicate that pro-secretory effects of UDCA in human colon are only apparent upon basolateral addition of relatively high concentrations (> 250 μM) and that these responses are indirectly mediated through activation of muscarinic nerves.

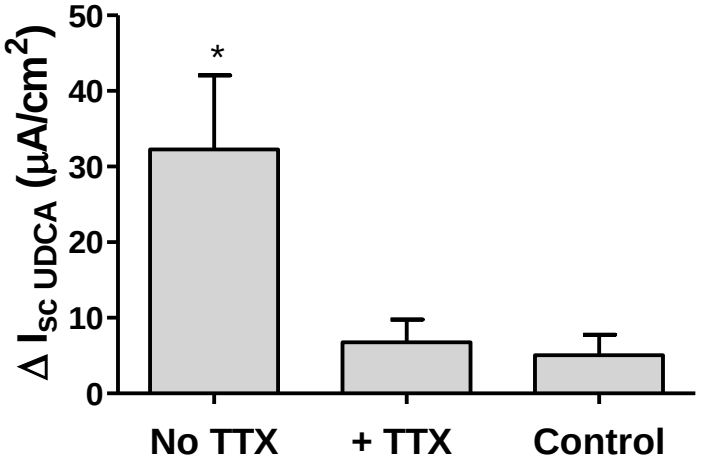


Figure 5.6: UDCA-induced I_{sc} responses in human colon are significantly reduced by TTX pre-treatment. Voltage-clamped sections of human colon were pre-treated with TTX (0.1 μM; 30 min) before addition of UDCA (basolateral; 500 μM; 15 min) and I_{sc} responses were measured and compared to control clamped human colon which was not treated with TTX. Responses to UDCA were significantly reduced by TTX pre-treatment from a rise of 32.2 ± 9.8 μA / cm^2 in response to UDCA in controls compared to only 6.8 ± 3.0 μA / cm^2 in TTX pre-treated tissues (n = 6). In TTX-pre-treated tissues, changes in I_{sc} after UDCA treatment were similar to spontaneous I_{sc} changes in the control. The control was human tissue treated with TTX alone with no addition of UDCA (n = 6; * $p < 0.05$).

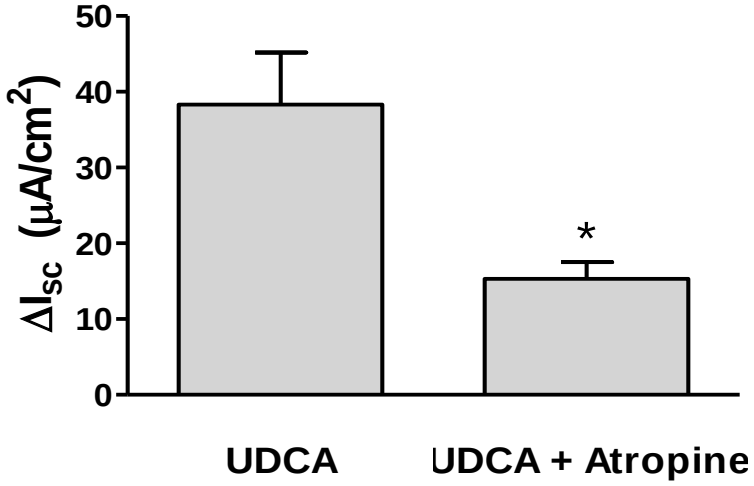


Figure 5.7: UDCA-induced I_{sc} responses in human colon are attenuated by the muscarinic antagonist, atropine. Human colonic tissues mounted in Ussing chambers were pre-treated with atropine (1 μM; 30 min; basolateral) and subsequent ΔI_{sc} responses to UDCA (500 μM, basolateral) were measured (n = 3, *$p < 0.05$).

5.3.4 UDCA exerts anti-secretory actions in TTX- pretreated human and rat colon

Next, the effects of UDCA on agonist-stimulated I_{sc} responses across human colonic tissue were assessed. In these experiments, all tissues were first pre-treated with TTX to remove the influence of enteric nerves. Interestingly, we found that under these conditions, UDCA exerts similar anti-secretory actions to those it exerts in cultured epithelial cells. In TTX-pre-treated tissues, UDCA (500 μM; bilateral; 15 min) significantly attenuated subsequent responses to CCh (100 μM) and FSK (10 μM) to 9.8 ± 4.0 and 25.1 ± 3.1 % of those in tissues treated with TTX alone ($n = 8$, $p < 0.01$, Figure 5.8). These anti-secretory effects were concentration-dependent with significant attenuation of both CCh- and FSK-induced responses apparent at a concentration of 100 μM UDCA (Figure 5.9).

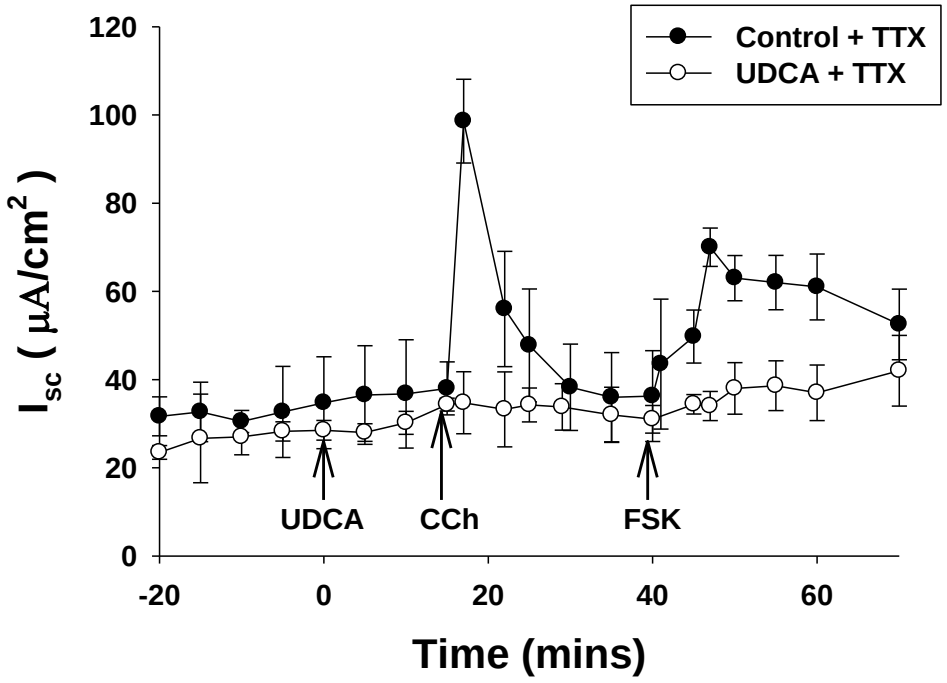


Figure 5.8: UDCA exerts anti-secretory effects in TTX- pre-treated human colonic mucosa. Muscle-stripped sections of human colon were mounted in Ussing chambers for measurements of I_{sc}. Both tissues were pre-treated with TTX (0.1 μM; 30 min), not shown, before treatment with UDCA (500 μM; 15 min). I_{sc} responses to CCh (100 μM; basolateral) and FSK (10 μM; apical) were then measured ($n = 8$, $p < 0.01$).

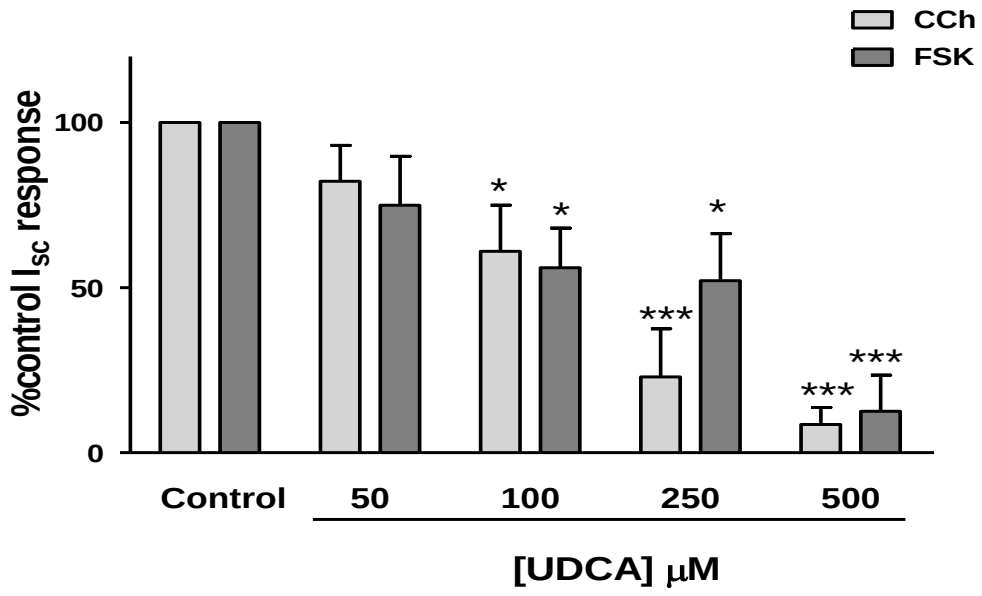


Figure 5.9: Anti-secretory actions of UDCA on CCh and FSK responses in human colon are concentration-dependent. Sections of human colonic mucosa were mounted in Ussing chambers and pre-treated with TTX before treatment with UDCA (50–500 μM; bilateral; 15 min) and subsequent agonist-induced responses were measured (n = 6; *p < 0.05, ***p < 0.001).

In a parallel series of experiments, we also examined the effects of UDCA on agonist-induced secretory responses across voltage-clamped rat colonic tissues. In these experiments, tissues were sequentially stimulated with CCh and FSK after I_{sc} responses to UDCA had resolved. Under these conditions, UDCA at concentrations ranging from 100 μM–500 μM significantly attenuated both CCh and FSK-induced secretory responses (n = 4; Figure 5.10).

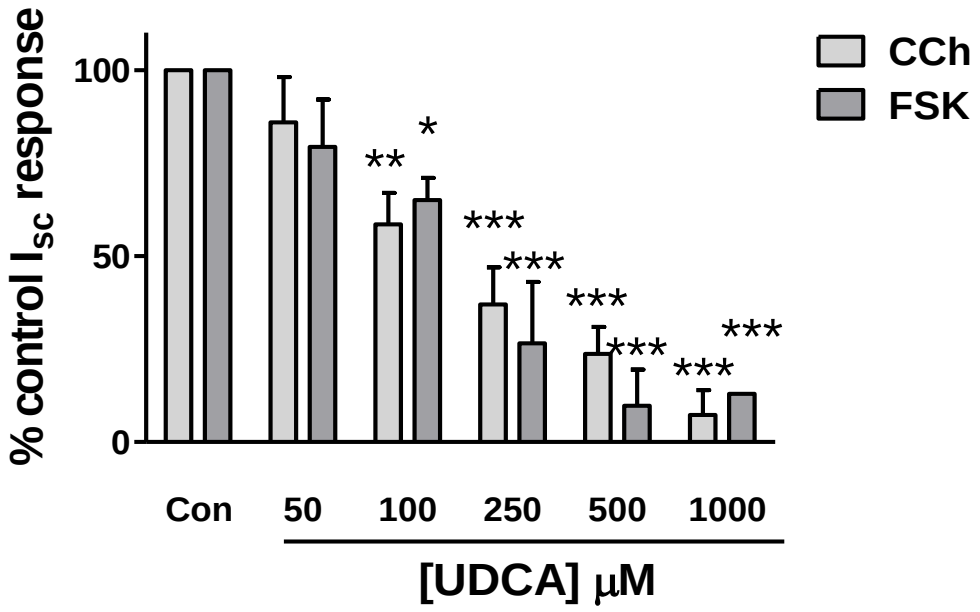


Figure 5.10: UDCA attenuates agonist-induced Cl^- secretion in a concentration-dependent manner in rat colon. Muscle stripped sections of rat colonic mucosa mounted in Ussing chambers were pre-treated with UDCA (50–500 μM; bilateral; 15 min) and subsequent responses to CCh (100 μM; basolateral) and FSK (10 μM; apical) were then measured. UDCA pre-treatment attenuated I_{sc} responses to both CCh (100 μM; basolateral) and FSK (10 μM; apical) in a concentration-dependent manner (n = 4; *p < 0.05, **p < 0.01, ***p < 0.001).

5.3.5 Anti-secretory actions of UDCA in human colonic mucosa are independent of the enteric nervous system.

To determine what effect, if any, TTX might play in mediating the anti-secretory effects of UDCA, a series of experiments was carried out in which human colonic mucosa was treated with UDCA (500μM, bilateral) in the presence and absence of TTX and ΔI_{sc} in response to CCh and FSK were compared. TTX pre-treatment did not significantly alter the anti-secretory effects of UDCA on agonist-stimulated secretory responses (n = 6, Figure 5.11, Panel A, n = 4, Panel B). Importantly, TTX pre-treatment alone did not significantly alter control CCh or FSK-induced responses, which in TTX-pre-treated tissues were 106 ± 33% (n = 5) and 111.8 ± 37 % (n = 4), respectively, of those in untreated controls.

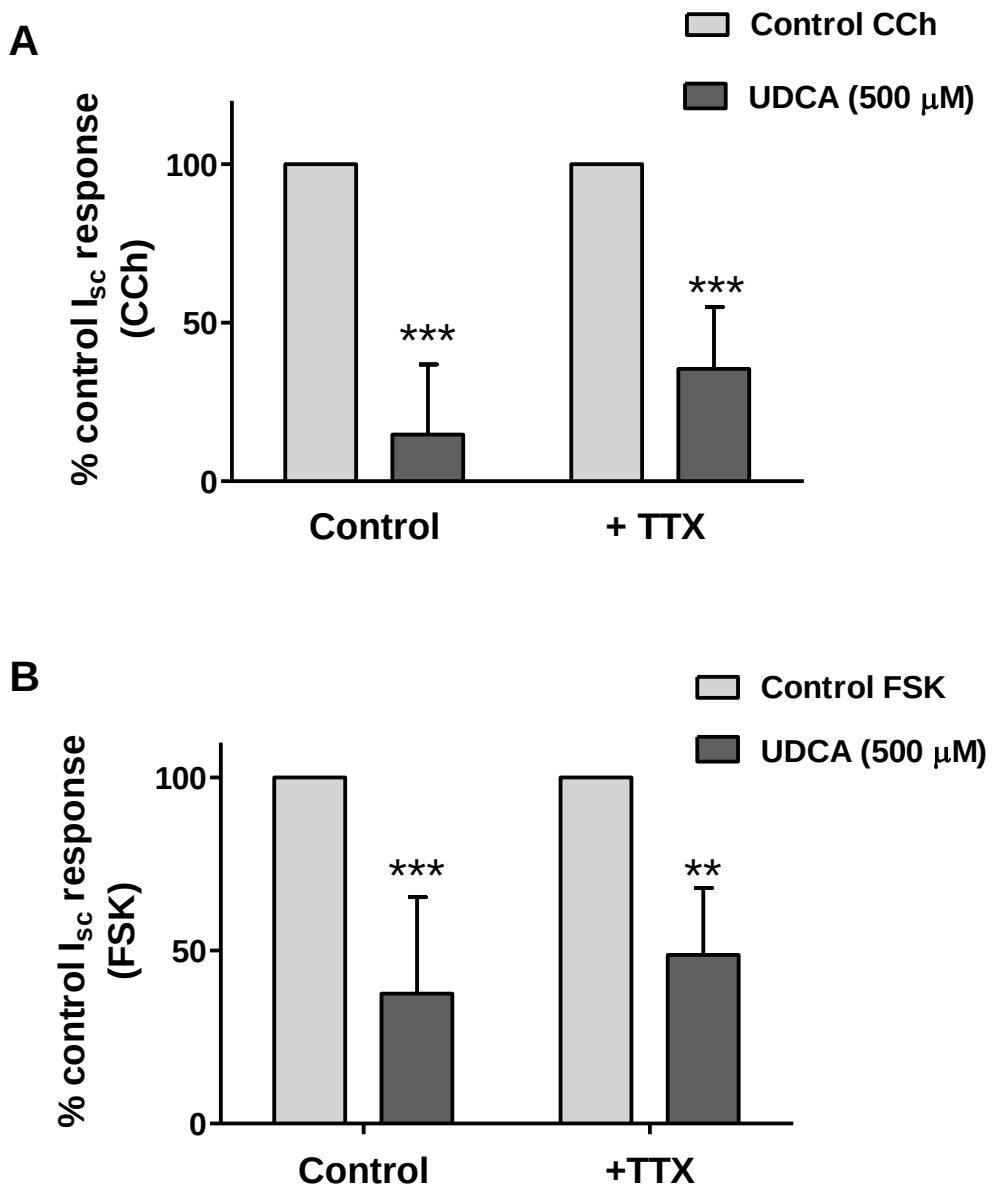


Figure 5.11: TTX pre-treatment does not alter the anti-secretory effects of UDCA in human colon. Voltage-clamped sections of muscle-stripped human colon were mounted in Ussing chambers and pre-treated with UDCA (500 μM) or PBS (control) in the presence or absence of TTX (0.1 μM; 30 min pre-treatment) before measuring I_{sc} responses to sequential addition of CCh (100 μM) and FSK (10 μM). Data were normalised and expressed as % control responses. There was no significant difference in the effect of UDCA pre-treatment on CCh or FSK-induced responses between control and TTX pre-treated tissues. Panel **A** illustrates % control responses to CCh in tissues pre-treated with UDCA in the presence or absence of TTX with CCh-induced responses being reduced to 14.7 ± 9.8 % of controls in untreated tissues and 35.2 ± 8.1% of controls in TTX pre-treated tissues (0.1 μM; 30 min; n = 6). Panel **B** illustrates % control responses to FSK in tissues pre-treated with UDCA in the presence or absence of TTX), while FSK-induced responses were reduced to 37.5 ± 9 % of controls in untreated tissues and 48 ± 13 % in TTX-pre-treated tissues (0.1 μM; 30 min; n = 4).

5.3.6 Basolateral UDCA is more effective in exerting anti-secretory actions in human colon

We previously found that UDCA exhibited more profound anti-secretory effects when added basolaterally to cultured monolayers of T_{84} cells (c.f. Chapter III; Figure 3.17). Thus, we next assessed the sidedness of UDCA effects on agonist-induced secretory responses across sections of human colon. We found that, similar to cultured epithelial cells, UDCA (500 μM) was significantly more effective at inhibiting CCh-induced secretory responses when added to the basolateral side of the tissue ($n = 4$; $p < 0.05$; Figure 5.12). Interestingly, although less effective than basolateral addition, the effects of apical UDCA on CCh-induced responses were significant compared to controls. Thus, the sidedness of anti-secretory actions of UDCA in human tissue is similar to that of its effects in cultured colonic epithelial monolayers.

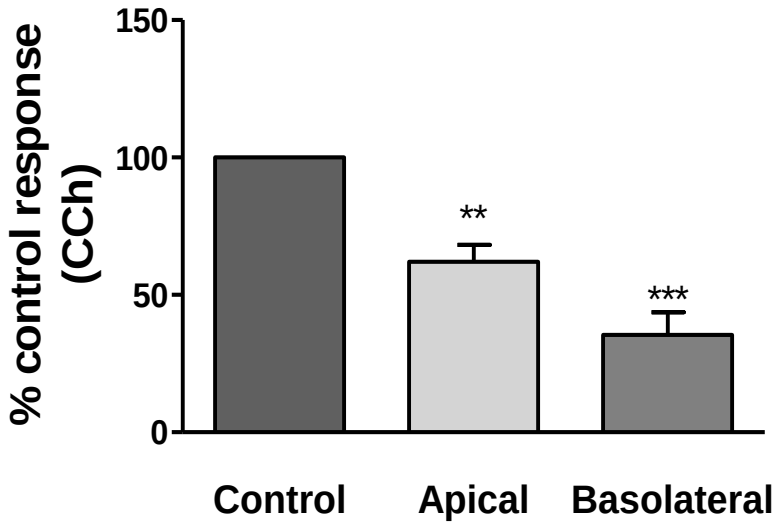


Figure 5.12: Sidedness of anti-secretory effects of UDCA in human colon. Voltage-clamped sections of muscle-stripped human colon were mounted in Ussing chambers and were pre-treated with UDCA (500 μM; 15 min) on the apical or basolateral side. I_{sc} responses to CCh (100 μM) were then measured. Data were expressed as % control responses. Basolateral addition of UDCA reduced CCh-induced responses to 36 ± 8.3% of controls, compared to a reduction to 62 ± 6.1 % of controls upon apical addition of the BA ($n = 4$; ***$p < 0.001$, **$p < 0.01$).

5.3.7 The effects of Lithocholic acid and 6α -MUDCA on I_{sc} responses in human colonic tissue.

As previously discussed in Chapter III, UDCA is rapidly metabolised *in vivo* to LCA in the colon by resident bacteria. We also found that, in contrast to the anti-secretory actions of UDCA, LCA pre-treatment enhanced subsequent responses to

secretagogues, such as CCh, in cultured epithelial cells. Thus, we also wished to examine the effects of LCA on CCh-induced secretory responses in human colon. We found that, similar to its effects on cultured epithelial cells, LCA (50 μM; 15 min pre-treatment) significantly enhanced I_{sc} responses to CCh in human colonic tissue to 130 ± 9% of those in control tissues (n = 3, *p < 0.05, Figure 5.13). Trans-mucosal conductances after LCA treatment were marginally higher than controls but not to a significant level (19.3 ± 1.3 $mS.cm^2$ in treated tissues compared to 12.2 ± 1.68 $mS.cm^{-2}$ in controls, n = 3).

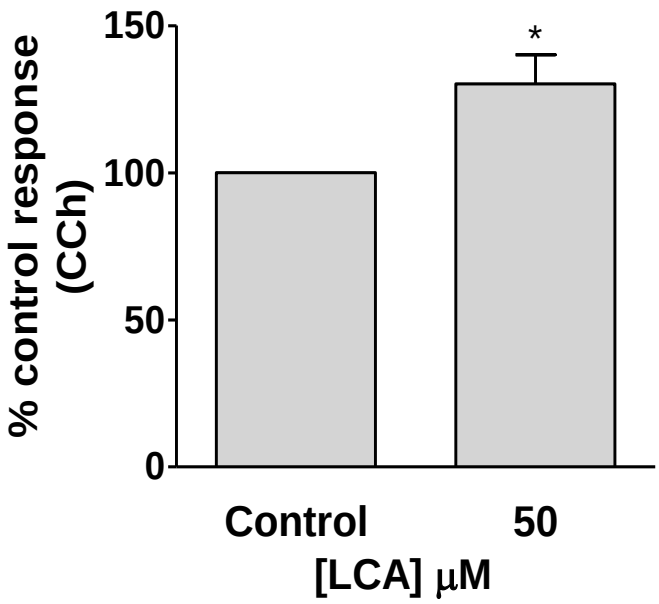


Figure 5.13: LCA potentiates CCh-induced I_{sc} responses in human colon. Voltage-clamped sections of muscle-stripped human colon were mounted in Ussing chambers and pre-treated with control (PBS; bilateral addition; 15 min) and LCA (50 μM; bilateral; 15 min) before I_{sc} responses to CCh (100 μM) were measured. Data are expressed as % control response (n = 3; *p < 0.05).

In Chapter 3, 6α-MUDCA, a non-metabolisable UDCA analogue, was found to have a similar potency to UDCA in exerting anti-secretory actions on cultured epithelial monolayers (cf. Chapter 3; Figure 3.15). We also demonstrated that 6α-MUDCA exerts anti-secretory actions in an *in vivo* mouse model. In order to determine if it also exerts anti-secretory effects in human colonic mucosa, tissues were pre-treated with 6α–MUDCA (500 μM; apical; 15 min) and subsequent CCh-induced responses were recorded. 6α–MUDCA (500 μM; apical; 15 min) significantly attenuated CCh-induced responses ($n = 3$, $p < 0.05$; Figure 5.14, Panel A). Responses to CCh were reduced to 16.6 ± 4.4 $\mu A/cm^2$ in 6α-MUDCA-treated tissues compared to 30 ± 2.3 $\mu A/ cm^2$ in untreated controls ($n = 3$, $p < 0.05$, Figure 5.14, Panel B). This represents a reduction of CCh-induced responses to 55 ± 6.3% of controls, which is comparable to the anti-secretory action of UDCA at similar concentrations. These experiments were done in the absence of TTX. It appears from these experiments that the pro-secretory action of 6α-MUDCA is much less than that of UDCA.

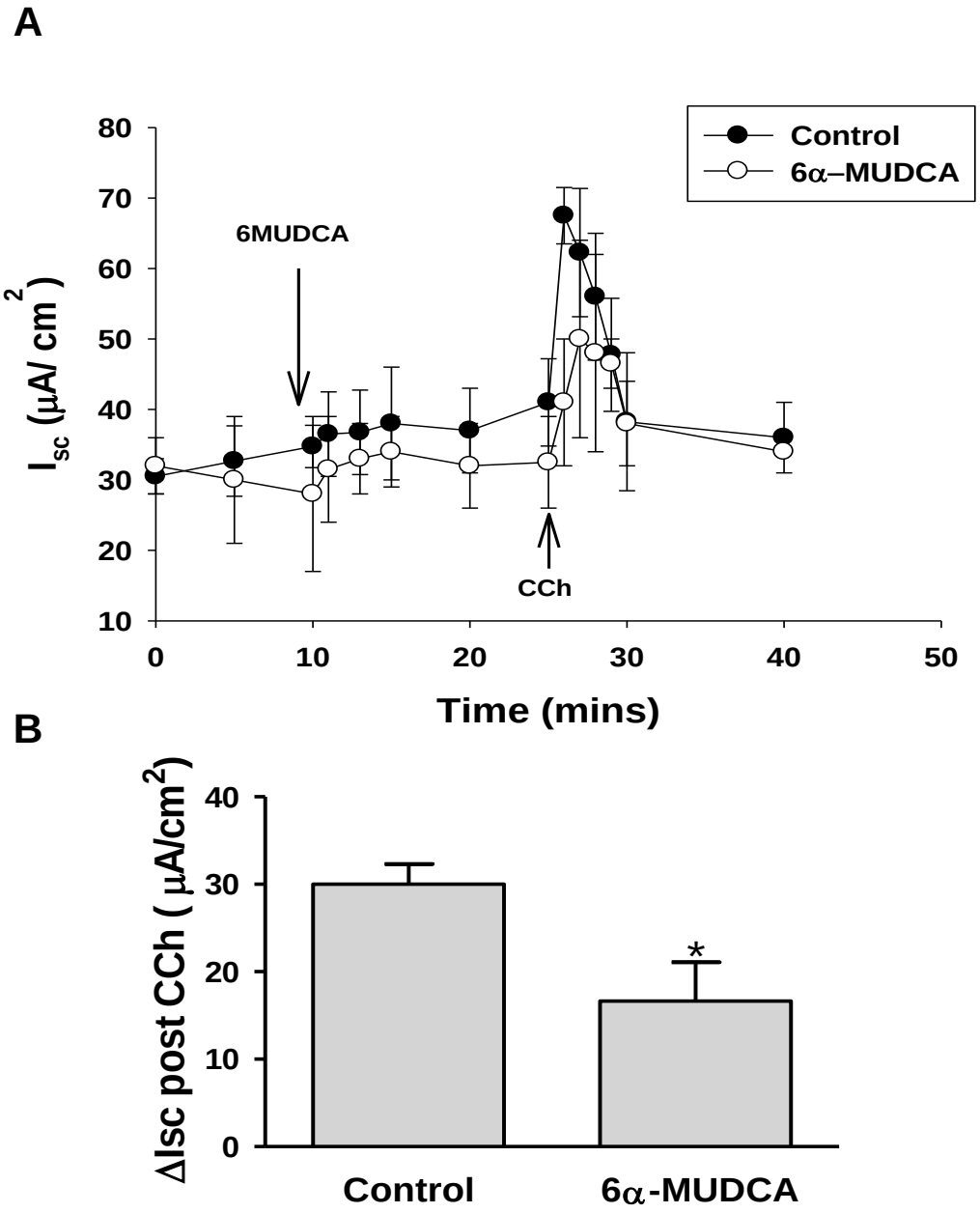


Figure 5.14: 6α-MUDCA inhibits CCh responses in human colon.A) Voltage-clamped sections of muscle-stripped human colon were mounted in Ussing chambers and pre-treated with 6α-MUDCA (500 μM; apical; 15 min) or PBS (control) before I_{sc} responses to CCh (100 μM) were measured. **B)** 6α-MUDCA attenuated subsequent CCh-induced responses to 55 ± 6.3% of those in controls (n = 3, *p < 0.05).

5.3.8 Effects of long-term oral UDCA therapy on colonic transport function.

Thus far in our studies, the acute effects of direct UDCA, LCA or 6α–MUDCA treatment of human colonic epithelial cells and mucosal tissues had been investigated. However, there is little data currently available regarding the long-term consequences of UDCA therapy on colonic secretory function in humans. Thus, experiments were carried out on voltage-clamped endoscopic samples obtained from patients receiving oral UDCA therapy (average dose of 8–10 mg/ kg), in order to determine if there were any alterations on basal or agonist-stimulated transport function. Control samples were obtained from patients who were deemed to have a normal endoscopic examination and who had no previous treatment with UDCA or bile acid sequestrants and no previous history of IBD or cancer.

Demographics of patients for this study are outlined in Table 5.3. Indications for colonoscopy in the UDCA group were; 1) surveillance for colorectal cancer in the setting of primary sclerosing cholangitis (PSC) and ulcerative colitis (UC) (n = 3), and 2) previous occurrence of polyps (n = 1). The indications for UDCA therapy were for treatment of PSC (n = 3) and for treatment of primary biliary cirrhosis (PBC) (n = 1). All patients included had macroscopically normal colons. In the case of those with a previous diagnosis of colitis only those with quiescent colitis were included. In the control group, 2 patients were examined due to a family history of colorectal cancer, and 2 had a previous history of polyps. All control colonoscopies were endoscopically normal and participants were asymptomatic at time of endoscopy.

Table 5.3 Demographics of patients included in a study of chronic UDCA treatment on colonic transport function

Demographic	Control (n = 4)	UDCA (n = 4)
Gender (M:F)	3:1	3:1
Mean Age (range)	39.2 (22-67)	39.7 (28-54)

Since the size of the tissues used in these studies was small, potential difference (PD) measurements were used as an index of electrogenic ion transport, since changes in I_{sc} were too small to measure using our apparatus (Grubb, 1994, Goerg , 1988). We found that there was no appreciable difference in basal PD between patients on UDCA therapy and controls. Basal PD in patients on UDCA therapy was 79 ± 24% of controls (n = 4, Figure 5.15, Panel A). Although CCh-induced PD responses were variable in both patient cohorts, no appreciable differences were noted. A significant CCh response was recordable in both groups compared to spontaneous changes in PD, which were minimal. Mean basal PD was 0.15 ± 0.07 mV. The mean CCh-induced ΔPD was 0.5 ± 0.10 mV in the control group, compared to 0.57 ± 0.16 mV in the UDCA-treated group (n = 4, Figure 5.15, Panel B).

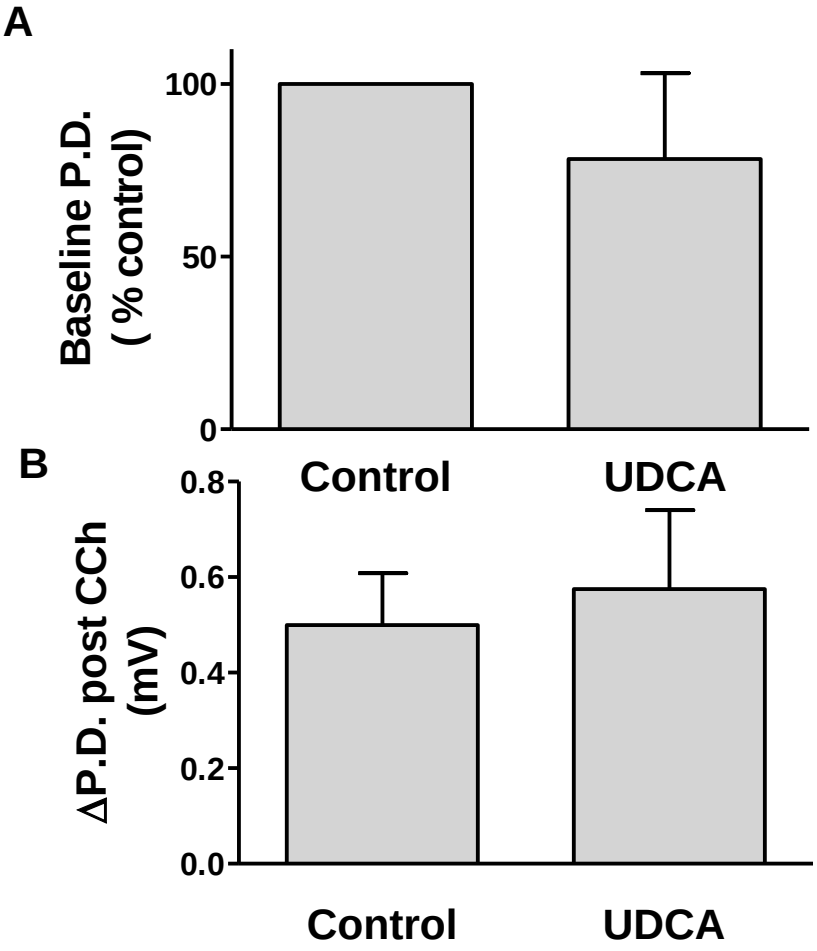


Figure 5.15: Long-term oral UDCA therapy does not alter basal or CCh-induced changes in trans-epithelial PD in human colon. Jumbo biopsies taken during endoscopic examination of patients on oral UDCA therapy or controls were mounted in Ussing chambers (window area = 0.03 cm^2). After allowing the tissues to equilibrate, basal PD was noted as a measure of basal Cl^- secretion and then CCh (100 μM)-induced changes in PD were measured. Data are expressed as % control response or in mV. **A)** There was no appreciable difference between basal PD measured in patients on UDCA therapy compared to healthy matched controls (Patient n = 8, 4 on UDCA treatment, 4 age and sex matched controls). **B)** Similarly, no differences in CCh-induced changes in PD were observed between the treatment and control groups (n = 4).

5.4 Discussion

The data described in this chapter are the first to characterize the effects of UDCA on basal and agonist-induced ion transport in human colon *in vitro*. First, we demonstrated that UDCA, when added basolaterally to either rat or human colonic tissue *in vitro*, gives rise to a monophasic increase in I_{sc} which is concentration dependent. These data are in direct contrast to our findings in T_{84} cells (*c.f.* Chapter III), where UDCA had no effect on basal I_{sc}. This suggests that the actions of UDCA in intact colonic tissue are likely to be mediated by indirect mechanisms, such as through activation of neural or immune cell populations in the mucosa. In this regard, we found that, similar to its effects in murine small intestine (Hardcastle, 2001), the effects of UDCA in human colon were inhibited by the neurotoxin, TTX, suggesting that they are neuronally-mediated. Thus, our data suggest that, similar to other dihydroxy bile acids, such as DCA, UDCA has the capability to recruit the enteric nervous system to regulate epithelial function (Karlstrom, 1986; Fihn, 2003). Furthermore, our studies with the muscarinic receptor antagonist, atropine, revealed that in contrast to its effects in mouse ileum (Hardcastle, 2001), the pro-secretory actions of UDCA in human colon are mediated through cholinergic activation of muscarinic receptors. Although, further investigation is required to identify the muscarinic receptor subtype involved in mediating responses to UDCA, previous studies suggest that it is likely to be the M_3 receptor (Cheng., 2002, Sun, 2004).

Our studies cannot rule out the possibility that other indirect effects could also be involved in mediating the actions of UDCA on colonic transport function *in vivo*. For example, BAs have been shown to activate mucosal mast cells in mouse intestine (Quist, 1991), with consequent histamine release contributing to BA-induced secretory responses (Gelbmann, 1995). Entero-endocrine L-cells are also stimulated by the presence of BAs (Parker, 2012), and it is therefore conceivable that BA effects on colonic secretion could also be regulated by entero-endocrine means. The sub-epithelial myofibroblastic sheet is also an important source of mediators, such as prostaglandins (Edwards, 2006, Powell, 1999), that can regulate epithelial transport function (Schultheiss, 2005). However, the role that UDCA plays in regulating mast cells, L-cells, and fibroblasts remains to be determined and could be the focus of future work.

Although experiments were not performed in the current studies to identify the charge carrying ion responsible for UDCA-induced increases in I_{sc} in human colon, it is likely that these responses are due to increased Cl^- secretion. This is based on the observation that responses to UDCA are abolished by atropine, which blocks the effects of ACh at the neuro-epithelial junction, and previous studies have shown that alterations in I_{sc} in response to ACh, or its analogue, CCh, are due to electrogenic Cl^- secretion (Dharmsathaphorn , 1986). Further experiments could be carried out in the future to confirm this, using loop diuretics, such as bumetanide, to inhibit NKCC1 or by substituting Cl^- in the bathing solution with impermeant anions, such as gluconate.

While basolateral UDCA stimulates Cl^- secretion in intact colon, our data also show that, similar to its effects on T_{84} cells, UDCA inhibits subsequent secretory responses to both Ca^{2+} and cAMP-dependent agonists. Interestingly, this anti-secretory effect is also apparent upon apical addition of UDCA, which does not stimulate pro-secretory responses. Furthermore, the anti-secretory effects of UDCA are not sensitive to TTX. Together these data confirm that, in contrast to other dihydroxy BAs, the direct effects of UDCA on colonic epithelial cells are anti-secretory. It is also important to note that the anti-secretory effects of UDCA occur at lower concentrations than do its pro-secretory actions. Thus, elevated levels of UDCA in the lumen could be expected to exert anti-secretory/anti-diarrhoeal actions.

However, when considering the data presented in this chapter, the question arises as to whether these anti-secretory concentrations of UDCA are achievable during therapy? It should be noted that the UDCA concentrations employed in the current studies were higher than physiological ranges of UDCA found in the colon, which have been reported to be in the range of 5–10 μM (Gleich, 1971; Adler, 1975). However, it is hypothesised that to achieve sufficiently high concentrations of UDCA for it to exert anti-secretory actions it would likely require either topical administration or the use of delayed release or bacterially-resistant analogues.

When considering the effects of UDCA on colonic epithelial secretory function, it is also important to consider its major metabolite, LCA, which has been shown to become the predominant colonic BA during UDCA therapy (Sinakos, 2010). In this

chapter we also present for the first time data illustrating the effects of LCA on human colonic epithelial secretion. LCA has directly contrasting effects to UDCA in that it potentiates secretagogue-induced responses, thus confirming our previous findings in T_{84} cells. This supports the idea that it is due to its rapid metabolism to LCA that underlies the tendency of UDCA to cause diarrhoea in patients. The mechanisms involved in mediating the effects of LCA are not yet known but could involve activation of specific bile acid receptors. In particular, LCA is an agonist of the TGR5 receptor. TGR5 is a cell surface receptor for BAs which has been shown to be expressed on enteric nerves (Camilleri, 2011). Unpublished studies from this laboratory have also found it to be expressed on colonic epithelial cells. TGR5 is a G_sPCR and it is coupled to elevations in intracellular cAMP (Kawamata, 2003, Li, 2011). Thus, activation of TGR5 by LCA, could serve to prime the epithelium for enhanced responsiveness to subsequent exposure to Ca^{2+}-dependent secretagogues. However we did not find an increase in basal I_{sc} with addition of LCA which may confound this theory somewhat. Future work in this area could investigate the molecular mechanisms involved in the actions of LCA, focussing on targets such as TGR5. Studies of the effects of LCA on transport protein activity and surface expression are also required in order to gain a deeper understanding of its actions on colonic secretory function. However, suffice as to say that our studies in intact human colonic tissue support the idea that in order to exploit the anti-secretory actions of UDCA in the clinic, a metabolically-stable analogue would be required.

The studies presented here have focussed specifically on the acute and direct effects of UDCA on colonic epithelial secretory function *in vitro*. In further studies we were interested in investigating if oral therapy with UDCA might alter colonic transport function. In this work, we found that long-term UDCA therapy did not appear to alter agonist-induced responses across colonic tissues from these patients. There are a number of possible reasons for this apparent lack of action in patients. Firstly, sufficient levels of the drug may not have reached the colon in the particular subset of patients studied. This could have been due to the doses used being too low, or the presence of inflammation in some patients could have caused a more rapid transit and excretion of the drug from the bowel. Alternatively, as discussed above, UDCA may have been metabolised too rapidly for its anti-secretory

effects to become apparent. Studies of the effects of drugs like 6α-MUDCA in humans would be useful to determine if this is the case.

In conclusion, the current studies demonstrate that UDCA has dual actions on ion transport across colonic mucosal tissues. At high concentrations, UDCA stimulates secretory responses through activation of cholinergic nerves, while at lower concentrations; it can exert direct anti-secretory effects. Our findings support the idea that oral administration of metabolically stable UDCA analogues could be useful for treating intestinal disorders associated with diarrhoea.

Chapter 6

An investigation of the anti-inflammatory effects of UDCA on colonic epithelial cells.

6.1 Introduction

6.1.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a common and debilitating immune-mediated inflammatory disorder which, as described in Chapter 1. Profuse and bloody diarrhoea along with abdominal pain and malnutrition are common features among patients with IBD. Regulation of the intestinal innate immune system in these diseases has been the subject of intense research and with the evolution of our understanding of the processes involved; it has become a target for development of new therapeutics in combating these debilitating conditions. The overall incidence per 100,000 for IBD in Europe, at ages 15–64 years, has been estimated as 10.4 for UC (95% CI: 7.6–13.1) and 5.6 (95% CI: 2.8–8.3) for CD. Rates of UC in Northern centres were 40% higher than those in the South (rate ratio (RR) = 1.4 (95% CI: 1.2–1.5)) and for CD they were 80% higher (RR = 1.8 (95% CI: 1.5–2.1), suggesting a regional variability independent of factors such as tobacco use and education (Shivananda, 1996).

6.1.2 Altered immunity in the pathogenesis of IBD

While the precise initiating factors in IBD are not well-defined, it is known that altered immune regulation leads to prolonged mucosal inflammation, which is amplified and perpetuated by recruitment of leucocytes from the blood. Up-regulation of nuclear transcription factors, such as NFκB, is likely to underlie the subsequent local release of cytokines, eicosanoids, and other mediators of inflammation, including reactive oxygen species, prostaglandins, and platelet activating factor, nitric oxide, proteases and growth factors. In UC, cytokine release from non T-helper lymphocytes generates a largely humoral response, while in Crohn's, a cell-mediated response is induced by T-helper 1 cells (Csutora, 2006).

Toll-like receptors (TLRs) play a key role in regulating innate immunity (Marshak-Rothstein, 2006). TLRs and their place in whole body immunity has been described in the General Introduction, so here the focus is on specific TLR- and cytokine-mediated effects in relation to the pathogenesis of IBD.

6.1.3 TLR recognition and IBD

Primary human IECs (intestinal epithelial cells) constitutively express TLR-3 and TLR-5 and low levels of TLR-2 and TLR-4 (Cario, 2000). However, expression of TLR-4 in the colon is increased in UC (Cario, 2000). In addition, a number of risk variants in the TLR-1/-2/-6 genes have been associated with distinct disease phenotypes of IBD (Cario, 2010). Monocytes from inflamed intestinal tissue also respond to LPS with increased expression of IL-1β, and a higher percentage of intestinal DCs express TLR-4 in IBD patients compared to controls (Hausmann, 2002). Challenge of MyD88/TRIF-deficient mice with the commensal bacterium, *Escherichia coli* K-12, resulted in recovery of more live bacteria from the spleen than in control mice (Slack, 2009), suggesting that prevention of bacterial invasion is dependent on intact TLR signalling. Furthermore, TLR signalling as outlined in Figure 6.1: has been shown to play a role in maintaining the integrity and function of the intestinal epithelium. Mechanisms involved are unclear but are currently being addressed (Lavelle, 2010). Since TLR-4 and TLR-2 over-expression and NOD-like receptor mutations have all been clearly associated with the pathogenesis of IBD, the current studies focussed on cytokine production in response to ligands of these receptors and the potential for UDCA in interfering with these responses.

TLR-2: TLR-2 is thought to play a particularly important role in maintenance of intestinal barrier integrity (Cario, 2007). Treatment of IECs with the TLR-2, agonist Pam3CSK, resulted in increased intercellular communication through the gap junctional connexion, Cx43, during cell injury (Ey, 2009). Among Caucasians, TLR -2 genetic mutations are associated with a more severe disease phenotype in UC patients (Pierik, 2006). Furthermore, in a neonatal rat model of necrotizing enterocolitis there is increased expression of both TLR-2 and TLR-4 (Le Mandat Schultz, 2007). Combined, these data highlight the importance of TLR-2 in intestinal barrier function. Although further characterisation is required, this evidence serves to promote the idea of targeting TLR-2 signalling as a potential therapeutic approach in intestinal inflammatory diseases.

TLR-4: Previous studies have shown that stimulation of IECs with TLR-4 agonists induces proliferation, NFκB activation, and the release of pro-inflammatory cytokines

(Otte, 2004). However, responses to LPS are variable in intestinal cells (Hornef, 2002) due to highly variable expression of TLR-4 (Cerovic, 2009), making it difficult to fully characterise responsiveness to TLR stimuli in *in vitro* studies. TLR-4 antagonists have been shown to inhibit the development of colitis in a DSS model as have antibodies to TLR-4 (Ungaro, 2009), while necrotising enterocolitis results in increased expression of TLR-4 compared to controls (Le Mandat Schultz, 2007). These data indicate an important role for TLR-4 in inflammation in the colonic mucosa.

NOD-like receptors: NOD-like receptors, as mentioned in the Introduction to this thesis, constitute a recently identified family of intracellular PRRs (Creagh and O'Neill, 2006). The physiological importance of NOD-1 and NOD-2 in immune responses is evident from the association of their mutations with inflammatory diseases in humans. Crohn's disease has a complex pathology, as already outlined, but there is an established link between the disease and NOD-2 mutations (Okumura, 2009). NOD-1 gene polymorphisms have also been shown to be associated with susceptibility to both CD and UC (McGovern, 2005). MDP has been shown to stimulate NOD-2 responses in colonic epithelial cells (Canto, 2009, Richardson, 2010). Interestingly, there may be inhibitory interactions between stimuli of NOD-2 and TLR-4, which may explain a protective role for NOD-2 that has been observed in some intestinal diseases, such as necrotizing enterocolitis of the new-born (Richardson, 2010).

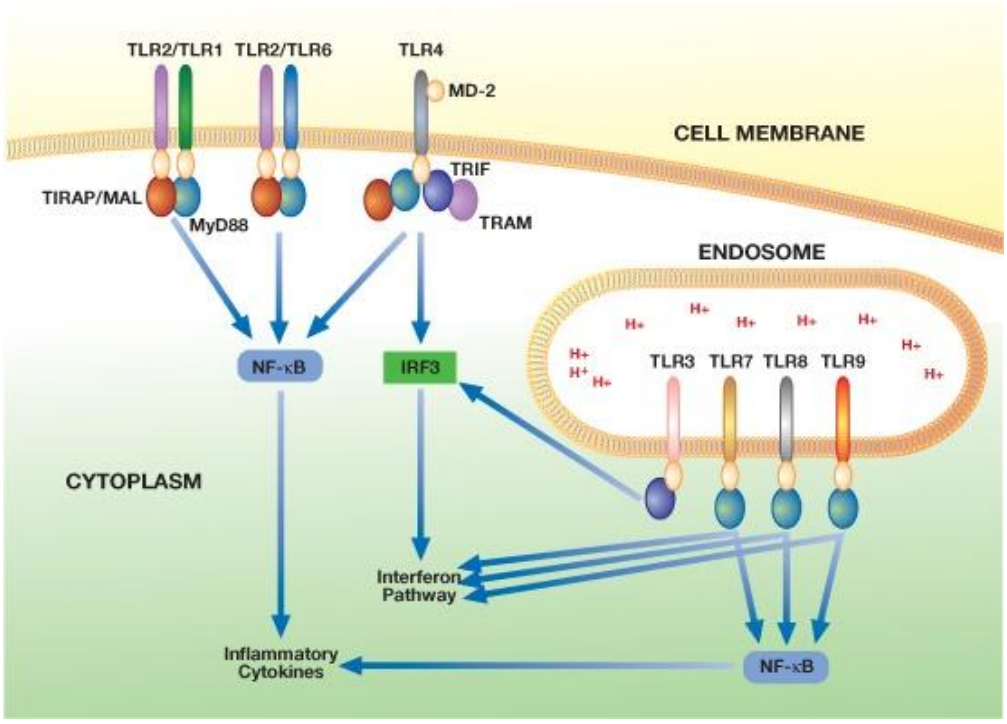


Figure 6.1: TLR signalling pathways. TLRs are transmembrane proteins that act alone or in heterologous pairs to detect invading microbes, triggering signalling pathways that ultimately lead to the release of inflammatory cytokines through the NFκB pathway or the release of interferons through the IRF3 pathway. TLRs can localize to the cell membrane or to endosomes and can differ in terms of the adaptor molecules (circles) that relay their intracellular signals. Some of the adaptor molecules (MyD88, TRIF, and TRAM) have been characterised, while others remain unidentified (Adapted from MacKichan 2005).

TLRs and cytokine release in the gut: The mucosal surfaces of the gastrointestinal tract are continually exposed to an enormous antigenic load of microbial and dietary origin, yet homeostasis is normally maintained. TLRs have a key role in maintaining the integrity and function of the epithelial barrier and in promoting maturation of the mucosal immune system and it is known that there is up regulation of a number of TLRs in intestinal inflammation (Hausmann, 2002). Under these conditions, TLR stimulation by pathogens may result in release of pro-inflammatory cytokines (Creagh, 2006). Prolonged, excessive, or inappropriate cytokine release, such as that which occurs in IBD, can result in damage to the epithelium. In experimental conditions, prolonged exposure of HT29 cells to various cytokines (e.g. IL-6, TNF-α, IFN-γ) reduced both epithelial barrier function and electrolyte transport (Hiribarren, 1993). Human colonic epithelial cells and cell lines are also known to produce IL-8 (Eckmann, 1993), a potent neutrophil chemo-attractant, and leukotrienes that may be involved in the initiation and regulation of mucosal inflammatory responses. Colonic epithelial cell lines can also respond to a broad array of cytokines (e.g.

TNFα, IL-I, IL-4, IL-6, IFN-γ) with altered gene expression and growth characteristics (Jung, 1995).

Cytokines play an essential role in intercellular communication by delivering signals which influence the activation, growth, differentiation or migration of their target cells. Alone and in combination, pro-inflammatory cytokines are known to attract and activate inflammatory cells and thus may provide an important early signalling system for the initiation and amplification of mucosal inflammatory responses in the early stages of bacterial infection (Jung, 1995). As mentioned previously, it is thought that it is excessive and prolonged pro-inflammatory cytokine release that is responsible for tissue damage in inflammatory bowel conditions.

6.1.4 Modulation of cytokine activity as a therapeutic option in IBD

The recognition that altered cytokine expression is important in the pathogenesis of IBD prompted the trial of anti-TNF-α antibodies, and IL-11 and IL-1 receptor antagonists, for treating the disease. Of these, only TNF-α antibodies have been fully-licensed for use in patients. Infliximab and Adalimumab are currently licensed for use in CD (Colombel, 2010), (Kamm, 2011), and Infliximab for moderate-severe UC (Feagan, 2007). With further research, it is likely that a panel of cytokine-based therapies will become available for use, especially in patients with refractory/non-responsive IBD (Sandborn, 2011, Toussirot, 2012, Klotz, 2005). Future strategies might also include gene transfer techniques for induction of protective cytokines, such as IL-4 and IL-10 (Csutora, 2006). Thus, the roles that different cytokines play in pathogenesis of IBD and their potential as targets for development as new therapeutics, continues to be a field of intense research interest. In the current studies, we selected a number of cytokines, known to be important in the development of IBD, for investigation.

RANTES:

RANTES, also known as CCL5 (C-C motif ligand 5), is an 8 kDa protein classified as a chemotactic cytokine, or chemokine. It is released upon activation of normal T cells and has a range of pro-inflammatory activities (Tawadrous, 2012, Alcendor, 2012, Hu, 2012). It is chemotactic for T cells, eosinophils, basophils, and leucocytes. It has

been shown to interact with the CC chemokine receptors; CCR-3 (Struyf, 2001), CCR-5 (Proudfoot, 2001), and CCR-1 (Proudfoot, 2001).

RANTES also activates the G-protein coupled receptor, GPR75. Along with IL-2 and IFN-γ, RANTES induces proliferation/activation of certain natural-killer cells and it is a HIV-suppressive factor, released from CD8+ T cells. The importance of RANTES is underlined by the fact that it has been shown to be up-regulated in more than 100, mainly granulomatous, human diseases, including IBD. It has been shown that there is increased expression of both RANTES and IL-8 in the epithelium of patients with both CD and UC (McCormack, 2001, Thomas, 2012), where it is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting neutrophils into inflammatory sites. In combination with other cytokines (i.e., IL-2 and IFN-γ) that are released by T cells, RANTES also induces the proliferation and activation of certain natural-killer (NK) cells to form CHAK (CC-chemokine-activated killer) cells.

IL-8:

IL-8 is a pro-inflammatory cytokine that is produced by immune cells and epithelial cells and primarily functions to induce chemotaxis. There are many receptors at the cell surface capable of binding IL-8; the most frequently studied being the G protein-coupled serpentine receptors, CXCR1, and CXCR2 (Bondurant, 2012). IL-8 is a member of the CXC chemokine family and is one of the major mediators of inflammatory responses. IL-8 and -10 other members of the CXC chemokine gene family form a cluster in a region mapped to chromosome 4q.Through a chain of biochemical reactions, IL-8 is secreted and its primary function is the induction of chemotaxis in its target cells (e.g. neutrophils). While neutrophil granulocytes are the primary target cells of IL-8, there is a relatively wide range of cells (endothelial cells, macrophages, mast cells, and keratinocytes) which also respond to this chemokine. IL-8 secretion is increased by a range of stimuli, including oxidant stress, causing the recruitment of inflammatory cells which induces a further increase in oxidant stress, making it a key mediator of localized inflammation (Vlahopoulos, 1999). UC is characterized by increased production of IL-8 (Arijs, 2011, Guimbaud, 1998), and recent studies in patients with IBD noted that expression of IL-8 was increased in patients with active ileal CD (Arijs, 2011). IL-8 is produced mainly in the colonic

lamina propria in IBD, and its expression correlates with the degree of mucosal inflammation (Daig, 1996).

6.1.5 Bile acids and intestinal inflammation

Many studies have shown that there is increased delivery of BAs to the colon in conditions of IBD. The most severe bile acid malabsorption (BAM) occurs in Crohn's patients after resection of the distal ileum, but BAM can occur in surgically untreated CD patients, regardless of disease localisation (Lenicek, 2011). Physiological studies in humans suggest that BAM may be caused by an abnormal negative feedback system, resulting in increased synthesis of bile salts and entry into the colon, thereby causing watery diarrhoea (Surawicz, 2010). Some patients with terminal ileitis suffer from significant BAM, even if the inflammation is locally limited. There are data to suggest that limited acute ileitis impairs active BA uptake in the terminal ileum. It also diminishes active BA and glucose absorption in more proximal segments of the small intestine, likely by a systemic effect (Stelzner, 2001). This systemic effect may aggravate BA malabsorption in patients with limited ileitis. The most prominent of the colonic BAs, DCA, has been well-established as being pro-inflammatory (Muhlbauer, 2004). Indeed, DCA is known to induce IL-8 secretion and to activate NFκB in HT29 cells (Lee, 2004), actions that are opposed by taurine conjugated UDCA (Muhlbauer, 2004). The stimulatory effect of DCA is reproduced in some but not all other intestinal cell lines (Payne, 1998).

BAs may also play a role in the pathogenesis of intestinal inflammation by activating the signalling pathways that control cell proliferation. CDCA, DCA and LCA all have stimulatory effects on the expression of the proto-oncogene, c-fos, in Caco-2 cells (Di Toro, 2000). Another mechanism by which DCA can aggravate inflammation is by disruption to the Golgi apparatus, an organelle critical for normal cell function. Analysis of Golgi architecture *in vivo* using tissue microarrays by Long et al., revealed Golgi fragmentation in both UC and colorectal cancer tissue. Interestingly, they further demonstrated that DCA can disrupt the structure of the Golgi. Inhibition of this DCA-induced Golgi fragmentation by UDCA was mediated via the GR (Byrne, 2010). This represents a potential mechanism of observed chemo-preventive effects of UDCA in benign and malignant disease of the colon. Thus, it can be extrapolated

that dihydroxy BAs such as DCA have pro-inflammatory effects in the intestine and that these effects may be counteracted by UDCA.

In contrast to the other dihydroxy BAs, there is significant evidence that UDCA exerts anti-inflammatory activity in the intestine. Studies have shown that UDCA treatment mildly reduces intestinal permeability and oxidative stress in the indomethacin model of ileitis in rats (Bernardes-Silva, 2004). Similarly, UDCA counteracts ibuprofen-induced intestinal ulceration in rats (Lloyd-Still, 2001). UDCA also inhibits IL-1β and DCA-induced activation of NF-κB and AP-1, and inhibits iNOS synthesis in human colon cancer cells (Shah, 2006, Invernizzi, 1997). More recently, a Belgian group have been studying the protective effects of TUDCA on intestinal inflammation in a DSS-induced colitis model, noting that large doses (500 mg/ kg) administered intra-peritoneally result in significant reductions in intestinal inflammation and this process is thought to be driven by epithelial IRE1 activation, thereby altering the contribution of the unfolded protein response to intestinal inflammation (Laukens, 2012).

BA effects on epithelial barrier function: The organization of the colonic epithelium is directed towards maintaining a continuous layer of cells with functional maturity at the surface, with a constant supply of epithelial cells migrating upwards from the crypts. Cells are lost by shedding at the surface or by undergoing apoptosis *in situ*, followed by shedding or phagocytosis. The nature of the predominant form of cell death under normal circumstances remains uncertain but probably involves multiple mechanisms. Death of abnormal cells in the crypts is an important process that prevents clonal expansion and tumour formation. Apoptosis of normal cells is largely secondary to the effects of exposure to luminal factors, such as short-chain fatty acids, or cytokine-induced death in association with inflammation. In IBD, there is known to be increased rates of epithelial cell death (Chen, 2010). In turn, this can lead to a compromised barrier function, impaired epithelial restitution, loss of stem cells and regenerative capacity, and can have relevance to carcinogenesis (Sturm, 2008).

BAs are well known to regulate apoptosis in the intestine. Toxic BAs induce apoptosis by activating both ligand-dependent and -independent death receptor

oligomerization. There have been several studies suggesting that it is the hydrophobicity of BAs that is the most important determinant of their pro-apoptotic activity (Powell, 2001). Gilmer et al recently studied relationships between lipophilicity and cytotoxicity using multiple derivatives of UDCA, which is less hydrophobic than other dihydroxy BAs (Roda, 1990), in an oesophageal cell line and found them to have no effects on cell viability (Shiraki, 2005; Sharma, 2010). This is in keeping with the findings of multiple other studies of UDCA actions on cell viability in various models (Invernizzi,1997;Bellentani, 2005).

UDCA and its taurine-conjugated form (TUDCA), both show cyto-protective properties. Indeed, these molecules have been described as potent inhibitors of classic pathways of apoptosis in liver cells, although their precise mode of action remains to be clarified (Amaral et al., 2009). UDCA is currently considered the first choice therapy for several forms of cholestatic immune syndromes. However, the beneficial effects of both UDCA and TUDCA have been tested in other experimental pathological conditions which involve dysregulated apoptosis, including neurological disorders, such as Alzheimer's, Parkinson's, and Huntington's diseases (Amaral, 2009). UDCA can also interfere with the death receptor pathway, inhibiting caspase-3 activation (Lim, 2010). DCA and LCA on the other hand, have been shown to promote proliferation in colon cancer lines which express M_3 receptors and EGFR (Cheng, 2005).

BAs and epithelial tight junctions: UDCA has been shown to have inhibitory effects on cytokine-induced changes in paracellular permeability in hepatocytes (Hanada, 2003). However, there is conflicting evidence regarding the effects of BAs on intestinal epithelial permeability. Both CDCA (0.5 mM) and UDCA (0.5 mM) have been shown to significantly increase mucosal permeability to lactulose in jejunum and ileum. In these studies, tight junctions appeared to be loosened by the addition of 1 mM CDCA, suggesting that this is a major site where BAs transiently increase small intestinal permeability (Fasano, 1990). Furthermore in Caco-2 cells, CA, DCA and CDCA, but not UDCA, decreased trans-epithelial electrical resistance and result in redistribution of tight junction proteins. CDCA and DCA also induce reversible EGFr phosphorylation and occludin dephosphorylation in this setting, suggesting that these BAs modulate intestinal permeability through interaction with these proteins

(Raimondi, 2008). Furthermore, BAs which activate the nuclear receptor, FXR, may be protective in the context of inflammation through their effects on TJs. FXR activation has been shown in a DSS mouse model of colitis to prevent inflammation, partly through inhibition of increases in epithelial permeability (Gadaleta, 2011).

6.1.6 Anti-inflammatory actions of UDCA

UDCA has been well-described to have anti-inflammatory actions (Hylemon, 2009), most notably in the liver where it has been widely used as a therapeutic in cholestatic immune diseases, such as primary biliary cirrhosis and primary sclerosing cholangitis (Pardi, 2003). Though still not completely understood, the anti-inflammatory effects of UDCA in the hepatobiliary tree are complex. Its mechanisms of action in PBC are mediated, at least in part, via a modulation of protein biosynthetic pathways (Chen, 2008), while in a cholangitis model, UDCA suppresses hepatocyte IL-2 production (Miyaguchi, 2005).

BAs are also important in progression of oesophageal disease. Reflux of gastro-duodenal contents (including BAs) and consequent inflammatory responses are associated with the development of Barrett's oesophagus (BO) and the promotion of oesophageal adenocarcinoma (OAC). DCA has been proposed to play an important role in the development of OAC through regulation of apoptosis and COX-2-regulated cell survival, suggesting that the balance between these 2 opposing signals may determine the transformation potential of DCA as a component of the refluxate (Looby, 2009). In contrast, UDCA has no such cytotoxic effects on oesophageal cells, as demonstrated in numerous studies (Bozikas, 2008, Huo, 2011). However, to date, clinical studies have not shown UDCA treatment to alter the pathologic appearance of BO, although it does exhibit some clinical efficacy in bile reflux related gastritis (Huo, 2011; Bozikas, 2008, Burnat, 2010, Rosman, 1987). Based on these previous studies and the ever-growing body of evidence that suggests UDCA to be primarily anti-inflammatory, there is a compelling case for further investigating whether this BA also exerts anti-inflammatory effects on intestinal epithelial cells, thereby providing a good target for the development of new drugs to treat IBD.

6.2 Aim:

The primary aim of this chapter was to investigate if UDCA exerts anti-inflammatory actions on colonic epithelial cells. Specifically, we aimed to determine:

i) The effects of UDCA on basal and TLR-stimulated cytokine release from colonic epithelial cells.

ii) The effects of UDCA on epithelial barrier function, cell growth, and apoptosis.

6.3 Results:

6.3.1 UDCA regulates basal cytokine release from T_{84} cell monolayers

Initial experiments were carried out to assess whether UDCA might affect basal cytokine release from colonic epithelial cells, T_{84} cells were grown to 70–80% confluency on 96 well plates and, after serum starvation for 24 h, were treated with UDCA (250 μM; 24 h). Levels of the pro-inflammatory chemokines, RANTES, IL-6 and IL-8, were then measured in the culture medium by ELISA and compared to levels in untreated controls. These experiments revealed that UDCA pre-treatment (250 μM; 24 h) significantly reduced basal release of RANTES from T_{84} cells, reducing levels to 42 ± 19% of controls ($n = 6$, $p < 0.05$, Figure 6.2, Panel A). In contrast, UDCA was found to have no significant effect on basal levels of either IL-8 ($n = 6$) or IL-6 ($n = 4$) (Figure 6.2, Panels B and C, respectively).

Having shown that UDCA can alter basal RANTES release from colonic epithelial cells, we next went on to assess whether the BA could affect cytokine release in response to common TLR agonists. Preliminary experiments were first carried out to determine which TLR agonists could induce cytokine release in our cell culture model, paying particular attention to those agonists most relevant to IBD (data not shown). Thus, a series of optimization experiments were performed with a variety of TLR ligands at a range of concentrations in the T_{84} cell line. We found that the optimal concentration for stimulation of TLR-4 with lipopolysaccharide (LPS) was 100 ng/ mL, which caused a 2.4 ± 0.31 fold increase in RANTES release compared to controls ($n = 6$, $p < 0.001$, Figure 6.3, Panel A). The optimal concentration for The optimal concentration for stimulation of TLR-2 with Pam3Cys was 10 µg/ mL, which induced a 2.01 ± 0.24 fold increase in RANTES levels compared to controls ($n = 6$, $p < 0.001$, Figure 6.4, Panel A). The optimal concentration noted for stimulation with muramyl dipeptide (MDP), the ligand for NOD stimulation, was 1 μg/ mL and induced a 1.62 ± 0.42 fold increase in RANTES levels compared to controls ($n = 8$, $p < 0.05$, , Figure 6.5 Panel A). Finally, stimulation of TLR-3 with Poly I: C was found to be 20 µg/ mL, which caused a 3.0 ± 0.09 fold increase in RANTES release above controls ($n = 6$, $p < 0.001$, Figure 6.6 Panel A).

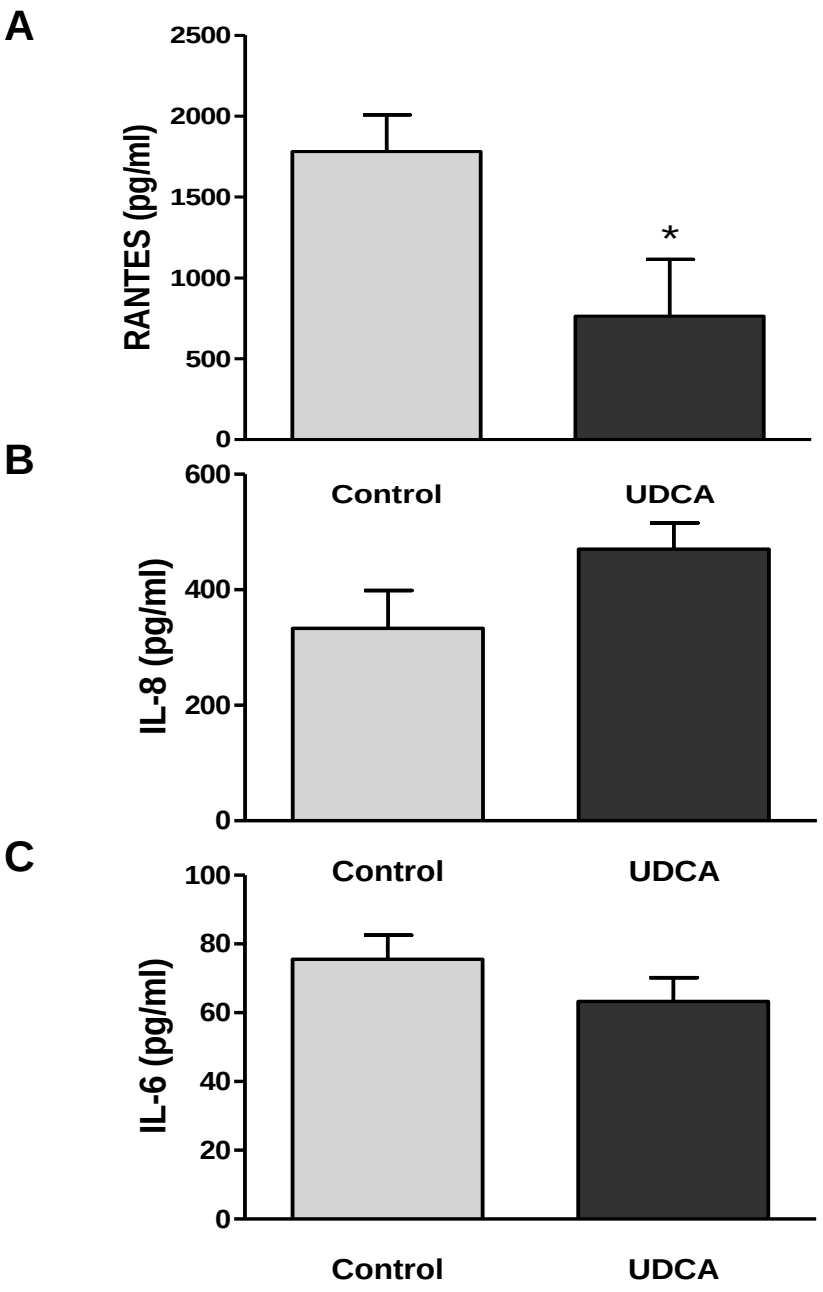


Figure 6.2: UDCA inhibits basal RANTES release from T_{84} cells. T_{84} cells were grown to 70-80% confluency in 96 well plates and then serum starved for 24 h. They were then exposed to UDCA (250 μM; 24 h) and levels of various pro-inflammatory cytokines (RANTES, IL-6 and IL-8) were measured by ELISA and compared to untreated control cells (n = 6, * $p < 0.05$).

6.3.2 UDCA downregulates TLR-driven RANTES secretion from T_{84} cells.

To assess the effects of UDCA on TLR-stimulated cytokine secretion, we initially focused on RANTES having noted a change in basal levels. UDCA pre-treatment (250 μM; 24 h) was found to significantly inhibit LPS-induced RANTES release to 39.1 ± 7.3% of that from control cells ($p < 0.001$, $n = 6$ Figure 6.3 Panel A). An analysis of the concentration dependence of UDCA for this effect demonstrated that at concentrations from 25 – 500 μM the BA caused significant reduction of RANTES release from LPS-stimulated cells (Figure 6.3, Panel B). UDCA (250 μM; 24 h) also inhibited TLR-2-induced RANTES release in response to Pam3Cys (10 µg/ml) to 34.3 ± 6.8% of that from control cells ($p < 0.001$, $n = 6$,Figure 6.4 Panel A). Analysis of the concentration-dependence of the BA revealed that concentrations ranging from 100–500 μM significantly reduced RANTES release from Pam3Cys-stimulated T_{84} cells (Figure 6.4, Panel B). UDCA pre-treatment (250 μM; 24 h) also inhibited NOD-2-induced RANTES release with muramyl dipeptide (MDP, 1 µg/ mL) to 27.9 ± 10.3% of that from controls ($p < 0.001$, $n = 6$, Figure 6.5, Panel A). Again, the effects of UDCA were concentration-dependent in the range of 100–500 μM but were not statistically significant (Figure 6.5, Panel B). In contrast, UDCA did not alter TLR-3 stimulated RANTES release with Poly I: C at concentrations ranging from 25 – 250 μM with some effect noted at 500 μM ($n = 12$, Figure 6.6) implying that its effects were predominantly affecting the membrane bound TLRs.

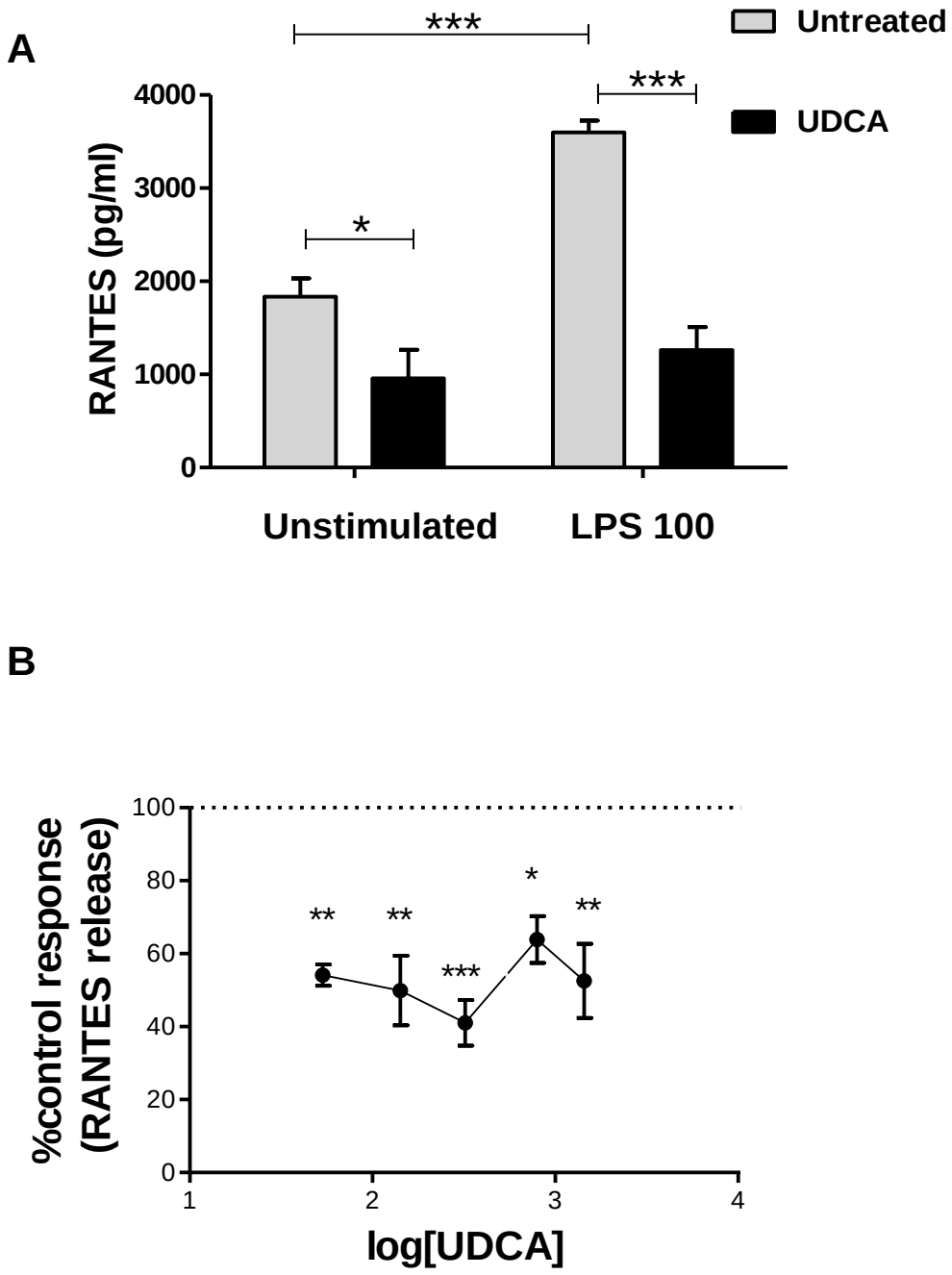


Figure 6.3: UDCA pre-treatment significantly attenuates TLR-4-driven RANTES secretion from T_{84} colonic epithelial cells. A) T_{84} cells grown to 70–80% confluency in 96 well plates were exposed to UDCA (250 μM) for 30 min before stimulating with LPS (100 ng/ mL) for a further 24 h (n = 6). **B)** UDCA pre-treatment at concentrations ranging from 25–500 μM significantly reduced RANTES release from LPS-stimulated T_{84} cells compared to control response. Graph represents relationship of log [UDCA] to percentage control response where control response is 100%. All concentrations used resulted in significantly lower % RANTES release. Calculated EC_{50} was 197 μM (n = 4; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

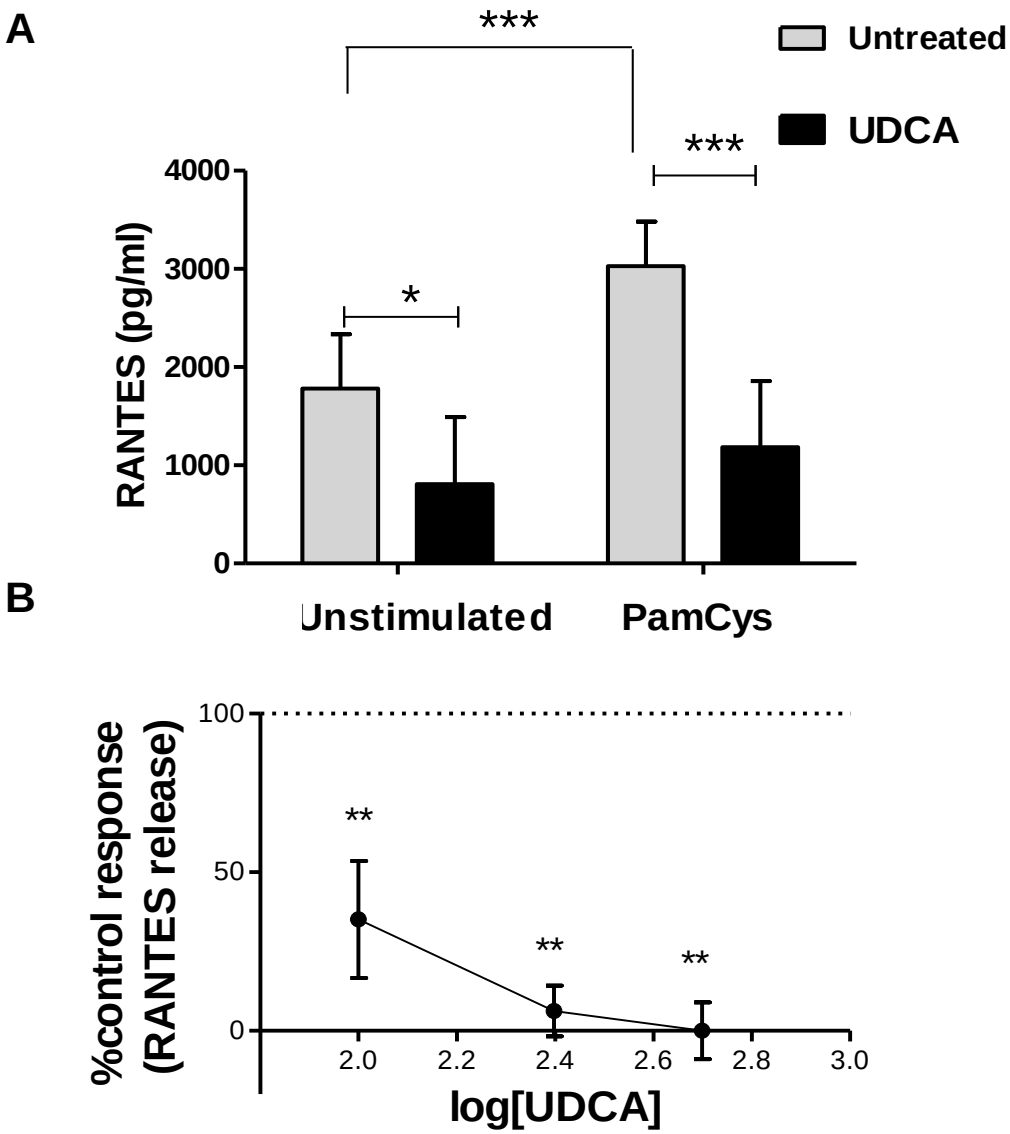


Figure 6.4: UDCA pre-treatment significantly attenuates TLR-2-driven RANTES secretion from T_{84} colonic epithelial cells. A) T_{84} cells grown to 70 - 80% confluency in 96 well plates were exposed to UDCA (250 μM; 30 min) then to Pam3Cys (10 μg/ mL; 24 h) and RANTES levels compared to unstimulated controls (n = 6). **B)** UDCA pre-treatment at concentrations ranging from 100–500 μM significantly reduced RANTES release from LPS-stimulated T_{84} cells. Graph represents relationship of log [UDCA] to percentage control response where control response is 100%. All concentrations used resulted in significantly lower % RANTES release. Calculated EC_{50} was 39 μM (n = 4; *p < 0.05, **p < 0.01, ***p < 0.001).

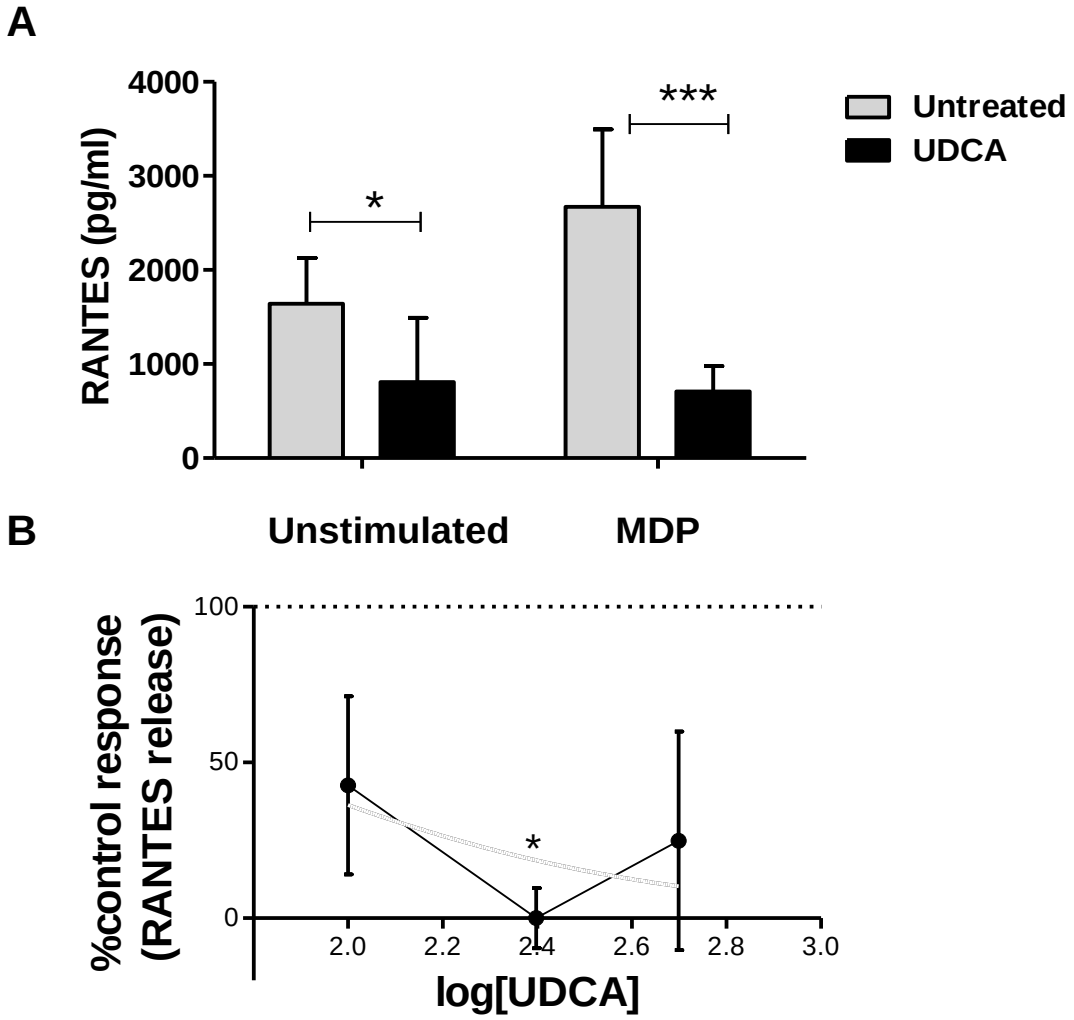


Figure 6.5: UDCA pre-treatment significantly attenuates NOD-driven RANTES secretion from T_{84} colonic epithelial cells. A) T_{84} cells grown to 70-80% confluency in 96 well plates were exposed to UDCA (250 μM; 30 min) followed by muramyl dipeptide (MDP 1μg/ mL; 24 h) and RANTES levels were compared to untreated controls (n = 6). B) UDCA at concentrations from 100 – 500 μM caused significant reduction in RANTES release from MDP-stimulated T_{84} cells Graph represents relationship of log [UDCA] to percentage control response where control response is 100%. Only 250 μM concentration resulted in significantly lower % RANTES release. Calculated EC_{50} was 79 μM (n = 6; *p < 0.01, ***p < 0. 001).

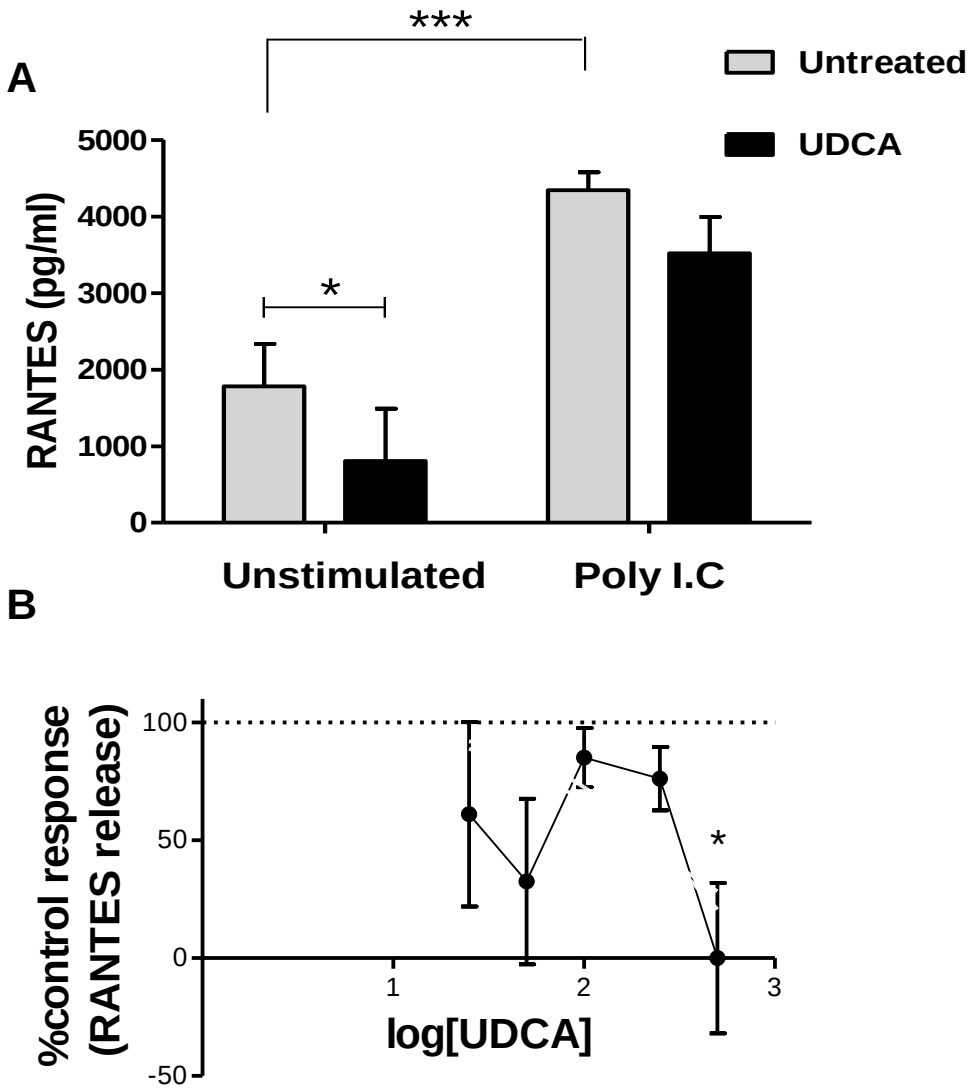


Figure 6.6: UDCA pre-treatment does not alter TLR-3-driven RANTES secretion from T_{84} colonic epithelial cells. A) T_{84} cells grown to 70-80% confluency in 96 well plates were exposed to UDCA (250 μM; 30 min pre-treatment) then to Poly I: C (20 μg/ mL; 24 h) and subsequent RANTES levels were measured and compared to controls (n = 6). **B)** UDCA did not alter Poly I: C-induced RANTES release at concentrations ranging from 25 - 250 μM (n = 12; *p < 0.05, ***p < 0.001). Graph represents relationship of log [UDCA] to percentage control response where control response is 100%. Only 500 μM concentration resulted in significantly lower % RANTES release. Calculated EC_{50} was 178μM (n = 12; *p < 0.05).

6.3.3 UDCA selectively regulates TLR-4-driven IL-8 secretion from T_{84} cells.

Having shown that UDCA pretreatment reduced not only basal RANTES release but also TLR-driven secretion of this pro-inflammatory chemokine, we next turned our attention to another important cytokine, IL-8. Although, UDCA does not appear to alter basal levels of cytokine production, we hypothesized that it may have a role to play in regulating agonist-induced release of the cytokine. Thus, we assessed the effects of UDCA pre-treatment on Poly I: C and LPS stimulated IL-8 release. Poly I: C (20 µg/ mL; 24 h) and LPS (100 ng/ mL; 24 h) induced 1.83 ± 0.07 and 2.9 ± 0.24 fold increases in IL-8 release ($n = 3$, $p < 0.01$), respectively (Figure 6 UDCA pre-treatment (250 µM; 24 h) inhibited LPS-induced IL-8 release to 67.3 ± 15 % of controls ($n = 3$, $p < 0.05$;Figure 6 Panel A). However, UDCA (250 µM; 24 h) was without significant effect on Poly I: C-induced IL-8 release ($n = 4$,Figure 6 , Panel B.

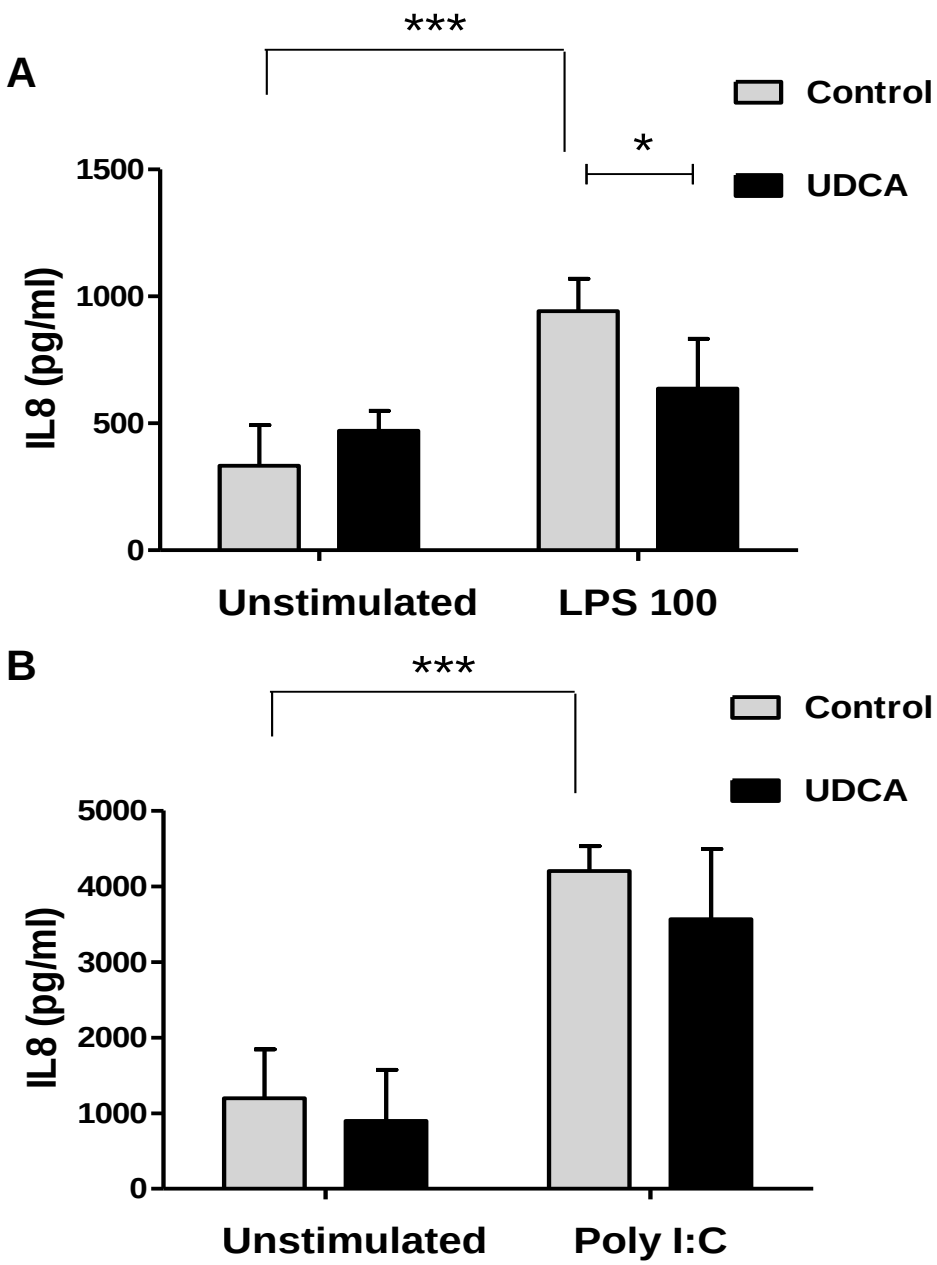


Figure 6.7: UDCA pre-treatment significantly attenuates TLR-4, but not TLR-3 driven, IL-8 secretion from T_{84} cells. T_{84} cells grown to 70–80% confluency in 96 well plates were exposed to UDCA for 30 min before treatment for 24 h with **A)** LPS (100 ng/ mL; n = 3) or **B)** Poly I: C (20 µg/ mL; n = 4. *p < 0.05; ***p < 0.001 compared to cells not treated with UDCA.

6.3.4 The effects of UDCA on TLR stimulated cytokine levels in human colon.

In order to characterise the effects of UDCA in a more physiological setting, experiments were next carried out to examine effects of UDCA on cytokine release from resected human colonic tissue. Firstly, a series of preliminary experiments were undertaken to determine the time course over which resected human tissue remains viable *in vitro* (Figure 6.8). Tissue was mounted in Ussing chambers and bathed in physiological Ringers' solution, aerated with O_2/CO_2 mix and maintained at 37 ^{0}C. Based on tissue conductance measurements, which were stable for 6 h, this time point was selected for subsequent studies (Figure 6.8 6.9). LPS stimulation (100 ng/ mL; 6 h) of human colonic mucosa resulted in increased basolateral RANTES release by 3-fold, compared to unstimulated controls ($n = 3$, $p < 0.001$). This response was abolished by pre-treatment with UDCA (250 μM; 30 min). Similarly, LPS stimulated IL-8 release was increased to 959 ± 22 pg/ mL compared to 435 ± 46 pg/ mL in unstimulated controls ($n = 3$, $p < 0.001$). This response was abolished by pre-treatment with UDCA (250 μM; Figure 6.9).

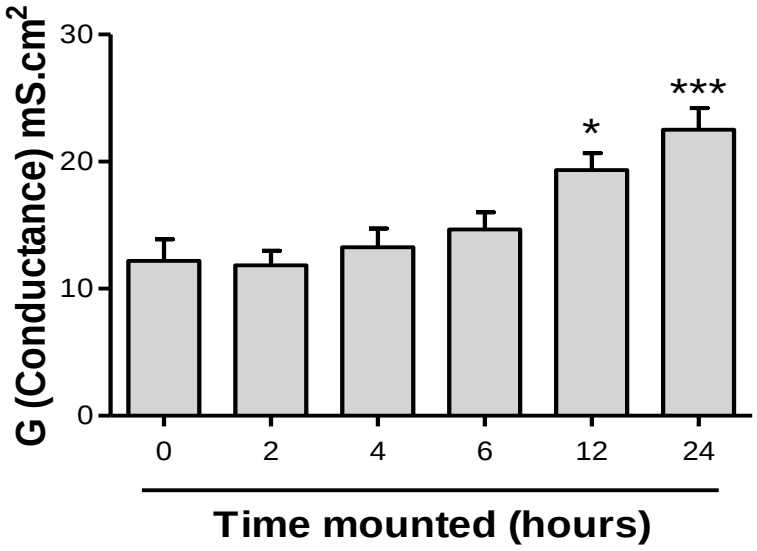


Figure 6.8: Time course of conductance changes across resected human colonic tissue in Ussing chambers. Under the experimental conditions used (37 ^{0}C, bathed in physiological Ringers solution and aerated with 5% O_2/ 95% CO_2 mix), control conductance across resected human colonic mucosa remains unchanged for 6 h, with a rise in conductance (denoting loss of barrier integrity and likely cell death) observed at the 12 and 24 h time-points ($n = 3$, *$p < 0.05$, ***$p < 0.001$). In view of this, all subsequent treatments were carried out for a maximum of 6 h

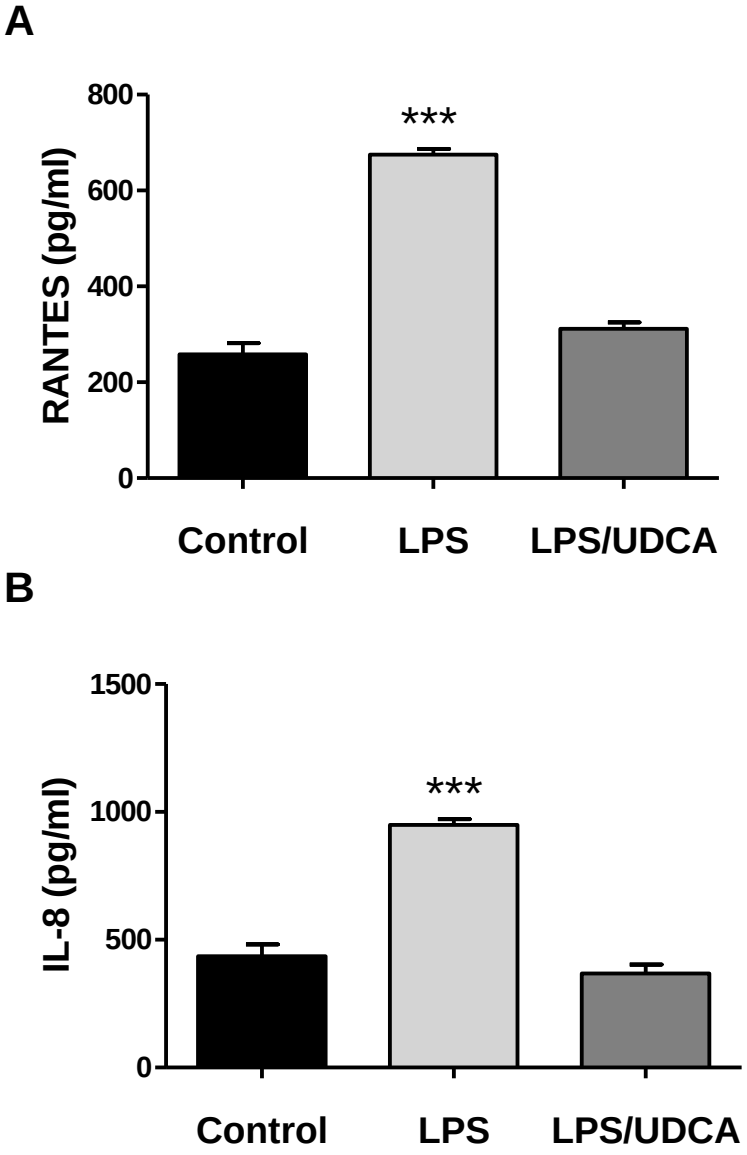


Figure 6.9: UDCA attenuates LPS-driven IL-8 and RANTES release human colonic mucosa *in vitro*. A) Resected human colon was mounted in Ussing chambers and exposed to UDCA (250 μM, 30 min pre-treatment) then to LPS (100 ng /mL; 6 h). RANTES levels were measured and compared to controls (n = 3; *** $p < 0.001$). **B)** Under similar conditions, IL-8 levels were measured (n = 3, ***$p < 0.001$).

6.3.5 The effects of UDCA on epithelial barrier function.

Having gained some insights into the effects of UDCA on cytokine release from colonic epithelial cells, we went on to investigate its effects on other aspects of intestinal innate immunity. Excessive increases in epithelial barrier permeability occur under inflammatory conditions and this breach in barrier function plays an important role in pathogenesis of disease (Taylor, 1998, Hering, 2012). It was thus important to determine if UDCA has the capacity to alter epithelial barrier integrity. One simple measure of monolayer integrity which has been widely validated in the T_{84} cell model is the use of trans-epithelial conductance measurements (Madsen, 1997). In experiments assessing the effects of UDCA treatment on conductance across confluent T_{84} cell monolayers, it was found that UDCA (50 μM –1 mM; 24 h) had no significant effect (cf. Chapter III, Figure 3.6, n = 6). Similarly, UDCA (250 μM; 15 min) did not alter conductance when added bilaterally to rat (Figure 6.10, Panel A, n = 6) or human colonic mucosal tissues (Panel B, n = 10). Interestingly, however, it was noted that when UDCA (100 mg/ kg) was injected intra-peritoneally into C57BL6 mice, subsequent conductance measurements across *ex vivo* colonic tissues 4 h later were 1.78 fold higher than those in control mice while in 6α- MUDCA treated animals, conductance levels were comparable to controls (Figure 6.11).

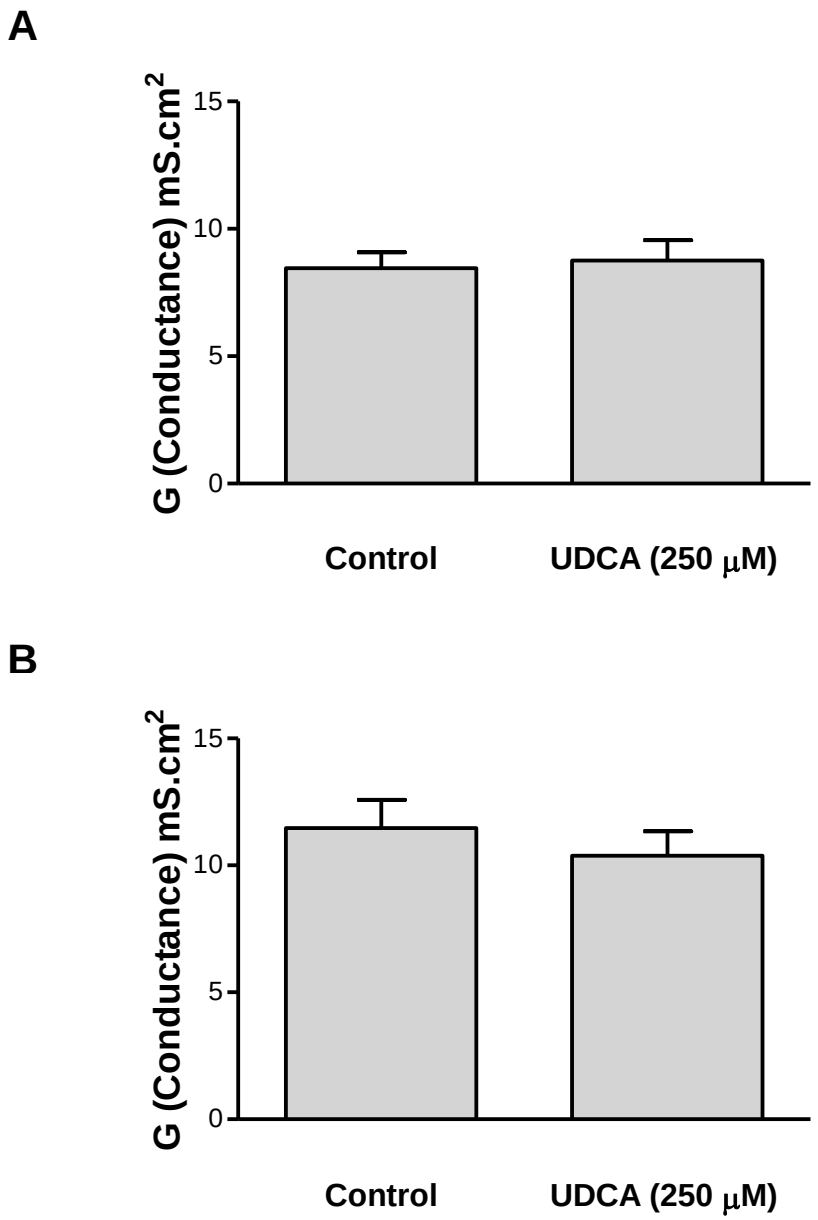


Figure 6.10: UDCA does not alter basal conductance across human or rat colonic tissue *in vitro*. Panel **A** shows that UDCA (250 μM; 30 min) does not significantly alter conductance across voltage clamped rat colonic mucosa (n = 6). Panel **B** shows that UDCA (250 μM; 30 min) does not alter conductance across human colonic mucosal tissues (n = 10).

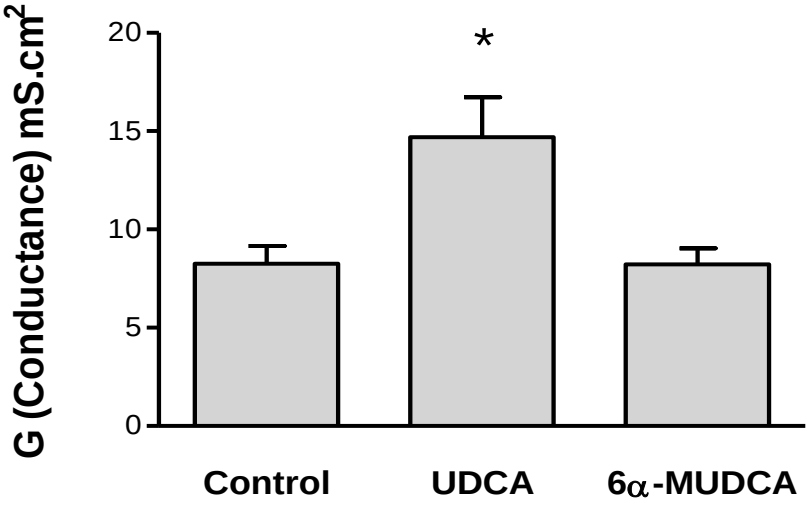


Figure 6.11: UDCA administration *in vivo* increases basal conductance across *ex vivo* colonic tissues. Male C57B6 mice were intra-peritoneally injected with either UDCA (25 mg/kg), 6α–MUDCA (25 mg/ kg) or vehicle control (PBS), and after 4 h were sacrificed. Muscle-stripped colonic mucosa was then mounted in Ussing chambers, TER was measured, and conductance (G) calculated (n = 5, *p < 0.05).Conductance was significantly higher in UDCA-treated animals while in 6α- MUDCA-treated animals conductance was comparable to controls.

6.3.6 UDCA effects on colonic epithelial cell growth and apoptosis.

UDCA has thus far been shown to exhibit marked anti-inflammatory properties by reducing cytokine release from colonic epithelium. However, intestinal innate immunity is a complex process involving multiple variables. Another crucial factor upon which intestinal barrier function depends is the appropriate balance between epithelial cell growth and death. Thus, experiments were conducted to assess the effects of UDCA on epithelial cell viability. T_{84} cells were seeded onto 96 well plates and allowed to attach. Cells were then treated with UDCA (10 μM–1 mM; 24 h). Using a commercially-available cell viability assay (c.f. Chapter II), no significant effect of UDCA was observed at any concentration tested (n = 3; Figure 6.1).

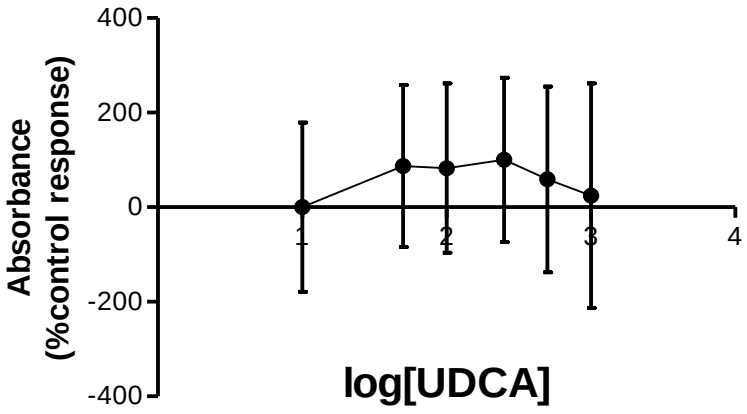


Figure 6.12: UDCA does not alter epithelial cell viability. The effects of pre-treatment with UDCA on cell viability were measured, using a commercially available XTT assay. Cells were treated with UDCA for 1 h, 4 h, 24 h, 48 h and 72 h, at concentrations ranging from 10 μM–1 mM. A representative graph for the 24 h treatment is shown. Graph demonstrates the % control response absorbance for log [UDCA] where control response is o. There was no significant difference noted at any concentration (n = 6).

Potential effects of UDCA on apoptosis were also investigated. Caco2 cells grown on 96 well plates were treated with UDCA 100 μM for 24 h. This was followed by treatment with the apoptotic stimulants cycloheximide (5 μg/ mL) and TRAIL (25 ng/ mL). The % of late-phase apoptotic cells was then measured by flow cytometric analysis (c.f. Chapter II). We found that pre-treatment with UDCA resulted in a significant reduction in the % of apoptotic cells to 34.6 ± 2.0 % in UDCA-treated group, compared to 63.7 ± 9.0% in cells exposed to TRAIL/CHX alone. The values for % apoptotic cells in the absence of any treatments was 4.5 ± 0.3% (Figure 6.1, n = 6).

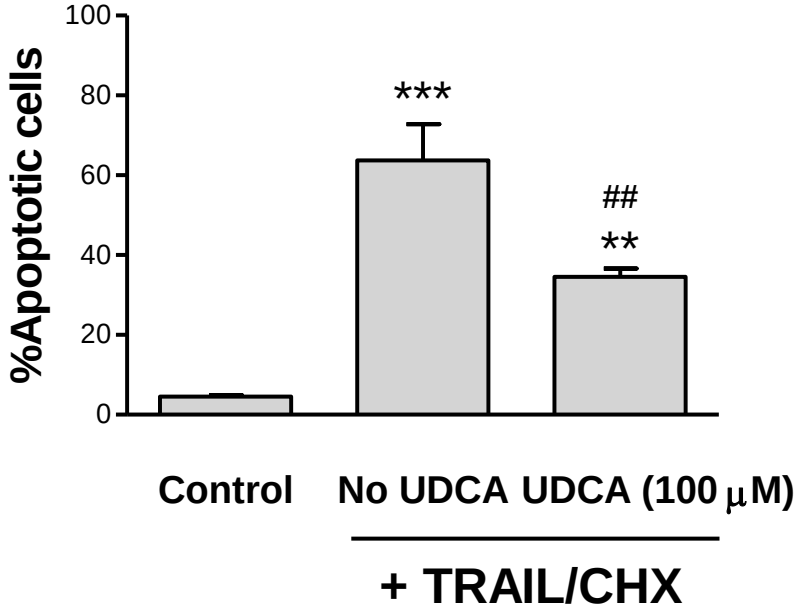


Figure 6.13: UDCA pre-treatment attenuates TRAIL/CHX-induced apoptosis in Caco- 2 cells. Cycloheximide (5 μg/ mL) and TRAIL (25 ng/ mL) were used to induce apoptosis in Caco- 2 cells and the % of apoptotic cells was measured by flow cytometry (n = 6, **$p < 0.01$, ***$p < 0.001$ compared to untreated cells; $^{##}p < 0.01$ compared to cells treated with TRAIL/CHX alone).

6.4: Discussion

Perturbed homeostasis, release of cytokines and disruption of cellular apoptotic pathways are critical determinants in the pathogenesis of IBD. TLR signalling plays an important role in maintaining intestinal homeostasis, with altered expression of TLR in IBD being first described 10 years ago (Cario, 2010). Since then, studies from many groups have led to the current concept that TLRs are key regulators of innate intestinal defense (Lavelle, 2010). Recent findings in murine models of colitis have helped to reveal the mechanistic importance of TLR dysfunction in IBD pathogenesis (Fukata, 2005, Ocampo, 2012, Hardenberg, 2012). It has become evident that environment, genetics, and host immunity form a multidimensional, and highly interactive, regulatory triad that controls intestinal TLR function. Imbalanced relationships within this triad may promote aberrant TLR signalling, thereby contributing to inflammatory processes in the intestine (Cario, 2010). Data presented in this chapter suggest that UDCA may have the ability to interfere with this regulatory triad by virtue of its ability to inhibit TLR-induced cytokine production.

Multiple factors regulate TLR signalling and expression at different anatomical sites within the intestine. Thus, in the current studies it was initially important to ascertain which TLR ligands regulate cytokine release from our colonic epithelial model. We found that the T_{84} cell line expresses relatively high basal levels of RANTES and lower levels of IL-8 and IL-6. Previous work has shown similar levels of basal RANTES and IL-8 release in T_{84} and HT-29 cells (Chen, 2006, Nandakumar, 2009). It is also now well-established that RANTES is released upon activation of normal T cells and that it exerts a range of pro-inflammatory activities (Alcendor, 2012; Hu, 2012; Tawadrous, 2012). Examples of RANTES activities include recruiting leucocytes into inflammatory sites and inducing proliferation and activation of certain natural-killer (NK) cells (Maghazachi, 1996). Importantly, it has been noted that mucosal expression of RANTES is increased in biopsies from patients with either CD or UC (McCormack, 2001), and that RANTES expression is also significantly up regulated in colonic epithelial cells of mice susceptible to colitis, such as Foxo4-null mice (Zhou, 2009). Thus, RANTES could provide a good target for developing new therapeutics for intestinal inflammation. Indeed, our current findings in T_{84} cells and

normal colonic mucosa indicate that UDCA may exert anti-inflammatory effects by reducing basal RANTES levels.

The effects of UDCA on basal cytokine secretion appear to be relatively cytokine specific, since even though it reduced RANTES release, it did not significantly alter basal IL-6 release from T_{84} cells. However, preliminary studies using TLR ligands also failed to show significant stimulation of IL-6 release from T_{84} cells. It should be noted that given the low basal levels of IL-6 present in the cell line, changes in response to UDCA may be difficult to detect. The low basal levels and lack of stimulation of IL6 release in our studies correlate with previous work investigating IL-6 levels in T_{84} cells (Ledesma, 2010). However, previously published data from other systems are not conclusive regarding the effects of UDCA on IL-6 levels. For example, in a clinical trial where UDCA (1.5 mg/ kg) was used to treat patients with heart failure, levels of TNF-α and IL-6 were found to be unchanged, or even increased, compared to placebo (von Haehling, 2012) In contrast, UDCA (50 μM) reduced IL-6 production in an *in vitro* model of ethanol-induced cytotoxicity using Hep G2 liver cells (Neuman, 1998). Interestingly, further studies by van Milligen *et al*, showed that baseline serum levels of IL-6 were normal and IL-8 levels were increased in patients with primary sclerosing cholangitis, but that neither of these parameters were significantly altered by UDCA therapy (van Milligen, 1999). Our current studies also found that UDCA had no significant effect on basal IL-8 levels released from cultured colonic epithelial cells. Again, it is possible that this apparent lack of effect could be due to the relatively low basal IL-8 levels released from T_{84} cells (Ledesma, 2010, Zheng, 2008), which could make it difficult to detect UDCA-induced changes. Notably however, our studies did reveal that UDCA significantly inhibited agonist-stimulated IL-8 and RANTES release from both cultured T_{84} cells and human tissue. Thus, the effects of UDCA on epithelial release of these cytokines might be particularly important in the context of IBD, when elevated levels of agonists for TLRs occur in the mucosa.

A particularly intriguing finding of the current studies was the apparent differential effects of UDCA on TLR-stimulated cytokine release. While, UDCA inhibited responses to agonists acting at membrane TLR-2 and TLR-4, it had no effect on

those to activation of endosomal TLR-3. This observation may help to define the mechanisms by which UDCA has its effects. This differential effect on TLR-4 and TLR-2 stimulation has particular relevance in IBD where both of these TLRs become up regulated (Frolova, 2008; Szebeni, 2008).

In terms of looking at the mechanism involved, it has been well established that activation of membrane TLR-2 or -4 stimulates an MyD88-dependent pathway (Takeda, 2004) leading to activation of NFκβ (Toubi, 2004). Indeed, previous studies have shown that taurine-conjugates of UDCA can oppose the effects of DCA on NFκB activation in HT-29 cells (Lee, 2004, Muhlbauer, 2004). There is thus a need to further investigate the potential role of NFκB inhibition in mediating the effects of UDCA on colonic epithelial cells.

We have also shown here, that UDCA may reduce NOD-2-stimulated cytokine release. Polymorphisms in the NOD-like receptor family confer genetic risk for IBD in a mouse population (Natividad, 2012). MDP, the NOD ligand used in these experiments, has been shown to induce experimental colitis in rabbits and interestingly, similar to what is seen in some patients with IBD, an associated pericholangitis (Kuroe, 1996). The implications of this are that UDCA, already known to ameliorate immune-mediated cholestasis, may also play a role in inhibiting MDP-mediated colonic inflammation. However, more work in disease models will be necessary to ascertain if this is truly the case.

In order to assess UDCA effects on barrier integrity, trans-epithelial conductance was used as a measure of epithelial barrier integrity (Taylor, 1998). Acute treatment with UDCA was found to have no adverse effects on epithelial monolayer integrity. This contrasts with the effects of other dihydroxy BAs, such as DCA and may be reflective of the unique physicochemical properties of UDCA. BA lipophilicity is believed to be the most important determinant of cytotoxicity, although this relationship is still not well-characterized. Roda *et al*., have demonstrated the order of lipophilicity amongst BAs to be DCA > CDCA > UDCA (Roda, 1990), which corresponds to their relative cytotoxicities (Perez, 2009), pro-secretory activity (Keely, 2007) and pro-inflammatory responses (Munch, 2007). Furthermore, Gilmer

et al recently studied relationships between lipophilicity and cytotoxicity using multiple derivatives of UDCA in an oesophageal cell line and found them to have no adverse effects on cell viability (Shiraki, 2005; Sharma, 2010). This is in keeping with the findings of multiple other studies assessing UDCA effects on cell viability (Invernizzi, 1997). Future studies could assess whether UDCA might have protective effects against agents which reduce TER in the colonic epithelia such as IFNγ and TNFα.

In addition to their cytotoxic effects, BAs are known to have important roles in regulating cell growth and apoptosis. Previous studies on the effects of UDCA and TUDCA on apoptosis in cholangiocytes share a number of common features with our current findings. For example, administration of UDCA or TUDCA prevented apoptosis in bile duct ligation-vagotomized rats (Marzioni, 2006). Shiraki et al noted that both DCA and UDCA reduced proliferation in of HT-29 colon cancer cells (Shiraki, 2005). Other *in vitro* work in colonic cell lines indicates that UDCA can suppress epithelial cell growth and mitogenic signalling through inhibiting the mitogenic activity of receptor tyrosine kinases, such as the EGFr, through increased receptor degradation (Feldman, 2009). In contrast, in the current studies, we found no effect of UDCA on basal T_{84} cell growth as determined using the XTT assay.

Colonic epithelial barrier function is determined by a balance between cell growth and apoptosis. Previous data demonstrate cytoprotective properties of UDCA (Amaral, 2009). Indeed, our data further support the idea that UDCA exerts cytoprotective effects by showing that it reduced the number of apoptotic cells under CHX/TRAIL-stimulated conditions. Such anti-apoptotic effects of UDCA are in direct contrast to other dihydroxy BAs (Saeki, 2012). For example, it has been previously shown that cytotoxicity associated with DCA is due to the induction of apoptosis, by a process requiring caspase-3 activity (Ignacio, 2011, Glinghammar, 2002). However, this is not always the case, since DCA can also induce cell death by necrosis (LaRue, 2000). As mentioned in the introduction to this chapter, there have been several studies suggesting that it is the hydrophobicity of BAs that determines their pro-apoptotic activity (Powell, 2001). The activation of death receptors by BAs invariably signals the mitochondrial pathway of apoptosis in type II cells, such as

hepatocytes. Hydrophobic BAs also partially activate death-receptor-dependent survival pathways, such as the NF-κB signalling pathway. In fact, Akt-dependent NF-κB activation is required for BAs to rescue colon cancer cells from stress-induced apoptosis, leading to resistance to chemotherapy and radiation (Shant, 2009).

Whatever the mechanism underlying its anti-apoptotic actions, our data suggest that UDCA may be a useful agent in preserving barrier function under conditions of inflammation. However, when considering such a use for UDCA, one must also consider the long-term risks of inhibiting apoptosis in terms of potential development of dysplasia and cancer.

Another potential limitation to the use of UDCA as treatment for IBD could be that bacterial degradation to LCA in the colon could limit its therapeutic activity. Thus, we propose that a metabolically stable analogue of UDCA, such as 6α-MUDCA could provide a more efficacious anti-inflammatory response. Future studies could be carried out in mouse models of colitis to determine if this is the case.

In summary, the data presented in this chapter suggest that UDCA can exert anti-inflammatory and cytoprotective effects on colonic epithelium. UDCA reduces TLR-stimulated cytokine release and maintains epithelial barrier integrity through inhibition of apoptosis. Although further studies are required to elucidate the mechanisms involved, our data suggests that UDCA should be beneficial in treatment of IBD. Esmaily *et al*., have recently shown that UDCA treatment concomitantly with silabinin is protective in a rat experimental model of colitis (Esmaily, 2011). However, even though UDCA has been widely used in patients with PSC and concomitant UC, it has not been reported to exert substantial anti-inflammatory effects in the colon. We hypothesise that this could be due to bacterial metabolism of UDCA to LCA, which would limit its therapeutic efficacy. Indeed, it has been proposed that since UDCA treatment dramatically increases colonic LCA levels, this may underlie the adverse effects and reduced survival observed in PSC patients treated with high dose UDCA (Sinakos, 2010). Thus, future studies examining the effects of metabolically stable analogues of UDCA in animal models of IBD could lead to the development of a new approach to treat these diseases.

Chapter 7

General Discussion

The work described in this thesis provides novel insights into the effects of UDCA, a widely used therapeutic BA, on colonic epithelial function. We have discovered new effects of UDCA on both colonic secretory function and innate immunity, as demonstrated by studies in cells, an animal model, and resected human tissues. While previous work has established that UDCA, unlike other dihydroxy BAs, lacks secretory actions in the colon (Mekjian 1971, Rahban, 1980, Gelbmann, 1995), we present data to show that it also exerts potent anti-secretory actions *in vitro*.

Intriguingly, we found that, in contrast to such anti-secretory actions *in vitro*, UDCA potentiated secretagogue-induced responses *in vivo*. We propose that such effects are due to metabolism to LCA, and may underlie the diarrhoeal side effects that can occur when UDCA is used therapeutically (Alberts, 2005). This is supported by data showing that 6α-MUDCA, a 6-methylated derivative of UDCA that is completely resistant to bacterial dehydroxylation (Roda, 1994) did not alter caecal LCA levels and significantly inhibited secretagogue-induced responses across voltage-clamped colonic tissues. These findings suggested to us that 6α–MUDCA could have several advantages over its mother compound, UDCA, as a targeted therapeutic for colonic disease. Cytotoxicity data in cells and resected tissue has proven it safe thus far; however further studies in animal disease models would help to clarify side effects or toxicity issues.

While 6α-MUDCA and UDCA have been demonstrated to exert potent effects when directly applied to the human colonic epithelium, it is unclear what the bioavailability might be upon oral administration to humans. One of the disadvantages of 6α–MUDCA is that, even though it is not metabolised by dehydroxylation, like other BAs, it too would be expected to become concentrated in the enterohepatic circulation, thereby lowering its delivery to the colon and increasing its propensity for systemic side effects. Thus, to further enhance efficacy, strategies using a colonic drug delivery system to enhance delivery to the desired target could be utilized. Such strategies would bypass the major absorptive pathways of the ileum (Assifaoui, 2013, Xiao, 2012). Slow release polysaccharide based matrices (Rehman, 2013, Mihaela, 2013) and nanoparticle technology, currently under development (Pertuit, 2007), offer the prospect of direct treatment at the disease site and, consequently,

lower dosing and reduction of systemic side effects. It is interesting in this context that the aim of this strategy is to bypass the enterohepatic circulation, which is conversely so advantageous in delivering bile acids for therapy in liver disease (Kubota, 1988).

As mentioned above, the use of 6α-MUDCA prevents formation of LCA in the colon. The consequences of this are still unclear and could also be a target for future investigation. Recently, it has been shown that activation of TGR5 (upon which LCA acts) is protective against production of pro-inflammatory cytokines in Crohn's Disease (Yoneno, 2013). LCA is also known to down-regulate NFκ B in colon cancer cell lines (Yoneno, 2013, Sun, 2008). Given these anti-inflammatory effects, this begs the question as to whether the metabolism of UDCA to LCA could in fact be beneficial to long-term intestinal health. ELISA studies similar to those performed with UDCA and 6α-MUDCA assessing the direct effect of LCA on cytokine production from colonic epithelium would be helpful, along with further investigations into the role of TGR5 activation (if any) in mediating the effects of UDCA and its metabolites on intestinal inflammation. On the other hand, there is also substantial evidence to suggest that LCA may be detrimental to colonic health, especially in terms of increasing colon cancer risk. LCA has been shown to stimulate invasion and proliferation of colonic epithelial cells through a number of oncogenic signalling pathways (Kozoni, 2000). Thus its carcinogenic potential must be weighed against its potential anti-inflammatory effects.

Furthermore, greater understanding of the role of bacterial degradation as demonstrated by the dehydroxylation of UDCA to LCA may help further enhance the efficiency of future drug delivery methods (Vadlamudi, 2012). As alluded to above, there may be numerous beneficial effects mediated by BAs in the colon. Probiotics could potentially be used to increase relative UDCA levels in the colon to enhance these effects. There have been recent studies in mouse models assessing the effects of probiotics and prebiotics on total faecal BA levels, and there is some discussion as to whether faecal BA measurements could be used to assess the effects of these dietary measures on the colonic environment. However, larger powered studies and more stringent measurements, including that of individual BA

levels, are needed to make more solid conclusions on the effects of probiotics on bioavailability of therapeutic BAs in the colon (Kuo, 2013).

Our work using a taurine conjugate of UDCA has provided some insights into how UDCA may exert its actions, and suggests that UDCA must enter the cells before exerting anti-secretory actions. We also found that upon entry into colonic epithelial cells, UDCA rapidly inhibits activity of some of the key transport proteins (the Na^+/K^+ ATPase pumps and basolateral K^+ channels) required for Cl^- secretion, without altering localization or abundance of these proteins. Further studies of the effects of UDCA on KCNQ1, TMEM16A and NKCC1 could provide further detailed insight into the mechanisms involved with the anti-secretory effects of UDCA. Work presented here suggests that UDCA has little effect on either ENaC-related fluid absorption or SGLT-1 activity, but the colon also absorbs NaCl via the coupled operation of apical NHE3 and Cl^-/HCO_3 exchanger SLC26A3 (DRA) (Talbot, 2010). In future studies, it is thus important to further assess the effects of UDCA on these exchanger proteins which play important roles in regulation of colonic Na^+ and fluid absorption.

Further investigation of the molecular mechanisms by which UDCA exerts its anti-secretory actions should also be interesting. These could include investigating the potential mechanism by which UDCA inhibits the activity of Na^+/K^+ ATPase pumps and K^+ channels. Several lines of research could be pursued. For example, UDCA may act by altering the generation of signals that activate these transport proteins, or alternatively could alter their association with regulatory proteins. As mentioned previously, several regulatory proteins are known to bind to the Na^+/K^+ ATPase and to modulate its activity, including FXYD proteins (Garty, 2006), translationally-controlled tumour protein (Jung, 2004), and modulator of Na^+/K^+ ATPase (Mao, 2005). The role of such proteins could be investigated by using immune-precipitations, confocal microscopy, or siRNA.

Our studies suggest that the effects of UDCA on transport protein activity may, in fact, be related to stimulation of Ca^{2+} influx, which in contrast to the pro-secretory actions of Ca^{2+} released from intracellular stores, appears to be anti-secretory. This observation would suggest that elevations in different pools of Ca^{2+} within the cell

can have drastically different consequences for epithelial secretory function. This is a novel and highly interesting finding. It would be interesting to assess whether the down-regulation of basolateral transport protein activity by UDCA is distinct from, or if it is a related process, to influx of extracellular Ca^{2+}. There is some precedence in the literature for this differential pooling of Ca^{2+} (Keely, 2003, Epple, 2001). In fact, it could be hypothesized that the anti-secretory effects of UDCA are mediated by reduced Ca^{2+} release from intracellular stores as a consequence of the preferential activation of the MAPK-dependent pathway of Ca^{2+} influx. These pathways could also represent new targets for development of future anti-secretory, anti-diarrhoeal agents.

While studies in cultured epithelial cells have yielded some intriguing insights, our work also suggests that responses to UDCA in human tissue are more complex. In addition to direct anti-secretory actions on colonic epithelial cells, UDCA can also induce transient Cl^- secretory responses through activation of the enteric nervous system. However, the interplay between the environment and the colonic epithelium is complex and further studies assessing the effects of other key factors, such as those released by paracrine cells and intestinal immune cells would yield valuable insights. It would be interesting to pursue the neuronal effects of UDCA and to further assess what type of nerves could be involved. Thus, some interesting questions raised by our work to date include, does the neuronally-mediated pro-secretory effect that occurs via nerves reduce the potential for UDCA analogues as anti-diarrhoeals? Furthermore, UDCA appears to activate the muscarinic nerve receptor as demonstrated by its lack of effect in the presence of atropine. In addition, what are the effects of UDCA on other cells found within the colonic mucosa? There is a possibility to build on the T_{84} reductionist model by using co-culture experiments to determine how UDCA affects epithelial interactions with cells, such as fibroblasts, macrophages, IELs, etc. (Toumi, 2003, Nishitani, 2013, Taylor, 1997, Beltinger, 1999).

Data presented in this thesis also suggest a potentially protective role for UDCA in intestinal inflammation by preventing pro-inflammatory cytokine release and epithelial apoptosis. Dysregulation of these processes are key factors underlying the

pathogenesis of IBD, and our findings therefore suggest potential clinical applications for stable analogues of UDCA in treatment of colonic inflammation or in prophylactic treatment for IBD-related colorectal cancer (Byrne, 2010, Serfaty, 2010; Pardi, 2003).

Future studies could be undertaken to delineate the precise mechanisms of UDCA down-regulation of cytokine secretion in the colonic epithelium. Such studies could be designed to more deeply investigate the regulation of inflammatory cytokine production by epithelial cells and there is great potential for the use of new tools for studying regulation of gene expression both at the transcriptional and posttranscriptional levels. There are a number of possible modes of transcriptional regulation by UDCA which could mediate its effects on cytokine production. These include transcriptional repression, production of a blocking factor, and potentially epigenetic modifications. There is also the possibility that these effects are due to posttranscriptional mechanisms, through effects on microRNA (miRNAs) production or through altered proteasomal degradation. Thus, an important next step in these studies it to measure changes in mRNA levels of various cytokines or inflammatory mediators in response to UDCA treatment. Future work could then also focus on investigation of miRNA involvement in regulation of epithelial responses to BAs. Recent studies in rat liver show that miR-21, may play a significant role in modulating proliferation and cell cycle progression genes and that interestingly, miR-21 is induced by UDCA in regenerating rat liver (Castro, 2010). There has also been a link between liver cell apoptosis and miR-34a/SIRT1/p53 signalling, which can also be modulated by UDCA (Castro, 2013). Thus, it would be interesting to investigate if UDCA may also modulate miRNA expression in the colonic epithelium to regulate cytokine production and barrier function.

While experiments carried out in cultured colonic epithelial cells and isolated human tissue are valuable tools in evaluating the potential efficacy of new therapies and are essential in elucidating molecular mechanisms underlying regulation of secretion and inflammation, future directions for this work should include the use of animal models of disease and eventually placebo controlled clinical trials. To this end, an analysis of the effects of UDCA could be undertaken in a number of IBD disease models, including the dextran sulphate (DSS) or TNBS-induced colitis models (Wirtz., 2007),

IL-10 knockout mice (Kang, 2013). Studies in models of diarrhoeal disease would also be useful, such as the mouse model of ova-albumin-induced allergic diarrhoea (Duncker,2012), or *C. difficile* infection (Sadighi, 2013) given the prevalence of this problem in health care facilities and the rising incidence of refractory cases (Ananthakrishnan, 2011). There are of course various advantages and disadvantages attached to each of these models. The DSS model for instance is widely used and is well-appreciated for its simplicity, offering many similarities to human IBD without huge expense or technical complexity (Perse, 2012). It is however somewhat of a “blunderbuss” or “destroy all in its path” approach and does not account for subtle variations in what is a finely regulated system which can lead to drastically different clinical manifestations. Thus, use of this model may need to be modified to determine effects of UDCA treatment in conditions of milder disease. The most widely used of the gene-targeted models is the IL-10 deficient mouse. IL-10 knockout mice develop a chronic inflammatory bowel disease which shares many characteristics of human IBD (Davidson, 1996). The advantages of the IL-$10^{-/-}$ model is that it is a well-established Th1-mediated model of trans-mural colitis, which is responsive to various therapeutic agents. However, disease onset and severity are variable and may require months to develop. The T cell transfer model is the best-characterized model of colitis induced by disruption of T cell homeostasis and causes inflammation in both large and small bowel making it useful for studies of Crohn’s disease in particular. However, the techniques are complex and expensive and there is a possibility that mice may develop little or no disease (Ostanin, 2009).

The effects of any new therapeutic agent must, however, be measured in patients in order to truly realise their potential. Already our research has shown safety and efficacy of UDCA in cell models, animal models, and in human colonic tissue. Further safety and efficacy analyses in animal models of disease could then logically be translated to assessment in control and disease patient samples as part of a series of controlled trials, with the ultimate goal of examining the effects of 6α–MUDCA, both clinically and biochemically.

In conclusion, it has been shown that UDCA has predominantly anti-secretory and anti-inflammatory effects in the colonic epithelium and with modification of its

structure and improved drug delivery methods, there is potential for these beneficial effects to be harnessed for therapeutic effect. Ultimately, it is our hope that the work carried out for this thesis contributes to our evolving knowledge of the role of BAs in intestinal homeostasis and will yield new molecular targets for the development of more effective and specific therapeutic tools for patients with intestinal disease.

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