Modulation of regulatory of mechanisms of intestinal ion secretion by TNFa and NPY

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Chapter one

General introduction

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Introduction

The transport of water, electrolytes and nutrients substrates is a key function of the intestinal tract. The amount of water present in the intestine is of great importance for a variety of processes and is therefore closely regulated. Water is necessary to maintain fluidity of the lumen for digestion and absorption. Furthermore, water is necessary to serve as a medium for bringing digestive enzymes into contact with food particles and to allow the diffusion of digested nutrients to the epithelial cells for absorption. The intestinal tract is loaded daily with nine liters of fluid. The intestine itself supplies a significant proportion of the daily fluid load to subserve the above-mentioned functions. Most of the fluid is absorbed by the small intestine. The jejunum and ileum absorb four and three and a half liters of fluid respectively, leaving 1500 ml for absorption in the large intestine, or colon. The colon regulates the final volume and electrolyte composition of the stool, leaving about 100 ml to be excreted in the stool.

Despite the large volume of water involved in the daily water movement across the intestinal wall, water transport is a passive process. (Non-) electrolytes, that are transported by active processes, are the driving force for water transport into and out of the intestinal lumen. The actively transported solutes, mainly electrolytes, cause water to move, so that iso-osmolarity between luminal and tissue compartments is maintained. Thus, water transport is indirectly dependent on the regulation of electrolyte transport. Endogenous as well as exogenous factors can affect active electrolyte transport in the epithelium. In addition, nutrients and non-absorbable substances also play an important role by their osmolarity or the osmolarity of their metabolites in the lumen, thereby affecting passive water transport.

Besides transepithelial water transport, motility plays an important role in the digestion and absorption of ingested nutrients. The motility of the intestine is a result of the contractions and relaxations of the external longitudinal and circular muscle layers in the intestinal wall that stand under neuronal control. The gastrointestinal tract is unique in having an intrinsic nervous system, the enteric nervous system, ENS. It plays an important role in the control of motility, blood flow, water and electrolyte transport.

Neurotransmitters released by enteric nerve endings can directly bind to epithelial cells and modulate electrolyte transport, as has been described for acetylcholine and VIP. However, neurotransmitters may also have indirect effects on ion transport through their ability to release secondary mediators from endocrine or immune cells (e.g. Substance P can induce the release of histamine from mast cells [1]). Neural regulation appears to be important in maintaining the ion transport tone of the intestine. Mental or physical stressors can influence this tone leading to increased secretory activity. This regulation may depend on vagus activity. However, the ENS plays a significant role and it is considered to have an intrinsic motorprogram. For instance, the timely integration of intestinal electrolyte transport and motility is coordinated by the enteric nervous system. In jejunum of humans and other mammals the muscular contraction correlates temporally with increased mucosal ion

transport which appears to be a coordinative function of the submucosal plexus [2]. Immunohistochemal studies have provided insight into the transmitters and neuropeptides present in the neurons of the ENS. It has even been demonstrated that the expression of transmitters is polarized, i.e. oral extension contains more VIP, whereas aboral extension contains more cholinergic transmitters [3].

Besides neural regulation, immune cells play an important role in regulating electrolyte transport in the intestine [1, 4, 5]. Immune mediators released from immune cells like mast cells and monocytes can either directly or indirectly regulate the intestinal ion transport. Among these immune mediators are histamine, tumor necrosis factor α (TNFα), interferon γ (IFN γ) and interleukins. The immune modulators may affect the intestinal epithelium directly or indirectly via release of subepithelial mediators like prostaglandins.

Enterochromaffin cells respond to changes in luminal contents and release mediators that influence local epithelial cells (paracrine regulation) [4]. In addition, epithelial cells themselves also release substances that modify epithelial function (autocrine regulation).

There is significant cross-talk between the regulatory mechanisms. Strong interactions exist between neuronal signaling and the immune cells in the lamina propria. Mast cell mediators appear to recruit the enteric nervous system secondarily to propagate the effect of mast cell mediators on electrolyte transport [1, 4, 5]. This complex control mechanism underscores the physiological importance of this response and allows for fine-tuning of fluid transport in the intestine.

Previous studies in our laboratory revealed that in rat intestinal tissue, the addition of carbachol, a stable acetylcholine derivative, results in an increase in permeability [6]. However, this increase was not evident in all experiments. It was proposed that the variable results may be related to the neurohormonal 'state' of the epithelium. E.g. it is possible that mediators, which are released by immunocytes in the lamina propria may influence the response to carbachol. Immunocytes can release cytokines, as described above. The cytokines TNFα and IFNγ have been described to affect the intestinal permeability when applied in high concentrations [7-12]. We considered the possibility that differing amounts of TNFα in the preparation may have an influence on the response to other messengers. Therefore we decided to study whether exposure of intestinal epithelial cells to the cytokine TNFα before application of a neurotransmitter could change the effect of that transmitter. It turned out that TNFα had a synergistic effect on the carbachol and histamine induced secretory responses in cell lines as well as in isolated distal colon of the mouse. At the concentrations used the permeability was not affected in the cell line.

To begin with we present an overview of the anatomical substratum and physiological processes involved in transepithelial transport in the paragraphs below.

1. Anatomy

The wall of the small and large intestine consists of four layers: the mucosa, submucosa, muscularis and serosa, as illustrated in Figure 1. The mucosa is divided into three sublayers. The surface epithelium contains enterocytes, Paneth cells, endocrine cells and goblet cells. The lamina propria contains regulatory cells like nerve cells and immune cells. The third layer is a thin smooth muscle layer, the muscularis mucosa.

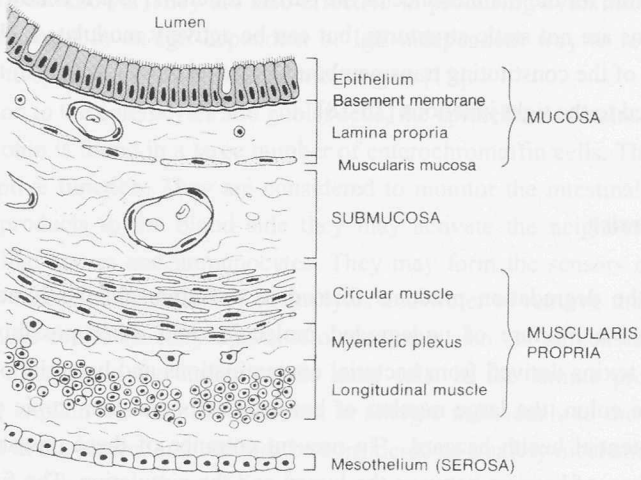

Figure 1. Generic organization of the intestinal wall. Adapted from [13].

The mucosa is supported by the submucosa, consisting of connective tissue, with blood and lymph vessels, nerve plexus (plexus of Meissner) and regulatory cells, including lymphocytes, mast cells and macrophages. The submucosa rests on the muscularis propria (tunica muscularis externa) and consists of two layers of circular and longitudinal muscles with an intermediate nerve plexus (plexus of Auerbach). The outer layer is the serosa, consisting mainly of connecting tissue.

It is thought that absorptive and secretory processes are carried out by two separate epithelial cell types. The absorptive cells are present only on the villus of the small intestine and on the surface of the large intestine. The secretory cells are known to be present in the crypts. Electrolyte transport and related transport processes are possible because the epithelial cells are polarized. This structural specialization allows cells to transport electrolytes in a vectorial or transcellular manner, generally involving a form of active transport on the one side, and passive transport on the other. Specific transporters are located either in the apical or basolateral membrane. The tight junctions are located near the apical pole of the cell. The mucosal and serosal sides of the cells are separated by the tight junctions, which form the barrier between the apical and basolateral sides of the epithelium. They also form a barrier

for diffusion of membrane proteins between apical and basolateral cell membranes. The tight junctions largely determine the transepithelial conductance for electrolytes. They are normally cation selective and are considered to have pores for non-electrolytes allowing the (very slow) passage of small molecules up to about 200 D. They restrict the back-diffusion of transported solutes. Although transepithelial resistance is usually considered a good indicator for transepithelial permeability, there are circumstances that a rather large increase in permeability for (non-) electrolytes does not show in a lowered resistance [14]. Recently, it has been shown that tight junctions are a product of an overall polarizing process and that they are responsible for the maintenance of the barrier function [15]. It is now recognized that the tight junctions are not static structures, but can be actively modulated, either directly by phosphorylation of the constituting transmembrane proteins or by changes in the cytoskeleton which is anchored to the tight junctions [16-18].

2. Epithelial barrier

In addition to the degradation products formed in the stomach, the lumen of the small intestine contains a mixture of undegraded molecules and other possibly immunogenic compounds and toxins derived from bacterial contaminations and bacterial overgrowth from the colon. In the colon, the large number of bacterial cells and their toxic products can be considered a potential health hazzard. To prevent entrance of these unwanted compounds there are a number of barriers between the lumen and the circulation. The first barrier is the mucus layer. The mucus in the lumen serves as a protection against bacteria and other invasive products. Mucus is synthesized by cells in the epithelium, the goblet cells. Mucus can act as a barrier by behaving as a viscous hydrated gel. The mucus molecules (mucins) possess carbohydrate groups that have the potential to bind bacterial surfaces and inhibit direct epithelial-bacterial binding which could otherwise lead to surface colonization. In addition, due to the extensive glycosylation, mucins can cross link bacteria and therefore serve to aggregate bacteria. Exposure to threats like bacteria or toxins results in a reflexive secretory release of mucins.

The epithelial surfaces in the gastrointestinal tract are loaded with the immunoglobulin secretory IgA. Secretory IgA acts as a barrier to antigens and is produced by subepithelial cells and transported to the intestinal lumen, where it can bind to luminal threats such as pathogenic bacteria or other antigens, e.g. cholera toxin. Besides preventing pathogen-epithelial interactions, it may enhance pathogen-epithelial interactions at selected sites such as the M-cells. These cells are primarily involved in uptake of macromolecules and delivery to the submucosal immunocytes.

The epithelial cells lining the lumen of the gastrointestinal tract, serve as a barrier and separate the lumenal compounds from the subepithelial fluids. The transcellular pathway through the epithelial cells is highly restrictive to passive flow of hydrophilic solutes. Small

molecules pass by selective transport or selective pores, large molecules can be endocytosed and then degraded or passed as undegraded molecules to the basolateral side. The paracellular pathway is a major pathway for passive solute permeation. It consists of the apical intercellular tight junction and the underlying paracellular space. The lamina propria contains a large number of immunocytes, which can be considered the last barrier. Recently it was discovered that one type of immune cells may play an important function in regulation of epithelial function and in passing signals from the lamina propria to the nervous system. These cells, called mast cells, contain a large number of preformed messengers of which histamine is the best known. They are also effective in producing cytokines like TNFα and they can be stimulated in an IgE-dependent or IgE-independent way to release preformed messengers and to synthesize newly formed messengers [19].

In addition to the enterocytes and goblet cells, the epithelium contains endocrine cells. Especially serotonin is found in a large number of enterochromaffin cells. These cells seems to have a receptive function. They are considered to monitor the intestinal content and by releasing their products to the blood side they may activate the neighboring cells in the lamina propria like nerves and immunocytes. They may form the sensors of a local reflex chain leading to increased secretion of electrolytes and water to remove the message in the lumen [20]. It was found recently that enterocytes may also act as sensors and relay the presence of an allergen in the lumen to the mast cells in the lamina propria [21]. This mechanism may explain the very fast reaction in allergic individuals to the presence of the specific allergen in the lumen, and can also be considered as a safety mechanism whereby the induced secretion flushes the allergen away.

3. Cellular mechanisms for transepithelial transport

A. Absorption

The predominant electrolyte that drives fluid absorption is Na^+. Sodium is absorbed mainly via electroneutral mechanisms. The Na^+-H^+-exchanger (NHE3) is coupled to Cl^-/HCO_3^- exchange (Figure 2). Coupling of the exchangers occurs through changes in pH. The Na^+ gradient is maintained by the Na^+/K^+ pump. The Na^+ gradient and the cell potential drives Na^+ into the cell, and H^+ is extruded by means of Na^+/H^+ exchange. This tends to rise the cellular pH, which energizes HCO_3^- efflux in exchange for Cl^-. The net reaction is Na^+ and Cl^- uptake in exchange for H^+ and HCO_3^- efflux. The coupling of these lumenal compounds to CO_2 and water means that the exchangers transport osmoticants from the lumen to the cells. Na^+ is pumped out of the cell by the Na^+-K^+-ATPase, mainly in the lateral intercellular space, and Cl^- follows, by a transporter that has as yet not been identified. Electrogenic Na^+

absorption takes place via the Na^+-glucose cotransporter (SGLT1) (Figure 3). This cotransporter is located in the apical membrane of the cells and brings 2 Na^+ into the cell together with 1 glucose molecule. Na^+ is again pumped out of the cell via the Na^+-K^+-ATPase. Glucose efflux is mediated by facilitated transport carriers GLUT-2.

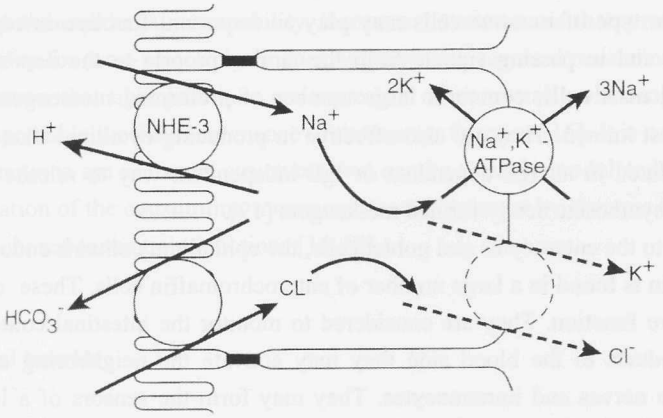

Figure 2: electroneutral NaCl absorption in small intestine and colon. Adapted from [22]

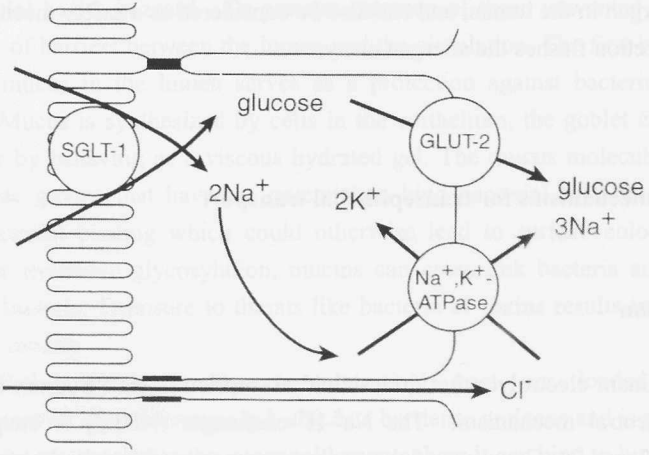

Figure 3: solute-coupled Na^+ absorption in small intestine. Adapted from [22].

Similar Na^+ cotransport mechanisms also exist for many amino acids and bile salts. Besides electroneutral and solute-coupled Na^+ absorption, electrogenic Na^+ transport occurs via an

apical Na^+-channel (ENaC) (Figure 4) in the distal colon. This channel is sensitive to amiloride and is upregulated by aldosterone.

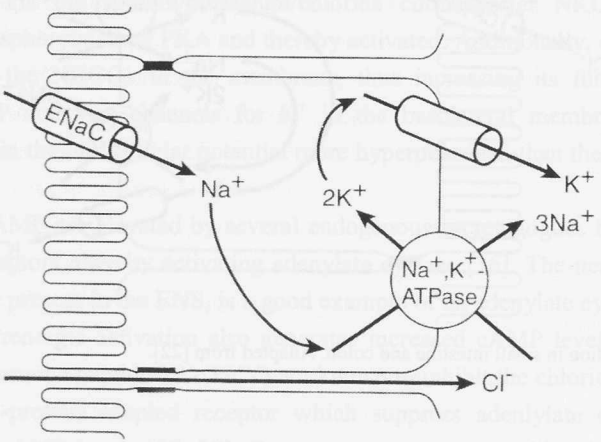

Figure 4. Electrogenic Na^+ absorption in distal colon. Adapted from [22].

B. Secretion

The predominant electrolyte driving fluid secretion is chloride. Chloride is taken up into the cell via the $Na^+K^+2Cl^-$ cotransporter (NKCC1) in the basolateral membrane and accumulated in the cell (Figure 5). The Na^+-K^+-ATPase, located in the basolateral membrane, provides the energy necessary for this process and recycles the sodium. A potassium conductance, located in the basolateral membrane, recycles potassium and keeps the membrane potential from the cell to a more negative value than the chloride equilibrium. A key function of the sodium-potassium-chloride transporter is to use the energy of the Na^+ gradient to accumulate Cl^- in the cells above its electrochemical equilibrium. As chloride is accumulated in the cell, upon opening of apical chloride channels by regulatory mechanisms, it will exit into the lumen leading to a driving force for cation diffusion from the blood to the lumen. The most important chloride channel in the intestinal epithelium is believed to be the cystic fibrosis transmembrane regulator (CFTR) which is the product of the CF gene. To keep electroneutrality sodium passively follows by paracellular flux through the tight junctions. This flux of electrolytes will be accompanied by water because of the osmotic activity of the transported salt into the crypt lumen.

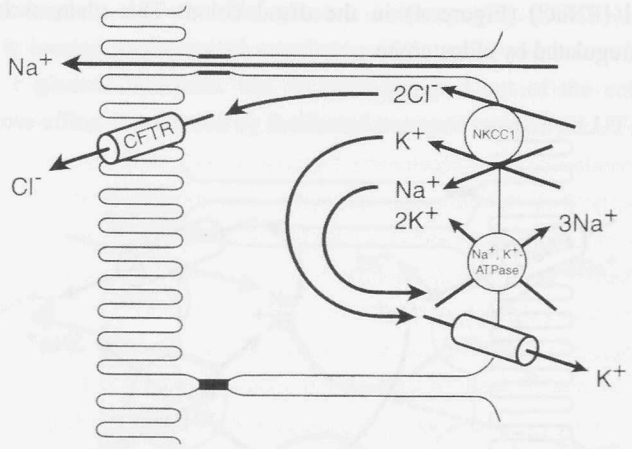

Figure 5. Cl^- secretion in small intestine and colon. Adapted from [22].

4. Regulation of ion transport

The epithelium can respond to signals from a variety of regulatory systems, including those supplied by the endocrine, neural and immune systems [1, 23, 24, 25]. There is substantial interplay between the mediators produced by the various controlling systems. Moreover, the lumenal contents may activate the epithelial cells directly as is the case for instance, for cholera toxins and *E. coli* toxins. Because the interaction between all extracellular messengers is at the level of intracellular effectors and intracellular messengers a brief summary of intracellular messenger systems related to electrolyte secretion is given below.

A. Intracellular messengers

(i) cAMP, cGMP

Increases in the level of cyclic AMP and cyclic GMP stimulate chloride secretion and inhibit the electroneutral NaCl absorption. The sodium-proton exchanger NHE has been described as being negatively regulated by increases in cGMP or cAMP levels [26, 27].

The main pathway for apical chloride exit is through the CFTR chloride channel in the apical membrane. CFTR is regulated in a two step process that involves covalent modification of the regulatory domain by cAMP-dependent protein kinase (PKA), which is followed by ATP binding to the nucleotide binding domain [28-30]. These two steps are necessary for the opening of the channel. The resulting chloride secretion is sustained in the

presence of the agonist [31]. This is an important characteristic, which allows to discriminate between several secretagogues based on their typical time course of Cl^- secretion.

Besides the direct increase in chloride channel activation, cAMP regulates the chloride secretion via the sodium/potassium/chloride cotransporter NKCC1 [32]. This cotransporter is phosphorylated by PKA and thereby activated. Additionally, cAMP increases the recruitment of the NKCC1 in the membrane, thus increasing its functionality [33]. Furthermore, cAMP activates channels for K^+ in the basolateral membrane, which are important to maintain the intracellular potential more hyperpolarized than the Cl^- equilibrium potential [34, 35].

Levels of cAMP are elevated by several endogenous secretagogues by activating G_s protein coupled receptors, thereby activating adenylate cyclase [36]. The neuropeptide VIP, which is abundantly present in the ENS, is a good example of an adenylate cyclase activating transmitter. Beta-adrenergic activation also generates increased cAMP levels. Somatostatin and NPY and the hormone peptide YY (PYY) are known to inhibit the chloride secretion, via coupling to the G_i-protein coupled receptor which suppress adenlylate cyclase activity thereby decreasing cAMP levels [37, 38]. Exogenous secretagogues like cholera toxin and thermolabile *E. coli* toxin have also been shown to stimulate intracellular cAMP levels [39]. Cyclic GMP is generated by membrane bound guanylate cyclase which is covalently linked to a transmembrane receptor. The receptor is activated by a recently discovered endogenous peptide, guanylin, and is also used by the thermostable form of *E. coli* toxin. An increased level of cGMP activates protein kinase G which can also activate CFTR [40]. Since cGMP also inhibits a specific isoform of cAMP-phosphodiesterase PDE3 it can increase the level of cAMP [40a].

(ii) IP_3, DAG, Ca^{2+}

Increases in intracellular calcium levels have been recognized to inhibit Na^+-Cl^- absorption and to stimulate chloride secretion. Phosphatidylinositol hydrolysis by phospholipase C results in the formation of IP_3 and diacylglycerol, DAG. IP_3 liberates calcium from the intracellular pools, thereby increasing the cytosolic calcium levels $[Ca]_i$. The change in intracellular Ca^{2+} level consists of a peak-change followed by a sustained increase which only decrease very slowly. Elevation of intracellular calcium levels have been shown to inhibit the sodium-proton exchanger via a PKC pathway [41, 42].

Calcium plays also an important role in the secretory mechanism in the crypt cells because it can activate basolateral K^+ channels. Thereby the basolateral membrane potential is driven to the K^+ equilibrium potential and is hyperpolarized. This increases the driving force for Cl^- efflux through the apical membrane. It is thought that the activation of the muscarinic receptor in the basolateral membrane of T84 cells by e.g. carbachol increases the K^+ conductance transiently. As a consequence, the carbachol induced increase in secretion is transient [43-45]. In HT29cl.19A cells, in addition to the increase of Ca^{2+} activation of PKC

occurs via the increased amount of DAG (see below) [46]. A confusing observation is that although intracellular Ca^{2+} levels remain high in the presence of carbachol, the basolateral K^+ conductance decreases. Some investigators favor the idea that a metabolite of inositolphosphate, namely inositol 3,4,5,6-tetraphopshate IP_4 is an endogenous inhibitor [47]. In other cells an inhibitory function of activated PKC has been found [48, 49].

In HT29cl.19A cells the first response to an increase of $[Ca]_i$ is the activation of calcium-dependent Cl^- channels in the apical and basolateral membrane. The change in conductances is usually larger in the basolateral membrane. This can be deduced from intracellular recordings of the fractional membrance resistances, but it is usually not seen on transepithelial registration except for a small serosa-negative change in potential. In trachea, but not in intestine, a calcium-dependent chloride channel in the apical membrane may substitute for CFTR and may contribute to the augmented chloride secretion induced by acetylcholine.

Several intestinal neurotransmitters and hormones, including acetylcholine, bradykinin, histamine, substance P and neurotensin have been demonstrated to regulate the chloride secretion by releasing IP_3 and subsequent increase of the intracellular calcium levels and by releasing DAG [4, 50]. In the second part of this thesis, we show that the secretory response to these secretagogues can be reduced by NPY.

The second intracellular messenger derived from PLC activation is DAG. This membrane-bound lipid can activate most of the protein kinase C isotypes. In the laboratory, the effect of DAG is commonly stimulated by the group of phorbol esters which bind to PKC at the same site as DAG. It should be mentioned here that the fatty acid tails of DAG may differ strongly depending on its substrate. Consequently, it is conceivable that PLC action on different substrates like PIP_2 and phosphatidylcholine activate different isotypes of PKC. Phorbol esters may activate all types of DAG-dependent PKC's. The Ca^{2+} dependent isotypes of PKC are activated by the simultaneous increase of intracellular Ca^{2+} because of increased IP_3 and the increase of DAG. Activation of PKC has been reported to inhibit electrolyte absorption via an inhibition of the sodium-proton exchanger [51, 52].

The role of PKC in the regulation of chloride secretion is 'controversial'. In some intestinal preparations phorbol esters do not induce secretion whereas they do in others. In HT29cl.19A cells, activation of PKC resulted in an increase in chloride secretion [53]. Based on the work of Bajnath, it has been hypothesized that increased PKC activity may lead to fusion of vesicles (which contain CFTR channels) with the apical membrane, thereby increasing the chloride conductance of the apical membrane [48]. On the other hand, activated PKC can limit secretory processes [49, 54-57]. This is ascribed to inhibition of basolateral transport sites including the sodium-potassium-chloride cotransporter NKCC and basolateral potassium channels [53]. As yet, it is unknown which isotype of PKC is responsible for each activity. However, some evidence from studies with HT29cl.19A cells suggests that PKCα is involved both the activation of CFTR [58], while in T84 cells, PKCε has been found to inhibit basolateral K^+ conductance [59].

From this brief summary it can be deduced that the secretory action of cAMP-mediated extracellular signals may be augmented by simultaneous activation of the PLC/Ca^{2+} pathway. Moreover, it is clear that signals which prolong the activity of intracellular messengers (e.g. effect of cGMP on cAMP-PDE) act synergistically with the secretagogues.

Considering the large number of secretagogues and their synergy it is somewhat surprising that only a few anti-secretory extracellular signals like somatostatin and NPY/PYY are present. It appears that the enterocytes rely mainly on internal feedback systems to end the effect of secretory signals as described above for the formation of IP_4, or the inhibitory effect of PKCε [50].

5. Water transport; diarrhea and constipation

It has been postulated that water movement is driven by local osmotic gradients within the tissue. The transport of solute across a water-permeable barrier induces water flow in the same direction.

Diamond & Bossert [60] proposed a model for water absorption. Their model is based on findings in gall bladder and they propose that transported solutes such as Na^+/Cl^- accumulate in the lateral intercellular space as a result of active transport. The resulting osmotic gradient causes a net flow of water from the lumen into these spaces. The resulting hydrostatic pressure pushes solution into the direction of the blood where water is absorbed as a result of the Starling forces - the sum of osmotic and hydrostatic forces between tissue water and capillary blood.

Lundgren et al. presented a model for water absorption, which involves a countercurrent multiplier system [61]. They proposed that during the absorption of sodium a high osmolality is generated in the interstitial fluid in the tip of the villi and maintained by the counter current blood flow in the villus. This osmotic gradient results in water absorption.

The secretion of water is also thought to occur via a local increase in osmolality. The transported solutes to the lumen accumulate in the crypt interstitial space, an unstirred region, which is covered by a mucus layer. A local increase in osmolality in the 'unstirred' region occurs and this results in the flow of water into the lumen [62].

The flow of water can be paracellular through the tight junctions [63] or transcellular. The transcellular movement of water is thought to occur via recently discovered water channels (aquaporins) in the membrane [64], via solute-coupled transport also called water pumps [65, 66].

If the transport of water is disturbed, pathophysiological conditions can occur. On the one hand, the lumen of the intestine can become too dry if too much water is absorbed or too little is secreted. On the other hand, excessive water secretion or defective water absorption results in excessive water in the lumen and hence to diarrhea.

A. Constipation

In patients suffering from cystic fibrosis, CF, the chloride channel CFTR is ineffective due to a mutation in the CF gene. A subgroup of these patients (appr. 15 %) show intestinal obstruction, which appears to result from the inability of secretory mechanisms to maintain appropriate viscosity of the lumen.

Other causes for constipation are often found to be of neuronal origin. Deficient neurons of the ENS result in decreased motility, thereby causing obstruction of function and lack of intestinal propulsion. As a consequence of the defect in the neuronal system, the regulation of the electrolyte secretion may also be affected as Greenwood [67] proposed. Decreased innervation, as found in patients with Hirschsprung's disease, is reflected in these patients by the unability to relax the contracted intestine. Hardy et al. showed that the secretory response to acetylcholine is strongly depressed in rectosigmoid colon in children suffering Hirschsprung's disease. This may be due to increased acetylcholinesterase levels, thereby decreasing the levels of acetylcholine [68]. These results suggest that a change in neurotransmitter ratio due to innervation disorders possibly result in a shifted electrolyte transport balance, in the direction of absorption. This results in the lumen of the intestine becoming too dry, which leads to constipation.

B. Diarrhea

On the contrary, when the balance between absorption and secretion is shifted towards secretion, excessive water secretion into the lumen occurs, leading to diarrhea. Several forms of diarrhea can be distinguished.

(i) Osmotic or malabsorption diarrhea

If the absorptive capacity of the small intestine is reduced, the daily volume presented to the colon can exceed its absorptive capacity of about four liters. Alternatively, if the colon is deranged so that it cannot absorb the one and a half liters presented to it by the normal small intestine, then this also results in diarrhea. The absorptive defect could be caused by a failure of absorption of Na^+ as the result of Na^+/H^+-exchanger deficiency. This congenital Na^+ diarrhea is a very rare disorder [69]. A defect in the sodium-glucose transporter called glucose-galactose malabsorption is more common. In these patients the amount of monosacharides presented to the colonic bacteria leads to osmotic secretion and diarrhea. Patients with a lactase deficiency have a similar problem because the unabsorbable lactose cannot be metabolized to absorbable glucose and galactose. The unabsorbed lactose can be metabolized by colonic bacteria. This results in an increased solute load, promoting water movement to the lumen [70, 71].

(ii) Secretory diarrhea

Secretory diarrhea is caused by an excessive chloride secretion into the lumen. This occurs when patients are infected with toxins or bacteria. Several bacteria express enterotoxins that can have a profound effect on chloride secretion. Infection with *Vibrio cholerae* remains a cause of life-threatening diarrhea. A subunit of the enterotoxin cholera toxin is transported into the cell and at the basolateral membrane it irreversibly activates the cAMP-signaling pathway. This results in a continuous stimulation of the chloride secretion and thus to excessive water secretion into the lumen [72]. This capacity to secrete large amounts of water is regarded as a defense mechanism designed to flush the lumen free of invading pathogens. In the case of cholera, however, the patients may die from excessive dehydration. The mechanism of cholera toxin induced diarrhea is not restricted to cAMP generation in the enterocytes. Lundgren et al. have shown that the toxin also activates the enteric nervous system. The neurotransmitters released induce secretion via their specific receptors on the enterocytes [20]. Another example of secretory diarrhea (nervous diarrhea) may be due to stress-induced increased motor function of the colon together with increased mucosal secretion.

(iii) Leak-flux induced diarrhea.

Due to a disturbed epithelial barrier function, back-leak of ions and water into the intestinal lumen can occur from the lateral intercellular spaces in the case of increased tight junction permeability. The intestinal 'structure' might be affected: a loss of epithelial cells will result in a decreased barrier. Moreover, when the tight junctions, responsible for the maintenance of the barrier, loose their tightness, the epithelium loses its ability to transport/absorb electrolytes and consequently the ability to transport water. An increase in permeability may result in an increased entrance of lumenal toxins, bacteria and antigens, which is a trigger to increase the secretory response as a defense mechanism.

6. Inflammation and intestinal dysfunction

Inflammatory bowel disease (IBD) encompasses a heterogeneous group of diseases including Crohn's Disease (CD) and Ulcerative Colitis (UC). These are chronic inflammatory diseases of the gastrointestinal tract. In CD, the total gastrointestinal tract may be affected from mucosa to serosa, whereas in UC only the mucosa and submucosa of the colon are inflamed. The dominant symptom in patients suffering IBD is diarrhea [73]. Although the etiology remains unclear, a role for enteric bacteria and their products, which initiate the chronic intestinal inflammatory process (in genetically susceptible individuals) resulting in decreased

intestinal functioning, has been proposed [74]. The pathophysiological mechanism underlying the inflammatory diarrhea has been investigated extensively.

Decreased colonic absorption has been described in human inflamed colonic tissue [75-79]. Also in a rat model, in which colitis was induced by intrarectal administration of 2,4,6-trinitrobenzenesulfonic acid TNBS [80], distal colon showed decreased absorption of water.

The secretory features of intestinal epithelium had also changed. A few reports describe that basal ion secretion is affected in inflamed mucosa tissue. In caecum from a mouse model of colitis (C3H/HeJBir substrain), the basal chloride secretion was higher than in control caecum [81]. Moreover, Crowe et al. showed that mucosa tissues from IBD patients show increased basal I_{sc}, implicating increased basal ion secretion [82]. However, in several animal models and human colonic inflamed tissue, secretory responses were reduced in inflamed tissue. Especially, the responses to agents like histamine, IBMX, prostaglandin E1 and E2, acetylcholine, bradykinin and electrical field stimulation (EFS), were diminished [82-87].

Additionally, the epithelial barrier appeared to be disturbed in experimental models of inflammation, as well as in tissues from UC and CD patients. In the animal model of TNBS-induced colitis, the permeability was increased [88] as was the case in another animal model of colitis, which had been induced by the chemical agent dextrane sulphate sodium (DSS model) [89]. Furthermore, in colonic mucosa from IBD patients the barrier proved to be disrupted as seen by decreased resistance and increased permeability [78, 90, 91]. A recent report states that the disruption of the barrier is due to an altered tight junction structure [90]. A disrupted epithelial barrier may affect intestinal function by two mechanisms. Firstly, the disruption of the barrier allows antigens and other noxious agents to penetrate the epithelium, thereby inducing or augmenting the inflammatory process in IBD. Secondly, it could lead to back-flux of electrolytes and non-electrolytes followed by water into the intestinal lumen, resulting in leak-flux diarrhea.

7. Inflammatory mediators

Under normal conditions, the gut immune response is characterized by a balance between pro- and anti-inflammatory factors. It has been hypothesized that chronic inflammation in IBD may be due to an imbalance between inflammatory and anti-inflammatory mediator production [92]. A large number of inflammatory mediators increase in the mucosa of patients with IBD, including eicosanoids, chemokines and cytokines, as reviewed by Ciancio and Chang [93]. The role of these immunomodulators in regulating epithelial physiology is being investigated extensively to gain more insight in the complex networks in the intestinal mucosa and their involvement in inflammatory diseases. Mast cells play an important role in the immune-related regulation of ion secretion [94]. They release mast cell mediators,

including histamine, serotonin, cytokines including TNFα [19], interleukins, prostaglandins, leukotriens and enzymes like mast cell protease II [1]. Firstly, the mediators could promote chloride secretion via a direct interaction with the epithelial cells. Secondly, mast cell mediators can augment the secondary synthesis or release of chloride secretagogues from other mucosal elements. Thirdly, mast cell mediators could alter the sensitivity of the epithelium to normal levels of endogenous hormones and neurotransmitters.

Histamine induces chloride secretion via direct as well as indirect effects on the epithelium. Histamine binds directly to H1 receptor on enterocytes, stimulating phospholipase C, which results in increased intracellular calcium levels [95]. Consequently, this leads to increased ion secretion. In addition, histamine and serotonin have been reported to indirectly increase chloride secretion in intestinal tissues via increased enteric nerve stimulation or prostaglandin release [96-100].

Prostaglandins and leukotriens are metabolized from arachidonic acid via two distinct mechanisms involving cyclooxygenase and lipoxygenase respectively, and are reported to increase chloride secretion in intestinal tissue [101, 102]. Prostaglandins are thought to stimulate chloride secretion directly via increasing cAMP levels [101, 103] and indirectly via stimulation of the enteric neurons to release acetylcholine [104, 105].

Mast cell products affect the barrier function of the intestinal epithelium. Mast cell protease II has been recognized to act on the epithelia via increasing the permeability in rat intestine [106].

More direct evidence that specific immune stimulation of mast cells alters ion secretion and permeability stems from studies with mutant mice. Intestine from immune-sensitized mast-cell deficient mice demonstrated small antigen-induced secretory responses, compared with responses in intestine from control mice [107]. More recently, Santos et al. showed that mast cell deficient rats do not respond to stress with an increase in permeability and ion secretion as do the control rats, suggesting an important role for mast cells [108]. Mast cell deficient rats, which were sensitized and challenged with the antigen, did not show an increased transepithelial flux of the antigen. Neither did they show increased conductance nor the presence of a macromolecule in the paracellular space, suggesting an important role for mast cells in the regulation of the permeability of the epithelial paracellular pathway [21].

8. TNFα - a pleiotropic messenger

Tumor necrosis factor α TNFα is a 17 kD cytokine, produced mainly by monocytes, macrophages and T cells and circulating as a homotrimer. Two specific transmembrane receptors have been identified, type I 55 kD and type II 75 kD [109]. These receptors can be released as soluble receptors and are thought to compete with the cell surface receptors for the binding of TNFα and act as antagonists [110]. TNFα was isolated on the basis of the ability to kill tumor cells [111]. It appeared to be identical to the earlier identified cachectin

[112]. TNFα is a powerful pro-inflammatory mediator [113, 114], a mediator of endotoxic shock [115] and it is recognized for its role in the induction of apoptosis [116]. TNFα is synthesized by mast cells as mentioned previously [19]. More recently it was found that all types of cells, including epithelial cells, can release TNFα [117].

A number of studies have revealed a pivotal role for TNFα in the pathogenesis of IBD. Levels of mRNA for TNFα as well as the immunoreactivity for TNFα are increased in bowel mucosa of patients with CD [118-120]. Furthermore, TNFα concentrations in stools have been shown to parallel disease activity [121]. Recently, it has been reported that treatment of CD patients with antibodies against TNFα can be successful against the symptoms of diarrhea in these patients [122, 123]. These findings imply an important role of TNFα in this disease. However, at what level TNFα is involved in the epithelial dysfunctioning remains uncertain. Many investigations focused primarily on the interference of TNFα with the innate and adaptive immune response, but the role of TNFα in affecting intestinal epithelial is now also being investigated by several groups. Kandil et al. and Schmitz et al. [124, 125] first reported that TNFα affects the ion secretion in porcine ileum and human distal colon, respectively. They state that TNFα increases ion secretion via enhanced release of subepithelial prostaglandins. Co-culture of the epithelial colon cell line with monocytes shows increased ion secretion responses. TNFα seems to account for this phenomenon by acting as an autocrine mediator on the monocytes [126]. Direct effects of TNFα on epithelial barrier function have been studied only in cell lines. In the intestinal epithelial cell lines Caco-2 BBE, HT29cl.19A and HT29/B6, TNFα was shown to decrease the transepithelial resistance, concomitant with increased paracellular permeability, except for Caco-2 BBE cells [7-9]. This disrupting effect of TNFα on the barrier function was only apparent after incubating the cells with high levels of TNFα (100 ng/ml). However, in the presence of IFNγ, low concentrations of TNFα did also affect the intestinal physiology, which is ascribed to an induction of more TNFα receptors by IFNγ [127].

The outline of the thesis

Part I

Information about the involvement of mediators and cytokines in electrolyte transport in IBD is based on prolonged inflammatory states of the tissues, or after long treatment with these cytokines. It is of interest to study the acute effects of cytokines. As far as we know, there is no information about the time course of the effect of TNFα, its effect at lower concentrations and the possible underlying mechanisms. It is also not clear whether changes in ion secretion or in transpeithelial permeability are related. Because of our knowledge that PKC actvation

affects both characteristics, i.e. increase and later inhibition of secretion and an increase in paracellular permeability with a rather long lag time [53, 127a], we speculated that this cytokines might exert its action via this pathway. Therefor we thought it of interest to study what the acute interaction is of the cytokine TNFα with the ion secretion in non-diseased material and to discuss what the contribution of the cytokine might be in the pathogenesis of the disease. The main goal of this thesis was to investigate the direct effect of a lower concentration of the cytokine on the intestinal properties of cell lines and native tissue. Moreover, the thesis describes which second messenger systems are involved in the regulatory mechanisms.

In Chapter 2, we present the effect of TNFα on the chloride secretion in HT29cl.19A cells. The electrophysiological measurements suggest that muscarinic receptor activation with carbachol involved the PLD pathway and that TNFα potentiated the response via this pathway by a protein synthesis-dependent process. In Chapter 3 we provide evidence on the basis of pospholipid analyses that muscarinic receptor activation stimulates the PLD mediated production of phosphatidic acid, PA. Furthermore, we demonstrate that the well-known β-adrenergic recetor antagonist propranolol effectively blocked the conversion of PA to DAG. The effect of exposure to TNFα appeared to be an increased turnover of PA to DAG by the enzyme phosphatidic acid phosphatase. To study whether the effect of TNFα on phospholipid metabolism was not only restricted to the particular cell line HT29cl.19A cells, we used another colonic epithelial cell line, T84 cells (Chapter 4). Besides electrophysiological experiments, phospholipid analysis was performed. In this chapter, the difference in effects of TNFα on the two cell lines is discussed. It is of great importance to determine whether results obtained in cell lines can be extrapolated to native tissue. Therefore, we compared the effects of the two cell lines with the effect of TNFα on chloride secretion in mouse distal colon. Since measurements in mouse distal colon resembled the results obtained in the HT29cl.19A cell line, we consider this cell line to be a representative model for studying chloride secretion and its regulatory mechanisms including the effect of TNFα.

Since we obtained evidence from intracellular electrophysiology that the intracellular pathway activated by the muscarinic receptor resembled the pathway activated by the histamine 1 receptor, we studied the effect of TNFα on chloride secretion induced by histamine in HT29cl.19A cells and mouse colon employing electrophysiological techniques. Moreover, the involvement of phospholipid messengers was studied by phospholipid analysis in the cell line.

Part II

This part of the thesis focuses on the interaction of secretagogues and antisecretory messengers with respect to the ion secretion in HT29cl.19A cells. As stated above, the

neurotransmitter VIP and acetylcholine are known to increase the chloride secretion in intestinal epithelium albeit via very different mechanisms. NPY and somatostatin are anti-secretory neuropeptides [128-132]. A well-known effect of both peptides is their inhibitive action on cAMP synthesis. The interesting fact however, is that somatostatin could also inhibit the secretory response to muscarinic receptor activation [133]. We hypothesized that NPY could have a similar inhibitory effect. The aim of the study was to elucidate the mechanism whereby NPY could inhibit both secretory pathways. In Chapters 6 and 7, we report on the effect of NPY on chloride secretion in HT29cl.19A cells. All evidence points to an additional effect of NPY on potassium channels. Further study of the mechanism of this inhibition was, however, no longer possible using this cell line because the cells lost their NPY receptor due to a change in serum supply. Attempts to use isolated colon crypts to continue this study were unsuccessful because it proved impossible to obtain stable (patch clamp) recordings from the human colon crypt cells.

In Chapter 8, we consider and discuss the conclusions from the preceding chapters with regard to their possible implications for pathophysiology.

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