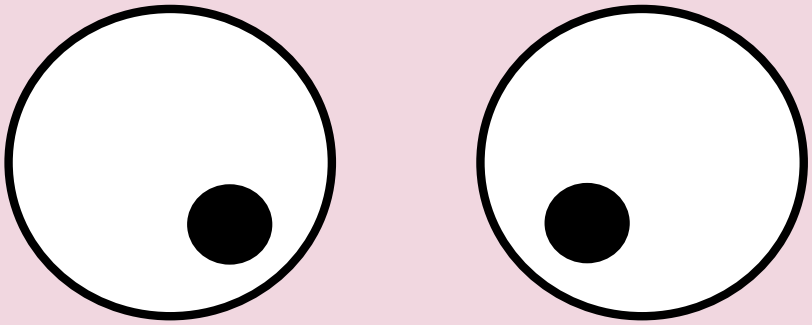
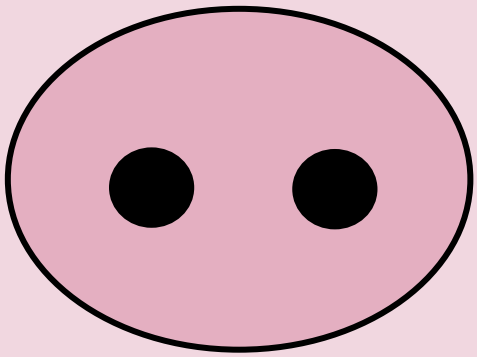
UNIVERSITEIT GENT



Cell-specific distribution and signal transduction control of 5-HT_4 receptors in the gastrointestinal tract

Evelien Priem



Promotor Prof. Dr. R.A. Lefebvre

Faculty of Medicine and Health Sciences

Heymans Institute of Pharmacology

2013

Cell-specific distribution and signal transduction control of 5-HT_4 receptors in the gastrointestinal tract

Evelien Priem

The research of this thesis was financially supported by grant G.0061.08 from the Fund for Scientific Research Flanders (FWO-Vlaanderen).

Thesis submitted as partial fulfillment of the requirements for the degree of Doctor in Medical Sciences

Cell-specific distribution and signal transduction control of 5-HT_4 receptors in the gastrointestinal tract

Evelien Priem

Promotor - Prof. Dr.R.A. Lefebvre
Faculty - Faculty of Medicine and Health Sciences
Department - Heymans Institute of Pharmacology
Year - 2013

”To be a star, you must shine your own light, follow your own path, and don’t worry about the darkness, for that is when the stars shine the brightest.”

Table of Contents

List of Abbreviations

5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
AC	adenylyl cyclase
ACh	acetylcholine
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene related peptide
CNS	central nervous system
CO	carbon monoxide
COPD	Chronic Obstructive Pulmonary Disease
CREB	cAMP response element binding protein
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
EC	enterochromaffin
ECL	enterochromaffin-like
EFS	electrical field stimulation
ENS	enteric nervous system
Epac	exchange protein directly activated by cAMP
ERK	extracellular signal-regulated protein kinase
FACS	fluorescence activated cell sorting
FGIDs	functional gastrointestine disorders
GABA	gamma aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GI	gastrointestinal
GIPs	G-protein coupled receptor interacting proteins
GMCs	giant migrating contractions
GPCR	G-protein coupled receptor
GRKs	G-protein-coupled protein kinases
GRP	gastrin-releasing peptide
GTP	guanosine triphosphate
H&E	hematoxyline and eosine
H_2S	hydrogen sulfide
HCl	hydrochloric acid
hERG	human ether-a-go-go related gene
IBMX	3-isobutyl-1-methylxanthine
ICCs	interstitial cells of Cajal
IFANs	intestinofugal afferent neurons
IHC	immunohistochemistry
IPANs	intrinsic primary afferent neurons
L-AADC	L-amino acid decarboxylase
LES	lower esophageal sphincter
LMPC	laser microdissection and pressure catapulting
L-NAME	N-nitro-L-arginine emthyl ester hydrochloride
LTP	long term potentiation

MAO	monoamine oxidase
MMCs	migrating motor complexes
MMP	muscular-myenteric plexus
nAChR	nicotinic receptors
NANC	non-cholinergic non-adrenergic
NC-IUPHAR	Nomenclature Committee of the International Union of Pharmacology
NO	nitric oxide
PACAP	pituitary adenylate cyclase activating peptide
PBS	phosphate buffered saline
PDE	phosphodiesterase
PDZ	PSD-95/Disc large/Zonula occludens-1
PEN	polyethylene naphtalate
$PGF_{2\alpha}$	prostaglandin F2α
PKA	protein kinase A
PKC	protein kinase C
PSS	physiological salt solution
RT-PCR	reverse transcriptase-polymerase chain reaction
RQI	RNA quality indicator
SERT	serotonin reuptake transporter
SEM	standard error of the mean
SK	small conductance
Src	sarcoma
TpH-1	tryptophan hydroxylase-1
TTX	tetrodotoxin
VIP	vasoactive intestinal peptide
VMAT	vesicular monoamine transporter

Chapter 1

LITERATURE SURVEY

Chapter 1 Literature survey

1. I. Gastrointestinal tract

The digestive system is the largest endocrine organ in the body and its overall function is primarily to break down food for absorption. This process occurs in five main phases: ingestion, digestion, propulsion, absorption and elimination of waste. The major physiological processes that contribute to this function are secretion, absorption and motility, which are defined by their own local intrinsic properties but also controlled by environmental factors, hormones and nerves in an integrated system (Johnson and Weisbrodt, 2007; Raybould et al., 2003; Young and Heath, 2000).

1.I.1. Anatomy and histology

1.I.1.1. Anatomy

The various organs of the digestive system can be divided into two main groups: the *gastrointestinal (GI) tract* and the *accessory digestive organs*.

The accessory digestive organs are the teeth, the tongue, the gallbladder and the digestive glands (the salivary glands, the liver and the pancreas), which lie external to the GI tract and are connected to the GI tract by ducts.

The GI tract winds through the body starting from the mouth, over pharynx, esophagus, stomach, small intestine and large intestine, ending in the anus. The organs of the digestive system perform the following five essential food-processing activities (**Fig 1.1**):

- ingestion
- digestion
- propulsion
- absorption
- defecation

Ingestion and initial fragmentation of food occur in the mouth, resulting in the formation of a bolus of food, which is transported along the esophagus to the stomach during swallowing, a coordinated action by the tongue, pharyngeal and esophageal muscles. Fragmentation and swallowing are facilitated by the secretion of saliva from the salivary glands and fragmentation is completed in the stomach, where digestion is initiated.

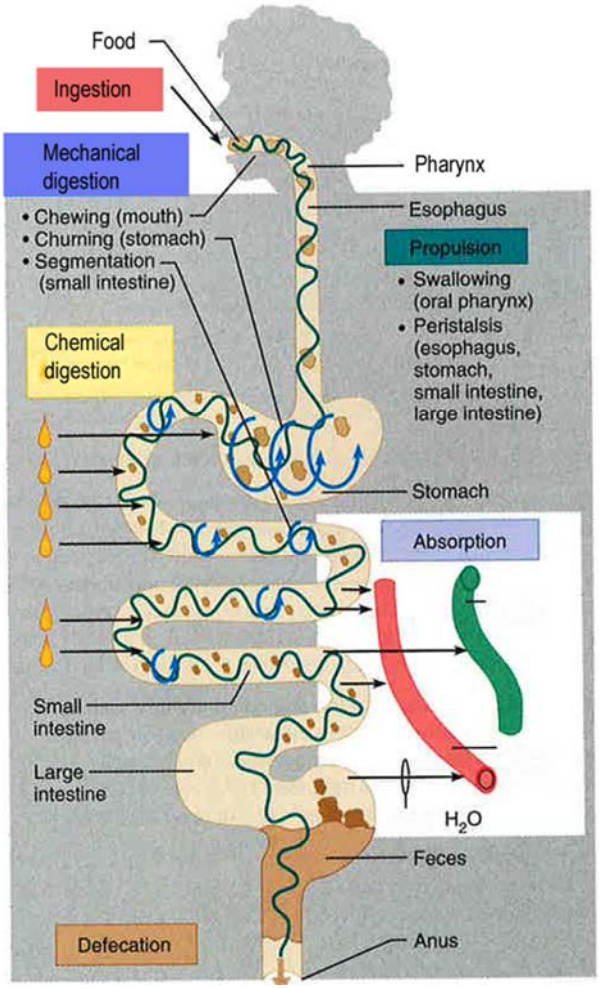


Figure 1.1. Schematic summary of digestive processes (from Marieb and Mallat, 2003).

Digestion is the process by which food is broken down into small pieces to be absorbed into the blood circulation. This process consists of *chemical digestion*, which is the enzymatic breakdown of macromolecules into smaller molecules and *mechanical digestion*, which is the fragmentation by an intense muscular action of the GI wall. The muscular layer of the GI wall also provides *propulsion*. The fragmented and partially digested food is squirted through a muscular sphincter, the pylorus, from the stomach into the duodenum through the rest of the small intestine, where the process of digestion is completed and the main absorptive phase occurs.

Although *absorption* occurs in the small and large intestine, the small intestine is the principal site for absorption, which is the transport of the digested end products, water and electrolytes from the lumen into the blood or lymph capillaries, located in the wall of the GI tract.

The non-absorbed rest of the liquid digested food passes from the small intestine through the ileocaecal valve, into the large intestine. Here, water is further absorbed so that the luminal content becomes progressively more solid as it passes towards the anus. The first part of the large intestine is called the caecum, from which projects the appendix. The next part of the large intestine is called the colon and is divided anatomically into ascending, transverse, descending and sigmoid segments, although histologically the segments are indistinguishable from one another.

The waste is then eliminated by the process of *defecation.* The large intestine and the rectum, hold the faeces until defecation occurs via the anal canal (Marieb and Mallat, 2003; Young and Heath, 2000).

1.I.1.2. Histology

The structure of the GI tract conforms to a systematic organization which is clearly evident from the esophagus to the anus. The tract is essentially a long tube with a wall composed of a number of tissue layers (**Fig 1.2**):

- *the inner mucosal layer*
- *the submucosal layer* with vascular and lymphatic components forming an important link with the mucosa
- *the muscle layers*
- *the outer layer*, covering the whole tube, called adventitia.

The arrangement of the major muscular components remains relatively constant throughout the tract, whereas the mucosa shows marked variations in the different regions of the tract (**Fig 1.3**) (Marieb and Mallat, 2003; Scratcherd and Grundy, 1984; Young and Heath, 2000).

Mucosa. The mucosa is made up of three components: the epithelium, the lamina propria, supporting the epithelium, and the muscularis mucosae, a thin smooth muscle layer which produces local movement and folding of the mucosa. At four points along the tract the mucosa undergoes abrupt transition from one form to another (**Fig 1.3**): the gastro-esophageal junction, the gastroduodenal junction, the ileocaecal junction and the recto-anal junction. Pathogenesis of several GI diseases starts at the level of the mucosa because the mucosal layer is the first barrier between the ingested food and the human body; therefore the majority of immune cells is present in this layer (Marieb and Mallat, 2003; Young and Heath, 2000).

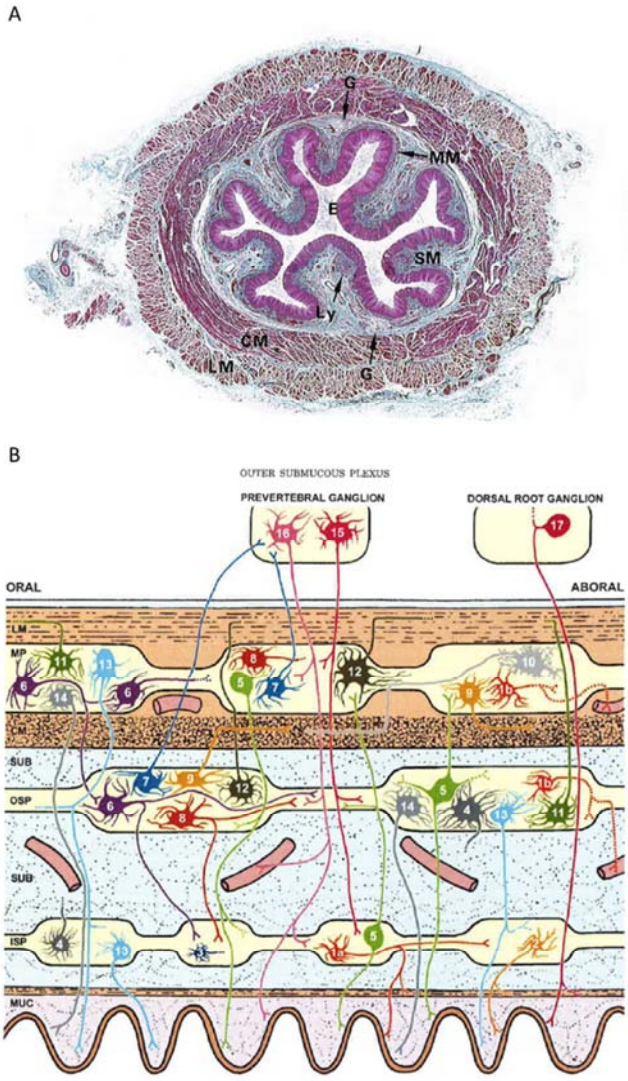


Figure 1.2.

(A) Components of the wall of the GI tract (Esophagus Masson's trichrome staining). **(E)** Epithelium **(MM)** Muscularis mucosae **(SM)** Submucosa **(G)** Ganglia **(CM)** Circular smooth muscle **(LM)** Longitudinal smooth muscle (from Young and Heath, 2000).

(B) Layers and enteric nervous system (ENS) of the GI tract in larger mammals (from esophagus through large intestine). **(MUC)** Mucosa **(ISP)** Inner submucous plexus **(SUB)** Submucosa **(OSP)** Outer submucous plexus **(CM)** Circular smooth muscle **(MP)** Myenteric plexus **(LM)** Longitudinal smooth muscle. The different neuron types depicted are 1a) Secretomotor neuron 1b) Possibly vasomotor neuron 2) Secretomotor neuron 3) Uncharacterized 4) Uncharacterized 5) Intrinsic primary afferent neuron (IPAN) 6) Interneuron 7) Intestinofugal neuron 8) Interneuron 9) Inhibitory motor neuron to CM 10) Excitatory motor neuron to CM 11) Excitatory motor neuron to LM 12) Inhibitory motor neuron to LM 13) Secretomotor neuron? 14) Secretomotor neuron? 15) Postganglionic sympathetic neuron 16) Postganglionic sympathetic neuron 17) Extrinsic primary afferent neuron (from Timmermans et al., 2001).

Submucosa. This layer of loose collagenous tissue contains the larger blood vessels, lymphatics and nerves. In the stomach, small and large intestine of larger mammals, at least two distinct ganglionic nerve networks exist in the submucosa: one located close to the muscularis mucosae, this submucosal plexus is called Meissner's plexus or inner submucous plexus (ISP) and the other is located near the circular layer of the muscularis propria, called Schabadasch (Henle's) plexus or outer submucous plexus (OSP). The neuron populations of the ISP and OSP largely differ from each other in both morphological and neurochemical features, but are intensely connected via interconnecting fibers. The ISP contains postganglionic sympathetic fibers arising from the superior mesenteric ganglion, intrinsic primary afferent neurons (IPANs) and secretomotor neurons. The OSP contains intestinofugal afferent neurons (IFANs), IPANs, secretomotor neurons, interneurons to the ISP and motor neurons to the smooth muscle layers of the muscularis propria (Brehmer et al., 2010; Marieb and Mallat, 2003; Timmermans et al., 1993; Timmermans et al., 2001; Young and Heath, 2000).

Muscularis propria. The muscular wall consists of smooth muscle cells which are usually arranged as an inner circular layer which is thicker and more powerful than the outer longitudinal layer. These smooth muscle layers have their own inherent rhythmicity which is modulated by the autonomic parasympathetic nervous system. The action of the two layers is the basis of peristaltic contraction and is orchestrated by the interstitial cell of Cajal (ICCs) and further regulated by the large clusters of parasympathetic ganglion cells with their pre- and postganglionic fibers and the postganglionic sympathic fibers that are found between the two layers. This plexus of nerve cell bodies and fibers between the two muscle layers is known as the myenteric plexus or Auerbach's plexus and contains IPANs, IFANs, secretomotor neurons, interneurons and motor neurons to the smooth muscle layers of the muscularis propria (Marieb and Mallat, 2003; Timmermans et al., 2001; Young and Heath, 2000).

Adventitia. This outer layer of loose supporting tissue is lined by a simple epithelium layer and conducts the major vessels and nerves. Where the gut lies within the abdominal cavity, the adventitia is referred to as the serosa (Marieb and Mallat, 2003; Young and Heath, 2000).

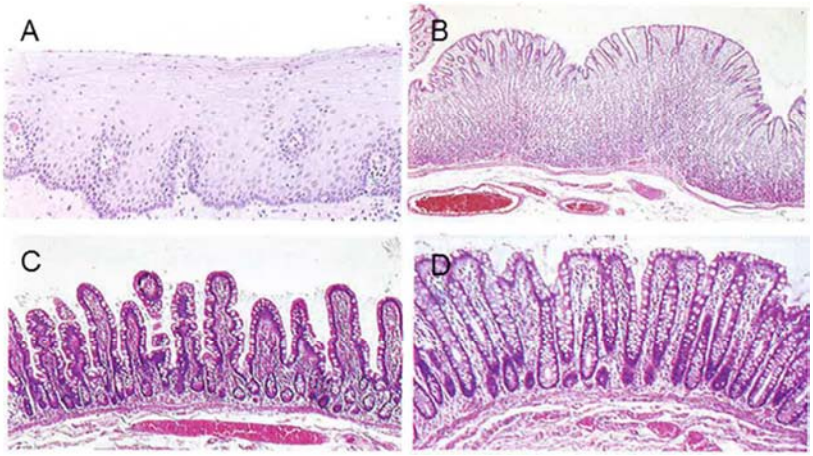


Figure I.3. Four basic mucosal types classified according to their main function (Hematoxylin and eosin staining). (A) Protective: this type is found in the oral cavity, pharynx, esophagus and anal canal. (B) Secretory: this type occurs only in the stomach. (C) Absorptive: this mucosal form is typical of the entire small intestine. (D) Absorptive/protective: this form lines the whole large intestine (from Young and Heath, 2000).

1.I.2. Gastrointestinal secretion

Every day, the digestive system secretes liters of fluid, delivering the following primary components for the chemical breakdown of food and protection of the luminal wall: ions, digestive enzymes, mucus and bile. About half of these fluids are secreted by the digestive glands (salivary glands, pancreas and liver) and the rest of the fluid is secreted by the GI epithelial cells. Although some chemical digestion of carbohydrates, proteins and fats can take place in the stomach, the final breakdown mainly occurs in the small intestine (Johnson and Weisbrodt, 2007; Rhoades and Bell, 2009; Young and Heath, 2000).

1.I.2.1. Gastric secretion

The major function of the stomach is storage, but another important function of the stomach is to prepare the food for digestion in the small intestine, hereby converting large solid particles into smaller pieces via a combined action of gastric secretion, peristaltic movements of the stomach and contraction of the pyloric sphincter (Rhoades and Bell, 2009; Schubert, 2010).

1.I.2.1.1. Mucosal cell types

Anatomically, the stomach is divided in different regions: fundus, corpus, antrum and pylorus. Functionally, the gastric mucosa is composed of pits and tubular glands, containing three main types of glands: cardiac, pyloric and oxyntic glands **(Fig 1.4)**. The gastric mucosa contains mainly columnar mucous cells, covering the surface or located in the gland, secreting mucus and HCO_3^- ions, which protect the stomach from the acid in the lumen. Apart from the mucous cells, the gastric mucosa also contains various neuroendocrine cells, found in the base of the gastric glands as well as stem cells, which divide continuously to replace epithelial cells and are mainly found in the neck of the gastric gland (Johnson and Weisbrodt, 2007; Rhoades and Bell, 2009).

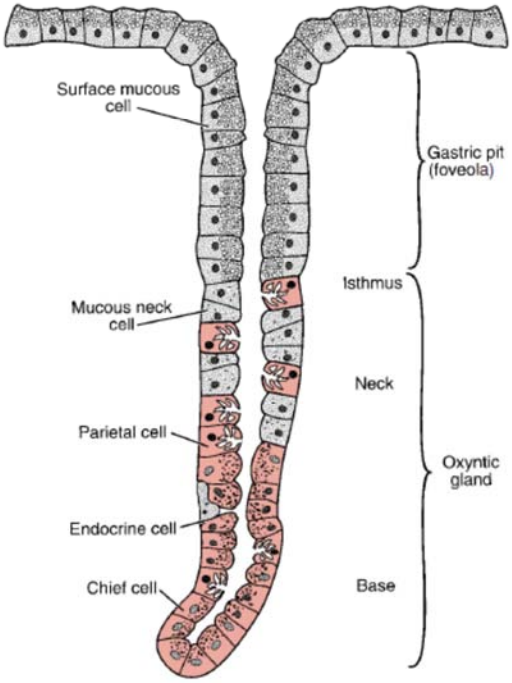


Figure 1.4. A simplified diagram of the oxyntic gland in the fundus and corpus of a mammalian stomach (from Rhoades and Bell, 2009).

The cardiac glands are located in a small area close to the esophagus and are mainly lined by mucous cells. The distal 20% of the gastric mucosa, called the pyloric gland mucosa is located close to the duodenum and contains G cells, D cells, A cells, enterochromaffin (EC) cells and mucous cells. A cells and G cells, secrete ghrelin and gastrin respectively, which stimulate gastric secretion. EC cells and D cells, secrete serotonin (5-hydroxytryptamine, 5-HT) and

somatostatin respectively, that both inhibit acid secretion (Johnson and Weisbrodt, 2007; Rhoades and Bell, 2009). The oxyntic gland mucosa is located in the proximal 80% of the stomach, including the fundus and the corpus and mainly secretes acid. The oxyntic glands contain parietal (oxyntic) cells, chief cells, A cells, mucous cells, EC cells and endocrine enterochromaffin-like (ECL) cells. A recent study confirmed the presence of 5-HT-producing EC cells in the human stomach (Penkova et al., 2010). The parietal cells principally secrete hydrochloric acid (HCl) and intrinsic factor, which is necessary for absorption of vitamin B_{12} in the terminal ileum, chief cells secrete the enzyme precursor pepsinogen. In some species the chief cells also secrete intrinsic factor. The chief cells of the oxyntic glands release inactive pepsinogen, which is activated by acid in the gastric lumen to form the active enzyme pepsin, which cleaves peptic bonds within protein molecules of the nutrients. Pepsin also catalyzes its own formation from pepsinogen. Next to activation of pepsinogen, the acidity of gastric juice poses also a barrier to invasion of the GI tract by microbes and parasites. The pH is low due to the secretion of acid, but the gastric mucosa is protected by the mucus gel layer covering the surface and the HCO_3^- ions, trapped in the mucus gel layer neutralizing diffusing acid, preventing damage to the mucosal cell surface (Johnson and Weisbrodt, 2007; Rhoades and Bell, 2009).

1.I.2.1.2. Regulation

Gastric acid secretion is a dynamic process regulated by neuronal (efferent and afferent), hormonal (gastrin) and paracrine (histamine, ghrelin, somatostatin) pathways, depending on the composition of the food.

The stimulation of acid secretion can be divided into three phases: the cephalic phase, the gastric phase and the intestinal phase. The *cephalic phase* is initiated when smelling, chewing and swallowing food. The central nervous system (CNS) sends impulses via vagal nerve fibers to the stomach releasing acetylcholine (ACh) and gastrin-releasing peptide (GRP) to the parietal cells and G cells, respectively. The *gastric phase* is mainly a result of gastric distention and chemical constituents of food, which affect acid secretion indirectly through stimulation of gastrin release. Distention of the stomach activates mechanoreceptors, stimulating the cholinergic neurons to release ACh acting directly on the parietal cell **(Fig 1.5)**.

ECL cells are believed to be the source of histamine, which activates parietal cells via histamine H_2 receptors. On the other hand, histamine, acting on H_3 receptors on D cells, amplifies the ability of secretagogues to stimulate acid secretion by suppressing somatostatin release from D

cells. During the *intestinal phase*, circulating amino acids and gastrin, secreted by intestinal G cells in response to products of protein digestion, stimulate the parietal cells.

When gastric digestion is finished and food is squirted through the pylorus to the duodenum, gastric secretion must be inhibited, because excess acid can damage the gastric and duodenal mucosa. Gastric luminal pH is a sensitive regulator of acid secretion. The food mass buffers secreted acid but as the stomach empties, pH drops. When pH drops below 3, endocrine D cells in the antrum secrete somatostatin, which is the main inhibitor of acid secretion by inhibiting the release of gastrin from G cells and the release of histamine from ECL cells, and inhibiting parietal cells directly (Lindström et al., 2001; Rhoades and Bell, 2009; Schubert, 2007; 2009; 2010).

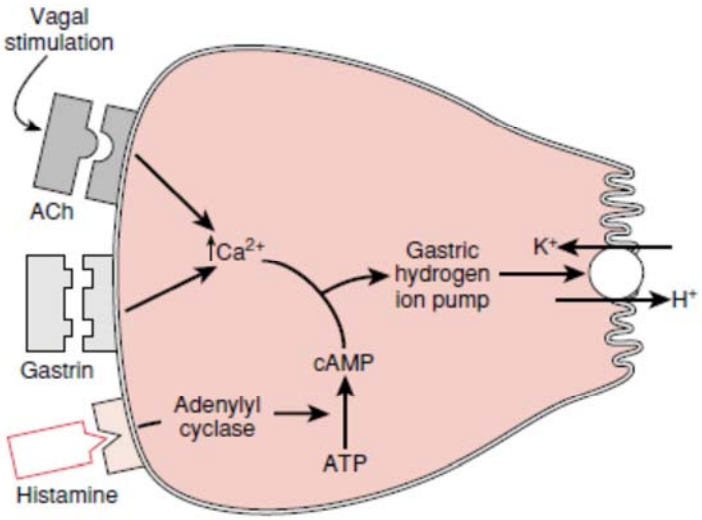


Figure 1.5. The stimulation of parietal cell acid secretion by histamine, gastrin and acetylcholine (ACh) (from Rhoades and Bell, 2009).

Another additional mechanism for inhibiting gastric secretion has been suggested. Studies in rat and dog stomach, demonstrated the ability of 5-HT to inhibit vagally stimulated gastric acid secretion but not basal acid output (Canfield and Spencer, 1983; Lai et al., 2009; LePard et al., 1996; LePard and Stephens, 1994). Although the mechanism mediating this 5-HT-induced inhibitory response is not yet clear, there are indications that 5-HT might inhibit acid secretion by activation of 5-HT_3 and/or 5-HT_1-like receptors (Lai et al., 2009; LePard 1994).

1.I.2.2. Intestinal secretion

Final breakdown of food occurs in the small intestine by hydrolytic enzymes. Both small and large intestine secrete water and electrolytes to maintain the fluidity of the digested food. The HCO_3^- in intestinal secretions protects the intestinal mucosa by neutralizing any H^+ ions present in the lumen (Johnson and Weisbrodt, 2007; Rhoades and Bell, 2009).

1.I.2.2.1. Mucosal cell types

Several cell types make up the intestinal epithelium of the small and large intestine, but the majority of cells that contribute to the digestive and absorptive physiology are the enterocytes and goblet cells. Enterocytes are the most numerous cell type and are tall columnar cells with surface microvilli, which increase the absorption area. Goblet cells produce mucin for lubrication of the intestinal contents and protection of the epithelium. The mucosa of the small intestine is made up of numerous finger-like projections called villi, whilst the mucosa between the bases of the villi is formed into crypts. The epithelium of the small intestine also includes other cell types than enterocytes and goblet cells, such as paneth cells and intraepithelial lymphocytes, which both have a defensive function, stem cells and neuroendocrine cells such as EC cells, I cells, S cells and M cells which produce 5-HT, cholecystokinin (CCK), secretin and motilin respectively, regulating motility and secretion. The mucosa of the large intestine only consists of the absorptive enterocytes and mucus-producing goblet cells (Rhoades and Bell, 2009; Xue et al., 2007; Young and Heath, 2000).

1.I.2.2.2. Regulation

A complex array of hormonal and neuronal mechanisms control secretion in the GI tract. *The small intestine*, comprising the duodenum, jejunum and ileum, is where most chemical digestion takes place. Many of the digestive enzymes that act in the small intestine are secreted by the exocrine pancreas and enter the small intestine via the pancreatic duct. The enzymes that digest smaller carbohydrate and peptide products, originating from *luminal digestion* by pancreatic enzymes, are secreted by the enterocytes and provide the single sugars and amino acids, ready to be absorbed, by *membranar digestion*. The pancreatic enzymes enter the small intestine in response to the hormone CCK, which is produced by the I cells in the small intestine in response to the presence of nutrients and gastric emptying. The hormone secretin, released in response

to low pH, stimulates pancreatic HCO_3^- release into the small intestine in order to neutralize the potentially harmful acid coming from the stomach (Rhoades and Bell, 2009).

Cl^- secretion is a major determinant of the net movement of fluid across the mucosal surface into the intestinal lumen of the small and large intestine. The players that determine Cl^- secretion are 1) the crypt epithelial cell, 2) the enteroendocrine cells and 3) the neurons in the neural circuits. (Wapnir and Teichberg, 2002; Xue et al, 2007). Two Cl^- are moved into the cell against the electrochemical gradient along with one Na^+ and one K^+ via the $NaKCl_2$ cotransporter. The entering Na^+ is extruded into the intestinal lumen by the Na^+ pump and K^+ diffuses back out. Extra Na^+ moves through paracellular pathways into the lumen, driven by the secretion of Cl^-. The chloride channels in the apical membrane, responsible for Cl^- secretion, are stimulated by ACh and vasoactive intestinal peptide (VIP) released by the enteric nervous system but also secretin and prostaglandin, stimulating the chloride channels by increase of cyclic adenosine monophosphate (cAMP). However, in several functional studies in human jejunum, 5-HT also stimulates Cl^- secretion and water movement into the intestinal lumen. For endogenous 5-HT, a neuronal pathway seems involved while the response to exogenous 5-HT is non-neuronal (Budhoo and Kellum, 1994b; Budhoo et al., 1996b; Kellum et al., 1999). Together with the motility responses to 5-HT, the accumulated fluid sweeps and flushes out the intestinal content (Rhoades and Bell, 2009; Wapnir and Teichberg, 2002; Xue et al, 2007). Secretion that is stimulated by GI hormones and neurotransmitters after a meal helps the digestion to maintain a liquid chyme during the process of absorption. The resulting secretion of Na^+ and Cl^- establishes an osmotic gradient that draws water into the lumen. Water secretion always occurs in response to osmotic forces produced by transport of organic solutes or ions (Johnson and Weisbrodt, 2007).

Proceeding from the duodenum to *the large intestine*, the Na^+ and Cl^- concentrations in the lumen progressively become lower than the plasma concentrations. In the colon, the Na^+ concentration decreases whereas the K^+ concentration increases by a net K^+ secretion. The apical and basolateral membranes of the enterocytes are permeable to K^+ and factors that promote Na^+ absorption such as aldosterone, increase K^+ secretion in exchange for Na^+. The major anions in the colonic lumen are Cl^- and HCO_3^-; the Cl^- concentration progressively decreases towards the distal end. HCO_3^- is secreted out of the cell in countertransport with Cl^- (Johnson and Weisbrodt, 2007). The large intestine is able to absorb as well as secrete electrolytes and fluids which is critical to maintain proper hydration of the organism. Regulation of water movement is important from physiological and immunological perspectives. If the process of intestinal hydration becomes disregulated, as during acute intestinal infections or in

the context of intestinal inflammation, diarrhea or constipation can result. In the colon, ion transport is regulated by acetylcholine via muscarinic receptors, located on enteric neurons and enterocytes, hormones, paracrine substances and gasotransmitters.
Gaseous molecules such as nitric oxide (NO), hydrogen sulfide (H_2S) or carbon monoxide (CO) are thought to be involved in the regulation of colonic water and salt transport (Hirota and McKay, 2006; Pouokam et al., 2011).

1.I.3. Gastrointestinal absorption

How and where digestion and absorption occurs depends on the composition of the food, but most nutrients, fluid and electrolytes from intestinal secretions are usually absorbed by the duodenum and jejunum. To ensure the optimal absorption of nutrients, vitamins, bile salts and water *in the small intestine* after a meal, a large absorption area is needed. This is ascertained by the length of the small intestine, circular folds of the mucosa and submucosa called plicae circulares, villi and crypts at the mucosal surface and microvilli at the luminal surface of the epithelial cells (Rhoades and Bell, 2009; Young and Heath, 2000). The enterocytes along the villi are involved in the absorption and the plasma membrane of the enterocyte is often considered the only factor restricting the free movement of substances from the gut lumen into the blood or lymph. However, this movement involves a complex pathway and includes subsequent passage of substances through the unstirred layer of fluid, the glycocalyx covering the microvilli, the apical cell membrane, the cytoplasm of the enterocyte, the basolateral cell membrane, the intercellular space, the basement membrane and the membrane of the capillary or lymph vessel.
The enterocyte membrane controls the flux of solutes and fluid between the lumen and blood, involving several mechanisms: passive diffusion, facilitated diffusion and active transport. In the case of passive diffusion, the epithelium behaves like an inert barrier and the particles traverse the cell layer through pores in the cell membrane or through intercellular spaces (Johnson and Weisbrodt, 2007).
The capacity of the human small intestine to absorb free sugars is enormous and there appears to be little physiologic control of sugar absorption. Under normal circumstances the major portion of sugar assimilation is complete in the proximal jejunum. In general, there is a large disaccharidase reserve in the small intestine, so much that the rate-limiting step in sugar assimilation is not digestion but the absorption of free hexoses following hydrolysis. Hexoses are absorbed by active or facilitated transport. At the apical membrane, 2 transporters are involved,

one for glucose and galactose and the other for fructose; at the basolateral membrane, the 3 hexoses are moved by a single transporter. Peptides are digested to single amino acids, that are absorbed via a set of separate transporters in the apical and basolateral membrane. The apical membrane also contains an oligopeptide transporter, allowing the absorption of di- and tripeptides, that are then metabolized to single amino acids by intracellular peptidases. Absorption of lipolytic products occurs mainly by diffusion through lipid portions of the membrane. Bile salts form water-soluble aggregates to enhance the diffusion of the poorly soluble products of lipolysis through the unstirred aqueous layer overlying the enterocytes (Johnson and Weisbrodt, 2007; Mourad and Saadé, 2011).

Water absorption occurs mainly in response to osmotic forces produced by the transport of organic solutes (amino acids, glucose) and/or ions (Na^+, K^+, Cl^-) in the small as well in the large intestine, however specific molecular water channels, known as aquaporins are also involved. Solutes are moved from the lumen into the cell to the capillaries, creating a local osmotic gradient, causing to move water from the gut lumen into the intercellular space. Absorption of water can also be externally regulated by the autonomic nervous system and agents such as dopamine, enkephalins and endorphins (Johnson and Weisbrodt, 2007).

The principal absorptive function of *the large intestine* is the recovery of water and salt from the faeces. Under healthy conditions there is a net colonic absorption of Na^+ and Cl^-, the two most important anorganic ions transported in the large intestine. The mucosa of the large intestine is folded in the non-distended state but it does not exhibit distinct plicae circulares or villi like those of the small intestine (Rhoades and Bell, 2009; Wapnir and Teichberg, 2002).

1.I.4. Gastrointestinal motility

Another important function of the GI tract is to transport the ingested food at an optimal rate and to mix the food for optimal exposure to digestive enzymes. This is achieved by the contractile activity of smooth muscle cells in the GI tract. GI motility is controlled by the presence of food, by autonomic nerves and by hormones. Feeding and the presence of food initiates swallowing, contractions of the stomach wall and subsequently gastric emptying and intestinal segmentation and peristalsis. During fasting, a specific motor activity -the migrating motor complexes (MMCs)- occur (Olsson and Holmgren, 2001).

The fasting period, is the period in between meals determined by spontaneous local contractions occurring in a certain pattern, moving along the stomach and small intestine; periods of relative rest alternate with periods of contractile activity. The MMCs can be divided into three phases:

phase I is quiescent and known as the time of relative rest, phase II is characterized by irregular single contractions and phase III by intensive rhythmic contractions (Costa et al., 2002; Johnson and Weisbrodt, 2007; Kunze and Furness, 1999; Olsson and Holmgren, 2001; Scratcherd and Grundy, 1984).

Fed patterns start when food is ingested. Once food is swallowed and passes through the esophagus, a reflex is initiated that relaxes the stomach and similarly, distention of the stomach by incoming food itself initiates reflexes that cause relaxation. This relaxation mainly occurs in the proximal part of the stomach i.e. the fundus and the proximal third of the corpus. The stomach then starts a series of contractions, initiated at the second part of the corpus and propagating towards the pylorus, necessary for emptying. During the remainder of the digestive state, pressure in the fundus and proximal third of the corpus increases due to tonic contractions, reducing the size of the stomach while emptying continues. The distal corpus and antrum exhibit peristaltic contractions towards the gastroduodenal junction at a frequency of 3/min. As contractions approach the gastroduodenal junction, they increase in force and velocity (Johnson and Weisbrodt, 2007; Kunze and Furness, 1999; Olsson and Holmgren, 2001; Raybould et al., 2003; Scratcherd and Grundy, 1984).

When the gastric peristaltic contractions reach the pylorus, the pylorus will relax allowing sufficiently small particles to be emptied in the duodenum, while greater particles will be retropulsed into the stomach. Once food arrives in the small intestine, food is further digested and absorbed and chyme is propelled towards the large intestine. Contractions in the small intestine perform at least three functions: 1) mixing of ingested food with digestive secretions and enzymes, 2) circulation of all intestinal contents to facilitate contact with the intestinal mucosa and 3) net propulsion of the intestinal content in aboral direction. To mix and locally circulate the contents for optimal digestion and absorption, non-coordinated rhythmic local contractions above and below the food content occur. Such contractions divide the bowel into segments, a process called *segmentation*. A section that was just contracted will relax and vice version allowing mixing of food with enzymes and contact with the mucosa. However, the small intestine is also capable of eliciting a highly coordinated contractile response that is propulsive in function, called peristalsis. *Peristalsis* depends on the interaction between the intrinsic nervous system and the circular and longitudinal muscle layers, which results in relaxation in front of the bolus and contraction distally of the bolus (**Fig. 1.6**). The peristaltic reflex is initiated by mucosal stimulation and/or GI wall distention by the bolus; 5-HT might play a role in triggering the nervous response to these stimuli. Contractile activity of the small intestine slows down towards

the ileum as mainly absorption occurs in the ileum compared to the digestion and absorption in the duodenum.

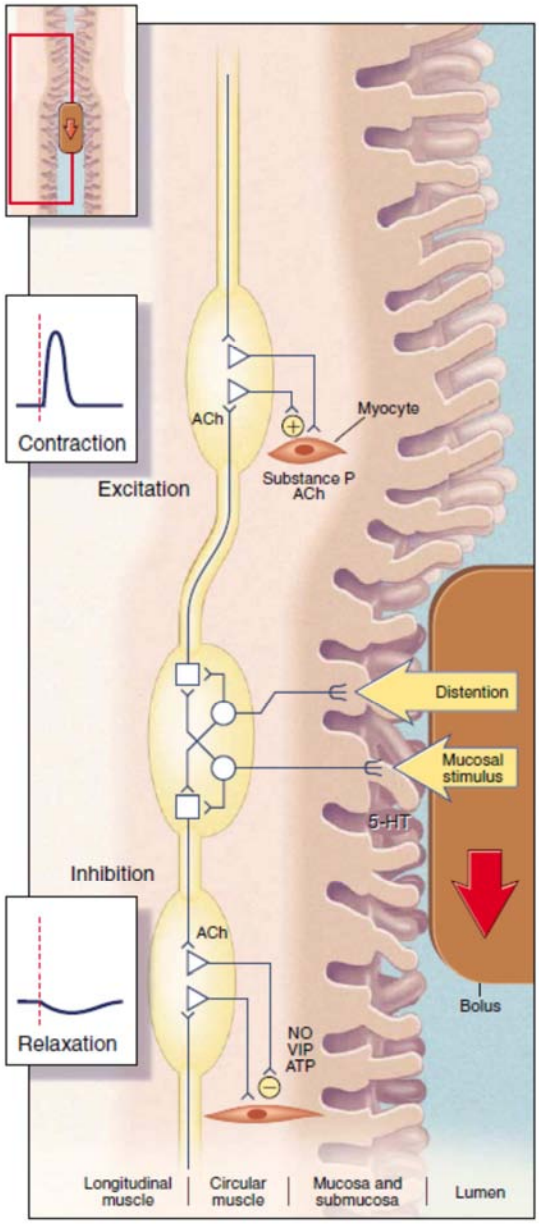


Figure 1.6. Intestinal Peristaltic Reflex. Peristalsis is the result of a series of local reflexes, each consisting of a contraction of intestinal muscle above an intraluminal stimulus and a relaxation of muscle below the stimulus. The release of 5-HT by mucosal stimulation or mechanical distention of the GI tract triggers activity in the intrinsic afferent neurons. Above the site of the stimulus, ascending cholinergic interneurons relay this signal to excitatory motor neurons containing acetylcholine (ACh) and substance P. As a result, the circular muscle layer above the stimulus contracts. At the same time, below the stimulus site, descending cholinergic interneurons activate inhibitory motor neurons that contain nitric oxide (NO),

vasoactive intestinal polypeptide (VIP) and ATP, causing relaxation. The resultant forces propel the bolus in an antegrade direction (from Goyal and Hirano, 1996).

Peristalsis is governed by a multitude of overlapping mechanisms through cooperation of sensory and motor neurons, smooth muscle cells, ICCs, endocrine or paracrine factors associated with secretory glands, secretory mucosal cells and intestinal flora. Peristalsis is generally not the consequence of independent actions of different control systems but rather depends on communication between the control systems, initiated by specific stimuli leading to a coordinated action of both smooth muscle layers (Huizinga and Lammers, 2009). Chemical and mechanical stimulation leads to activation of the peristaltic reflex and anal propagation, that involves integrative mechanisms between the circular and longitudinal smooth muscle layers, resulting in an oral excitation and anal inhibition of the muscle layers (Smith et al., 2007). The main control mechanisms of intestinal peristalsis are: 1) ICCs as mediators of the communication between the circular and longitudinal muscle layers 2) sensory neurons and intrinsic motor neurons that orchestrate the motor patterns in the intestine (Huizinga and Lammers, 2009).

Sensory neurons activate ascending and descending interneurons that in turn activate excitatory and inhibitory motor neurons respectively. In the large intestine, the circular and longitudinal smooth muscle layers are synchronously activated, this is because the excitatory and inhibitory motor neurons innervating the muscle layers are activated by common ascending and descending neurons (Smith et al., 2007).

Contractions of the large intestine are organized to allow optimal absorption of water and electrolytes, net aboral movement of contents, and storage and elimination of waste. Once material reaches the proximal large intestine, it is acted on by 3 types of contractions. A majority of these are segmental in nature and occur independently, resulting in slow back and forth movement of the content, mixing and exposing it to the mucosa for absorption. Also the peristaltic mechanism occurs propelling the content over a short distance. However, propulsion in the large intestine occurs mainly during a characteristic mass movement termed giant migrating contractions (GMCs). This is a powerful peristaltic contraction propelling the content over a larger part of the large intestine. By the time material reaches the descending and sigmoid colon, it has changed from liquid to a semisolid state. The waste is stored in the rectum and can be emptied by the defecation process (Johnson and Weisbrodt, 2007; Kunze and Furness, 1999).

1.I.4.1. Smooth muscle activity

Generally, the smooth muscle cells are long thin cells, connected to each other by gap junctions. Electrical stimuli can spread between the smooth muscle cells through the gap junctions, causing parts of the muscle layer to act as one single unit. The level of muscular activity depends on intrinsic myogenic activity, modulated by fluctuating levels of free intracellular calcium (Ca^{2+}) entering the cell by voltage-dependent Ca^{2+} channels in response to changes in membrane potentials as well as on extrinsic factors. With the exception of the esophagus and the proximal part of the stomach, GI smooth muscle shows rhythmic electrical activity (Berridge, 2008; Johnson and Weisbrodt, 2007; Olsson and Holmgren, 2001):

- *slow waves* are cyclic changes in membrane potential due to activation and inactivation of different ion channels or pumps, resulting in slow increases in Ca^{2+} lasting a few seconds. These depolarizations of the smooth muscle membrane occur without triggering phasic contractile events. The waves arise from the muscle itself and are paced by pacemaker cells, the ICCs.
- *action potentials or spikes* are produced by a rapid increase in the calcium concentration through voltage-dependent calcium channels within the smooth muscle cell resulting in powerful contraction with a duration of a few seconds. These spikes are superimposed on the slow wave where they usually occur in bursts and when the membrane potential reaches a critical value. It is the function of the slow wave not only to set the frequency of intestinal contraction, but to move the membrane potential towards the threshold for firing.

1.I.4.2. Regulation of gastrointestinal motility

Although the activity of the smooth muscle is mainly determined by its intrinsic myogenic activity, other mechanisms such as ICCs, hormonal action and the intrinsic and extrinsic nervous system are involved to control motility. The control by the nervous system will be discussed in a separate section.

As described by Barajas-López et al. (1989), Langton et al., (1989), Rumessen et al., (1993) ICCs are mesenchymal cells, spindle shaped and are typically situated between muscle cells within both circular and longitudinal muscle layers from the esophagus to the anus or between neuronal plexuses and the muscle layers. The ICCs are coupled to each other and to smooth muscle cells, mainly via gap junctions. ICCs act mainly as pacemakers in the GI wall, by

developing spontaneous slow waves, which spread passively to the smooth muscle cells (Hansen, 2003a; Olsson and Holmgren, 2001).

The pacemaker property of ICCs is believed to be restricted to ICCs situated in the myenteric plexus (ICC-MY) and in the submucosal plexus (ICC-SMP), acting as the pacemakers of the slow waves generated in smooth muscle cells. The slow waves are propagated by the intramuscular ICCs (ICC-IM); these are distributed in between the muscle layers and make close contact with nerve endings innervating the muscle layers, to help transmitting the neuronal signals to the smooth muscle cells (Tamada and Komuro, 2012; Wouters et al., 2007; Zhang et al., 2011). ICC-IM have shown to express receptors for inhibitory (VIP, NO, ATP) (Burnstock and Lavin, 2002; Epperson et al., 2000; Shuttleworth et al., 1993) transmitters as well as excitatory transmitters (ACh and tachykinins) (Epperson et al., 2000; Portbury et al., 1996), hereby mediating both inhibitory and excitatory transmission from the enteric neurons to the muscle (Ward et al., 1998; Wouters et al., 2007). There are indications of 5-HT receptors present on ICCs, however so far only 5-HT_3 and 5-HT_4 receptors have been described on rodent ICCs and the role of these receptors is unknown (Wouters et al., 2007).

The distribution and role of ICCs in the GI tract are heterogeneous. In the stomach, the ICC-MY serve mainly as the pacemakers of the slow waves, while the ICC-IM propagate the slow waves (Wouters et al., 2007; Zhang et al., 2011). ICC-SMP of the stomach have been proposed to be involved with the regulation of specific mucosal functions, such as secretion, absorption and transport of fluids (Kunisawa and Komuro, 2008). In the colon, slow waves appear to originate mainly from ICC-SMP enhancing absorption, whereas the ICC-MY may drive the cyclic depolarizations responsible for the GMCs (Huizinga et al., 2011; Tamada and Komuro, 2012; Wang et al., 2003; Ward and Sanders, 2001; Wouters et al., 2007).

In addition to the involvement of ICCs, hormones are also involved in the control of GI motility. They modulate motility by activating receptors on smooth muscle cells, sensory fibers, the extrinsic and intrinsic primary afferent neurons (IPANs) and again back on the endocrine cells in an autoregulatory fashion (Hansen, 2003a; Olsson and Holmgren, 2001). The hormonal influence and its interplay with the enteric nervous system (ENS), takes place after and in between meals. The hormones are released locally, usually from endocrine cells in the mucosa of the GI tract. E.g. gastrin, CCK and motilin, but also other hormones like insulin, neurotensin and glucagon-like peptides influence GI motility. E.g. CCK causes delay in gastric emptying and direct contraction of the muscle cells in the gallbladder (Hansen, 2003a). Another important messenger in GI motility is 5-HT, locally released from EC cells in the mucosa.

1.I.4.3. Nervous system

The neuronal regulation of GI motility involves intrinsic as well as extrinsic nerves. The intrinsic innervation involves the ENS, which consists of ganglionated and non-ganglionated plexi. The GI tract and the ENS are connected to the CNS through the autonomic nervous system (the brain-gut axis). The ENS is a center of integrative neuronal activity that is able to regulate the behavior of the gut, even in the absence of input from the CNS, and is engaged in a two-way dialogue with the CNS. The ENS is capable of autonomously controlling GI reflexes. The CNS is able to modulate, but not entirely control, the motor activity by sending instructions via the 2 components of the extrinsic nervous system: the sympathetic and parasympathetic nervous system. This extrinsic innervation involves the vagus nerve and splanchnic nerves to the stomach and upper intestine, while the pelvic nerves supply the distal intestine. The ENS is integrated with the CNS via afferent (vagal, splanchnic and pelvic nerves) and efferent nerves (**Fig. 1.7**) (Furness et al, 2003; Grundy et al., 2006; Gershon and Tack, 2007; Hansen, 2003b; 2003c; Harrington et al., 2010; Johnson and Weisbrodt, 2007).

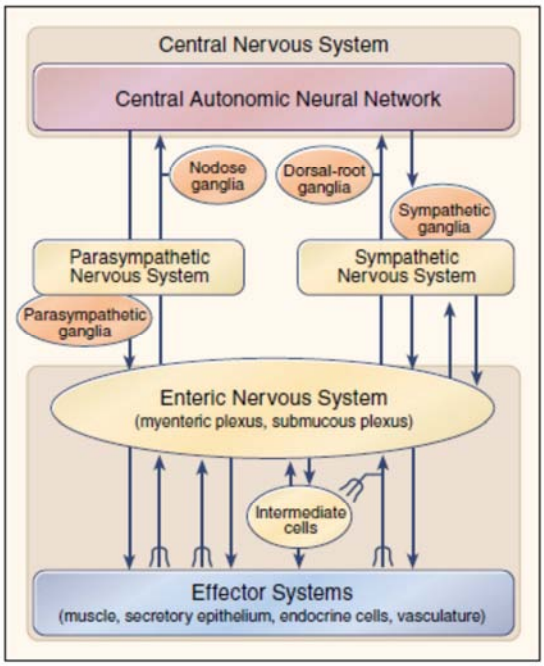


Figure 1.7. Innervation of the GI tract. The neural plexuses in the gut represent an independently functioning network known as the enteric nervous system (ENS), which is connected to the central nervous system (CNS) by parasympathetic and sympathetic nerves. The ENS may influence the effector systems in the GI tract directly or may do so indirectly through its action on intermediate cells, which include endocrine cells and ICCs. The cell bodies of the primary vagal and primary splanchnic afferent neurons are located in the nodose ganglia and the dorsal-root ganglia (from Goyal and Hirano, 1996).

1.I.4.3.1. Extrinsic neurons

The extrinsic pathways act independently of the ENS, yet use components of the ENS as effectors. Extrinsic nerves initiate and modulate peristalsis, MMCs and segmentation. The extrinsic nervous system is divided into parasympathetic and sympathetic branches:

Parasympathetic innervation is supplied by the vagus and pelvic nerves. These preganglionic nerves enter the various organs of the GI tract where they synapse mainly with cells of the ENS. In addition, these same nerve bundles contain many afferent nerves whose receptors lie within the various tissues of the gut. These nerves project to the brain and spinal cord to provide sensory input for integration.

Sympathetic innervation is supplied by nerves that run between the spinal cord and the prevertebral ganglia and between these ganglia and the organs of the gut. Preganglionic efferent fibers arise within the cord and end in the prevertebral ganglia. Postganglionic fibers from these ganglia innervate primarily the elements of the enteric nervous system. Few fibers end directly on secretory, absorptive or muscle cells. Afferent fibers are also present within the sympathetic division. These nerves project back to the prevertebral ganglia and/or the spinal cord (**Fig. 1.7**) (Johnson and Weisbrodt, 2007; Olsson and Holmgren, 2001).

1.I.4.3.2. Intrinsic neurons

Elements of the enteric nervous system include all neurons with their cell bodies situated within the GI wall; they are mainly grouped into two ganglionated plexuses: the myenteric plexus and the submucosal plexus. The majority of efferent myenteric neurons are inhibitory and stimulatory motor neurons that project to the circular as well as the longitudinal layers, mediating muscle activity and controlling digestive motility such as MMCs, peristalsis, segmentation, GMCs and sphincter activity. However, a small population of myenteric neurons innervates the submucosal plexus influencing secretion and facilitating transfer of intrinsic sensory information for local coordinated activity. Submucosal efferent neurons are mainly secretomotor, principally innervating the mucosal glands, where they regulate ion secretion but also villus movements of the mucosa to optimize absorption of the GI contents. In addition, the submucosal nerves innervate submucosal blood vessels regulating blood supply to the mucosa (Furness et al, 2004; Harrington et al., 2010; Olssen and Holmgren, 2001). Neurons of the ENS can be classified according to their morphological, neurochemical, electrophysiological or functional properties. In this survey, the intrinsic neurons are described according to their functional properties: sensory neurons, intestinofugal neurons, interneurons, and motor neurons.

1.I.4.3.2.1. Sensory neurons

The principal sensory neurons in the GI tract are IPANs which comprise an important group of mechanosensory neurons (Furness et al., 2006). Their cell bodies are located in the myenteric plexus and in the OSP. Enteric reflexes are initiated by nerve endings of the IPANs in the mucosa or in the muscle layers. These are stimulated chemically by substances such as fatty acids, lipids and bile salts or mechanically by distortion of the mucosa. The receptor regions on the nerve endings transform changes in the stimulus level into signals transmitted as action potentials along the nerve fibers of the IPANs. The IPANs transmit the signal to interneurons, innervating inhibitory and excitatory motor/secretomotor neurons in the myenteric respectively submucosal plexus. Communication between the IPANs themselves and their target neurons is mediated by ACh acting via nicotinic receptors and tachykinins acting on NK receptors (Clerc and Furness, 2004; Furness et al., 2004; Holzer et al., 2001; Olsson and Holmgren, 2001).

1.I.4.3.2.2. Intestinofugal neurons

IFANs are another unique subset of neurons which relay mechanosensory information from the myenteric plexus or OSP to sympathetic prevertebral ganglion neurons (Furness, 2006; Hibberd et al., 2012; Szurszewski et al., 2002). IFANs form a part of the entero-enteric inhibitory reflex pathway including passing information from the distal small intestine to the proximal small intestine, from the proximal small intestine back to the same region, from the colon to the small intestine and from the distal colon to the proximal colon (Furness et al., 2000). The cell bodies and dendritic fibers of IFANS lie within the intestinal wall but their axons leave the intestine to form synapses in the prevertebral ganglia (Szurszewski et al., 2002). Under some circumstances IFANs both function as slowly adapting mechanoreceptors which are activated by intestinal distention in response to circular muscle stretch as well as receive cholinergic synaptic input from connecting descending interneurons (Ermilov et al., 2003; Hibberd et al., 2012; Lomax et al., 2000). In rat and guinea-pig, only a few IFANs occur in the stomach, and the frequency increases more distally in the intestine. IFANs in the pig, rat and guinea-pig small and large intestine have a proximo-distal gradient of frequency of occurrence. The only significant difference between species seems to be that the cell bodies of IFANs in guinea-pig and rat are all in the myenteric plexus, whereas in the pig there are also many IFANs in the OSP (Furness et al., 2000; Timmermans et al., 1993). The targets of IFANs neurons are mainly the sympathetic prevertebral ganglion neurons but they target also the secretomotor inhibiting neurons and the motility inhibiting neurons but not the vasomotor neurons (Furness, 2003).

1.I.4.3.2.3. Interneurons

Interneurons are connected by synapses into networks that process sensory information and control the behavior of motor neurons (Wood et al, 1999). At least one type of ascending and 3 types of descending interneurons have been characterized in the small and large intestine of the guinea pig as a model for human enteric neurons. Transmission between ascending interneurons is mainly via ACh release acting at nicotinic receptors (nAChR), but tachykinins may be involved too (Bornstein et al., 2004 ; Lecci et al., 2006). The descending interneurons have each their own characteristic set of inputs and outputs. They have a complex chemical coding including ACh, NO, VIP, 5-HT and somatostatin. ACh/NO/VIP/somatostatin neurons are involved in local motility reflexes, while ACh/5-HT neurons are involved in local secretomotor reflexes (Bornstein et al., 2004; Furness, 2000; Kunze and Furness, 1999).

1.I.4.3.2.4. Motor neurons

Motor neurons are the final common pathways for transmission of control signals to the effector systems. In the GI tract, motor signals may initiate, sustain, suppress the behavior of the effector depending on the kind of transmitter released. The motor neuron pool of the ENS consists of excitatory and inhibitory motor neurons, secretomotor/vasodilator neurons and neurons innervating enteroendocrine cells (Furness, 2000; Hansen, 2003b; Wood et al., 1999).
Excitatory and inhibitory motor neurons innervate the longitudinal and circular muscle and the muscularis mucosae throughout the GI tract, coordinating contractions and relaxations.
The balance of epithelial transport processes and blood flow is accomplished by the intrinsic secretomotor/vasodilator neurons through local reflex circuits. The secretomotor neurons innervating the parietal cells of the stomach to release gastric acid are cholinergic. It has been shown that enterochromaffin cells receive direct innervation of cholinergic motor neurons as well as neurons containing both VIP and pituitary adenylate cyclase-activating polypeptide (PACAP), both able to control the release of 5-HT (Furness, 2000; Hansen, 2003b; Holzer et al., 2001; Kunze and Furness., 1999).

1.I.4.3.2.4.1. Excitatory motor neurons

Excitatory motor neurons release neurotransmitters that evoke muscle contractions and mucosal secretion. ACh is the main neurotransmitter released from excitatory motor neurons, called cholinergic neurons, to evoke contraction of the muscles, but also tachykinins are released from excitatory motor neurons, which represent the excitatory non-cholinergic non-adrenergic (NANC) neurotransmitters (Furness, 2000; Kunze and Furness, 1999; Lecci et al., 2006). Excitatory secretomotor neurons involved in secretion from intestinal crypts release ACh and VIP (Furness, 2000; Wood et al., 1999).

1.I.4.3.2.4.2. Inhibitory motor neurons

Inhibitory motor neurons release inhibitory NANC neurotransmitters that suppress contractile activity of the muscle. Many inhibitory neurons in the human GI tract contain NO synthase and release NO (Boeckxstaens et al., 1993; Murr et al., 1999; Tonini et al., 2000). However, there is also clear evidence that NO is not the sole transmitter. Depending on the region and species, different inhibitory co-neurotransmitters have been reported with most evidence for ATP and VIP. The role of ATP as a co-neurotransmitter is reported in the human jejunum (Xue et al., 1999) and colon (Benkó et al., 2007; Boeckxstaens et al., 1993). A joint role of NO and VIP is reported in human stomach (Tonini et al., 2000). Other substances have also been proposed as inhibitory NANC transmitters eg. gamma aminobutyric acid (GABA) has been suggested as a inhibitory neurotransmitter at neuromuscular junctions in the GI tract of rodents (Bayer et al., 2002; Kaputlu and Sadan, 1996), but was not examined in humans. CO and H_2S are gaseous messengers, which have recently been reported to also elicit inhibitory smooth muscle responses in mammalian intestine (Hirota and McKay, 2006; Pouokam et al., 2011).

The functional significance of the inhibitory motor neurons is related to the specialized physiology of the muscle cells which behave as an electrical syncytium and the presence of ICCs that function as pacemaker cells, generating forces of propulsion. The activity state of inhibitory motor neurons determines when the omnipresent slow waves are able to initiate a contraction, as well as the distance and direction of propagation once the contraction has begun (Wood et al., 1999). Normally the inhibitory motor neurons are switched off in the aboral direction during the peristaltic reflex, resulting in contractile activity that propagates in the aboral direction (Hansen, 2003b).

1.I.1.5. Functional gastrointestinal disorders

All diseases that pertain to the digestive system, interfering with the functions of the intestine are labeled as digestive diseases. This includes all diseases of the esophagus, stomach, small intestine and large intestine and GI cancers but also the diseases associated with the accessory digestive glands. In this section only non-cancerous disorders of the GI tract will be discussed; they are categorized into two categories: inflammatory and functional disorders (Chang et al., 2000; Longstreth et al., 2006; Podolsky, 1991a; 1991b).

Inflammatory GI disorders involve chronic inflammation of all or parts of the digestive tract that has been defined by clinical, endoscopic and radiological features. Disorders like gastritis and inflammatory bowel disease (Crohn's disease and ulcerative colitis) are categorized under this section (Xavier and Podolsky, 2007).

Functional GI disorders (FGIDs) are characterized by symptoms and not by sign of disease or injury. To separate these chronic conditions from transient GI symptoms, several clinical entities have been delineated based on the symptom patterns, for which there is no established pathophysiological mechanism (De Ponti and Malagelada, 1998; Drossman, 2006; Longstreth et al., 2006). Symptom-based classification has great importance in reliably diagnosing these conditions, particularly for use in clinical trials and prescribing specific treatment. Therefore the Rome III criteria were developed internationally to help investigators and physicians to diagnose FGIDs based on the patient's history and symptoms (Drossman and Dumitrascu, 2006). In the Rome III classification, FGIDs are diagnosed by characteristic symptoms that have occurred for at least 6 months prior to diagnosis and the symptoms must be currently present for 3 months in the absence of an abnormal structural or biochemical change. FGIDs are further classified in a pediatric and an adult category. The adult category is subdivided into six major domains (Drossman and Dumitrascu, 2006):

- Esophageal (category A)
- Gastroduodenal (category B)
- Bowel (category C)
- Functional Abdominal Pain Syndrome (category D)
- Biliary (category E)
- Anorectal (category F)

The gastroduodenal disorders include: (B1) Functional dyspepsia, (B2) Belching disorders, (B3) Nausea and vomiting disorders, (B4) Rumination syndrome. The functional bowel disorders (FBD) (category C) include: Irritable bowel syndrome (C1), Functional bloating (C2), Functional constipation (C3), Functional diarrhea (C4) (Drossman, 2006; Longstreth et al., 2006).

FGIDs are often characterized by multiple symptoms (pain, nausea, vomiting, bloating, diarrhea, constipation) and the perception of these symptoms may be amplified by physiological and sociocultural factors. These symptoms are experienced as severe, troublesome or threatening, with a subsequent impact on daily life activities. Furthermore, these chronic illnesses are characterized by long-term courses, unpredictable symptom episodes, and disabling effects that are often accompanied by minimally effective treatments. In the Western world, discomfort of FGIDs cause a burden not only to the patient but also to the economy due to the associated consuming health care resources (Camilleri et al., 2002; Gershon and Tack, 2007; Manabe et al., 2010; Rao et al., 2011).

In some FGIDs such as chronic constipation, IBS and functional dyspepsia, GI dysmotility can be the underlying cause of symptoms. GI dysmotility is identified in about 30% of FGID patients. Also other digestive diseases that show a clear pathophysiological abnormality in the smooth muscle or ENS are categorized under GI motility disorders, such as gastroparesis, postoperative ileus, gastroesophagal reflux, Hirschsprung's disease, chronic intestinal pseudo-obstruction, achalasia and scleroderma (Boeckxstaens et al., 2002; De Ponti and Malagelada, 1998; Gershon and Tack, 2007; Longo and Vernava, 1993; Manabe et al., 2010; Sanger, 2008; Wood et al., 1999). For patients with GI motility disorders, where there are no well-defined pathophysiological abnormalities, treatment should be aimed at symptom improvement and individualized patient care.

Prokinetics and antispasmodics have been widely used in the treatment of dysmotility. Although treatment options for chronic constipation (CC) are still centered on laxatives, non-laxative prokinetic agents have emerged, such as cisapride, tegaserod, mosapride, levosulpiride, domperidone, metoclopramide and prucalopride. None of these therapies have attained worldwide acceptance and availability (Camilleri and Deiteren, 2010; Cremonini et al., 2011; Dubois et al., 2010; Tack et al., 2009). The currently marketed prokinetics target mainly 5-HT_4 receptors, the most recent one prucalopride being a highly selective 5-HT_4 receptor agonist. The physiological role of 5-HT in the GI tract has still not been completely elucidated, partly because of the presence of multiple 5-HT receptor classes (1a, 2, 3, 4, 7) in the GI wall and partly because suitable ligands for in vivo studies are lacking (Gershon and Tack, 2007; Kindt and Tack, 2007). Still, it is clear that 5-HT is a critical signaling molecule in normal and abnormal GI function (Manabe et al., 2010; Neal and Bornstein, 2006).

1. II. 5-hydroxytryptamine (5-HT) and its receptors

1.II.1. Distribution of 5-HT in the body

The monoamine neurohumoral messenger, serotonin (5-hydroxytryptamine, 5-HT) has been implicated in the pathophysiology of a wide range of neuropsychiatric conditions including depression, anxiety disorders, obsessive-compulsive disorder, psychosis, eating disorders, and substance abuse and dependence. However, despite the importance of 5-HT in the CNS, 5-HT also has a role outside of its traditional place in neuropsychiatry (Coupar et al., 2007; Veenstra-VanderWeele et al., 2000). Peripherally, 5-HT modulates GI reflexes, but it is also functional in the bladder and adrenal gland. Most of the body's 5-HT (~95%) resides in the gut, where it is mainly synthesized, stored and released by EC cells, found in the mucosal crypts of the GI tract and to some extent also by descending interneurons of the myenteric plexus which project to other myenteric ganglia and/or submucous ganglia (De Ponti, 2004; Hansen, 2003c; Li et al., 2011). The amount of 5-HT present in intestinal neurons is very low in comparison to the amount present in EC cells (Li et al., 2011). In the brain, central serotonergic neurons are the main source of 5-HT; they are separated from the periphery by the blood-brain barrier, which is impermeable to 5-HT. The amount of 5-HT that is produced centrally is dependent on the amount of tryptophan, that is available peripherally to cross the blood-brain-barrier by active transport (Gershon and Tack, 2007; Jonnakuty and Gragnoli, 2008).

1.II.2. Synthesis, storage and metabolism of 5-HT

The EC cells in the GI tract produce and store 5-HT in granules until release. 5-HT is synthesized in the EC cells from the dietary amino acid L-tryptophan using the rate-limiting enzyme tryptophan hydroxylase-1 (TpH-1), whereas enteric and central serotonergic neurons contain another enzyme, TpH-2, which is a different gene product (**Fig. 1.8**) (Bertrand and Bertrand, 2009; Gershon and Tack, 2007; Walther and Bader, 2003). Mast cells also show immunoreactivity for TpH1 and contain 5-HT, indicating that mast cells can produce 5-HT on their own (Li et al., 2011; Penissi et al., 2003; Walther and Bader, 2003; Yu et al., 1999). TpH-1 in the EC cells converts L-tryptophan to 5-hydroxytryptophan (5-HTP) and the non-rate limiting enzyme L-amino acid decarboxylase (L-AADC) converts 5-HTP further to 5-HT.

Newly produced 5-HT is packaged into granules/vesicles by the vesicular monoamine transporter 1 (VMAT1); this isoform is specific for EC cells (Bertrand and Bertrand, 2009; Jonnakuty and Gragnoli, 2008).

In the serotonergic neurons of the CNS and ENS, VMAT2 is primarily expressed and responsible for packaging and transport of 5-HT (Anlauf et al., 2003; De Giorgio et al., 1996; Erickson et al., 1996; Wimalasena, 2011). 5-HT is released mainly from the granules stored near the basal border of the EC cell; this process is calcium-dependent suggesting an exocytotic release mechanism, but some studies have also identified granules near the apical membrane (Bertrand and Bertrand, 2009; Schwörer and Ramadori, 1998). Close contact between EC cells and other structures is not common, thus 5-HT must be released from the vesicles in high concentrations in a paracrine manner to enter the lamina propria where it can interact with nerve terminals and immune cells. EC cells produce and secrete far more 5-HT than either central or peripheral serotonergic neurons, such that the 5-HT secreted by EC cells overflows to reach the GI lumen and blood circulation. Overflowing 5-HT from EC cells is taken up by enterocytes, enteric neurons (Gershon and Tack, 2007), mast cells (Grundy, 2008; Kushnir-Sukhov et al., 2007) and platelets (Mercado and Kilic, 2010) via the 5-HT reuptake transporter (SERT). Platelets lack TpH and can thus not produce 5-HT on their own but are virtually the sole source of 5-HT in the blood (Gershon and Tack, 2007).

The primary targets of 5-HT secreted by EC cells are 1) the mucosal projections of intrinsic and extrinsic sensory nerves, which transmit sensations and discomfort to the central nervous system, 2) submucosal IPANs, initiating peristaltic and secretory reflexes and 3) myenteric IPANs (Bertrand and Bertrand, 2009; Gershon and Tack, 2007; Grider et al., 1998; Liu 2005; Racké et al., 1996).

Because of the continuous secretion of 5-HT, effective mechanisms for its inactivation are needed as 5-HT is positively charged at physiologic pH and thus passive diffusion cannot fulfill this task. Therefore, at different levels, versatile mechanisms such as enzyme-mediated catabolism of signaling molecules and transporter-mediated 5-HT uptake help to inactivate 5-HT and to regulate the timing of its action (Gershon and Tack, 2007; Jonnakuty and Gragnoli 2008). In the brain, the action of 5-HT is terminated by uptake via the transporter located in the membrane of serotonergic axon terminals and returned to presynaptic terminals where it is metabolized (Jonnakuty and Gragnoli 2008).

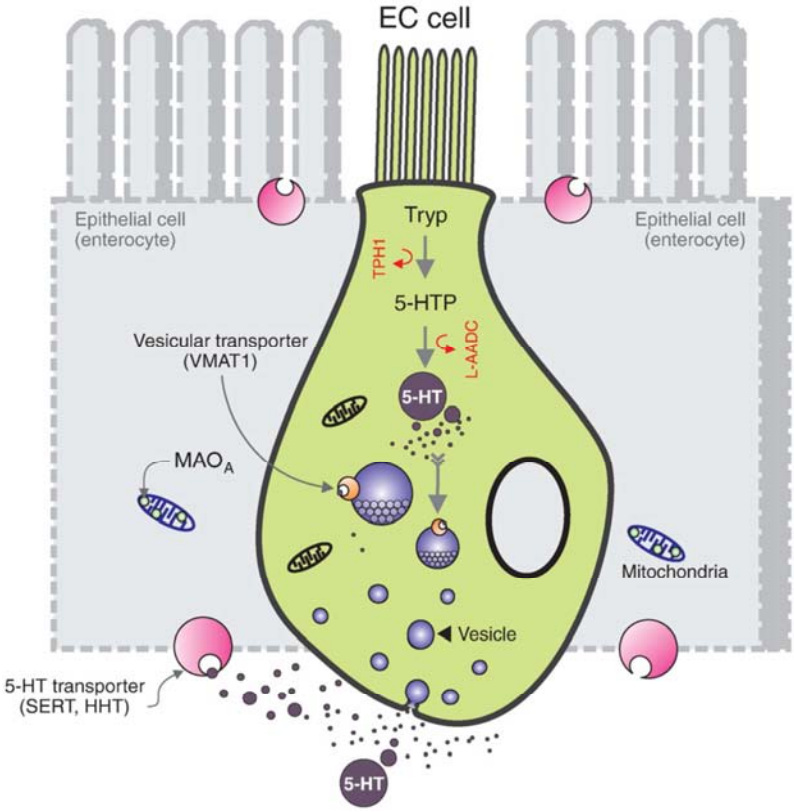


Figure 1.8. The synthesis of 5-HT by the enterochromaffin (EC) cell. The beginning of the synthesis pathway for 5-HT is represented at the top of the cell where dietary tryptophan (Tryp) is converted to 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase 1 (TpH1). 5-HTP is then converted to 5-HT by the enzyme L-amino acid decarboxylase (L-AADC). Newly produced 5-HT is packed into vesicles by the vesicular monoamine transporter 1 (VMAT1). Once released, 5-HT can be transported into surrounding epithelial cells by the 5-HT reuptake transporter (SERT) and degraded to 5-hydroxyindoleacetic acid (5-HAA) by monoamine oxidase A (MAO-A) (from Bertrand and Bertrand, 2009).

Via the SERT, 5-HT is actively transported in the platelets, enterocytes and enteric neurons, where 5-HT is catabolized to 5-hydroxyindoleacetic acid (5-HAA) by intracellular enzymes such as monoamine oxidases and glucuronyl transferases. Two isoforms of monoamine oxidase (MAO-A and –B) exist. In platelets, only MAO-B is present and in enterocytes only MAO-A. Both MAO-A and -B exist in neurons (Bertrand and Bertrand, 2009; De Ponti, 2004; Jonnakuty and Gragnoli, 2008).

1.II.3. 5-HT receptors

5-HT produces its effects through a variety of membrane-bound 5-HT receptors (Hansen, 2003c; Gershon and Tack, 2007). The 5-HT receptors have been divided into 7 classes, based upon their pharmacological profiles, cDNA-deduced sequences and signal transduction mechanisms. Although each 5-HT receptor can be potently activated by 5-HT itself, the differences in protein structure, and consequent affinities for different synthetic chemicals, provide a basis for identifying selective ligands, either agonist or antagonist, for each receptor variant (Hoyer et al., 1994). According to the current appellation of the receptor Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR), the 7 classes are indicated as 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-ht_5, 5-HT_6 and 5-HT_7 receptor. The 5-ht_5 receptor is at present not yet functionally identified and its transductional characteristics are unknown; as such it cannot be fully characterized and can only be provisionally classified retaining its lower case appellation (Hannon and Hoyer, 2008; Hoyer et al., 1994; Hoyer et al., 2002).

Some of the 7 classes of 5-HT receptors include multiple subtype receptors, which share similar structural and effector properties, but display different operational profiles (Coupar, 2007; Kindt and Tack, 2007). This relatively large number of 5-HT receptors is attributed to the long evolutionary history of the 5-HT signaling system, which predates the separation of vertebrates and invertebrates, some 600 million years ago. Consequently, there has been abundant time for gene duplications, followed by mutations and sequence shifts to form the different genes encoding for the different subtypes. It has been speculated that the ancestral 5-HT receptors functioned to facilitate cell to cell connections and to promote growth and differentiation. All 5-HT receptors belong to the superfamily of G-protein-coupled receptors containing a predicted seven-transmembrane structure, with the exception of the 5-HT_3 receptor which forms a ligand-gated cation channel.

5-HT and its receptors are found both in the central and peripheral nervous system, as well as in a number of non-neuronal tissues in the GI tract, cardiovascular system, bladder, adrenal gland and lung. 5-HT receptors have been described in the CNS (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5ht_5, 5-HT_6, 5-HT_7 receptor), GI tract (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_7 receptor), cardiovascular system (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_7 receptor), lung (5-HT_2 and 5-HT_3 receptor), platelets (5-HT_2 and 5-HT_3 receptor), bladder (5-HT_3 and 5-HT_4 receptor) and adrenal gland (5-HT_4 receptor) (Hannon and Hoyer, 2008; Hoyer et al., 1994; Hoyer et al., 2002; Hoyer and Martin, 1997; Uphouse, 1997).

1.II.4. 5-HT receptors in the GI tract

5-HT receptors that are known to affect gut motor function belong to the 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7 classes. Their distribution is shown in **figure 1.9** (Chetty et al., 2009; De Ponti, 2004; Hannon and Hoyer, 2008; Irving et al., 2007; Wouters et al., 2007).

Neuronal 5-HT receptors may inhibit or enhance transmitter release and include 5-HT_{1A} (inhibitory), 5-HT_3 and 5-HT_4 (both excitatory) classes. Smooth muscle 5-HT receptors may contract or relax effector cells and include 5-HT_{2A} (mediating contraction), 5-HT_4 and 5-HT_7 classes (both mediating relaxation). In healthy controls and dyspeptic patients, 5-$HT_{1B/D}$ receptor stimulation seems to have an effect on gastric motility, causing a delay in gastric emptying as a result of a significant relaxation of the gastric fundus, hereby enabling accommodation of larger volume. However, direct evidence for the presence of 5-$HT_{1B/D}$ receptors in the human GI tract is still lacking. 5-HT_{1A} receptors are largely distributed throughout the CNS; a possible role of 5-HT_{1A} receptors in modulating GI motility has been suggested in animal and human studies.

5-HT_{1A} receptor activation is associated with inhibition of electrically evoked contractions in guinea pig ileum and in stomach circular muscle and induces relaxation in dog proximal stomach and mouse fundus. In patients with functional dyspepsia, 5-HT_{1A} receptor agonists induced gastric relaxation. However in healthy volunteers, 5-HT_{1A} receptor agonists have no effect and data on the pharmacological effect of 5-HT_{1A} receptor activation in humans are rather limited (Beattie and Smith, 2008).

5-HT_{2A} receptor mRNA and immunoreactivity have been localized to myenteric and submucosal neurons, enterocytes, and longitudinal and circular muscle cells of the GI tract (Beattie and Smith, 2008). In rat and guinea pig gastric antrum and corpus, 5-HT_{2A} receptor activation results in contraction, while in the rat fundus in relaxation. 5-HT_{2A} receptors also mediate part of the contractile effects of 5-HT in the guinea pig ileum, contraction of longitudinal muscle in canine and guinea pig colon and contraction of longitudinal muscle in human small intestine. In human and rat isolated colonic mucosa, 5-HT_{2A} receptor activation mediates secretory responses (Beattie and Smith, 2008; Hannon and Hoyer, 2008; Hoyer et al., 2002).

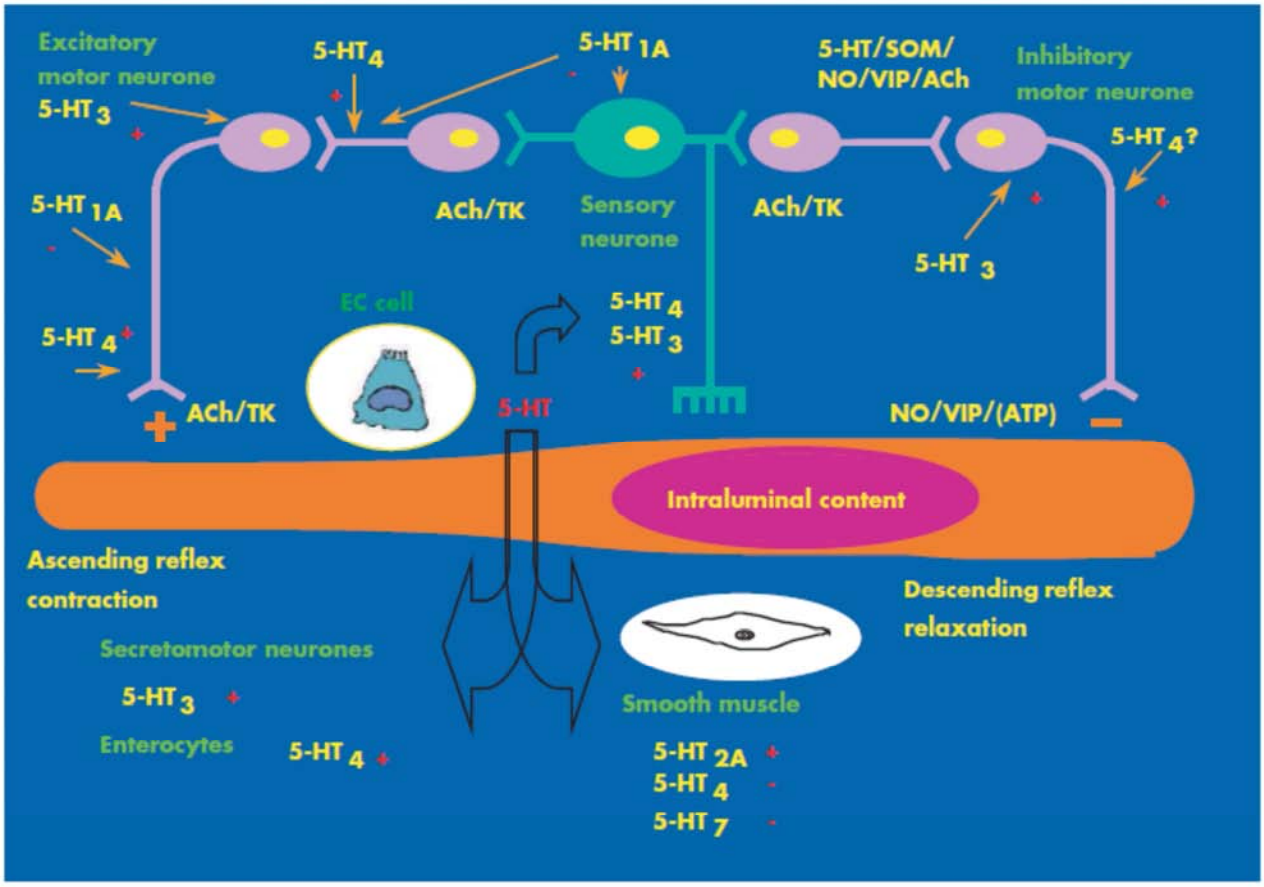


Figure 1.9. Modulation of intestinal function by 5-hydroxytryptamine (5-HT) receptors. Distention by intraluminal contents stimulates sensory neurons (intrinsic afferent neurons) which trigger an ascending excitatory reflex (leading to contraction) and a descending inhibitory reflex (leading to relaxation); 5-HT may be released by interneurons involved in the descending inhibitory reflex. 5-HT released from enterochromaffin (EC) cells may affect several subtypes of enteric neurons (sensory, motor, and secretomotor neurons) as well as final effector cells (smooth muscle cells and enterocytes) +, stimulation; -, inhibition, ?, circumstantial evidence (from De Ponti, 2004).

5-HT_{2B} receptors are predominantly found in the longitudinal and circular smooth muscle layers and in the myenteric nerve plexus throughout the colon where they cause neuronally mediated contractile responses of the longitudinal muscle (Chetty et al.,, 2009; Wouters et al., 2007). In rat stomach fundus, 5-HT_{2B} receptor stimulation causes fundic smooth muscle contraction (Beattie and Smith, 2008; Hannon and Hoyer, 2008; Hoyer et al., 2002). In human colon, 5-HT_{2B} receptor mRNA and protein are present in the longitudinal and, to a lesser extent, circular muscle layers and in myenteric neurons. 5-HT has been shown to produce 5-HT_{2B} receptor-mediated contraction of the longitudinal smooth muscle of the human ileum and augmentation of neuronally mediated contraction of human colonic smooth muscle (Beattie and Smith, 2008; Wouters et al., 2007).

mRNA expression analysis revealed that 5-HT_3 receptors are expressed in the human GI tract and several subunits have been identified (5-HT_{3A}, 5-HT_{3B}, 5-HT_{3C}, 5-HT_{3D}, 5-HT_{3E}). In the ENS, 5-HT_3 receptor immunoreactivity is expressed on neuronal cell bodies of the myenteric and submucosal plexuses, and nerve fibers in the circular and longitudinal muscle layers, submucosa and mucosa (Beattie and Smith, 2008; Wouters et al., 2007). 5-HT, released from EC cells by chemotherapy and abdominal radiotherapy, stimulates 5-HT_3 receptors on the GI nerve endings of extrinsic afferent neurons, leading to activation of the vomiting center in the CNS. This explains the efficacy of 5-HT_3 receptor antagonists in chemotherapy- and radiotherapy-induced nausea and vomiting (Beattie and Smith, 2008; Hoyer et al., 2002). In guinea pig and mouse stomach, 5-HT_3 receptor activation is associated with increased contractility. In guinea pig ileum, also mucosal processes of myenteric IPANs are excited via 5-HT_3 receptor activation and play a role in initiation or enhancement of myenteric reflexes (Beattie and Smith, 2008; Zhou and Galligan, 1999).

With respect to rodent and human GI motility effects, 5-HT_3 receptor antagonists are associated with reductions in colonic and whole GI tract motility, increased fluid absorption, stool consistency and visceral sensitivity (Beattie and Smith, 2008; De Ponti, 2004; Hoyer et al., 2002). 5-HT_{3A} and 5-HT_{3B} receptors are localized to the submucous plexus ganglion cells and are expressed in the myenteric nerve plexus in the human colon (Chetty et al., 2009). In patients with severe diarrhea predominant IBS, 5-HT_3 receptor antagonists inhibit 5-HT_3 receptors located on sensory, ascending and descending neuronal pathways involved in peristalsis, hereby slowing down colonic transit and inhibiting the colonic motor response to a meal (De Ponti, 2004). 5-HT_3 receptors are also expressed on sensory nerve endings where they contribute to sensations of bloating and pain. Several animal and human studies point to a role of 5-HT_3 receptors in modulating visceral sensitivity through an action on intrinsic or extrinsic

primary afferent neurons. In addition, 5-HT_3 receptor antagonists may also modulate perception of visceral sensations by acting at central 5-HT_3 receptors (De Ponti, 2004). The use of 5-HT_3 receptor agonists in therapeutics is still questionable, as stimulation of 5-HT_3 receptors can on the one hand exert a prokinetic effect but it can also stimulate nausea and vomiting.

The 5-HT_4 receptors mediate several responses in the gut, but will be extensively described in the next section.

The 5-HT_7 receptor is present in the ENS and is postulated to play a role in GI physiology. 5-HT_7 receptor immunoreactivity is shown in myenteric and submucosal IPANs and smooth muscle cells. In human colon and guinea pig ileum, 5-HT acts directly on smooth muscle 5-HT_7 receptors to induce relaxation (Beattie and Smith, 2008; Chetty et al., 2009). 5-HT_7 receptors also have a role in inhibition of peristalsis by 5-HT. Blockade of 5-HT_7 receptors may increase the threshold pressure to trigger intestinal peristalsis and decrease the compliance of the intestinal wall (De Ponti, 2004; Irving et al., 2007; Wouters et al., 2007).

1.III. 5-HT_4 receptors

5-HT_4 receptors are considered to have a particularly important physiological role in the regulation of GI function. In the upper gut, 5-HT_4 receptors facilitate gastric emptying and peristalsis, whereas in the colon, 5-HT_4 receptors modulate both smooth muscle relaxation and contraction. In the small and large intestine, they may stimulate mucosal secretion of water and electrolytes (Beatie and Smith, 2008; Tonini et al., 1999).

As for the extra-GI distribution, 5-HT_4 receptors have also been identified in a wide variety of tissues and species (Hoyer et al., 1994). In the CNS, the receptor appears to be located on nerve cells where they enhance neurotransmitter release and hence synaptic transmission, possibly playing a role in memory enhancement. In the heart, 5-HT_4 receptor activation evokes tachycardia in the right atria and a positive inotropic effect in left atria of humans, pigs and monkeys. Activation of the 5-HT_4 receptor in the urinary bladder of man enhances smooth muscle contraction and in the adrenocortical cells, the 5-HT_4 receptor mediates steroid secretion (Hoyer et al., 1994; Hoyer et al., 2002; Tonini et al., 1999).

1.III.1. Structure

The basic structure of the 5-HT_4 receptor, belonging to the G-protein coupled receptor (GPCR) family, is similar to that proposed for nearly all of the GPCRs. GPCRs are conserved molecules which have been central, during evolution to recognize and transduce external (e.g. odors, gustative molecules and light) as well as cell-cell communication signals (hormones, neurotransmitters, growth factors). They comprise 2% of the human genome and remain central for the understanding of human physiology and human diseases. Forty to 50% of all marketed drugs act directly to modulate GPCRs even if less than 20% of GPCRs (non-odorant receptors) are currently targeted. 5-HT_4 receptors reached their place in GPCR pharmacology almost 20 years ago and remain the matter of intense research especially in brain and gastro-intestinal systems. Indeed, the hope that 5-HT_4 receptor agonists could be successful drugs in gastro-intestinal pathologies and in particular for chronic idiopathic constipation is high (Bockaert et al., 2011).

The 5-HT_4 receptor is an integral membrane protein that possess seven hydrophobic transmembrane domains connected by three intracellular loops and three extracellular loops. The amino terminus is oriented toward the extracellular space, whereas the carboxyl terminus (C-terminus) is oriented toward the cytoplasm (**Fig. 1.10**). In some cases, the C-terminus is tethered to the plasma membrane through palmitoylation of cysteine residues, stimulated by receptor activation (Bockaert et al., 2004). The 5-HT_4 receptor also possesses common sites for posttranslational modifications. These include extracellular signal sequences, extracellular glycosylation domains, extracellular cysteine residues that participate in structurally significant disulfide bonds and intracellular domains for interacting with G proteins and other regulatory proteins, and sites for phosphorylation (Roth, 2006). Once formed, 5-HT_4 receptors are transported and embedded in the cell-surface, where they take on their functions, responding to their ligands (Coupar et al., 2007).

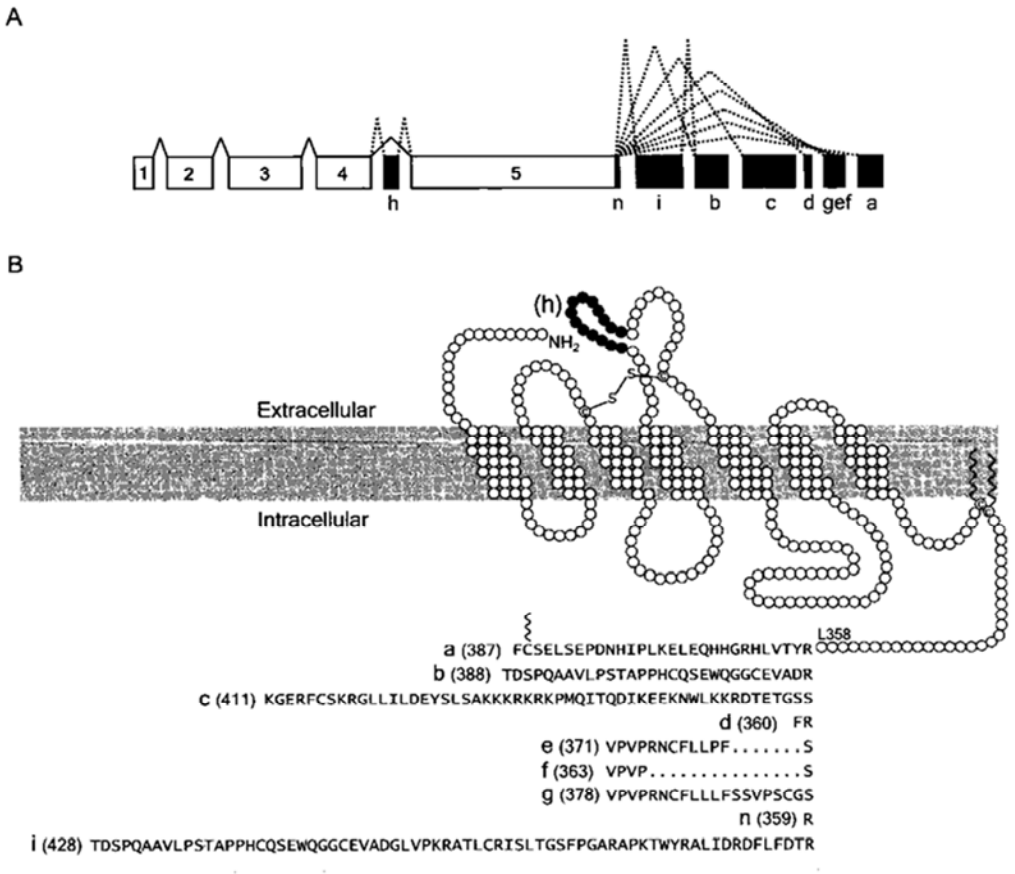


Figure 1.10. A) Organization of the coding part of the human *HTR4* gene. Schematic representation of the exons present in the coding region. B) Human 5-HT_4 receptor splice variants. C-terminal amino acid sequences are shown for the different splice variants. The 5-HT_4 receptor splice variants have identical sequences up to L^{358} and differ by length and composition of their C-terminus. The 5-$HT_{4(h)}$ receptor variant has an insertion in the second extracellular loop (from Bockaert et al., 2004).

1.III.2. Gene and splice variants

Among the GPCR genes, the *HT4R* gene is one of the largest (700kb, 38 exons). The human 5-HT_4 receptor is mapped to chromosome 5q31-33. The *HT4R* gene is highly complex: it contains multiple introns and exons, which create after transcription and alternative splicing several different splice variants, in most species including man (**Fig. 1.10**) (Bockaert et al., 2004; Hannon and Hoyer, 2008; Hoyer et al., 2002).

From the *HT4R* gene different products can be generated by the combination of alternative forms of particular exons. This process is referred to as 'alternative splicing' and translated products are called 'splice variants' or more commonly 'isoforms'. The discovery that particular splice variants are tissue specific (or prominent) has highlighted their potential as future drug targets. Therefore, just as the discovery of different receptor subgroups opened up vast opportunities to develop new drug treatments, the discovery of splice variants promises to further expand and refine these opportunities (Coupar et al., 2007).

Whether in mice, rats or humans, the splice variants (except 5-HT_{hb}) differ at their carboxyl termini after Leu^{358}, and the length and the composition of the rest of the C-terminal tail is specific for each variant (Hannon and Hoyer, 2008). To date, there are four C-terminal splice variants in rat (a, b, e, c1) and four in mouse (a, b, e, f) . There are at least 11 human 5-HT_4 receptor splice variants, ten C-terminal (a, b, c, long c, d, e, f, g, i, n) and one internal (h) (**Fig. 1.10**) (Nedi et al., 2011). The C-terminal end is very short for the 5-$HT_{4(d)}$ and 5-$HT_{4(n)}$ human isoforms (Hannon and Hoyer, 2008). The a, b, c, d, g and i isoforms of the 5-HT_4 receptor are all present in the human intestine (Wouters et al., 2007). The human 5-$HT_{4(d)}$ isoform appears to be unique, because in contrast to other isoforms, it has not been described in any other species yet and its expression appears to be restricted to the gut (Hannon and Hoyer, 2008; Hoyer et al., 2002).

The 5-$HT_{4(hb)}$ variant features a 14 residue isoform in the second extracellular loop and is in human only been found in the association with the b isoform (Bender et al., 2000). Tissue distribution studies revealed some degree of specificity of the splice variants in man. For instance, 5-$HT_{4(a)}$, 5-$HT_{4(b)}$, 5-$HT_{4(c)}$ and 5-$HT_{4(g)}$ receptors are all expressed in the heart (atrium), brain and intestine. The 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ receptor subtypes are the only receptors present in the bladder and adrenal gland (Hannon and Hoyer, 2008).

All the 5-HT_4 receptor splice variants have the same transmembrane domain core and therefore the same primary binding site for 5-HT as well as for most of the synthetic ligands. In addition, 5-HT_4 receptor splice variants uniformly are able to stimulate adenylate cyclase (AC) activity and raise intracellular cAMP levels in the presence of 5-HT or agonists.

However when functional studies with transfected 5-HT_4 receptor splice variants are considered, some interesting differences in efficacy and potency upon splice variant stimulation have been observed (Bockaert et al., 2004; Ponimaskin et al., 2005; Roth, 2006). Differences may extend to the type of G proteins to which the various splice variants are coupled. It is generally accepted that 5-HT_4 receptors are positively linked with G_s protein (Nedi et al., 2011). However, recent studies showed that the 5-$HT_{4(a)}$ receptor is coupled to both $G_{\alpha s}$ and $G_{\alpha 13}$ proteins, whereas the 5-$HT_{4(b)}$ receptor activates G_i and G_s proteins (Ponimaskin et al., 2005). On the other hand, the constitutive activity of the variants, depending on the sequence of the splice variants and differences in desensitization as a result of the action of G-protein-coupled protein kinases (GRKs), can play a role in different efficacy and potency (Bockaert et al., 2004). Interestingly, the short C-terminal isoforms (5-$HT_{4(e)}$, 5-$HT_{4(f)}$) have a higher constitutive activity than the long C-terminal isoforms (Bockaert et al., 2004). The 5-$HT_{4(e)}$ and 5-$HT_{4(f)}$ receptors induce significantly more agonist-independent increases in AC activity than do some other 5-HT_4 receptor splice variants. It is likely that 5-HT_4 receptor splice variants differ in their expression of distinct PDZ (PSD-95/Disc large/Zonula occludens-1) domains which might alter their signaling properties or other functions. For example, ten proteins are identified as potential binding partners of the 5-$HT_{4(a)}$ receptor, including seven with at least one PDZ domain. It is noteworthy that many of these proteins are known to be involved in membrane targeting or protein trafficking. In contrast, only three proteins were associated with the 5-$HT_{4(e)}$ receptor carboxyl terminus. Interestingly the 5-$HT_{4(b)}$ receptor carboxyl terminus, which lacks a PDZ domain, did not interact with any of the proteins that interacted selectively with the 5-$HT_{4(a)}$ or 5-$HT_{4(e)}$ receptors. So it seems likely that 5-HT_4 receptor-interacting proteins can selectively participate in membrane localization, trafficking and possibly signal transductions of the various isoforms (Roth, 2006).

1.III.3. Signal transduction

In the absence of agonists, 5-HT_4 receptors are dynamic and constitutive receptors which continuously change their conformational state, however agonist-mediated 5-HT_4 receptor signaling requires the isomerization of the receptor to a high-affinity agonist-binding conformation (Bockaert et al., 2004). 5-HT_4 receptors undergo a conformational change upon ligand binding that facilitates interaction between an intracellular domain of the receptor and a heterotrimeric guanine nucleotide binding protein (G protein). This interaction results in the activation of the G protein by facilitating the exchange of bound guanosine diphosphate (GDP) with guanosine triphosphate (GTP) and changing the G protein conformation.

The activated G protein then dissociates from the receptor into the active GTP bound G_α subunit and $G_{\beta\gamma}$ dimer. In their activated form the G protein subunits activate primary effectors which modulate ion or second messenger concentrations that are subsequently responsible for cellular response. The G_α unit remains active until GTP is hydrolyzed resulting in the inactive, GDP-bound G_α unit that eventually re-associates with the $G_{\beta\gamma}$ unit (Woehler and Ponimaskin, 2009). As a member of the GPCR family, the 5-HT_4 receptor couples preferentially to G_s and promotes cAMP formation, by activation of AC (Nedi et al., 2011). cAMP as an intracellular messenger interacts with various targets, such as the phosphorylating enzyme protein kinase A (PKA), but also cyclic nucleotide-gated ion channels, leading to the modulation of calcium ion flux and membrane excitability (Hannon and Hoyer, 2008; Nedi et al., 2011; Roth, 2006). PKA phosphorylates cAMP-responsive transcription factors, such as the cAMP response element binding protein (CREB), which leads to changes in gene expression, and thus may promote long term changes in gene expression and cellular responses. Although coupling to G proteins remains a central event in 5-HT_4 receptor mediated signaling, evidence indicates that the regulation of their functional status also depends on interactions with a variety of additional protein partners named GPCR interacting proteins (GIPs) (Bockaert et al., 2006; 2011). These interactions create additional opportunities for not only G-protein-dependent signaling but also for G-protein-independent signaling. Several proteins have been identified to interact directly with the C-terminus of 5-HT_4 receptor splice variants, most through PDZ-domain-containing proteins. The acronym PDZ derives from the three first proteins (PSD-95/Disc large/Zonula occludens-1) in which these domains have been described. PDZ proteins are involved in the scaffolding of multiprotein complexes that contribute to the targeting, the trafficking and fine tuning of signaling properties of membrane-bound 5-HT_4 receptors. These PDZ proteins bind generally to a conserved sequence located at the extreme C-terminus. Specific sets of PDZ proteins interacting with 5-$HT_{4(a)}$ and 5-$HT_{4(e)}$ receptor have already been characterized (Bockaert et al., 2006).

Further, cAMP seems to interact with a family of cAMP sensors called Epac (exchange protein directly activated by cAMP), which mediate the PKA-independent Src/ERK (sarcoma/extracellular signal-regulated protein kinase) pathway (Hannon and Hoyer, 2008; Nedi et al., 2011). 5-HT_4 receptor stimulation mediates G-protein independent pathways by ERK activation through phosphorylation and activation of the 5-HT_4 receptor-associated non-receptor tyrosine kinase Src (Bockaert et al., 2011).

5-HT_4 receptors also regulate a variety of channels: 1) activation of voltage sensitive Ca^{2+} channels, responsible for the prokinetic actions of the 5-HT_4 receptors in the gut, 2) positive inotropic effect in the atria by activating cardiac L-type Ca^{2+} channels, 3) regulation of the Ca^{2+}-activated K^+ current in adult hippocampal neurons. In human atrial myocytes, activation of L-type Ca^{2+} channels by the 5-HT_4 receptor occurs via an elevation of intracellular cAMP levels and stimulation of PKA (Roth, 2006). In mouse colliculi neurons and rat hippocampus, the 5-HT_4 receptor mediates increase in cAMP concentration after application of agonist, leading to the inhibition of voltage-gated K^+ channels, including Ca^{2+}-activated K^+ channels (Bockaert et al., 2006; Taniyama et al., 2000). In human atrial myocytes, 5-HT_4 receptor mediates contraction as a result of an increase in Ca^{2+} current, subsequent to PKA-dependent phosphorylation of L-type Ca^{2+} channels (Galindo-Tovar et al., 2009; Hegde and Eglen, 1996; Ouadid et al., 1992).

1.III.4. 5-HT_4 receptors in the GI tract

5-HT_4 receptors are considered to have an important role in the regulation of GI functions. In general, studies showed expression of 5-HT_4 receptors on EC cells, enteric neurons, ICCs, smooth muscle cells and enterocytes. In **figure 1.9**, the 5-HT_4 receptor locations are shown in a generalized scheme, pooling data obtained in different animals. Most knowledge on 5-HT_4 receptor involvement in GI motility and secretion is obtained in laboratory animals. Although many similarities exist between these animals and man, an understanding of the differences is important during the drug discovery process when interpreting experimental data obtained in animal models and when extrapolating to the human situation.

1.III.4.1. Animal studies

GI propulsion is dependent on local peristaltic reflexes, leading to an ascending contraction and a descending relaxation, evoked in response to GI tract distention or mucosal stimulation. This peristaltic reflex is coordinated by 5-HT_4 receptor activation triggering the release of acetylcholine from myenteric cholinergic neurons, but also release of neurotransmitters from IPANs (De Ponti, 2004; Gershon and Tack, 2007; Greenwood-van Meerveld, 2007; Greenwood-van Meerveld et al., 2006; Poole et al., 2006). In addition, several studies in the GI tract of rodents have revealed that 5-HT_4 receptors located on smooth muscle cells contribute to the descending relaxation of the peristaltic reflex (Komada and Yano, 2007; Liu et al., 2005; Poole et al., 2006; Sakurai-Yamashita et al., 1999; Takada et al., 1999).

Next to the effect of 5-HT_4 receptor agonists on GI motility, activation of 5-HT_4 receptors has also an effect on fluid secretion. 5-HT_4 receptor mediated secretory responses in the small and large intestine are well-known in laboratory animals. However, a functional role in mucosal secretion of the stomach is not yet known. In rats and mice, 5-HT induced HCO_3^- secretion in the duodenum and Cl^- and HCO_3^- secretion in the colon, which were both mediated by 5-HT_4 receptors located on the enterocytes (Albuquerque et al., 1998; Budhoo and Kellum, 1994a; Budhoo et al., 1996a; Grider, 2003; Ning et al., 2004; Säfsten et al., 2006; Tuo et al., 2004). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis confirmed the presence of 5-HT_4 receptors in the duodenal mucosa of mice (Tuo et al., 2004). Contradictory results have been reported about the effect of 5-HT_4 receptor activation on EC cells in the ileum. In guinea pig ileum, 5-HT_4 receptors on EC cells inhibit the release of 5-HT (Gebauer et al., 1993), whereas in rat ileum it has been reported that 5-HT release was stimulated via activation of 5-HT_4 receptors on the EC cells (Minami et al., 1995). A recent immunohistochemical study in the mucosa of mouse colon and ileum indicated, that enteroendocrine-like cells, at the base of the crypts of the ileum and both EC cells and goblet cells in the colon, express the 5-HT_4 receptor; in this study, mucosal 5-HT_4 receptor activation in the murine colon elicited 5-HT and Cl^- release and mucus secretion (Hoffman et al., 2012).

Apart from the presence of 5-HT_4 receptors on neurons, EC cells and enterocytes, 5-HT_4 receptors have also been described on rodent ICCs. Evidence for expression of 5-HT_4 receptors in ICCs comes from studies carried out in the mouse and guinea pig small intestine, but whether activation of 5-HT_4 receptors play a role in the survival of ICCs or is involved in the generation and propagation of the electric slow wave by increasing cAMP levels remains to be elucidated, as no functional studies have been carried out in laboratory animals. Also data regarding the expression and function of 5-HT_4 receptors on human ICCs are still lacking (Wouters et al., 2007).

1.III.4.2. Human studies

In the human GI tract, activation of the differently localized 5-HT_4 receptors affects GI motility and secretion in analogy to what has been observed in different animal models. At the level of secretion in the human small intestine, 5-HT-induced Cl^- secretion is controlled via a non-neuronal pathway by 5-HT_4 receptors located on enterocytes (Borman and Burleigh, 1993; Budhoo and Kellum, 1994b; Budhoo et al., 1996b; Burleigh and Borman, 1993; Kellum et al., 1994; Säfsten et al. 2006).

In contrast, the presence of inhibitory 5-HT_4 receptors on EC cells, reducing 5-HT release, has also been reported in the human small intestine (Schwörer and Ramadori, 1998). More recently, data of 5-HT_4 receptor mRNA expression in the mucosa of human stomach, small intestine (Hoffman et al., 2012; van Lelyveld et al., 2007) and colon (Chetty et al., 2009; Hoffman et al., 2012) confirmed the presence of 5-HT_4 receptors in the mucosa of human GI tissues. Functional evidence for 5-HT_4 receptors involved in 5-HT-induced secretion has already been reported in the human ascending colon (Borman and Burleigh, 1996), yet the cellular distribution and functional implications of 5-HT_4 receptors within the epithelial layer of human stomach and colon must be further elucidated.

On the other hand, 5-HT_4 receptors located on IPANs evoke a neuronal reflex that also stimulates Cl^- secretion, activated by 5-HT released in response to mucosal stroking of the human jejunum (Foxx Orenstein et al., 1996; Grider et al., 1998; Kellum et al., 1999). Whether 5-HT_4 receptors are located on IPANs in the human colon, mediating colonic secretion and/or visceral sensitivity, is not clear. Yet, studies with the partial 5-HT_4 receptor agonist, tegaserod, showed a decrease in colonic and/or rectal sensitivity suggesting that 5-HT_4 receptors located on IPANs are involved (Camilleri, 2001; Coffin et al., 2003).

Next to the involvement of 5-HT_4 receptor activation in human GI secretion, in the human lower esophageal sphincter (LES) (Tomita et al., 1997) and in the circular muscle layer of the human stomach (Leclere and Lefebvre, 2002) 5-HT_4 receptor activation induces facilitation of cholinergic neurotransmission enhancing contractile responses. In the human jejunum, 5-HT_4 receptor activation leads to a peristaltic reflex with an ascending contraction and descending relaxation (Foxx-Orenstein et al., 1996; Grider et al., 1998). In the human colon 5-HT_4 receptor activation might be involved in the peristaltic reflex at several sites. Direct activation of 5-HT_4 receptors located on circular smooth muscle (McLean et al., 1995; McLean and Coupar, 1996a; 1996b; Tam et al., 1995) and activation of 5-HT_4 receptors on nitrergic neurons was reported to induce circular muscle relaxation (Cellek et al., 2006). On the other hand, 5-HT_4 receptor activation stimulates acetylcholine release from the cholinergic neurons towards both muscle layers of the colon, leading to contraction. This effect is considered to be the main mechanism of action of the clinically used 5-HT_4 receptor agonists (Prins et al., 2000; Leclere et al., 2005).

1.III.4.3. Facilitation of gastrointestinal release of ACh by 5-HT_4 receptors

ACh is the excitatory transmitter substance of cholinergic enteric nerves distributed in the GI tract, depolarizing the membrane and producing contraction in GI smooth muscle cells, through activation of atropine-sensitive muscarinic receptors (Kodama et al., 2010). Release of ACh and modulation of release may utilize different mechanisms. Irrespective of the primary event that modulates release, the ultimate reaction on presynaptic receptor activation is a change in the amount of transmitter that is released (Cooper and Meyer, 1984). Exocytosis of synaptic vesicle contents is the predominant mechanism for the regulated secretion of ACh. However, alternative mechanisms of secretion have also been proposed (de Castro et al., 2009) suggesting that ACh is released from cholinergic nerve terminals in a Ca^{2+} independent non-vesicular manner (Cooper and Meyer, 1984), but further evidence is still lacking.

Synaptic vesicle transmission is initiated when an action potential induces the opening of voltage-dependent Ca^{2+} channels, and the resulting Ca^{2+} transient stimulates vesicle exocytosis by triggering fusion-pore opening of the vesicles with the plasma membrane. In preparation for neurotransmitter release, synaptic vesicles dock at the active zone and are converted to become Ca^{2+} responsive (Cooper and Meyer, 1984; Südhof, 2004). The mechanism underlying the exocytosis seems to be mediated by the increase in cytosolic free Ca^{2+} resulting from PKA potentiation of Ca^{2+} entry (Borges et al., 2002). However, involvement of protein kinase C (PKC) in exocytosis has also been indicated by Borges et al. (2002). In addition, Ca^{2+} levels can also be elevated by a voltage-independent process e.g. Na^{+}/Ca^{2+} exchange or a direct effect on an intraneuronal calcium channel, then modulation can be effected without a change in membrane potential (Cooper and Meyer, 1984).

The link between ACh release and excitation of cholinergic neurons or receptor activation on cholinergic neurons is not completely understood. Still in the guinea pig ileum, it has been shown that stimulating adenylate cyclase or inhibiting cAMP breakdown, stimulates ACh release from myenteric cholinergic neurons, indicating that cAMP is involved in the ACh release mechanism (Reese and Cooper, 1984; Yau et al., 1987). In addition, a potential role of PKC has been shown to enhance vesicular release of acetylcholine from enteric neurons of the guinea pig small intestine (Hashimoto et al., 1988).

The cellular mechanism by which 5-HT_4 receptors couple to increase ACh release from cholinergic neurons is not fully established. Neuronal 5-HT_4 receptors in the GI tract are positively coupled to adenylate cyclase and cAMP (**Fig. 1.11**). cAMP has been shown to depolarize myenteric neurons by activation of cAMP-dependent PKA, which may result in closure of potassium channels, leading to increased cell excitability and to enhanced acetylcholine release from myenteric cholinergic neurons (Eglen et al., 1995; Hegde and Eglen, 1996; Taniyama et al., 2000). Although 5-HT_4 receptor activation in myenteric neurons does not change neuronal excitability at the level of the cell body, still 5-HT_4 receptor agonists do increase ACh release, suggesting that the receptor and its signaling cascade are located in the nerve terminals. In guinea pig ileum, 5-HT_4 receptor-mediated facilitation of electrically evoked ACh release is coupled to increases in cAMP and depends on activation of PKA. cAMP acting directly on ion channels or effects independent on PKA signaling pathways were not seen in this study (Galligan et al., 2003).

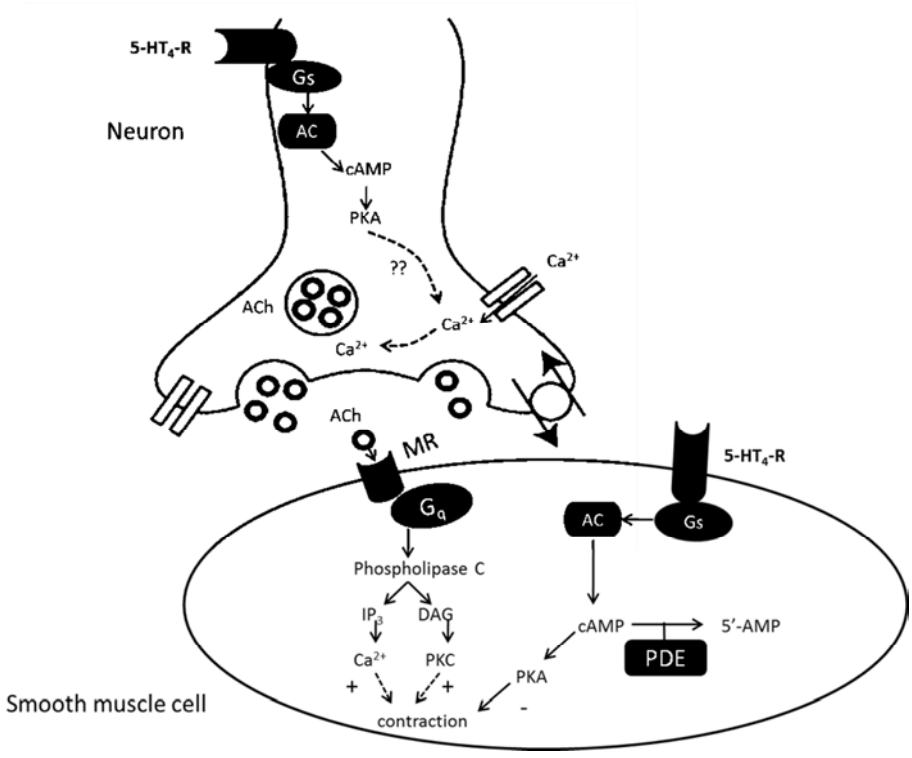


Figure 1.11. Schematic representation of neuronal 5-HT_4 receptor activation, leading to facilitation of acetylcholine release in cholinergic neurons and muscular 5-HT_4 receptor activation, leading to relaxation. Acetylcholine (ACh); adenylate cyclase (AC); muscarinic receptor (MR); inositol 1,4,5-triphosphate (IP_3); diacylglycerol (DAG); protein kinase C (PKC); cyclic adenosine monophosphate (cAMP); protein kinase A (PKA); phosphodiesterase (PDE). The mechanism of PKA to induce Ca^{2+} increase is not yet clear (??).

1.III.4.4. Signal transduction of 5-HT_4 receptors on gastrointestinal smooth muscle

Also muscular GI 5-HT_4 receptors are positively coupled to AC. In the tunica muscularis mucosae of the rat esophagus, it has been shown that 5-HT stimulates relaxation through cAMP formation via 5-HT_4 receptors (Ford et al., 1992). In human colonic smooth muscle, 5-HT_4 receptors couple to the G_s subtype of the G-protein in response to 5-HT with activation of adenylate cyclase resulting in accumulation of intracellular cAMP and subsequent activation of PKA (**Fig. 1.11**) (McLean and Coupar, 1996a; 1996b). PKA acts downstream to induce relaxation by inhibiting Ca^{2+}-mobilization, induced by excitatory substances such as ACh (**Fig. 1.11**). Together with cAMP, cyclic guanosine monophosphate (cGMP), induced by guanylate cyclase activation by NO, is a relaxant second messenger in the GI smooth muscle. Intracellular levels of the cyclic nucleotides (cAMP and cGMP) are controlled by a balance between their synthesis and breakdown and PDEs are the sole enzymes in mammalian cells responsible for their catabolism (Murthy, 2006). These PDEs exist as several families that vary in their substrate preference, sensitivity to endogenous activators and inhibitors, and tissue and intracellular distribution (Barnette et al., 1993). It is well established that PDEs also control the levels of cAMP and cGMP in the GI smooth muscle (Murthy, 2006). In the guinea pig ileum, longitudinal muscle contraction was inhibited by PDE5 inhibition through increase in cGMP and subsequent decrease in intracellular Ca^{2+} levels (Kaneda et al., 1997). In rabbit stomach, PDE3 and PDE4 inhibitors augmented cAMP levels in an additive manner, resulting in relaxation (Murthy et al. 2002). In canine colon, only PDE4 appeared to be functionally important in reducing contractile activity (Barnette et al., 1993). To what extent PDEs contribute to the control of cAMP in GI neurons is not well established.

1.III.5. 5-HT_4 receptors in the porcine GI tract

The pig is an omnivorous mammalian species with similar GI morphology and physiology to the human GI tract, suggesting that it could function as a good animal model (Miller and Ullrey, 1987). As for the human stomach, the stomach of the pig is of the glandular type and is lined with a cardiac, gastric and pyloric mucosa. However, the porcine stomach is 2 to 3 times larger and the cardiac mucosa occupies a greater portion of the stomach compared to the human stomach (Kararli, 1995). The porcine colon appears to be divided into 3 regions similar to the human colon: the ascending, the transverse and descending colon. However, how the colon winds through the body is different compared to the human intestine.

The porcine ascending colon is the largest part of the colon, occupies most of the abdomen and winds first spirally downwards in a centripetal coil and back upwards in a centrifugal coil. The transverse colon is short, crossing the abdomen from the left to the right side and continues in the descending colon, which descends straight down in the abdomen behind the ascending colon ending downwards in the rectum (Dyce and Wensing, 2010; Eurell and Frappier, 2006).

In view of its similarities, the porcine intestine has been employed as a suitable experimental model for studying the human ENS. Because of the limited availability of human material for neurogastroenterological studies, intensive investigations of ENS structure and function and their relationship to GI disorders should be conducted in animal models, such as the pig, having high homology to the human GI tract (Brown and Timmermans, 2004; Hens et al., 2000; Leclere et al., 1998; Sevcencu et al., 2005); this will also allow to develop the pig as a model for testing drugs in development.

Little information is yet available on 5-HT_4 receptor distribution and regulation in the GI tract of the pig. Activation of neuronal 5-HT_4 receptors induced Cl^- secretion from the mucosa of the porcine jejunum (Hansen, 1994; 1995). In addition, the presence of inhibitory 5-HT_4 receptors on EC cells was also shown in the porcine small intestine (Schwörer and Ramadori, 1998). As for motility, a recent study in porcine stomach showed the presence of a facilitating 5-HT_4 receptor on the cholinergic neurons towards the gastric longitudinal muscle in analogy with the human stomach, showing that the pig is a good animal model for the human stomach (De Maeyer et al., 2006a). The pig as a model for the study of 5-HT_4 receptors is further corroborated by the fact that it is the only suitable animal model, together with the monkey, to study cardiac effects of 5-HT_4 receptor agonists, as those are the only two species where 5-HT_4 receptors are detected in the heart under physiological conditions (Brattelid et al., 2004; De Maeyer et al., 2006a; 2006b; Galindo-Tovar et al., 2009; Kaumann, 1990; Kaumann and Levy, 2006; Krobert et al., 2005; Mader et al., 2006).

1.IV. REFERENCES

Albuquerque FC Jr, Smith EH, Kellum JM. 5-HT induces cAMP production in crypt colonocytes at a 5-HT4 receptor. J Surg Res. 1998; 77: 137-140

Anlauf M, Schäfer MK, Eiden L, Weihe E. Chemical coding of the human gastrointestinal nervous system: cholinergic, VIPergic, and catecholaminergic phenotypes. J Comp Neurol. 2003; 459: 90-111

Barajas-López C, Berezin I, Daniel EE, Huizinga JD. Pacemaker activity recorded in interstitial cells of Cajal of the gastrointestinal tract. Am J Physiol. 1989; 257: C830-C835

Barnette MS, Manning CD, Price WJ, Barone FC. Initial biochemical and functional characterization of cyclic nucleotide phosphodiesterase isozymes in canine colonic smooth muscle. J Pharmacol Exp Ther. 1993; 264: 801-812

Bayer S, Crenner F, Aunis D, Angel F. Effects of GABA on circular smooth muscle spontaneous activities of rat distal colon. Life Sci. 2002; 71: 911-925

Beattie DT, Smith JA. Serotonin pharmacology in the gastrointestinal tract: a review. Naunyn Schmiedebergs Arch Pharmacol. 2008; 377: 181-203

Bender E, Pindon A, van Oers I, Zhang YB, Gommeren W, Verhasselt P, Jurzak M, Leysen J, Luyten W. Structure of the human serotonin 5-HT4 receptor gene and cloning of a novel 5-HT4 splice variant. J Neurochem. 2000; 74: 478-489

Benkó R, Undi S, Wolf M, Vereczkei A, Illényi L, Kassai M, Cseke L, Kelemen D, Horváth OP, Antal A, Magyar K, Barthó L. P2 purinoceptor antagonists inhibit the non-adrenergic, non-cholinergic relaxation of the human colon in vitro. Neuroscience. 2007; 147: 146-152

Berridge MJ. Smooth muscle cell calcium activation mechanisms. J. Physiol 2008; 586(Pt 21): 5047-5061

Bertrand PP, Bertrand RL. Serotonin release and uptake in the gastrointestinal tract. Auton Neurosci. 2010; 153: 47-57

Bockaert J, Claeysen S, Bécamel C, Dumuis A, Marin P. Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. Cell Tissue Res. 2006; 326: 553-572

Bockaert J, Claeysen S, Compan V, Dumuis A. 5-HT4 receptors. Curr Drug Targets CNS Neurol Disord. 2004; 3: 39-51

Bockaert J, Claeysen S, Compan V, Dumuis A. 5-HT_4 receptors, a place in the sun: act two. Curr Opin Pharmacol. 2011; 11: 87-93

Boeckxstaens GE, Bartelsman JF, Lauwers L, Tytgat GN. Treatment of GI dysmotility in scleroderma with the new enterokinetic agent prucalopride. Am J Gastroenterol. 2002; 97: 194-197

Boeckxstaens GE, Pelckmans PA, Herman AG, Van Maercke YM. Involvement of nitric oxide in the inhibitory innervation of the human isolated colon. Gastroenterology. 1993; 104: 690-697

Borges R, Machado JD, Betancor G, Camacho M. Pharmacological regulation of the late steps of exocytosis. Ann N Y Acad Sci. 2002; 971: 184-192

Borman RA, Burleigh DE. Evidence for the involvement of a 5-HT4 receptor in the secretory response of human small intestine to 5-HT. Br J Pharmacol. 1993; 110: 927-928

Borman RA, Burleigh DE. Human colonic mucosa possesses a mixed population of 5-HT receptors. Eur J Pharmacol. 1996; 309: 271-274

Bornstein JC, Costa M, Grider JR. Enteric motor and interneuronal circuits controlling motility. Neurogastroenterol Motil. 2004; 16 Suppl 1: 34-38

Brattelid T, Qvigstad E, Lynham JA, Molenaar P, Aass H, Geiran O, Skomedal T, Osnes JB, Levy FO, Kaumann AJ. Functional serotonin 5-HT4 receptors in porcine and human ventricular myocardium with increased 5-HT4 mRNA in heart failure. Naunyn Schmiedebergs Arch Pharmacol. 2004; 370: 157-166

Brehmer A, Rupprecht H, Neuhuber W. Two submucosal nerve plexus in human intestines. Histochem Cell Biol**.** 2010; 133: 149-161

Brown DR, Timmermans JP. Lessons from the porcine enteric nervous system. Neurogastroenterol Motil. 2004; 16 Suppl 1: 50-54

Budhoo MR, Harris RP, Kellum JM. The role of the 5-HT4 receptor in Cl- secretion in human jejunal mucosa. Eur J Pharmacol. 1996b; 314: 109-114

Budhoo MR, Harris RP, Kellum JM. 5-Hydroxytryptamine-induced Cl- transport is mediated by 5-HT3 and 5-HT4 receptors in the rat distal colon. Eur J Pharmacol. 1996a; 298: 137-144

Budhoo MR, Kellum JM. The 5-HT4 receptor mediates 5-hydroxytryptamine-induced rise in short circuit current in the human jejunum in vitro. Surgery. 1994b; 116: 396-400

Budhoo MR, Kellum JM. Evidence for a 5-HT4 receptor pathway mediating chloride secretion in the rat distal colon. J Surg Res. 1994a; 57: 44-48

Burleigh DE, Borman RA. Short-circuit current responses to 5-hydroxytryptamine in human ileal mucosa are mediated by a 5-HT4 receptor. Eur J Pharmacol. 1993; 241: 125-128

Burnstock G, Lavin S. Interstitial cells of Cajal and purinergic signalling. Auton Neurosci. 2002; 97: 68-72

Camilleri M. Review article: tegaserod. Aliment Pharmacol Ther. 2001; 15: 277-289

Camilleri M. Drugs targeting functional bowel disorders: lessons from drug trials. Curr Opin Pharmacol. 2002; 2: 684-690

Camilleri M, Deiteren A. Prucalopride for constipation. Expert Opin. Pharmacother. 2010; 11: 451-461

Canfield SP, Spencer JE. The inhibitory effects of 5-hydroxytryptamine on gastric acid secretion by the rat isolated stomach. Br J Pharmacol. 1983; 78: 123-129

Chang L, Munakata J, Mayer EA, Schmulson MJ, Johnson TD, Bernstein CN, Saba L, Naliboff B, Anton PA, Matin K. Perceptual responses in patients with inflammatory and functional bowel disease. Gut. 2000; 47: 497-505

Cellek S, John AK, Thangiah R, Dass NB, Bassil AK, Jarvie EM, Lalude O, Vivekanandan S, Sanger GJ. 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. Neurogastroenterol Motil. 2006; 18: 853-861

Chetty N, Coupar IM, Tan YY, Desmond PV, Irving HR. Distribution of serotonin receptors and interacting proteins in the human sigmoid colon. Neurogastroenterol Motil. 2009; 21: 551-558

Clerc N, Furness JB. Intrinsic primary afferent neurones of the digestive tract. Neurogastroenterol Motil. 2004; 16 Suppl 1: 24-27

Coffin B, Farmachidi JP, Rueegg P, Bastie A, Bouhassira D. Tegaserod, a 5-HT4 receptor partial agonist, decreases sensitivity to rectal distension in healthy subjects. Aliment Pharmacol Ther. 2003; 17: 577-585

Cooper JR, Meyer EM. Possible mechanisms involved in the release and modulation of release of neuroactive agents. Neurochem Int. 1984; 6: 419-433

Costa M, Wattchow D, Brookes S. Neuronal control in gastrointestinal disease. Eur. J. Surg. 2002; 587: 39-46

Costedio MM, Hyman N, Mawe GM. Serotonin and its role in colonic function and in gastrointestinal disorders. Dis. Colon Rectum 2006; 50: 376-388

Coupar IM, Desmond PV, Irving HR. Human 5-HT(4) and 5-HT(7) receptor splice variants: are they important? Curr Neuropharmacol. 2007; 5: 224-231

Cremonini F, Chiarioni G, Lembo A. Evolving concepts in chronic constipation in Europe and elsewhere: not worlds apart. Neurogastroenterol Motil. 2011; 23: 693-696

de Castro BM, De Jaeger X, Martins-Silva C, Lima RD, Amaral E, Menezes C, Lima P, Neves CM, Pires RG, Gould TW, Welch I, Kushmerick C, Guatimosim C, Izquierdo I, Cammarota M, Rylett RJ, Gomez MV, Caron MG, Oppenheim RW, Prado MA, Prado VF. The vesicular acetylcholine transporter is required for neuromuscular development and function. Mol Cell Biol. 2009; 29: 5238-5250

De Giorgio R, Su D, Peter D, Edwards RH, Brecha NC, Sternini C. Vesicular monoamine transporter 2 expression in enteric neurons and enterochromaffin-like cells of the rat. Neurosci Lett. 1996; 217: 77-80

De Maeyer JH, Aerssens J, Verhasselt P, Lefebvre RA. Alternative splicing and exon duplication generates 10 unique porcine 5-HT 4 receptor splice variants including a functional homofusion variant. Physiol Genomics. 2008; 34: 22-33

De Maeyer JH, Prins NH, Schuurkes JA, Lefebvre RA. Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. J Pharmacol Exp Ther. 2006a; 317: 955-964

De Maeyer JH, Straetemans R, Schuurkes JA, Lefebvre RA. Porcine left atrial and sinoatrial 5-HT(4) receptor-induced responses: fading of the response and influence of development. Br J Pharmacol. 2006b; 147: 140-157

De Ponti F. Pharmacology of serotonin: what a clinician should know. Gut. 2004; 53: 1520-1535

De Ponti F, Malagelada JR. Functional gut disorders: From motility to sensitivity disorders. A review of current and investigational drugs for their management. Pharmacol. Ther. 1998; vol. 80: 49-88

Drossman DA. The functional gastrointestinal disorders and the Rome III process. Gastroenterology 2006; 130: 1377-1390

Drossman DA, Dumitrascu DL. Rome III: New standard for functional gastrointestinal disorders. J Gastrointestin Liver Dis. 2006; 15: 237-241

Dubois D, Gilet H, Viala-Danten M, Tack J. Psychometric performance and clinical meaningfulness of the Patient Assessment of Constipation-Quality of Life questionnaire in prucalopride (RESOLOR) trials for chronic constipation. Neurogastroenterol Motil. 2010; 22: 54-63

Dyce K.M., Wensing C.J.G. Chapter 34: Abdomen of the pig. In textbook of Veterinary anatomy (Saunders Elsevier). 2010; p. 765-771

Eglen RM, Wong EH, Dumuis A, Bockaert J. Central 5-HT4 receptors. Trends Pharmacol Sci. 1995; 16: 391-398

Epperson A, Hatton WJ, Callaghan B, Doherty P, Walker RL, Sanders KM, Ward SM, Horowitz B. Molecular markers expressed in cultured and freshly isolated interstitial cells of Cajal. Am J Physiol Cell Physiol. 2000; 279: C529-C539

Erickson JD, Schafer MK, Bonner TI, Eiden LE, Weihe E. Distinct pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human vesicular monoamine transporter. Proc Natl Acad Sci U S A. 1996; 93: 5166-5171

Ermilov LG, Miller SM, Schmalz PF, Hanani M, Lennon VA, Szurszewski JH. Morphological characteristics and immunohistochemical detection of nicotinic acetylcholine receptors on intestinofugal afferent neurones in guinea-pig colon. Neurogastroenterol Motil. 2003; 15: 289-298

Eurell J.A., Frappier B.L.. Chapter 10: Digestive system. In textbook of Veterinary histology. 2006; p.170

Ford AP, Baxter GS, Eglen RM, Clarke DE. 5-Hydroxytryptamine stimulates cyclic AMP formation in the tunica muscularis mucosae of the rat oesophagus via 5-HT4 receptors. Eur J Pharmacol. 1992; 211: 117-120

Foxx-Orenstein AE, Kuemmerle JF, Grider JR. Distinct 5-HT receptors mediate the peristaltic reflex induced by mucosal stimuli in human and guinea pig intestine. Gastroenterology. 1996; 111: 1281-1290

Furness JB. Types of neurons in the enteric nervous system. J Auton Nerv Syst. 2000; 81: 87-96

Furness JB. Intestinofugal neurons and sympathetic reflexes that bypass the central nervous system. J Comp Neurol. 2003; 455: 281-284

Furness JB. Novel gut afferents: Intrinsic afferent neurons and intestinofugal neurons. Auton Neurosci. 2006; 125: 81-85

Furness JB, Clerc N, Vogalis F, Stebbing M. The enteric nervous system and its intrinsic connections. In textbook of gastroenterology (Lipincott Williams and Wilkins) 2003; p12-34

Furness JB, Jones C, Nurgali K, Clerc N. Intrinsic primary afferent neurons and nerve circuits within the intestine. Prog Neurobiol. 2004; 72: 143-164

Furness JB, Koopmans HS, Robbins HL, Lin HC. Identification of intestinofugal neurons projecting to the coeliac and superior mesenteric ganglia in the rat. Auton Neurosci. 2000; 83: 81-85

Galligan JJ, Pan H, Messori E. Signalling mechanism coupled to 5-hydroxytryptamine4 receptor-mediated facilitation of fast synaptic transmission in the guinea-pig ileum myenteric plexus. Neurogastroenterol Motil. 2003; 15: 523-529

Galindo-Tovar A, Vargas ML, Escudero E, Kaumann AJ. Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT(4) receptors. Br J Pharmacol. 2009; 156: 237-249

Gebauer A, Merger M, Kilbinger H. Modulation by 5-HT3 and 5-HT4 receptors of the release of 5-hydroxytryptamine from the guinea-pig small intestine. Naunyn Schmiedebergs Arch Pharmacol. 1993; 347: 137-140

Gershon MD and Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology. 2007; 132: 397-414

Goyal RK, Hirano I. The enteric nervous system. N Engl J Med. 1996; 334: 1106-1115

Greenwood-Van Meerveld B. Importance of 5-hydroxytryptamine receptors on intestinal afferents in the regulation of visceral sensitivity. Neurogastroenterol Motil 2007; 19: 13–18

Greenwood-Van Meerveld B, Venkova K, Hicks G, Dennis E, Crowell MD. Activation of peripheral 5-HT receptors attenuates colonic sensitivity to intraluminal distension. Neurogastroenterol Motil. 2006; 18: 76-86

Grider JR. Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. J Pharmacol Exp Ther. 2003; 307: 460-467

Grider JR, Foxx-Orenstein AE, Jin JG. 5-Hydroxytryptamine4 receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. Gastroenterology. 1998; 115: 370-380

Grundy D. 5-HT system in the gut: roles in the regulation of visceral sensitivity and motor functions. Eur Rev Med Pharmacol Sci. 2008; 12 Suppl 1: 63-67

Grundy D, Al-Chaer ED, Aziz Q, Collins SM, Ke M, Taché Y, Wood JD. Fundamentals of neurogastroenterology: basic science. Gastroenterology. 2006; 130: 1391-1411

Hannon J, Hoyer D. Molecular biology of 5-HT receptors. Behav Brain Res. 2008; 195: 198-213

Hansen MB. ICS 205-930 reduces 5-methoxytryptamine-induced short-circuit current in stripped pig jejunum. Can J Physiol Pharmacol. 1994; 72: 227-232

Hansen MB. Tropisetron and octreotide reduce serotonin-induced fluid hypersecretion in pig jejunum. Pharmacol Toxicol. 1995; 76: 136-140

Hansen MB. Neurohumoral control of gastrointestinal motility. Physiol Res. 2003a; 52: 1-30

Hansen MB. The enteric nervous system I: organization and classification. Pharmacol Toxicol. 2003b; 92:105-113

Hansen MB.The enteric nervous system II: gastrointestinal functions. Pharmacology and Toxicology 2003c; 92: 249-257

Harrington AM, Hutson JM, Southwell BR. Cholinergic neurotransmission and muscarinic receptors in the enteric nervous system. Prog Histochem Cytochem. 2010; 44: 173-202

Hashimoto S, Shuntoh H, Taniyama K, Tanaka C. Role of protein kinase C in the vesicular release of acetylcholine and norepinephrine from enteric neurons of the guinea pig small intestine. Jpn J Pharmacol. 1988; 48: 377-385

Hegde SS, Eglen RM. Peripheral 5-HT4 receptors. FASEB J. 1996; 10: 1398-1407

Hens J, Schrödl F, Brehmer A, Adriaensen D, Neuhuber W, Scheuermann DW, Schemann M, Timmermans JP. Mucosal projections of enteric neurons in the porcine small intestine. J Comp Neurol. 2000; 421: 429-436

Hibberd TJ, Zagorodnyuk VP, Spencer NJ, Brookes SJ. Identification and mechanosensitivity of viscerofugal neurons. Neuroscience. 2012; 225: 118-129

Hirota CL, McKay DM. Cholinergic regulation of epithelial ion transport in the mammalian intestine. Br J Pharmacol. 2006; 149: 463-479

Hoffman JM, Tyler K, MacEachern SJ, Balemba OB, Johnson AC, Brooks EM, Zhao H, Swain GM, Moses PL, Galligan JJ, Sharkey KA, Greenwood-Van Meerveld B, Mawe GM. Activation of colonic mucosal 5-HT(4) receptors accelerates propulsive motility and inhibits visceral hypersensitivity. Gastroenterology 2012; 142: 844-854

Holzer P, Michl T, Danzer M, Jocic M, Schicho R, Lippe IT. Surveillance of the gastrointestinal mucosa by sensory neurons. J Physiol Pharmacol. 2001; 52: 505-521

Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). Pharmacol Rev. 1994; 46: 157-203

Hoyer D, Hannon JP, Martin GR. Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav. 2002; 71: 533-554

Hoyer D, Martin G. 5-HT receptor classification and nomenclature: towards a harmonization with the human genome. Neuropharmacology. 1997; 36: 419-428

Huizinga JD, Lammers WJ. Gut peristalsis is governed by a multitude of cooperating mechanisms. Am J Physiol Gastrointest Liver Physiol. 2009; 296: G1-8

Huizinga JD, Martz S, Gil V, Wang XY, Jimenez M, Parsons S. Two independent networks of interstitial cells of cajal work cooperatively with the enteric nervous system to create colonic motor patterns. Front Neurosci. 2011; 5: 93

Irving HR, Tan YY, Tochon-Danguy N, Liu H, Chetty N, Desmond PV, Pouton CW, Coupar IM. Comparison of 5-HT4 and 5-HT7 receptor expression and function in the circular muscle of the human colon. Life Sci. 2007; 80: 1198-1205

Johnson LR, Weisbrodt NW. Regulation: nerves and smooth muscle. In Gastrointestinal physiology (Mosby Elsevier) 2007; p.13-22 [book]

Jonnakuty C, Gragnoli C. What do we know about serotonin? J Cell Physiol. 2008; 217: 301-306

Kaneda T, Shimizu K, Nakajyo S, Urakawa N. Effects of various selective phosphodiesterase inhibitors on muscle contractility in guinea pig ileal longitudinal smooth muscle. Jpn J Pharmacol. 1997; 75: 77-85

Kaputlu I, Sadan G. Evidence that nitric oxide mediates non-adrenergic non-cholinergic relaxation induced by GABA and electrical stimulation in the rat isolated duodenum. J Auton Pharmacol. 1996; 16: 177-182

Kararli TT. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharm Drug Dispos. 1995; 16: 351-380

Kaumann AJ. Piglet sinoatrial 5-HT receptors resemble human atrial 5-HT4-like receptors. Naunyn Schmiedebergs Arch Pharmacol. 1990; 342: 619-622

Kaumann AJ, Levy FO. 5-hydroxytryptamine receptors in the human cardiovascular system. Pharmacol Ther. 2006; 111: 674-706

Kellum JM, Albuquerque FC, Stoner MC, Harris RP. Stroking human jejunal mucosa induces 5-HT release and Cl- secretion via afferent neurons and 5-HT4 receptors. Am J Physiol. 1999; 277: G515-G520

Kellum JM, Budhoo MR, Siriwardena AK, Smith EP, Jebraili SA. Serotonin induces Cl- secretion in human jejunal mucosa in vitro via a nonneural pathway at a 5-HT4 receptor. Am J Physiol. 1994; 267: G357-G363

Kindt S, Tack J. Mechanisms of serotonergic agents for treatment of gastrointestinal motility and functional bowel disorders. Neurogastroenterol Motil. 2007; 19 Suppl 2: 32-39

Kodama Y, Iino S, Shigemasa Y, Suzuki H. Properties of acetylcholine-induced relaxation of smooth muscle isolated from the proximal colon of the guinea-pig. J Smooth Muscle Res. 2010; 46: 185-200

Komada T, Yano S. Pharmacological characterization of 5-Hydroxytryptamine-receptor subtypes in circular muscle from the rat stomach. Biol Pharm Bull. 2007; 30: 508-513

Krobert KA, Brattelid T, Levy FO, Kaumann AJ. Prucalopride is a partial agonist through human and porcine atrial 5-HT4 receptors: comparison with recombinant human 5-HT4 splice variants. Naunyn Schmiedebergs Arch Pharmacol. 2005; 371: 473-479

Kunisawa Y, Komuro T. Interstitial cells of Cajal associated with the submucosal plexus of the Guinea-pig stomach. Neurosci Lett. 2008; 434: 273-276

Kunze WA, Furness JB. The enteric nervous system and regulation of intestinal motility. Annu Rev Physiol. 1999; 61: 117-142

Kushnir-Sukhov NM, Brown JM, Wu Y, Kirshenbaum A, Metcalfe DD. Human mast cells are capable of serotonin synthesis and release. J Allergy Clin Immunol. 2007; 119: 498-499

Lai YC, Ho Y, Huang KH, Tsai LH. Effects of serotonin on acid secretion in isolated rat stomach: the role of 5-HT3 receptors .Chin J Physiol. 2009; 52: 395-405

Langton P, Ward SM, Carl A, Norell MA, Sanders KM. Spontaneous electrical activity of interstitial cells of Cajal isolated from canine proximal colon. Proc Natl Acad Sci U S A. 1989; 86: 7280-7284

Lecci A, Capriati A, Altamura M, Maggi CA. Tachykinins and tachykinin receptors in the gut, with special reference to NK2 receptors in human. Auton Neurosci. 2006; 126-127: 232-249

Leclere PG, Lefebvre RA. Investigation of the interaction between cholinergic and nitrergic neurotransmission in the pig gastric fundus. Br J Pharmacol. 1998; 125: 1779-1787

Leclere PG, Lefebvre RA. Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. Br J Pharmacol 2002; 135: 135-142

Leclere PG, Prins NH, Schuurkes JA, Lefebvre RA. 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. Neurogastroenterol Motil. 2005; 17: 366-375

Lepard KJ, Chi J, Mohammed JR, Gidener S, Stephens RL Jr. Gastric antisecretory effect of serotonin: quantitation of release and site of action. Am J Physiol. 1996; 271: E669-E677

LePard KJ, Stephens RL Jr. Serotonin inhibits gastric acid secretion through a 5-hydroxytryptamine1-like receptor in the rat. J Pharmacol Exp Ther. 1994; 270: 1139-1144

Li Z, Chalazonitis A, Huang YY, Mann JJ, Margolis KG, Yang QM, Kim DO, Côté F, Mallet J, Gershon MD. Essential roles of enteric neuronal serotonin in gastrointestinal motility and the development/survival of enteric dopaminergic neurons. J Neurosci. 2011; 31: 8998-9009

Lindström E, Chen D, Norlén P, Andersson K, Håkanson R. Control of gastric acid secretion: the gastrin-ECL cell-parietal cell axis. Comp Biochem Physiol A Mol Integr Physiol. 2001; 128: 505-514

Liu M, Geddis MS, Wen Y, Setlik W, Gershon MD. Expression and function of 5-HT4 receptors in the mouse enteric nervous system. Am J Physiol Gastrointest Liver Physiol. 2005; 89: G1148-G1163

Lomax AE, Zhang JY, Furness JB. Origins of cholinergic inputs to the cell bodies of intestinofugal neurons in the guinea pig distal colon. J Comp Neurol. 2000; 416: 451-460

Longo WE, Vernava AM. Prokinetic agents for lower gastrointestinal motility disorders. Dis Colon Rectum. 1993; 36: 696-708

Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. Gastroenterology 2006; 130: 1480-1491

Mader R, Kocher T, Haier J, Wieczorek G, Pfannkuche HJ, Ito M. Investigation of serotonin type 4 receptor expression in human and non-human primate gastrointestinal samples. Eur. J. Gastroenterol. Hepatol. 2006; 18: 945-950

Manabe N, Wong BS, Camilleri M. New-generation 5-HT4 receptor agonists: potential for treatment of gastrointestinal motility disorders. Expert Opin. Investig. Drugs 2010; 19: 765-775

Marieb EN, Mallat J. The digestive system. In Human Anatomy (Pearson Education) 2003; p.634-673

McLean PG, Coupar IM, Molenaar P. A comparative study of functional 5-HT4 receptors in human colon, rat oesophagus and rat ileum. Br J Pharmacol. 1995; 115: 47-56

McLean PG, Coupar IM. Stimulation of cyclic AMP formation in the circular smooth muscle of human colon by activation of 5-HT4-like receptors. Br J Pharmacol. 1996a; 117: 238-239

McLean PG, Coupar IM. Further investigation into the signal transduction mechanism of the 5-HT4-like receptor in the circular smooth muscle of human colon. Br J Pharmacol. 1996b; 118: 1058-1064

Mercado CP, Kilic F. Molecular mechanisms of SERT in platelets: regulation of plasma serotonin levels. Mol Interv. 2010; 10: 231-41

Miller ER, Ullrey DE. The pig as a model for human nutrition. Annu Rev Nutr. 1987; 7: 361-382

Minami M, Tamakai H, Ogawa T, Endo T, Hamaue N, Hirafuji M, Yoshioka M, Blower PR. Chemical modulation of 5-HT3 and 5-HT4 receptors affects the release of 5-hydroxytryptamine from the ferret and rat intestine. Res Commun Mol Pathol Pharmacol. 1995; 89: 131-142

Mourad FH, Saadé NE. Neural regulation of intestinal nutrient absorption. Prog Neurobiol. 2011; 95: 149-162

Murr MM, Balsiger BM, Farrugia G, Sarr MG. Role of nitric oxide, vasoactive intestinal polypeptide, and ATP in inhibitory neurotransmission in human jejunum. J Surg Res. 1999; 84: 8-12

Murthy KS. Signaling for contraction and relaxation in smooth muscle of the gut. Annu. Rev. Physiol. 2006; 68: 345-374

Murthy KS, Zhou H, Makhlouf GM. PKA-dependent activation of PDE3A and PDE4 and inhibition of adenylyl cyclase V/VI in smooth muscle. Am J Physiol Cell Physiol. 2002; 282: C508-C517

Neal KB, Bornstein JC. Serotonergic receptors in therapeutic approaches to gastrointestinal disorders. Current opinion in Pharmacology 2006; 6: 547-552

Nedi T, White PJ, Coupar IM, Irving HR. Tissue dependent differences in G-protein coupled receptor kinases associated with 5-HT4 receptor desensitization in the rat gastro-intestinal tract. Biochem Pharmacol. 2011; 81: 123-133

Ning Y, Zhu JX, Chan HC. Regulation of ion transport by 5-hydroxytryptamine in rat colon. Clin Exp Pharmacol Physiol. 2004; 31: 424-428

Olsson C, Holmgren S. The control of gut motility. Comp Biochem Physiol A Mol Integr Physiol. 2001; 128: 481-503

Ouadid H, Seguin J, Dumuis A, Bockaert J, Nargeot J. Serotonin increases calcium current in human atrial myocytes via the newly described 5-hydroxytryptamine4 receptors. Mol Pharmacol. 1992; 41: 346-351

Penkova NI, Baltadjiev GA, Koeva YA, Atanassova PK, Andonov VN, Trichkova VA. Serotonin-producing cells in human gastric mucosa--immunohistochemical and electron microscopic study. Folia Med (Plovdiv). 2010; 52: 31-37

Penissi AB, Rudolph MI, Piezzi RS. Role of mast cells in gastrointestinal mucosal defense. Biocell. 2003; 27: 163-172

Podolsky DK. Inflammatory bowel disease (1). N Engl J Med. 1991a; 325: 928-937

Podolsky DK. Inflammatory bowel disease (2). N Engl J Med. 1991b; 325: 1008-1016

Ponimaskin E, Dumuis A, Gaven F, Barthet G, Heine M, Glebov K, Richter DW, Oppermann M. Palmitoylation of the 5-hydroxytryptamine4a receptor regulates receptor phosphorylation, desensitization, and beta-arrestin-mediated endocytosis. Mol Pharmacol. 2005; 67: 1434-1443

Poole DP, Xu B, Koh SL, Hunne B, Coupar IM, Irving HR, Shinjo K, Furness JB. Identification of neurons that express 5-hydroxytryptamine4 receptors in intestine. Cell Tissue Res. 2006; 325: 413-422

Portbury AL, Furness JB, Young HM, Southwell BR, Vigna SR. Localisation of NK1 receptor immunoreactivity to neurons and interstitial cells of the guinea-pig gastrointestinal tract. J Comp Neurol. 1996; 367: 342-351

Pouokam E, Steidle J, Diener M. Regulation of colonic ion transport by gasotransmitters. Biol Pharm Bull. 2011; 34: 789-793

Prins NH, Akkermans LM, Lefebvre RA, Schuurkes JA. 5-HT(4) receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. Br J Pharmacol. 2000; 131: 927-932

Racké K, Reimann A, Schwörer H, Kilbinger H. Regulation of 5-HT release from enterochromaffin cells. Behav Brain Res. 1996; 73: 83-87

Rao SS, Camilleri M, Hasler WL, Maurer AH, Parkman HP, Saad R, Scott MS, Simren M, Soffer E, Szarka L. Evaluation of gastrointestinal transit in clinical practice: position paper of the American and European Neurogastroenterology and Motility Societies. Neurogastroenterol Motil. 2011; 23: 8-23

Raybould HE, Pandol SE, Yee H. The integrated response of the gastrointestinal tract and liver to a meal. In Textbook of gastroenterology 2003: p2-11

Reese JH, Cooper JR. Stimulation of acetylcholine release from guinea-pig ileal synaptosomes by cyclic nucleotides and forskolin. Biochem Pharmacol. 1984; 33: 3007-3011

Rhoades RA, Bell DR. In textbook Medical Physiology: Principles for clinical medicine. 2009 p. 530 (Chapter 27)

Roth BL. The Serotonin Receptors: From molecular pharmacology to human therapeutics 2006; p.143 (chapter 5, 5-HT Receptor Signal Transduction Pathways) [book]

Rumessen JJ, Mikkelsen HB, Qvortrup K, Thuneberg L. Ultrastructure of interstitial cells of Cajal in circular muscle of human small intestine. Gastroenterology. 1993; 104: 343-350

Säfsten B, Sjöblom M, Flemström G. Serotonin increases protective duodenal bicarbonate secretion via enteric ganglia and a 5-HT4-dependent pathway. Scand J Gastroenterol. 2006; 41: 1279-1289

Sakurai-Yamashita Y, Yamashita K, Kanematsu T, Taniyama K. Localization of the 5-HT(4) receptor in the human and the guinea pig colon. Eur J Pharmacol. 1999; 383: 281-285

Sanger GJ. 5-Hydroxytryptamine and the gastrointestinal tract: where next? Trends Pharmacol. Sci. 2008; 29: 7465-7471

Schubert ML. Gastric secretion. Curr Opin Gastroenterol. 2010; 26: 598-603

Schubert ML. Stomach and duodenum. Curr Opin Gastroenterol. 2009; 25: 527-528

Schubert ML. Gastric secretion. Curr Opin Gastroenterol. 2007; 23: 595-601

Schwörer H, Ramadori G. Autoreceptors can modulate 5-hydroxytryptamine release from porcine and human small intestine in vitro. Naunyn Schmiedebergs Arch Pharmacol. 1998; 357: 548-552

Scratcherd T, Grundy D. The physiology of intestinal motility and secretion. Br J Anaesth.1984; 56: 3-18

Sevcencu C, Rijkhoff NJ, Gregersen H, Sinkjaer T. Propulsive activity induced by sequential electrical stimulation in the descending colon of the pig. Neurogastroenterol Motil. 2005; 17: 376-387

Shuttleworth CW, Xue C, Ward SM, de Vente J, Sanders KM. Immunohistochemical localization of 3',5'-cyclic guanosine monophosphate in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons. Neuroscience. 1993; 56: 513-522

Smith TK, Spencer NJ, Hennig GW, Dickson EJ. Recent advances in enteric neurobiology: mechanosensitive interneurons. Neurogastroenterol Motil. 2007; 19: 869-878

Südhof TC. The synaptic vesicle cycle. Annu Rev Neurosci. 2004; 27: 509-547

Szurszewski JH, Ermilov LG, Miller SM. Prevertebral ganglia and intestinofugal afferent neurones. Gut. 2002; 51 Suppl 1: i6-i10

Tack J, van Outryve M, Beyens G, Kerstens R, Vandeplassche L. Prucalopride (Resolor) in the treatment of severe chronic constipation in patients dissatisfied with laxatives. Gut. 2009; 58: 357-365

Takada K, Sakurai-Yamashita Y, Yamashita K, Kaibara M, Hamada Y, Nakane Y, Hioki K, Taniyama K. Regional difference in correlation of 5-HT4 receptor distribution with cholinergic transmission in the guinea pig stomach. Eur J Pharmacol. 1999; 374: 489-494

Tam FS, Hillier K, Bunce KT, Grossman C. Differences in response to 5-HT4 receptor agonists and antagonists of the 5-HT4-like receptor in human colon circular smooth muscle. Br J Pharmacol. 1995; 115: 172-176

Tamada H, Komuro T. Ultrastructural characterization of interstitial cells of Cajal associated with the submucosal plexus in the proximal colon of the guinea pig. Cell Tissue Res. 2012; 347: 319-326

Taniyama K, Makimoto N, Furuichi A, Sakurai-Yamashita Y, Nagase Y, Kaibara M, Kanematsu T. Functions of peripheral 5-hydroxytryptamine receptors, especially 5-hydroxytryptamine4 receptor, in gastrointestinal motility. J Gastroenterol. 2000; 35: 575-582

Timmermans JP, Barbiers M, Scheuermann DW, Stach W, Adriaensen D, De Groodt-Lasseel MH. Occurrence, distribution and neurochemical features of small intestinal neurons projecting to the cranial mesenteric ganglion in the pig. Cell Tissue Res. 1993; 272: 49-58

Timmermans JP, Hens J, Adriaensen D. Outer submucous plexus: an intrinsic nerve network involved in both secretory and motility processes in the intestine of large mammals and humans. Anat Rec. 2001; 262: 71-78

Tomita R, Tanjoh K, Munakata K. The role of motilin and cisapride in the enteric nervous system of the lower esophageal sphincter in humans. Surg Today. 1997; 27: 985-992

Tonini M, De Ponti F, Di Nucci A, Crema F. Review article: cardiac adverse effects of gastrointestinal prokinetics. Aliment Pharmacol Ther. 1999; 13: 1585-1591

Tonini M, De Giorgio R, De Ponti F, Sternini C, Spelta V, Dionigi P, Barbara G, Stanghellini V, Corinaldesi R. Role of nitric oxide- and vasoactive intestinal polypeptide-containing neurones in human gastric fundus strip relaxations. Br J Pharmacol. 2000; 129: 12-20

Tuo BG, Sellers Z, Paulus P, Barrett KE, Isenberg JI. 5-HT induces duodenal mucosal bicarbonate secretion via cAMP- and Ca2+-dependent signaling pathways and 5-HT4 receptors in mice. Am J Physiol Gastrointest Liver Physiol. 2004; 286: G444-G451

Uphouse L. Multiple serotonin receptors: too many, not enough, or just the right number? Neurosci Biobehav Rev. 1997; 21: 679-698

van Lelyveld N, Ter Linde J, Schipper ME, Samsom M. Regional differences in expression of TPH-1, SERT, 5-HT(3) and 5-HT(4) receptors in the human stomach and duodenum. Neurogastroenterol Motil 2007; 19: 342-348

Veenstra-VanderWeele J, Anderson GM, Cook EH Jr. Pharmacogenetics and the serotonin system: initial studies and future directions. Eur J Pharmacol. 2000; 410: 165-181

Walther DJ, Bader M. A unique central tryptophan hydroxylase isoform. Biochem Pharmacol. 2003; 66: 1673-1680

Wang, XY; Paterson, C; Huizinga, JD. Cholinergic and nitrergic innervation of ICC-DMP and ICC-IM in the human small intestine. Neurogastroenterol Motil 2003; 15: 531-543

Wapnir RA, Teichberg S. Regulation mechanisms of intestinal secretion: implications in nutrient absorption. J Nutr Biochem. 2002; 13: 190-199

Ward SM, Morris G, Reese L, Wang XY, Sanders KM. Interstitial cells of Cajal mediate enteric inhibitory neurotransmission in the lower esophageal and pyloric sphincters. Gastroenterology 1998; 115: 314-329

Ward SM, Sanders KM. Interstitial cells of Cajal: primary targets of enteric motor innervation. Anat Rec. 2001; 262: 125-135

Wimalasena K. Vesicular monoamine transporters: structure-function, pharmacology, and medicinal chemistry. Med Res Rev. 2011; 31: 483-519

Weninger S, De Maeyer JH, Lefebvre RA. Study of the regulation of the inotropic response to 5-HT4 receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium. Naunyn Schmiedebergs Arch Pharmacol. 2012; 385: 565-577

Woehler A, Ponimaskin EG. G protein--mediated signaling: same receptor, multiple effectors. Curr Mol Pharmacol. 2009; 2: 237-248

Wood JD, Alpers DH, Andrews PL. Fundamentals of neurogastroenterology. Gut. 1999; 45: II6-II16

Wouters MM, Farrugia G, Schemann M. 5-HT receptors on interstitial cells of Cajal, smooth muscle and enteric nerves. Neurogastroenterol Motil 2007; 19: 5-12

Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007; 448: 427-434

Xue J, Askwith C, Javed NH, Cooke HJ. Autonomic nervous system and secretion across the intestinal mucosal surface. Auton Neurosci. 2007; 133: 55-63

Xue L, Farrugia G, Sarr MG, Szurszewski JH. ATP is a mediator of the fast inhibitory junction potential in human jejunal circular smooth muscle. Am J Physiol. 1999; 276: G1373-G1379

Yau WM, Dorsett JA, Youther ML. Stimulation of acetylcholine release from myenteric neurons of guinea pig small intestine by forskolin and cyclic AMP. J Pharmacol Exp Ther. 1987; 243: 507-510

Young B, Heath JW. Gastrointestinal tract. In Functional Histology (Churchill Livingstone) 2000; p.249-273 [book]

Yu PL, Fujimura M, Okumiya K, Kinoshita M, Hasegawa H, Fujimiya M. Immunohistochemical localization of tryptophan hydroxylase in the human and rat gastrointestinal tracts. J Comp Neurol. 1999; 411: 654-665

Zhang RX, Wang XY, Chen D, Huizinga JD. Role of interstitial cells of Cajal in the generation and modulation of motor activity induced by cholinergic neurotransmission in the stomach. Neurogastroenterol Motil. 2011; 23: e356-e371

Zhou X, Galligan JJ. Synaptic activation and properties of 5-hydroxytryptamine(3) receptors in myenteric neurons of guinea pig intestine. J Pharmacol Exp Ther. 1999; 290: 803-810

Chapter 2

AIMS

Chapter 2 Aims

The primary function of the GI tract is the digestion and absorption of ingested nutrients and the maintenance of homeostasis between meals. It is generally accepted that to achieve this, integration of secretion, motility and absorption is required, with the result that these functions are interrelated and coordinated in a complex fashion. Motility and secretion are mediated by regulatory systems, such as the endocrine, paracrine and nervous system that monitor these events within the body (Greenwood and Davison, 1987; Johnson and Weisbrodt, 2007). 5-HT is an essential messenger in the coordinated movement of food along the GI tract, by interacting with a wide array of 5-HT receptors. Most 5-HT in the body resides in the GI tract, synthesized by EC cells found in the epithelium of the small intestine and to some extent by myenteric interneurons. 5-HT, released from these cells activates neural reflexes by activation of various 5-HT receptors located on intrinsic and extrinsic sensory neurons. The 5-HT receptors are classified into seven receptor classes of which the role of 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7 receptors has been studied in the gut (De Ponti, 2004; Gershon and Tack, 2007; Hansen, 2003a; 2003b; Jonnakuty and Gragnoli, 2008).

Within the GI wall, 5-HT regulates smooth muscle activity by activating 5-HT receptors located on smooth muscle cells or on intrinsic and extrinsic sensory neurons, resulting in regulation of peristalsis and communication between the brain and the GI tract (Jonnakuty and Gragnoli, 2008). Four types of 5-HT receptors have been demonstrated on smooth muscle cells: 5-HT_{2A} and 5-HT_{2B} receptors are likely to contract, while 5-HT_4 and 5-HT_7 receptors relax the smooth muscle cells. Neuronally located 5-HT receptors enhance or inhibit transmitter release hereby modulating peristalsis: 5-HT_{1A} receptors inhibit, while 5-HT_3 and 5-HT_4 receptors excite transmitter release (Hansen, 2003b). The 5-HT_4 receptor is expressed on excitatory cholinergic motor neurons in the GI tract, facilitating ACh release hereby stimulating GI motility. This presynaptic facilitation is thought to be the principal mechanism for the prokinetic action of 5-HT_4 receptor agonists, explaining their therapeutic use in GI dysmotility related disorders such as chronic constipation, gastroparesis and gastroesophagal reflux disease. For increasing gastric emptying in patients with gastroparesis, the non-selective 5-HT_4 receptor agonist cisapride was used, until it was withdrawn because of cardiac adverse effects. These cardiac side effects were not linked to activation of cardiac 5-HT_4 receptors, which have been shown to be functionally present in human (Blondel et al., 1997; Krobert et al., 2005) and pig atrium (De Maeyer et al., 2006a; 2006b; Galindo-Tovar et al., 2009; Kaumann, 1990; Schoemaker et al., 1992; Weninger et al., 2012), inducing inotropic and chronotropic effects.

The cardiac arrhythmias seen with cisapride were linked to peculiar characteristics in its molecular structure. Cisapride prolongs the action potential duration through a blockade of human ether-a-go-go related gene (hERG)-encoded voltage-dependent potassium channels hereby delaying cardiac repolarization (Mohammad et al., 1997; Tonini et al., 1999). In human and pig ventricles, 5-HT_4 receptor mRNA was detected (Bach et al., 2001; Brattelid et al., 2004; Qvigstad et al., 2005) but in porcine ventricle, 5-HT_4 receptor activation showed only to sustain a stable response in the presence of a PDE inhibitor, indicating the involvement of PDEs in the cardiac 5-HT_4 receptor signaling pathway (Brattelid et al., 2004). Also the in porcine left atrium, the selective 5-HT_4 receptor agonist prucalopride had only a weak and transient positive inotropic effect when compared to the pronounced enhancing effect of prucalopride on human colonic motility and the cardiac effect became only prominent and sustained upon PDE inhibition (De Maeyer et al., 2006b), PDE3 and PDE4 being involved (Galindo-Tovar et al., 2009; Weninger et al., 2012).

Prucalopride is approved for female patients with laxative-resistant chronic constipation and targets 5-HT_4 receptors located on cholinergic neurons towards human colonic circular (Leclere et al., 2005) and longitudinal (Prins et al., 2000a) smooth muscle. Next to stimulating colonic motility, prucalopride also accelerates gastric emptying in man (Bouras et al., 2001) by facilitating ACh release from cholinergic neurons towards human gastric circular muscle (Leclere and Lefebvre, 2002). As prucalopride has already a clear and sustained enhancing effect on the cholinergic contractions in the human stomach and colon, it had not been investigated whether PDEs are involved in the receptor signaling pathway of the GI neuronal 5-HT_4 receptors.

Aside the presence of 5-HT_4 receptors on cholinergic neurons in the GI tract, their localization has also been suggested on other cell types within the colon and stomach. In the human colon 5-HT_4 receptor agonists lead to smooth muscle relaxation by stimulating 5-HT_4 receptors on smooth muscle cells (McLean et al., 1995; Prins et al., 2000b) and possibly 5-HT_4 receptors located on nitrergic neurons (Cellek et al., 2006). 5-HT_4 receptor mRNA expression has also been reported in the pig gastric and colonic mucosa (De Maeyer et al., 2008) as well as in human gastric (Hoffman et al., 2012; van Lelyveld et al., 2007) and colonic mucosa (Chetty et al., 2009). In the human colon, it has been demonstrated that 5-HT induces secretion via 5-HT_{2A} and/or 5-HT_4 receptors (Borman and Burleigh, 1996). In the rat colon, it has been suggested that 5-HT-induced mucosal secretion is mediated by 5-HT_4 receptors (Albuquerque et al., 1998; Budhoo et al., 1996; Budhoo and Kellum, 1994; Ning et al., 2004). In addition, a recent immunohistochemical and functional study, showed that mucosal 5-HT_4 receptor activation in

the murine colon elicited 5-HT and Cl^- release and mucus secretion (Hoffman et al., 2012). But the cellular 5-HT_4 receptor distribution and the functional implication within the epithelial layer has not yet been investigated.

Importance of identifying site specific gene expression has already been shown by investigating distribution of 5-HT_3 receptor splice variants in enteric ganglia and epithelium of the human colon (Böttner et al., 2010; Kapeller et al., 2011). Therefore, more detailed information on the expression and localization of 5-HT_4 receptors and their signaling pathways would lead to a better understanding of the role of 5-HT_4 receptors in the GI motility and secretion. Possibly new therapeutic approaches might be developed to treat GI disorders by targeting site specific 5-HT_4 receptors or by targeting the second messengers involving the 5-HT_4 receptor signaling pathway. In order to determine whether the functions of 5-HT_4 receptors evaluated on enteric neuronal subpopulations, smooth muscle cells and epithelial cells in laboratory animals, such as the pig, can be extrapolated to the human colon and stomach, further investigation of the localization and control of 5-HT_4 receptors within the pig colon and stomach is justified.

The **aims** of the work described in this thesis were therefore to:

1. investigate the influence of the selective 5-HT_4 receptor agonist prucalopride on the cholinergic neurotransmission of the pig gastric circular smooth muscle and the possible regulation of this effect by phosphodiesterases **(Chapter 3)**.
2. evaluate the pig colon descendens as a possible animal model for the human colonic 5-HT_4 receptors involved in the regulation of motility **(Chapter 4)**.
3. investigate whether phosphodiesterases have also a potential role in the porcine colonic 5-HT_4 receptor pathway on cholinergic neurons **(Chapter 5)**.
4. investigate the mucosal expression of 5-HT_4 receptors in the pig stomach and colon by laser microdissection **(Chapter 6)**.

The most relevant compounds used in the studies described in chapters 3 to 5 are listed in **Table 2.1**.

5-HT receptor agonists	
5-HT	Non-selective 5-HT receptor agonist
Prucalopride	Selective 5-HT_4 receptor agonist
5-HT receptor antagonists	
GR113808	5-HT_4 receptor antagonist
Granisetron	5-HT_3 receptor antagonist
Methysergide	5-HT_1, 5-HT_2, 5-ht_5, 5-HT_6 and 5-HT_7 receptor antagonist
Phosphodiesterase inhibitors	
IBMX	Non-selective PDE inhibitor
Vinpocetine	PDE1 inhibitor
EHNA	PDE2 inhibitor
Cilostamide	PDE3 inhibitor
Rolipram	PDE4 inhibitor
Zaprinast	PDE5 inhibitor
Inhibitors of relaxant neurotransmitters	
L-NAME	NO synthase inhibitor
Apamin	Small conductance Ca^{2+}-dependent K^+ channel blocker
MRS2179	$P2Y_1$ receptor antagonist

Table 2.1. List of most relevant compounds used.

2.I. REFERENCES

Albuquerque FC Jr, Smith EH, Kellum JM. 5-HT induces cAMP production in crypt colonocytes at a 5-HT4 receptor. J Surg Res. 1998; 77: 137-140

Bach T, Syversveen T, Kvingedal AM, Krobert KA, Brattelid T, Kaumann AJ, Levy FO. 5HT4(a) and 5-HT4(b) receptors have nearly identical pharmacology and are both expressed in human atrium and ventricle. Naunyn Schmiedebergs Arch Pharmacol. 2001; 363(2): 146-160

Blondel O, Vandecasteele G, Gastineau M, Leclerc S, Dahmoune Y, Langlois M, Fischmeister R. Molecular and functional characterization of a 5-HT4 receptor cloned from human atrium. FEBS Lett. 1997; 412(3): 465-474

Böttner M, Bär F, Von Koschitzky H, Tafazzoli K, Roblick UJ, Bruch HP, Wedel T. Laser microdissection as a new tool to investigate site-specific gene expression in enteric ganglia of the human intestine. Neurogastroenterol Motil. 2010; 22: 168-172

Borman RA, Burleigh DE. Human colonic mucosa possesses a mixed population of 5-HT receptors.Eur J Pharmacol. 1996; 309(3): 271-274

Bouras EP, Camilleri M, Burton DD, Thomforde G, McKinzie S, Zinsmeister AR. Prucalopride accelerates gastrointestinal and colonic transit in patients with constipation without a rectal evacuation disorder. Gastroenterology. 2001; 120: 354-360

Brattelid T, Qvigstad E, Lynham JA, Molenaar P, Aass H, Geiran O, Skomedal T, Osnes JB, Levy FO, Kaumann AJ. Functional serotonin 5-HT4 receptors in porcine and human ventricular myocardium with increased 5-HT4 mRNA in heart failure. Naunyn Schmiedebergs Arch Pharmacol. 2004; 370(3): 157-166

Budhoo MR, Harris RP, Kellum JM. 5-Hydroxytryptamine-induced Cl- transport is mediated by 5-HT3 and 5-HT4 receptors in the rat distal colon. Eur J Pharmacol 1996; 298: 137-144

Budhoo MR, Kellum JM. Evidence for a 5-HT4 receptor pathway mediating chloride secretion in the rat distal colon. J Surg Res. 1994; 57: 44-48

Cellek S, John AK, Thangiah R, Dass NB, Bassil AK, Jarvie EM, Lalude O, Vivekanandan S, Sanger GJ. 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. Neurogastroenterol Motil. 2006; 18(9): 853-861

Chetty N, Coupar IM, Tan YY, Desmond PV, Irving HR. Distribution of serotonin receptors and interacting proteins in the human sigmoid colon. Neurogastroenterol Motil 2009; 21: 551-558, e14-e15

De Maeyer JH, Aerssens J, Verhasselt P, Lefebvre RA. Alternative splicing and exon duplication generates 10 unique porcine 5-HT 4 receptor splice variants including a functional homofusion variant. Physiol Genomics 2008; 34: 22-33

De Maeyer JH, Prins NH, Schuurkes JA, Lefebvre RA. Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. J Pharmacol Exp Ther. 2006a; 317(3): 955-964

De Maeyer JH, Straetemans R, Schuurkes JA, Lefebvre RA. Porcine left atrial and sinoatrial 5-HT(4) receptor-induced responses: fading of the response and influence of development. Br J Pharmacol. 2006b; 147(2): 140-157

De Ponti F. Pharmacology of serotonin: what a clinician should know. Gut. 2004; 53(10): 1520-1535

Galindo-Tovar A, Vargas ML, Escudero E, Kaumann AJ. Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT(4) receptors. Br J Pharmacol. 2009; 156(2): 237-249

Gershon MD and Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology. 2007; 132(1): 397-414

Greenwood B, Davison JS. The relationship between gastrointestinal motility and secretion. Am J Physiol. 1987; 252: G1-G7

Hansen MB. The enteric nervous system II: gastrointestinal functions. Pharmacology and Toxicology 2003a; 92: 249-257

Hansen MB. Neurohumoral control of gastrointestinal motility. Physiol Res. 2003b; 52(1): 1-30

Hoffman JM, Tyler K, MacEachern SJ, Balemba OB, Johnson AC, Brooks EM, Zhao H, Swain GM, Moses PL, Galligan JJ, Sharkey KA, Greenwood-Van Meerveld B, Mawe GM. Activation of colonic mucosal 5-HT(4) receptors accelerates propulsive motility and inhibits visceral hypersensitivity. Gastroenterology 2012; 142: 844-854

Johnson LR, Weisbrodt NW. Regulation: nerves and smooth muscle. In Gastrointestinal physiology (Mosby Elsevier) 2007; p.13-22 [book]

Jonnakuty C, Gragnoli C. What do we know about serotonin? J Cell Physiol. 2008; 217(2): 301-306

Kapeller J, Möller D, Lasitschka F, Autschbach F, Hovius R, Rappold G, Brüss M, Gershon MD, Niesler B. Serotonin receptor diversity in the human colon: Expression of serotonin type 3 receptor subunits 5-HT3C, 5-HT3D, and 5-HT3E. J Comp Neurol. 2011; 519(3): 420-432

Kaumann AJ. Piglet sinoatrial 5-HT receptors resemble human atrial 5-HT4-like receptors.Naunyn Schmiedebergs Arch Pharmacol. 1990; 342(5): 619-622

Krobert KA, Brattelid T, Levy FO, Kaumann AJ. Prucalopride is a partial agonist through human and porcine atrial 5-HT4 receptors: comparison with recombinant human 5-HT4 splice variants. Naunyn Schmiedebergs Arch Pharmacol. 2005; 371(6): 473-479

Leclere PG, Lefebvre RA. Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. Br J Pharmacol 2002; 135: 135-142

Leclere PG, Prins NH, Schuurkes JA, Lefebvre RA. 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. Neurogastroenterol Motil. 2005; 17: 366-375

McLean PG, Coupar IM, Molenaar P. A comparative study of functional 5-HT4 receptors in human colon, rat oesophagus and rat ileum. Br J Pharmacol. 1995; 115(1): 47-56

Mohammad S, Zhou Z, Gong Q, January CT. Blockage of the HERG human cardiac K+ channel by the gastrointestinal prokinetic agent cisapride. Am J Physiol. 1997; 273(5 Pt 2): H2534-H2538

Ning Y, Zhu JX, Chan HC. Regulation of ion transport by 5-hydroxytryptamine in rat colon. Clin Exp Pharmacol Physiol. 2004; 31: 424-428

Prins NH, Akkermans LM, Lefebvre RA, Schuurkes JA. 5-HT(4) receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. Br J Pharmacol. 2000a; 131: 927-932

Prins NH, Shankley NP, Welsh NJ, Briejer MR, Lefebvre RA, Akkermans LM, Schuurkes JA. An improved in vitro bioassay for the study of 5-HT(4) receptors in the human isolated large intestinal circular muscle. Br J Pharmacol. 2000b; 129(8): 1601-1608

Qvigstad E, Brattelid T, Sjaastad I, Andressen KW, Krobert KA, Birkeland JA, Sejersted OM, Kaumann AJ, Skomedal T, Osnes JB, Levy FO. Appearance of a ventricular 5-HT4 receptor-mediated inotropic response to serotonin in heart failure. Cardiovasc Res. 2005; 65(4): 869-878

Schoemaker RG, Du XY, Bax WA, Saxena PR. 5-Hydroxytryptamine increases contractile force in porcine right atrium but not in left ventricle. Naunyn Schmiedebergs Arch Pharmacol. 1992; 346(5): 486-489

Tonini M, De Ponti F, Di Nucci A, Crema F. Review article: cardiac adverse effects of gastrointestinal prokinetics. Aliment Pharmacol Ther. 1999; 13(12): 1585-1591

van Lelyveld N, Ter Linde J, Schipper ME, Samsom M. Regional differences in expression of TPH-1, SERT, 5-HT(3) and 5-HT(4) receptors in the human stomach and duodenum. Neurogastroenterol Motil 2007; 19: 342-348

Weninger S, De Maeyer JH, Lefebvre RA. Study of the regulation of the inotropic response to 5-HT4 receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium. Naunyn Schmiedebergs Arch Pharmacol. 2012; 385(6): 565-577

Chapter 3

THE FACILITATING EFFECT OF PRUCALOPRIDE ON CHOLINERGIC NEUROTRANSMISSION IN PIG GASTRIC CIRCULAR MUSCLE IS REGULATED BY PHOSPHODIESTERASE 4

Evelien K.V. Priem[a], Inge Van Colen[a], Joris H. De Maeyer[b] and Romain A. Lefebvre[a]

[a]Heymans Institute of Pharmacology, Ghent University, Gent, Belgium

[b]Shire-Movetis NV, Turnhout, Belgium

Based on

Neuropharmacology 2012; 62: 2126-2135

Chapter 3 The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4

3.I. Abstract

The influence of the selective 5-HT$_4$ receptor agonist prucalopride on acetylcholine release from cholinergic nerve endings innervating pig gastric circular muscle and the possible regulation of this effect by phosphodiesterases (PDE) was investigated. Circular muscle strips were prepared from pig proximal stomach and either submaximal cholinergic contractions or tritium outflow after incubation with [^{3}H]-choline, induced by electrical field stimulation, were studied. Prucalopride concentration-dependently increased the amplitude of submaximal cholinergic contractions and of acetylcholine release induced by electrical field stimulation. The effect of the highest concentration tested (0.3 µM) on cholinergic contractions was antagonized by the selective 5-HT$_4$ receptor antagonist GR113808 but not by granisetron or methysergide; the antagonism of prucalopride by GR113808 was confirmed in the release assay. The non-selective PDE inhibitor 3-isobutylmethylxanthine (IBMX) concentration-dependently reduced the amplitude of the cholinergic contractions; 3 µM IBMX reduced the cholinergic contractions maximally by 16 % but it enhanced the facilitating effect of prucalopride from 51 to 83%. IBMX (10 µM) induced and enhanced the facilitating effect of prucalopride on electrically induced acetylcholine release. The selective inhibitors vinpocetine (PDE1), EHNA (PDE2) and cilostamide (PDE3) did not influence the effect of prucalopride on acetylcholine release but the PDE4-inhibitor rolipram (1 µM) induced the facilitating effect of prucalopride to the same extent as IBMX. These results demonstrate that 5-HT$_4$ receptors are present on the cholinergic nerves towards the pig gastric circular muscle, facilitating acetylcholine release; the intracellular transduction pathway of this facilitation is regulated by PDE4. Combination of a 5-HT$_4$ receptor agonist with selective inhibition of the PDE involved in this regulation of transmitter release might enhance the prokinetic effect of the 5-HT$_4$ receptor agonist.

3.II. Introduction

The highly selective 5-HT$_4$ receptor agonist prucalopride accelerates colonic transit and increases stool frequency in healthy volunteers (Emmanuel et al., 1998; Bouras et al., 1999) and is efficient in patients with severe chronic constipation resistant to laxative treatment (Camilleri et al., 2008; Tack et al., 2009). In 2009, it was approved by the European Medicines Agency for the symptomatic treatment of chronic constipation in women in whom laxatives fail to provide adequate relief. The main mechanism for the above cited effects of prucalopride is thought to be interaction with facilitatory 5-HT$_4$ receptors on cholinergic nerve endings towards colonic smooth muscle, which have been shown in different species also at other levels of the gastrointestinal tract (Gershon and Tack, 2007). In human colon, these receptors were shown to be present on cholinergic nerve endings towards the longitudinal (Prins et al., 2000a) and circular muscle (Leclere et al., 2005; Cellek et al., 2006).

5-HT$_4$ receptors are also present on circular smooth muscle from human colon, inducing relaxation (McLean et al., 1995; McLean and Coupar, 1996; Prins et al., 2000b; Irving et al., 2007) although this could not be systematically confirmed (Cellek et al., 2006) possibly because of low 5-HT$_4$ receptor expression levels in the human colon, as demonstrated by Irving et al. (2007) who showed that 5-HT$_4$ receptor-mediated relaxation occurs at low to undetectable levels of tissue 5-HT$_4$ receptor mRNA. Cellek et al. (2006) could not confirm the relaxation of human colon circular muscle through muscular 5-HT$_4$ receptors but they identified a second mechanism by which 5-HT$_4$ receptor agonists can relax human colon circular muscle, i.e. facilitation of nitrergic neurotransmission. Whether 5-HT$_4$ receptor-mediated facilitation of nitrergic neurotransmission also occurs in the colon of other species or at other levels of the gastrointestinal tract has not yet been reported. Until it was withdrawn because of the potential of cardiac dysrhythmias, the non-selective 5-HT$_4$ receptor agonist cisapride was worldwide used for treatment of gastro-esophageal reflux and for increasing gastric emptying in gastroparesis, and less so for constipation. Facilitatory 5-HT$_4$ receptors are indeed also present on cholinergic nerve endings in human stomach circular muscle (Leclere and Lefebvre, 2002) similar to those described in the stomach of other species such as guinea-pig (Takada et al., 1999) and dog (Prins et al., 2001a). Also prucalopride indeed accelerates gastric emptying in man (Bouras et al., 2001).

The pig is a good model for the study of gastrointestinal issues in view of similar morphology and physiology of the gastrointestinal tract (Miller and Ullrey, 1987). As for porcine gastrointestinal 5-HT$_4$ receptors, we have shown before that 5-HT$_4$ receptor agonists facilitate electrically

induced cholinergic contractions of proximal stomach longitudinal muscle, suggesting the presence of 5-HT_4 receptors on cholinergic neurons controlling longitudinal muscle.

But the presence of 5-HT_4 receptors on cholinergic neurons innervating the circularly oriented muscle in porcine stomach was not yet investigated; nor was their possible presence on nitrergic neurons in this tissue (Lefebvre et al., 1995). When comparing the effect of prucalopride on electrically induced cholinergic contractions in porcine proximal stomach longitudinal muscle with that in electrically paced porcine left atrial pectinate muscles, a clear and sustained enhancement of the cholinergic contractions in proximal stomach was observed but the positive inotropic effect of prucalopride was transient and very weak and only became prominent and sustained upon phosphodiesterase (PDE) inhibition with 3-isobutyl-1-methylxanthine (IBMX), illustrating a regulatory role of PDEs on 5-HT_4 receptor function in atrium (De Maeyer et al., 2006a, b), which has since been shown to be related to PDE3 and PDE4 activity (Galindo-Tovar et al., 2009). The robust and sustained effect of prucalopride in porcine stomach makes it unclear whether regulatory PDEs are involved. We did not test IBMX versus prucalopride in porcine stomach (De Maeyer et al., 2006a) but all central nervous system neurons express multiple PDEs (Menniti et al., 2006) and a recent detailed immunohistochemical study of PDE2A distribution in different species including dog, monkey and man, showed that the strongest immunoreactivity for PDE2A outside the central nervous system was present in enteric ganglia from the stomach to the colon (Stephenson et al., 2009).

We therefore investigated whether prucalopride is able to facilitate cholinergic neurotransmission towards circularly oriented muscle in porcine proximal stomach, by measuring its effect both on electrically induced cholinergic contractions and on electrically induced [^{3}H]-acetylcholine release. Additionally we investigated whether prucalopride was able to influence electrically induced nitrergic relaxations. As our results demonstrated that prucalopride has a clear-cut facilitating effect on acetylcholine release towards circular muscle in porcine stomach but not on nitrergic neurotransmission, we investigated the influence of IBMX on this cholinergic effect, showing that PDE inhibition is able to enhance the facilitating effect of prucalopride on acetylcholine release or to induce it for a sub-effective concentration of prucalopride. This effect of the non-selective inhibitor IBMX was mimicked by the PDE4-inhibitor rolipram, but not by inhibitors of PDE1 (vinpocetine), PDE2 (EHNA) and PDE3 (cilostamide). Although being much more effective than left atrial 5-HT_4 receptors, the 5-HT_4 receptors on cholinergic neurons in porcine gastric circular muscle are thus also under regulatory control of PDEs, which seems solely related to PDE4 activity.

3.III. Methods

3.III.1. Animals

In the first part of the study, stomachs were obtained from approximately 6 months old healthy castrated male pigs, slaughtered at a local abattoir; the stomachs were transported to the laboratory in ice-chilled physiological salt solution. In the second part of the study (experiments with PDE-inhibitors), approximately 2 months old male piglets (Line 36, weighing approximately 20 kg) were obtained from Rattlerow Seghers (Lokeren, Belgium). On the morning of the experiment, the piglets were anesthetized with an intramuscular injection of 5 ml Zoletil 100 (containing 250 mg tiletamine and 250 mg zolazepam). After exsanguination, the entire stomach was dissected. The use of the piglets for this project was approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

For preparation of the smooth muscle strips, the stomach was cut open along the lesser curvature and placed in physiological salt solution (PSS) at room temperature (composition in mM : 112 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11.5 glucose and 25 $NaHCO_3$ as described by Mandrek and Milenov [1991; PSS I]; or 118 NaCl, 4.69 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 2.51 $CaCl_2$, 11.1 glucose, 25 $NaHCO_3$ [Krebs-Henseleit; PSS II). After removal of the mucosa, 4 to maximum 12 muscle strips of approximately 1.5 cm in length and 0.3 cm in width were prepared from the proximal stomach in the direction of the circular muscle layer; up to 6 strips were obtained from the ventral side cutting from the great curvature towards the small one; the additional strips were prepared at the same level cutting in the direction of the circular muscle layer over the great curvature so that these strips were partially from the ventral and partially from the dorsal side. Strips used for release experiments were always obtained from the ventral side. All strips were used on the day of preparation. For functional experiments with measurement of contractility, the strips were mounted under a load of 2 g between 2 platinum plate electrodes in classic organ baths containing 10 ml of PSS I (first part of the study), or 5 (experiments with PDE-inhibitors other than IBMX) or 7 ml (experiments with IBMX) of PSS II (second part of the study) at 37° C and gassed with carbogen (95% O_2 / 5% CO_2). Mechanical activity was recorded auxotonically via a Grass force-displacement transducer FT03 coupled in series with a 1g cm^{-1} spring on a Graphtec linearcorder F WR3701 in the first part of the study; in the second part of the study, mechanical activity was recorded isometrically via a Grass force-displacement transducer FT03 (experiments with IBMX) or a MLT050/D force transducer from ADInstruments (experiments with other PDE-inhibitors) on a PowerLab/8 sp data recording system (ADInstruments) with Chart software.

For release experiments, strips were mounted between 2 platinum wire electrodes under a load of 2 g in 2 ml organ baths containing PSS I, to which also 0.0015 mM choline and 0.057 mM ascorbic acid was added. Electrical field stimulation was performed by means of a Grass S88 stimulator with a constant voltage unit or a 4 channel custom-made stimulator.

3.III.2. Contractility study

In all series in the first part of the study where electrically induced contractions were studied, the PSS I continuously contained 4 µM guanethidine and 300 µM N^G-nitro-L-arginine methyl ester (L-NAME) to avoid noradrenergic and nitrergic responses respectively; additionally it contained 10 µM indomethacine to avoid spontaneous progressive contraction due to release of prostaglandins. After at least 1 h of equilibration with rinsing every 15 min, the tissues were contracted with 3 µM carbachol to test the contractile reactivity of the strip; this was followed by rinsing every 10 min during 30 min. Electrical field stimulation (EFS) was then applied twice at an interval of 5 min (10 s train at supramaximal voltage, 0.5 ms and 4 Hz). This yielded reproducible contractions after which 10 s trains of EFS were applied at 5 min interval with decreasing voltage until the voltage yielding a contraction amplitude of approximately 50% of that obtained at supramaximal voltage (V50%C) was reached. EFS was then stopped for 30 min with rinsing every 10 min. EFS was then started again and 10 s trains at V50%C, 0.5 ms and 4 Hz were repeated at 5 min interval until stabilization. After a further 5 trains, 0.03, 0.1 or 0.3 µM prucalopride was added to 3 parallel tissues and 10 further trains were registered; a fourth tissue received the solvent of prucalopride (control). To test antagonists versus the effect of prucalopride, the antagonist was added after 5 trains at V50%C; 6 further trains were then obtained before adding 0.3 µM prucalopride and registering 10 further trains; a parallel control strip received the solvent of the antagonist. To evaluate the neurogenic and cholinergic nature of the EFS-induced contractions, the influence of 3 µM tetrodotoxin and 1 µM atropine was tested. To test the possible influence of prucalopride on contractions induced by exogenous acetylcholine, a cumulative concentration-response curve to acetylcholine was constructed with half log unit ascending concentration increments from 1 nM onwards; after rinsing for 1 h at 10 min intervals, 0.03, 0.1 or 0.3 µM prucalopride was incubated for 15 min and the concentration-response curve to acetylcholine was repeated.

In the series of experiments during the first part of the study to evaluate the influence of prucalopride on electrically induced NANC relaxations, the PSS I contained 1 µM atropine and 4 µM guanethidine. After equilibration, 0.3 µM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was added inducing a sustained tonic contraction. Once the plateau was reached, a 10 s train of EFS (supramaximal voltage, 0.1 ms and 4 Hz) was applied inducing relaxation.

EFS was then repeated at 5 min interval with decreasing voltage to determine the voltage yielding a relaxation amplitude of approximately 50% of that obtained at supramaximal voltage (V50%R). EFS was then stopped and the tissue rinsed for 1 h at 10 min intervals. $PGF_{2\alpha}$ (0.3 µM) was readministered and EFS (V50%R) was applied with 10 s trains at 5 min intervals. After 5 stimulation trains 0.03, 0.1 or 0.3 µM prucalopride was added and 10 further trains registered. To study the neurogenic and nitrergic nature of the EFS-induced relaxations, the influence of 3 µM tetrodotoxin and 300 µM L-NAME was tested.

In the second part of the study, the PSS II continuously contained 100 µM N^G-nitro-L-arginine methyl ester (L-NAME) and 1 µM indomethacine. The initial part of the protocol with carbachol and EFS to determine the V50%C was as described above except that trains of EFS were administered every 3 min. Once EFS was started again at V50%C (0.5 ms, 4 Hz, 10s) and 5 stable responses were obtained, 2 types of experiments were performed. 1). The influence of the PDE-inhibitors IBMX, vinpocetine, EHNA, cilostamide, rolipram and zaprinast on the half maximal electrically induced contractions was investigated by adding them in half log unit ascending concentrations, starting after the 5^{th} train and registering the response to 6 trains after addition of each concentration. The influence of cilostamide plus rolipram was tested by adding 1 µM cilostamide, registering 10 trains, then adding 1 µM rolipram and registering another 20 trains; in half of the tissues the order of administration was reversed. 2) The influence of IBMX and rolipram versus prucalopride was studied as follows. A total of 33 to 35 trains (10s, V50%C, 0.5 ms, 4 Hz) was delivered at 3 min intervals. After 5 trains, 1, 3 or 10 µM IBMX was administered and after 15 trains 0.01 µM prucalopride; control tissues only received prucalopride or solvent. Similarly, 1 µM rolipram was added after 5 trains and 0.01, 0.03 or 0.1 µM prucalopride was added after 15 trains; in a small number of tissues, rolipram was added after 20 trains in the presence of prucalopride had been obtained.

3.III.3. Release study

The same method was used as described before (Leclere and Lefebvre, 2001). Strips were equilibrated for 1 h with superfusion of PSS I at 2 ml min^{-1} (Gilson Minipuls, France) and continuous EFS (40 V, 1 ms, 0.5 Hz) was applied for the last 20 min. Superfusion was stopped and the strips were incubated for 30 min with [^{3}H]-choline (5 µCi ml^{-1}) under continuous EFS (40 V, 1 ms, 2 Hz). EFS was stopped and the tissues were then superfused (2 ml min^{-1}) for 90 min to remove loosely bound radioactivity with PSS I, from now on also containing 10 µM hemicholinium-3 to prevent re-uptake of choline, 10 µM physostigmine to prevent hydrolysis of acetylcholine and 1 µM atropine to prevent auto-inhibition of acetylcholine release. After washout, the organ bath was filled with 1 ml of PSS. This was collected and replaced at 3 min intervals for a total of 37 samples.

The strips were stimulated twice (S1 and S2) at 15 V, 1 ms and 4 Hz for 2 min starting at the 13th (sample 5) and 73rd (sample 25) min after the end of the washout period. Prucalopride (0.03, 0.1 or 0.3 µM) was added 15 min (sample 20) before S2. The 5-HT_4 receptor antagonist GR113808 (1, 10 or 100 nM) was tested versus 0.3 µM prucalopride by adding it 21 min (sample 13) before prucalopride. In the second part of the study, the influence of 10 µM IBMX, added from sample 13 onwards, was tested versus 0.01 or 0.03 µM prucalopride, added from sample 20 onwards. In the same protocol, the influence of 10 µM vinpocetine, 10 µM EHNA, 1 µM cilostamide and 1 µM rolipram was tested versus 0.01 µM prucalopride. At the end of the experiment, the tissues were blotted and weighed. For each sample, 0.5 ml was mixed with 2 ml of the scintillator containing solution Ultima Gold (Perkin Elmer, USA). Radioactivity of all samples was measured by liquid scintillation counting (Packard Tri-Carb 2100 TR, Packard Instrument Company, USA); external standardization was used to correct for counting efficiency.

3.III.4. Data analysis

In the contractility study, the average contraction (or relaxation) to 5 trains of EFS before treatment was taken as 100 % and contractions (or relaxations) induced by EFS in the presence of the treatment were related to this reference value. In the release study, EFS evoked an increase in tritium overflow not only in samples 5 (S_1) and 25 (S_2) but also in up to maximally the 6 subsequent samples. Basal tritium overflow during the period with stimulation-induced increase of tritium overflow was calculated by fitting a regression line through the 4 samples just before stimulation and the 4 values starting from where overflow had returned to basal values after stimulation.

The stimulation-induced increase in tritium overflow was then determined by subtracting basal tritium overflow from the values in the samples with increased overflow. The S_2/S_1 ratio was then calculated.

Results are expressed as means ± SEM, n referring to tissues from different animals except for one series (see **Fig. 3.7**). Data obtained in parallel tissue groups were compared by an unpaired t-test (2 groups) or for more than 2 groups by ANOVA, followed by a post-hoc t-test corrected for multiple comparisons (Bonferroni). The influence of the increasing concentrations of the PDE-inhibitors on the electrically induced submaximal contractions was assessed by repeated measures ANOVA. P values of less than 0.05 were considered significant.

3.III.5. Drugs used

Acetylcholine chloride, l-ascorbic acid, atropine sulphate, choline chloride, guanethidine sulphate, indomethacine, prostaglandin $F_{2\alpha}$, zaprinast (Sigma), carbamoylcholin-chlorid (Fluka), hemicholinium-3-bromide (RBI or Sigma-Aldrich), 3-isobutyl-methyl-xanthine (Sigma-Aldrich), cilostamide, erythro-9-(2-hydroxyl-3-nonyl)adenine hydrochloride (EHNA), rolipram, vinpocetine (Tocris), [methyl-^{3}H]-choline chloride (NEN or Perkin Elmer), [1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1-H-indole-3-carboxylate maleate (GR 113808; GlaxoWellcome), methysergide maleate (Sandoz), physostigmine salicylate (Federa or Sigma Aldrich), N^G-nitro-L-arginine methyl ester (Sigma or Sigma-Aldrich), tetrodotoxin (Alomone Labs), prucalopride hydrochloride (Janssen Research Foundation), prucalopride succinate (Movetis). Drugs were dissolved in deionized water except for 3-isobutyl-methyl-xanthine (10 mM stock solution in 50 % ethanol), cilostamide, rolipram, vinpocetine (dissolved in DMSO) and indomethacine (dissolved in 10% $NaHCO_3$ or DMSO before adding it to the physiological salt solution). For granisetron commercially available ampoules (Kytril, Roche) were used and diluted with 0.9% NaCl solution till the required concentration.

3.IV. Results

3.IV.1. Influence of prucalopride on EFS-induced submaximal cholinergic contractions and nitrergic relaxations

The circular muscle strips of the pig proximal stomach did not show spontaneous phasic activity and basal tone remained constant during the course of the experiment. EFS-induced contractions at V50%C attained an amplitude of 67 ± 10 % (n = 6) of that induced by 3 µM carbachol at the beginning of the experiment; the contractions were neurogenic and cholinergic as they were abolished by 3 µM tetrodotoxin (n = 4) and 1 µM atropine (n = 4). In control tissues, the amplitude of the EFS-induced contractions at V50%C remained stable upon repetitive stimulation (amplitude of the contraction by a 15^{th} stimulation train was 100 ± 5 % of the mean response to trains 1 to 5; n = 6). Prucalopride did not influence the basal tone of the strips but it progressively enhanced the amplitude of the EFS-induced contractions (**Fig. 3.1A**) coming close to the maximal effect for a given concentration at the 5^{th} stimulation train in its presence; the facilitating effect of prucalopride was concentration-dependent for the concentration range studied (0.03, 0.1 or 0.3 µM; **Fig. 3.2A**). Cumulative administration of acetylcholine induced sustained and concentration-dependent contractions from 0.1 µM onwards; the contraction amplitude at 100 µM attained 95 ± 3 % (n = 4) of that induced by 3 µM carbachol at the beginning of the experiment.

Concentration-response curves of acetylcholine obtained in the presence of 0.03, 0.1 or 0.3 µM prucalopride did not differ from that in its absence within the same strips (n = 4 for each concentration). GR113808 (1, 10 and 100 nM) per se did not influence the EFS-induced contractions but concentration-dependently inhibited the facilitating effect of 0.3 µM prucalopride (**Fig. 3.2B**).

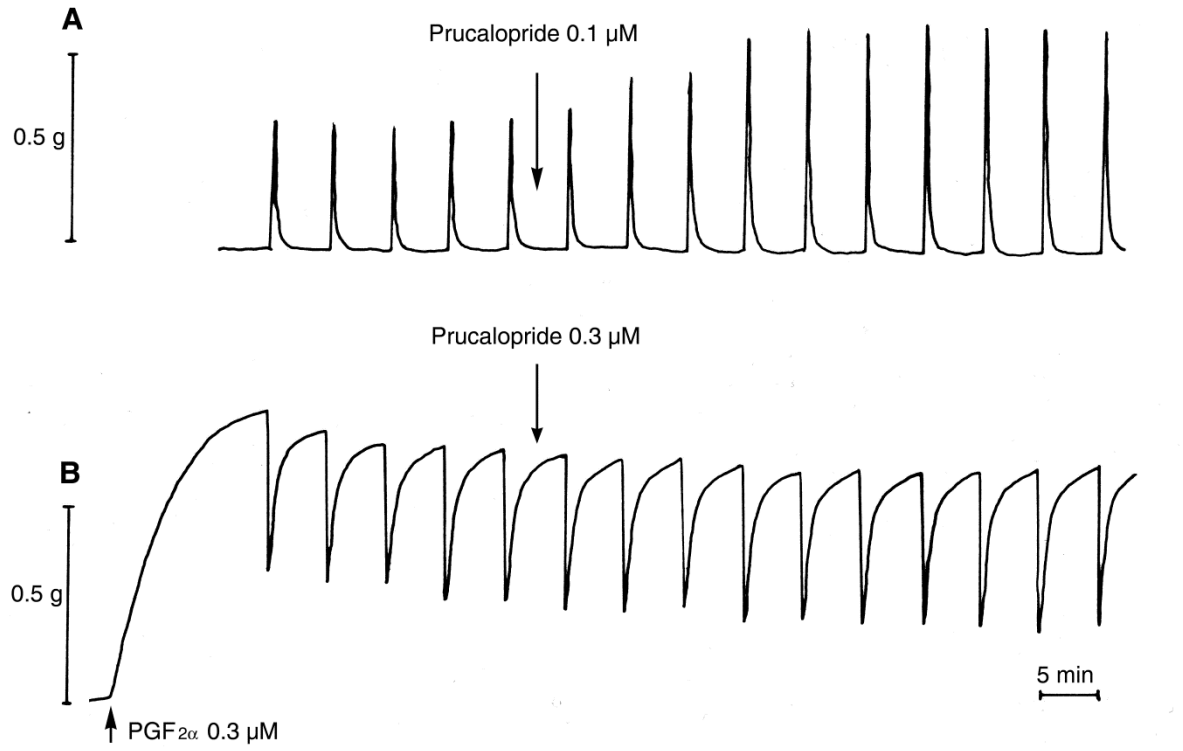


Figure 3.1

Representative traces (auxotonic registration) showing the facilitating effect of 0.1 µM prucalopride on submaximal EFS-induced contractions in the presence of 300 µM L-NAME (A) and the non-effect of 0.3 µM prucalopride on submaximal EFS-induced relaxations in the presence of 1 µM atropine (B) in pig gastric circular muscle strips. In B, the tissue was contracted with 0.3 µM prostaglandin $F_{2\alpha}$ to allow registration of relaxant responses.

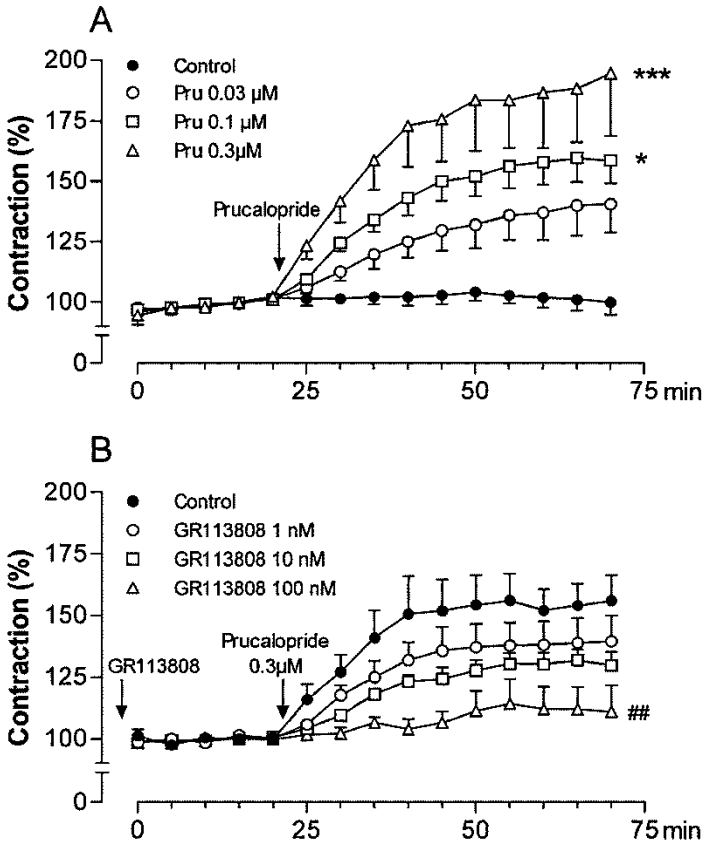


Figure 3.2

Enhancing effect of increasing concentrations of prucalopride (Pru) on EFS-induced submaximal contractions (A) and concentration-dependent antagonism by GR 113808 of the enhancing effect of 0.3 µM prucalopride (B). Responses are expressed as percentage of the mean of the 5 contractions before adding prucalopride. Means ± SEM of n = 6 tissues are shown. *** $P < 0.001$, * $P < 0.05$: significant difference of the final response versus that in control tissues without prucalopride; ## $P < 0.01$: significant difference of the final response versus that in control tissues only treated with prucalopride.

Granisetron (1 µM) per se did not influence the EFS-induced contractions nor did it influence the facilitating effect of 0.3 µM prucalopride, that enhanced the EFS-induced contractions to 125 ± 10 % (n = 6) in the absence of granisetron and to 134 ± 7 % (n = 6) in the presence of granisetron. Methysergide (10 µM) per se enhanced the amplitude of the EFS-induced contractions to 162 ± 11 % (n = 6) of that before it was added; but 0.3 µM prucalopride still enhanced the EFS-induced contractions in the presence of methysergide (**Fig. 3.3**).

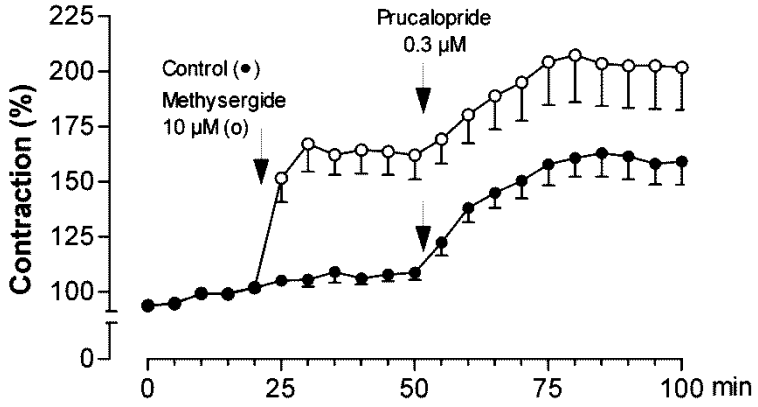


Figure 3.3

Evaluation of the influence of methysergide (10 µM) on the enhancing effect of 0.3 µM prucalopride on EFS-induced submaximal contractions. Responses are expressed as percentage of the mean of the 5 contractions before adding methysergide or its solvent (control). Means ± SEM of n = 6 tissues are shown.

In the presence of atropine and guanethidine and after contracting the tissues with $PGF_{2\alpha}$, EFS induced non-adrenergic non-cholinergic relaxations. At V50%R, the electrically induced submaximal relaxations were reproducible (**Fig. 3.1B**), of neuronal nature as abolished by 3 µM tetrodotoxin (n = 6) and mainly nitrergic (reduction by 300 µM L-NAME to 7 ± 3 %, n = 6). Prucalopride did not influence the submaximal nitrergic relaxations (see **Fig. 3.1B** for 0.3 µM prucalopride). The amplitude of the relaxation induced by the 10th stimulation train in the presence of 0.3 µM prucalopride was 93 ± 6 % (n = 6) of the mean response to the 5 stimulation trains before administration of prucalopride; in controls, this value was 90 ± 3 % (n = 6).

3.IV.2. Influence of PDE-inhibitors per se on EFS-induced submaximal cholinergic contractions

The non-selective PDE-inhibitor IBMX induced a concentration-dependent reduction of the contractions from 3 µM onwards and in the presence of 30 µM IBMX, the contractions were nearly abolished (**Fig. 3.4B**). None of the selective PDE-inhibitors was able to mimick the effect of IBMX. The PDE1-inhibitor vinpocetine (0.01-10 µM), the PDE2-inhibitor EHNA (1-30 µM) and zaprinast (0.01-10 µM) (did not significantly influence the submaximal cholinergic contractions (n = 6 for each agent; data not shown), nor did the PDE4-inhibitor rolipram (1-30 µM; **Fig. 3.4D**).

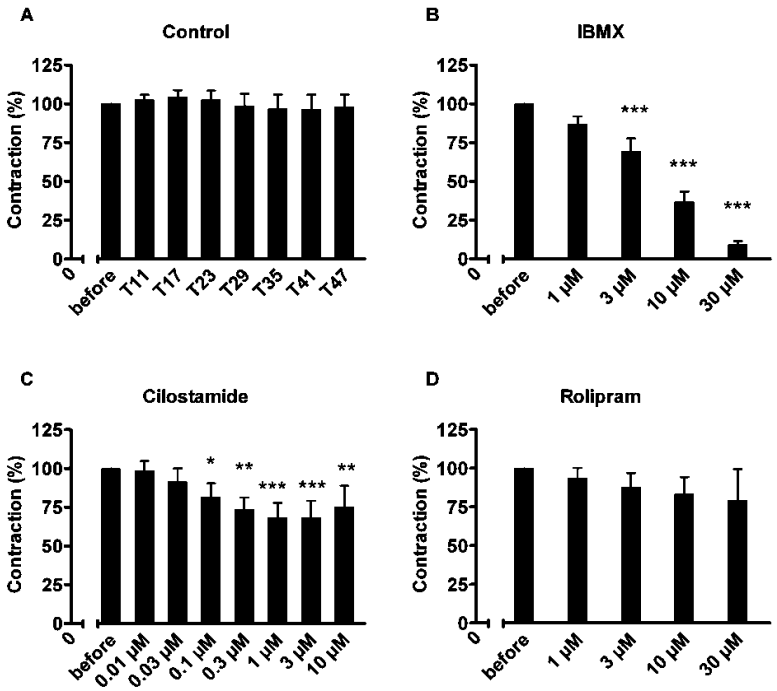


Figure 3.4

Influence of increasing concentrations of the PDE-inhibitors IBMX (B), cilostamide (C) and rolipram (D) on EFS-induced submaximal contractions. Six trains of EFS were applied in the presence of each concentration of PDE-inhibitor and the response to the 6th train was expressed as percentage of the mean of the 5 contractions before adding the lowest concentration of the PDE-inhibitor. Control tissues (A) were stimulated 47 times and the response was measured at each 6th train from train 11 (T11) on. Means ± SEM of n = 6-8 tissues are shown.

*** P < 0.001, ** P < 0.01, * P < 0.05 : significant difference versus the response before.

The PDE3-inhibitor cilostamide (0.01-10 µM) reduced the contractions from 0.1 µM onwards but the maximal depression obtained was much smaller than with IBMX (reduction to 68 ± 11 % with 3 µM cilostamide; **Fig. 3.4C**). When 1 µM cilostamide was added after 1 µM rolipram, it nearly abolished the electrically induced contractions (**Fig. 3.5A**); the response to the 10th stimulation train in the combined presence of rolipram and cilostamide only attained 13 ± 1 % (n = 4) of the response before adding the PDE-inhibitors. Also when the order of administration was reversed, electrically induced contractions were as good as abolished. After first adding 1 µM cilostamide, the contraction decreased to 59 ± 13 % at the 10th stimulation train in its presence; when further adding 1 µM rolipram, the contraction further decreased to 10 ± 5 % at the 10th stimulation train in their combined presence.

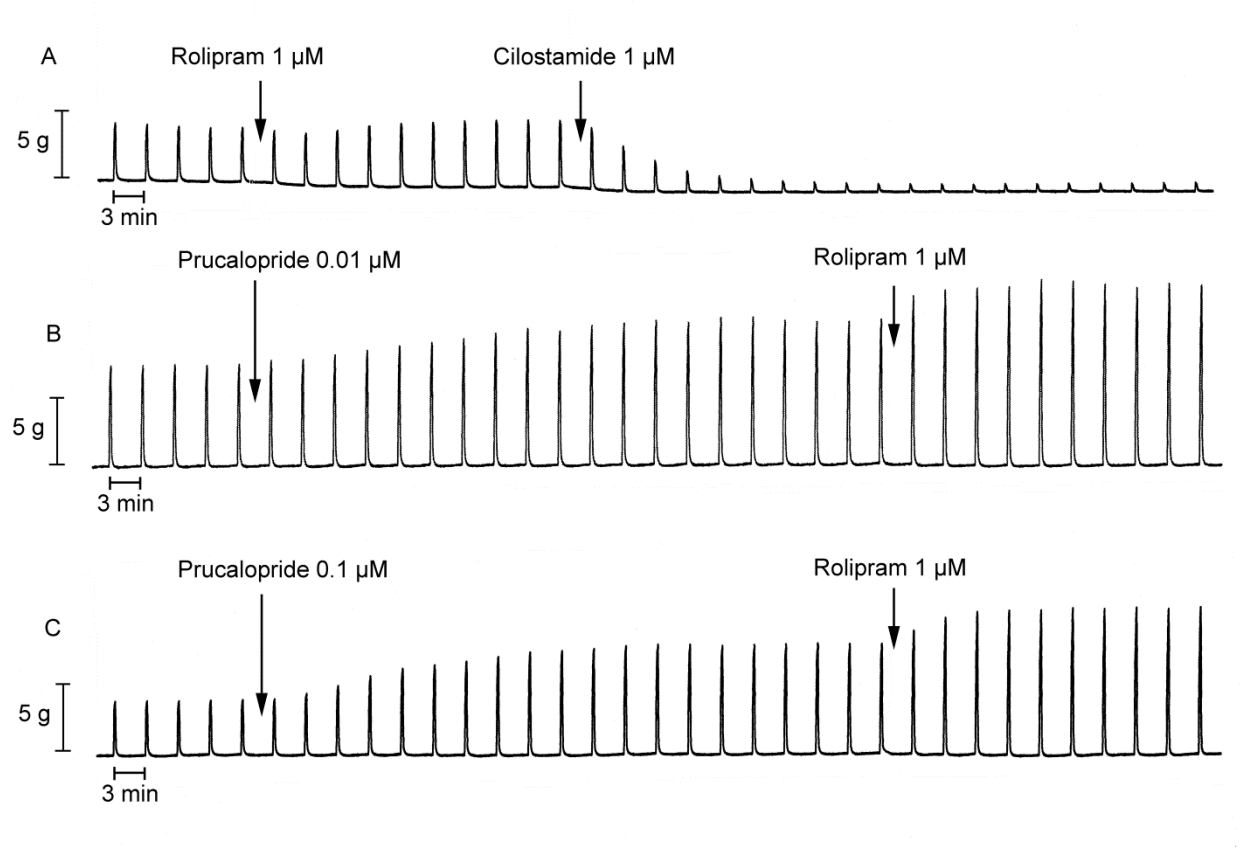


Figure 3.5

Representative traces (isometric registration) showing the influence on submaximal EFS-induced contractions of consecutive administration of 1 µM rolipram and 1 µM cilostamide (A), and of 0.01 µM (B) or 0.1 µM (C) prucalopride and 1µM rolipram.

3.IV.3. Influence of PDE inhibitors on the effect of prucalopride on EFS-induced submaximal cholinergic contractions

In the gastric circular muscle strips of piglets, prucalopride (0.01 µM) enhanced the EFS-induced contractions but this effect developed clearly slower than in the first part of the study (**Fig. 3.6**). IBMX, 1 and 3 µM, per se concentration-dependently decreased the EFS-induced contractions (maximally to 84 ± 2 %, n = 6, in the presence of 3 µM IBMX; **Fig. 3.6A**).

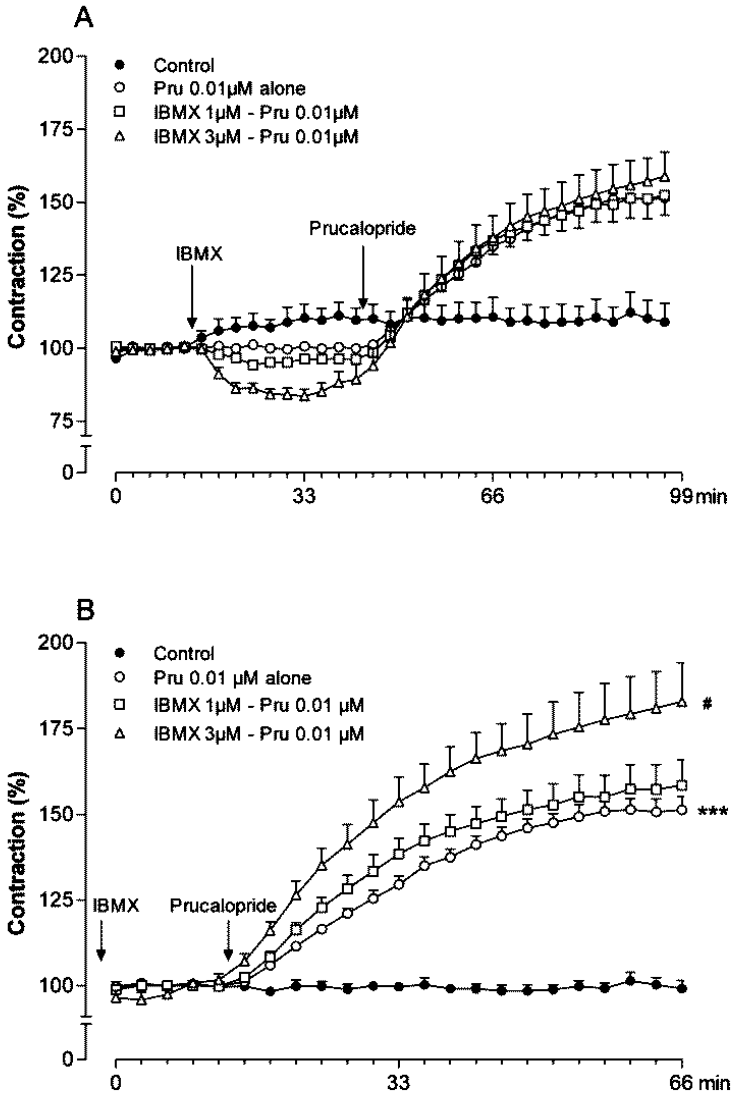


Figure 3.6

Influence of IBMX (1 or 3 µM) on the enhancing effect of 0.01 µM prucalopride (Pru) on EFS-induced submaximal contractions. In (A) responses are expressed as % of the mean of the first 5 responses ie the contractions induced before adding IBMX. In (B) responses are expressed as % of the mean of the 5 contractions before adding prucalopride. Means ± SEM of n = 6 tissues are shown. In panel A, the SEM is not shown on all mean data points for clarity. *** $P < 0.001$: significant difference of the final response versus that in control tissues without prucalopride; $^{\#}$ $P < 0.05$: significant difference of the final response versus that in tissues only treated with prucalopride.

To evaluate the effect of prucalopride, EFS-induced contractions in the presence of prucalopride were therefore expressed as % of the mean of the last 5 EFS-induced contractions in the presence of IBMX just before adding prucalopride (**Fig. 3.6B**).

This showed a significant enhancement of the facilitating effect of prucalopride by 3 µM IBMX. In an additional series, the influence of 10 µM IBMX was studied. This concentration of IBMX reduced the EFS-induced contractions by approximately 50 % (**Fig. 3.7A**); in the tissues that received only IBMX, this reduction was maintained till the end of the experiment. When added in the presence of 10 µM IBMX, prucalopride clearly enhanced the EFS-induced contractions and when expressed as % of the mean of the last 5 EFS-induced contractions just before adding prucalopride, this enhancement was more pronounced than for prucalopride in the absence of IBXM, although this did not reach significance (**Fig. 3.7B**) Rolipram (1 µM) was tested versus 0.01, 0.03 and 0.1 µM prucalopride (**Fig. 3.8**). In this series, the mean contractile response to the 10^{th} stimulation train in the presence of rolipram was somewhat increased in comparison to the response before its administration: to 114 ± 8 % (n = 8) before 0.01 µM prucalopride (**Fig. 3.8A**), 115 ± 8 % (n = 8) before 0.03 µM prucalopride (**Fig. 3.8B**) and 122 ± 9 % (n = 8) before 0.1 µM prucalopride (**Fig. 3.8C**). This was due to an increase in the response to stimulation in the presence of rolipram in some tissues. Eg in the tissues where 0.03 µM prucalopride was going to be added, the individual contractile response to the 10^{th} stimulation in the presence of rolipram was 96, 111, 137, 155, 93, 102, 101 and 128 %. Prucalopride alone significantly increased the electrically induced contractions to 162 ± 11 % (n = 7; 0.01 µM; **Fig. 3.8A**), 171 ± 15 % (n = 8; 0.03 µM; **Fig. 3.8B**) and 206 ± 10 % (n = 7; 0.1 µM; **Fig. 3.8C**). When rolipram had been added before prucalopride, the facilitating effect of prucalopride was increased to 181 ± 7 % (n = 8; 0.01 µM), 206 ± 24 % (n = 8; 0.03 µM) and 243 ± 23 % (n = 8; 0.1 µM). This increase did not reach significance. Still, when 1 µM rolipram was added after 20 stimulations in the presence of prucalopride had been obtained and the facilitating effect of prucalopride was stabilized, rolipram induced a clear-cut further increase of the electrically induced contractions (n = 2 for the 3 concentrations of prucalopride; illustrated in **Fig. 3.5B** for 0.01 and in **Fig. 3.5C** for 0.1 µM prucalopride).

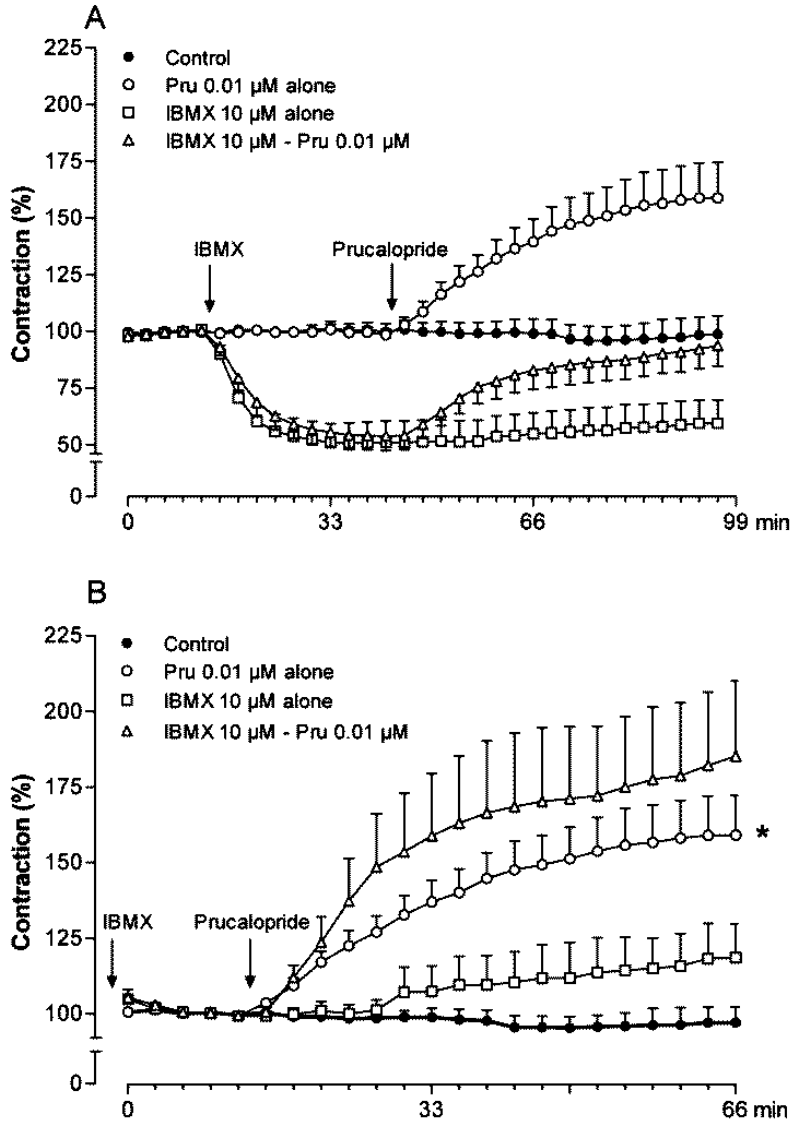


Figure 3.7

Influence of IBMX (10) µM) on the enhancing effect of 0.01 µM prucalopride (Pru) on EFS-induced submaximal contractions. In (A) responses are expressed as % of the mean of the first 5 contractions ie the contractions induced before adding IBMX. In (B) responses are expressed as % of the mean of the 5 contractions before adding prucalopride. Means ± SEM of n = 6-8 tissues from 5 animals are shown. * $P < 0.05$: significant difference of the final response versus that in control tissues without prucalopride.

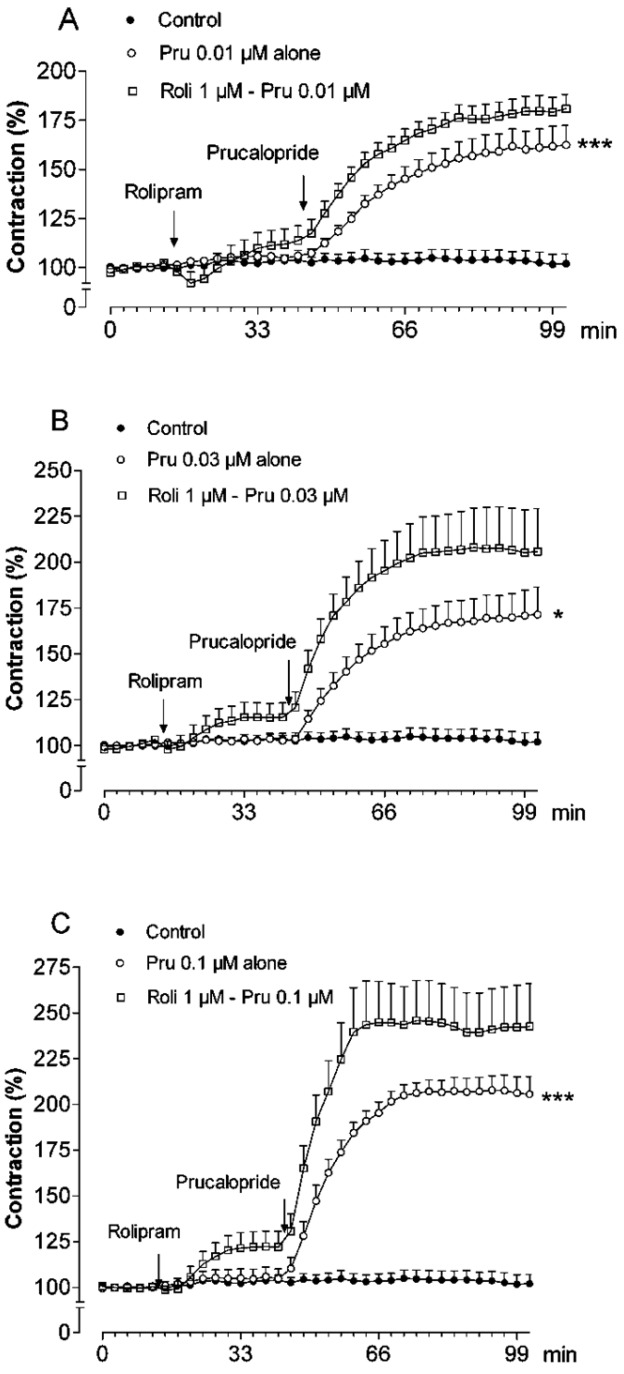


Figure 3.8

Influence of 1 µM rolipram on the enhancing effect of 0.01 (A), 0.03 (B) and 0.1 (C) µM prucalopride on EFS-induced submaximal contractions. Responses are expressed as percentage of the mean of the 5 contractions before adding rolipram. Means ± SEM of n = 7-8 tissues are shown. *** $P < 0.001$, * $P < 0.05$: significant difference of the final response versus that in control tissues without prucalopride.

3.IV.4. Influence of prucalopride on EFS-induced acetylcholine release

We have previously shown by separation with HPLC of the radioactive compounds in EFS-induced tritium outflow from pig stomach circular muscle strips after incubation with [^{3}H]-choline, that changes in [^{3}H]-acetylcholine parallel those in total tritium so that total tritium outflow can be considered as a marker for acetylcholine release (Leclere and Lefebvre, 2001).

EFS caused a clear-cut increase in tritium outflow above basal not only in the sample with stimulation but also in up to 6 further samples. The response induced by the second stimulation train was less pronounced yielding a S2/S1 ratio of 0.7 (**Table 3.1**). Prucalopride (0.03, 0.1 and 0.3 µM) did not influence basal outflow but it enhanced the tritium outflow induced by the second stimulation train leading to a concentration-dependent increase of the S2/S1 ratio with an S2/S1 ratio of 1.05 for 0.3 µM prucalopride (**Table 3.1**). In an additional series, the influence of 1 µM prucalopride was tested but this did not induce a more pronounced effect than 0.3 µM prucalopride (S2/S1 ratio: 0.74 ± 0.05 for controls, n = 5; 1.04 ± 0.05 for 1 µM prucalopride, n = 6; P < 0.01). GR 113808 (1, 10, 100 nM) did not influence basal outflow but concentration-dependently antagonized the facilitating effect of 0.3 µM prucalopride (**Table 3.1**).

Table 3.1 EFS-induced outflow of total radioactivity

Influence of prucalopride				
S1	50952 ± 3496	68328 ± 11006	91698 ± 24563	61343 ± 11445
Prucalopride (µM)	- (Control)	0.03	0.1	0.3
S2	35494 ± 3025	62398 ± 8272	91877 ± 21668	61498 ± 9813
S2/S1	0.70 ± 0.04	0.97 ± 0.14	1.02 ± 0.03*	1.05 ± 0.09*

Influence of GR113808 on effect of prucalopride				
S1	50543 ± 3791	42314 ± 3744	45180 ± 10235	49850 ± 8210
GR113808 (nM)	- (Control)	1	10	100
Prucalopride (µM)	0.3	0.3	0.3	0.3
S2	52591 ± 2950	43860 ± 4122	39273 ± 9533	47590 ± 8293
S2/S1	1.05 ± 0.03	1.05 ± 0.08	0.86 ± 0.04	0.74 ± 0.05$^{\#\#}$

Total radioactivity (tritium) is expressed in dpm g^{-1} tissue. For S1 and S2, the sum of radioactivity above baseline in sample 5 (S1) and sample 25 (S2), respectively, and the following samples with values above baseline is given. Means ± SEM of n = 5 to 6 tissues are given. * P < 0.05 versus control without prucalopride; $^{\#\#}$ P < 0.01 versus control without addition of GR 113808 before prucalopride.

3.IV.5. Influence of PDE-inhibitors on the effect of prucalopride on EFS-induced acetylcholine release

The influence of IBMX (10 µM) was first tested versus 0.01 µM prucalopride, a concentration expected to be minimally effective on acetylcholine release. Indeed, 0.01 µM prucalopride did not significantly increase EFS-induced tritium outflow versus control tissues (**Fig. 3.9**); the S2/S1 ratio was 0.68 ± 0.04 in tissues where 0.01 µM prucalopride was administered before S2 (n = 6) versus 0.59 ± 0.01 in control tissues (n = 6; **Fig. 3.10A**). IBMX (10 µM) per se did not influence basal outflow (**Fig. 3.6**) nor did it influence EFS-induced tritium outflow (**Fig. 3.10A**).
However, when IBMX was administered before prucalopride, a clear-cut significant increase in EFS-induced tritium outflow was obtained (**Fig. 3.10A**).

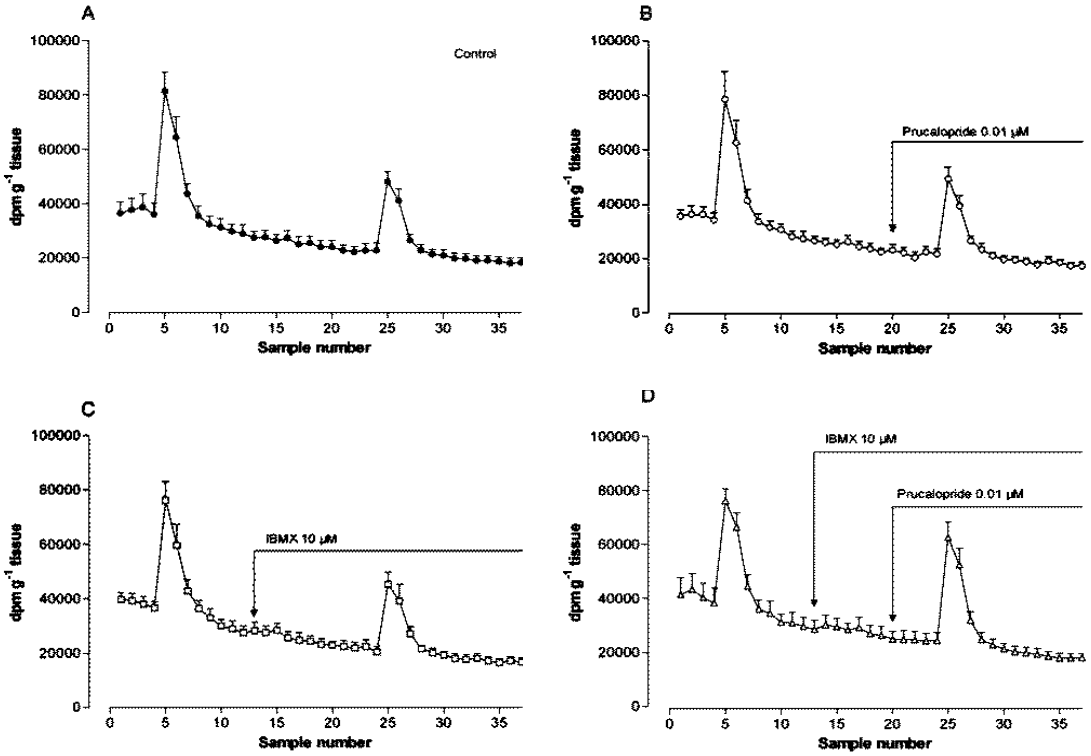


Figure 3.9

Influence of prucalopride (0.01 µM, B), IBMX (10 µM, C) and prucalopride in the presence of IBMX (D) on EFS-induced release of total radioactivity; in A, parallel control tissues are shown. The content of the organ bath was collected in 3 min samples for measurement of total radioactivity (37 samples in total). Tissues were stimulated twice (15 V, 1 ms, 4 Hz, 2 min), at the 5th (S1) and 25th (S2) sample. Prucalopride and IBMX were added before S2 at the time points indicated. Means ± SEM of n = 6 tissues are shown.

In a second series, 0.03 µM prucalopride per se significantly enhanced EFS-induced tritium outflow. IBMX (10 µM) per se again did not influence EFS-induced tritium outflow significantly but it further increased the facilitating effect of prucalopride (**Fig. 3.10B**). EHNA (10 µM) did not influence basal nor EFS-induced tritium outflow; it did also not induce a facilitating effect of 0.01 µM prucalopride (S2/S1 ratio in control tissues : 0.53 ± 0.02; with 10 µM EHNA: 0.51 ± 0.05; with 0.01 µM prucalopride : 0.63 ± 0.04; with EHNA and prucalopride : 0.58 ± 0.03; n = 4-6).

A small series was then started where 0.01 µM prucalopride was added before S2, either alone or preceded by vinpocetine (10 µM), cilostamide (1 µM) or rolipram (1 µM). None of these PDE-inhibitors influenced basal tritium outflow but in the presence of rolipram and prucalopride, the S2/S1 ratio (0.98 ± 0.02) was significantly enhanced ($P < 0.01$) versus that in the presence of prucalopride alone (0.70 ± 0.03); the S2/S1 ratio for vinpocetine plus prucalopride was 0.64 ± 0.05; for cilostamide plus prucalopride 0.69 ± 0.06 (n = 4 for each series).

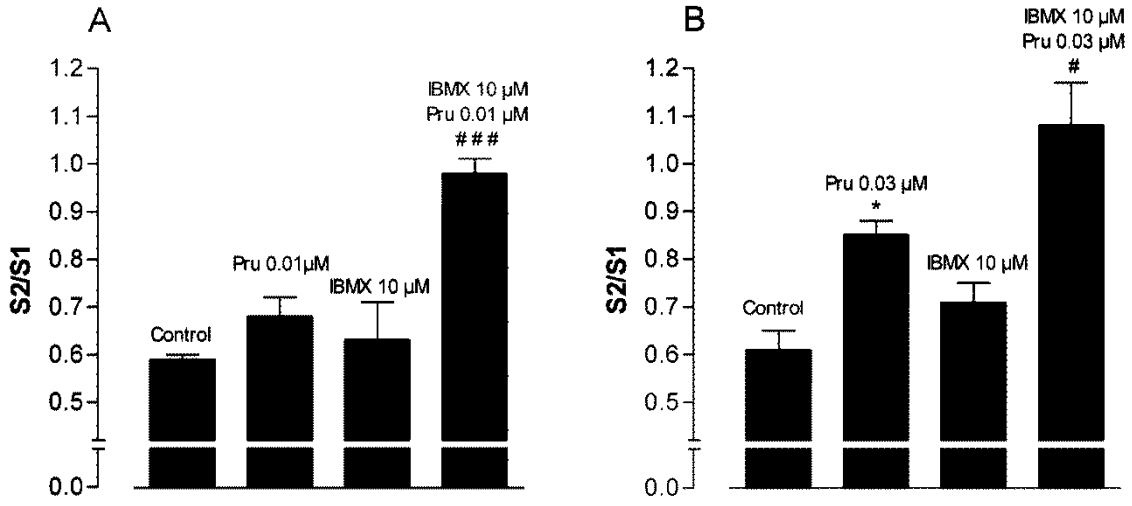


Figure 3.10

Influence of prucalopride (Pru; 0.01 µM, A; 0.03 µM, B), IBMX and prucalopride in the presence of IBMX on the S2/S1 ratio of EFS-evoked total radioactivity release. Tissues were stimulated twice (S1 and S2; 15V, 1 ms, 4 Hz, 2 min); IBMX was added 36 min and prucalopride 15 min before S2. The EFS-induced efflux of total radioactivity above baseline by S2 is expressed as a ratio of that by S1. Means ± SEM of n = 5 to 6 tissues are shown. * $P < 0.05$: significantly different from control; $^{\#}$ $P < 0.05$, $^{\#\#\#}$ $P < 0.001$: significantly different from prucalopride alone.

A series was then set up including control tissues and the addition of rolipram alone before S2. Rolipram (1 µM) per se tended to increase the S2/S1 ratio but this was not significant. The S2/S1 ratio in the presence of rolipram plus 0.01 µM prucalopride (0.98 ± 0.07; n = 6) was significantly enhanced versus that in the presence of prucalopride alone (0.65 ± 0.03; n = 6; **Fig. 3.11**) similar to the level obtained in the presence of IBMX plus 0.01 µM prucalopride (**Fig. 3.10A**).

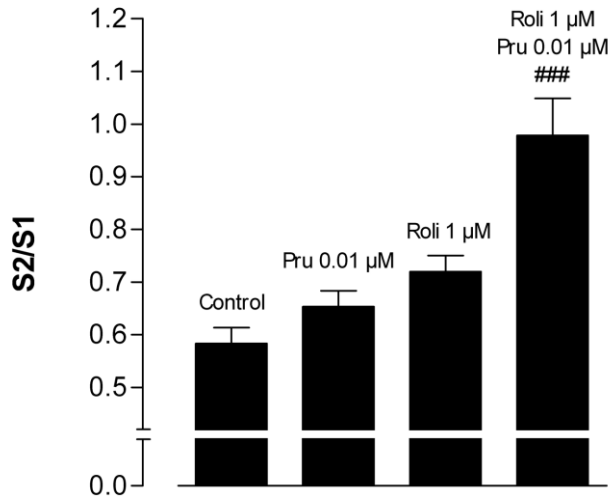


Figure 3.11

Influence of prucalopride (Pru, 0.01 µM), rolipram (Roli, 1 µM) and prucalopride in the presence of rolipram on the S2/S1 ratio of EFS-evoked total radioactivity release. Tissues were stimulated twice (S1 and S2; 15V, 1 ms, 4 Hz, 2 min); rolipram was added 36 min and prucalopride 15 min before S2. The EFS-induced efflux of total radioactivity above baseline by S2 is expressed as a ratio of that by S1. Means ± SEM of n = 6 tissues are shown. $^{###}$ P < 0.001 : significantly different from prucalopride alone.

3.V. Discussion

The key objective of this study was to investigate the influence of the 5-HT$_4$ receptor agonist prucalopride on cholinergic neurotransmission towards pig stomach circular muscle and whether this effect is influenced by PDE inhibition; additionally it was investigated whether prucalopride is able to facilitate nitrergic neurotransmission towards pig gastric circular muscle as has been proposed for human colon circular muscle.

3.V.1. Facilitation of cholinergic but not nitrergic neurotransmission by 5-HT_4 receptor activation in pig gastric circular muscle

The data presented illustrate that cholinergic nerves in pig gastric circular muscle are indeed endowed with facilitating 5-HT_4 receptors promoting acetylcholine release and cholinergic contractions. Neuronally induced submaximal cholinergic contractions, as evidenced by their blockade with the neuronal action potential propagation inhibitor tetrodotoxin and the muscarinic receptor antagonist atropine, were concentration-dependently enhanced by prucalopride, while cholinergic contractions induced by direct stimulation of muscarinic receptors with acetylcholine were not influenced. This suggests a presynaptic site of action of prucalopride with facilitation of acetylcholine release, which was confirmed by measuring acetylcholine release directly. The concentration range used lies within the EC_{50}-EC_{100} region of the concentration-response curve for the enhancing effect of prucalopride through presynaptic 5-HT_4 receptors on electrically induced submaximal cholinergic contractions in canine and pig gastric longitudinal muscle preparations and the enhancing effect of the highest concentration tested (0.3 µM; 95 %; see **Fig. 3.2A**) corresponds to an enhancing effect of 80-90 % with the same concentration in these 2 preparations (Prins et al., 2001b; De Maeyer et al., 2006a). The enhancing effect of 0.3 µM prucalopride both on contractions and acetylcholine release was concentration-dependently antagonized by the 5-HT_4 receptor antagonist GR113808 (Gale et al., 1994). GR113808 has a high affinity for 5-HT_4 receptors with reported pK_B estimates of 9.1 at 5-HT_4 receptors on cholinergic nerve endings in canine stomach (Prins et al., 2001b) and of 9.36 at 5-HT_4 receptors on human colon circular muscle cells (Irving et al., 2007). At 100 nM GR113808 can thus be expected to close to abolish the effect of a near maximal concentration of a 5-HT_4 receptor agonist which was the case with 0.3 µM prucalopride in both the functional and release assays corroborating its interaction with 5-HT_4 receptors. This was further underlined by the lack of effect of the 5-HT_3 receptor antagonist granisetron and the 5-HT_1, 5-HT_2, 5-ht_5, 5-HT_6 and 5-HT_7 receptor antagonist methysergide on the enhancement of cholinergic contractions by prucalopride. We have no clear-cut explanation for the effect per se of methysergide on the electrically induced cholinergic contractions, that we also observed before in pig gastric longitudinal muscle (De Maeyer et al., 2006a). Further investigation of this effect of methysergide was, however, not within the aim of this study.

Similar to the human colon, the pig stomach thus has 5-HT_4 receptors on the cholinergic nerve endings towards both the circular and longitudinal muscle layer and pig gastric circular muscle can thus be considered as a model for the 5-HT_4 receptors on the cholinergic nerves innervating circular muscle in the human stomach (Leclere and Lefebvre, 2002).

The longitudinal muscle layer of the stomach contracts in 1:1 correlation with the circular muscle layer (Sarna, 1993) but it can be expected that the thicker circular muscle layer contributes most to changes in intragastric pressure and gastric motility, so that it might be preferable to investigate the effect of new 5-HT$_4$ receptor agonists, developed for stimulating gastric motility and emptying, at 5-HT$_4$ receptors in the most important muscle layer.

In human colon circular muscle, 5-HT$_4$ receptors are present on cholinergic nerves and on smooth muscle cells with an opposite effect ie facilitation of cholinergic contraction versus induction of relaxation. It seems obvious that the stimulatory effect of prucalopride on human colonic transit (Emmanuel et al., 1998; Bouras et al., 1999) must be related to a balanced activation of the 5-HT$_4$ receptors at the neuronal and muscular level. Cellek et al. (2006) were within their experimental conditions not able to show the presence of muscular 5-HT$_4$ receptors in human colonic circular muscle, but provided evidence for 5-HT$_4$ receptors on nitrergic nerves facilitating nitrergic neurotransmission and thus relaxation. Again, balanced activation of the 5-HT$_4$ receptors on cholinergic respectively nitrergic neurons by prucalopride will then be required to lead to enhanced colonic transit. We therefore also investigated in pig gastric circular muscle the possible influence of prucalopride on electrically induced submaximal nitrergic relaxations. As the pig gastric circular muscle preparation is a low tone preparation, tissues had to be contracted with $PGF_{2\alpha}$ to observe relaxations. In this condition, prucalopride did not influence the submaximal nitrergic relaxations illustrating that nitrergic nerves in pig gastric circular muscle are not endowed with 5-HT$_4$ receptors.

3.V.2. Regulatory control by PDEs of the 5-HT$_4$ receptor on cholinergic neurons in pig gastric circular muscle

5-HT$_4$ receptors are adenylyl cyclase coupled receptors generating cAMP and the facilitating effect of the 5-HT$_4$ receptor agonist renzapride on acetylcholine release from guinea pig small intestinal myenteric neurons was reported to be related to activation of the adenylyl cyclase-protein kinase A pathway (Ren et al., 2008). Cellular cyclic nucleotide levels are regulated by PDEs which catalyse their breakdown. The positive inotropic effect of 5-HT$_4$ receptor agonists in porcine left atrium becomes only prominent and sustained under conditions of PDE inhibition (De Maeyer et al., 2006b; Galindo-Tovar et al., 2009) illustrating an important role of PDEs in the control of 5-HT$_4$ receptor-induced cardiac cAMP levels. The enhancing effect of prucalopride on submaximal cholinergic contractions in pig gastric longitudinal (De Maeyer et al., 2006a) and circular (this study) muscle is sustained in the absence of PDE inhibition but this does not

exclude a regulatory role of PDEs. A recent very thorough evaluation of the mRNA distribution for the 11 PDE isoenzymes in human brain and peripheral tissues showed all PDE isoenzymes to be expressed in the stomach except for PDE6A (Lakics et al., 2010). Also in gastrointestinal smooth muscle, cyclic nucleotides are essential mediators of relaxation and their intracellular concentration is regulated by PDEs; a major part of the PDE mRNA expressed in human stomach may thus have been derived from the smooth muscle cells. In our experiments, the non-selective PDE-inhibitor IBMX concentration-dependently reduced the amplitude of the electrically induced cholinergic contractions (**Fig. 3.4**). This corresponds to the inhibitory effect of non-selective PDE-inhibitors versus contractions induced by exogenously administered agonists (Barnette et al., 1993; Barbier and Lefebvre, 1995; Tomkinson and Raeburn, 1996) and electrical field stimulation (Park et al., 2003) in gastrointestinal muscle preparations and illustrates that also in porcine gastric circular muscle, PDEs are controling the cyclic nucleotide concentrations.

Still, a careful study of PDE2A distribution by immunohistochemistry revealed prominent expression in enteric ganglia from stomach to colon (Stephenson et al., 2009). We therefore investigated the influence of the non-selective PDE inhibitor IBMX on the facilitating effect of prucalopride on cholinergic contractions in the functional assay. As the pig stomach provision from the slaughter house was no longer available by then, commercially available piglets were now used. In order to be able to observe a possible facilitating influence of IBMX on the effect of prucalopride, 0.01 µM prucalopride (3 times lower than the lowest concentration in part I) was used expecting a mild influence on submaximal cholinergic contractions. However, this concentration increased the cholinergic contractions to approximately 150% in the gastric circular muscle strips of the piglets; this higher sensitivity might be related to the younger age of the animals. The time interval before reaching a stable effect of prucalopride was also increased but this might be related to the smaller interval in between stimulation trains (3 min instead of 5); cfr our results reported for pig gastric longitudinal muscle where trains of EFS at 3 min interval were shown to induce stable contractions (De Maeyer et al., 2006a), we had reduced the train interval to 3 min for the second part of the study. The interpretation of the results when studying IBMX versus prucalopride in the functional assay was hampered by the relaxing effect of IBMX per se. This functional antagonism by IBMX was maintained throughout the experiment as evident from the study of the effect of 10 µM IBMX alone (**Fig. 3.7**). When prucalopride was added in the presence of IBMX, it was able to enhance the electrically induced contractions, the effect of prucalopride in the presence of IBMX being more pronounced than with prucalopride alone, reaching significance for 3 µM IBMX (**Fig. 3.6B**).

This suggests that, notwithstanding the negative influence on the effect of prucalopride by functional antagonism of released acetylcholine at the muscular level, IBMX has a positive influence on the facilitating effect of prucalopride at the cholinergic nerves leading to a higher enhancement of acetylcholine release.

This was confirmed in the release assay. IBMX had no influence per se on basal outflow and on electrically induced acetylcholine release. As a xanthine, IBMX can antagonize A_{2A} receptors (Baraldi et al., 2008). In rat myenteric neurons, electrically induced acetylcholine release is influenced by inhibitory A_1 and facilitatory A_{2A} receptors, endogenous adenosine showing a predominantly tonic facilitatory effect via the prejunctional A_{2A} receptors (Duarte-Araujo et al., 2004). The observation that IBMX per se had no influence on electrically induced acetylcholine release in pig gastric circular muscle thus suggests that there is no facilitation of acetylcholine release by endogenous adenosine in this tissue. IBMX induced a significant facilitating effect on a per se subeffective concentration of prucalopride (0.01 µM) on acetylcholine release and it further increased the already significant effect of 0.03 µM prucalopride. In the release assay, 0.01 µM prucalopride indeed only tended to increase the acetylcholine release while it had a clear-cut effect on submaximal cholinergic contractions in the functional assay. However, one should realize that the release assay only measures overflow from acetylcholine out of the tissue in the organ bath medium while the functional assay measures the direct smooth muscle response to acetylcholine released from the cholinergic nerves. Additionally, acetylcholine release was measured after 15 min of incubation with prucalopride while the maximal enhancing effect on cholinergic contractions (50 % increase) in part II of the functional study was measured after a total incubation of prucalopride for 54 min (18 trains of EFS at 3 min interval). Our results indicate that the stimulatory effect of 5-HT_4 receptors on acetylcholine release from cholinergic nerves innervating circular muscle of the pig stomach is regulated by neuronal PDEs. This does not correlate with data of Kilbinger et al. (1995), who reported that in longitudinal muscle-myenteric plexus preparations of guinea pig ileum, IBMX did not increase the enhancing effect of 5-HT_4 receptor agonists on acetylcholine release. In contrast, Yau et al. (1987) reported that the adenylate cyclase activator forskolin increased acetylcholine release in the same set-up and this effect was enhanced in the presence of the non-selective PDE inhibitors IBMX and theophylline suggesting that cAMP-mediated influences on acetylcholine release are also in guinea pig ileum regulated by PDEs.

3.V.3. PDE subtype involved in the regulatory control of the 5-HT$_4$ receptor on cholinergic neurons in pig gastric circular muscle

Of the classic PDE subtypes 1-5 (Beavo and Reifsnyder, 1990; Maurice et al., 2003), PDE4 is cAMP specific, PDE5 is cGMP specific and PDE1, 2 and 3 have dual enzymatic activity, so that PDE1, 2, 3 or 4 might be involved in the control of the adenylate cyclase-cAMP-linked 5-HT$_4$ receptor on the cholinergic neurons in pig gastric circular muscle. We therefore studied the influence of selective PDE-inhibitors and first concentrated on PDE2 in view of its distribution in gastric enteric ganglia of different species (Stephenson et al., 2009). However EHNA, which has been shown to selectively inhibit PDE2 with an IC50 in the low micromolar range (Rivet-Bastide et al., 1997), did not influence the effect of prucalopride on electrically induced acetylcholine release in a concentration of 10 µM.

To evaluate the possible role of PDE1, 3 or 4, we investigated the influence of the inhibitors vinpocetine (PDE1), cilostamide (PDE3) and rolipram (PDE4) (Alexander et al., 2009). None of these PDE inhibitors per se mimicked the near abolition of electrically induced contractions seen with IBMX, as vinpocetine and rolipram were without significant effect, while cilostamide inhibited the contractions maximally by 32 %. As IBMX also inhibits the cGMP specific PDE5 and this enzyme can be present in gastrointestinal muscle, inhibition of PDE5 by IBMX might contribute to its pronounced inhibitory influence on electrically induced contractions; but the PDE5-selective inhibitor zaprinast (0.01-10 µM) did not influence the cholinergic contractions (results not shown). When cilostamide and rolipram were added together, near full inhibition of the electrically induced contractions was obtained, suggesting a redundant role of PDE3 and PDE4 in the control of the cyclic nucleotide levels in pig gastric circular muscle with PDE3 being predominant as cilostamide alone had some effect. The PDEs involved in the control of cyclic nucleotide levels and thus the contractile degree of gastrointestinal smooth muscle differs between species and regions of the gastrointestinal tract (see Barnette el al., 1990; 1993; Tomkinson and Raeburn, 1996). The role of both PDE3 and PDE4 in pig gastric circular muscle resembles the joint role of these 2 PDE subtypes in the control of canine and human respiratory smooth muscle (Torphy et al., 1991; Schmidt et al., 2000) and of the 5-HT$_4$ receptor-mediated inotropic response to 5-HT in porcine atrium (Galindo-Tovar et al., 2009).

When studying the influence of vinpocetine, cilostamide and rolipram versus the effect of prucalopride on acetylcholine release, only rolipram facilitated prucalopride. In a concentration of 1 µM, which is able to inhibit all PDE4 isozymes (Wang et al., 1997), it potentiated the effect of 0.01 µM prucalopride on acetylcholine release to the same extent as IBMX, suggesting that PDE4 is the sole PDE subtype controling the response to 5-HT_4 receptor activation in the cholinergic neurons of pig gastric circular muscle. PDEs are widely distributed in the central nervous system and PDE inhibitors were shown to enhance learning and synaptic plasticity (Reneerkens et al., 2009). Particular attention in this regard has been attracted by the cAMP specific PDE4, with rolipram facilitating long-term potentiation (LTP) and improving memory in mice (Barad et al., 1998). The enhancing effect of rolipram on 4-aminopyridine-induced glutamate release from prefrontocortical synaptosomes (Wang, 2006) suggests that facilitation of glutamate release might be involved in the effect of PDE4-inhibition on LTP in view of the important role of the central neurotransmitter glutamate in LTP; but this result certainly is another illustration that PDE4 can be involved in the regulation of neuronal transmitter release.

As rolipram per se did not reduce electrically induced cholinergic contractions in porcine gastric muscle, but enhanced the facilitating effect of prucalopride on acetylcholine release, it was expected to also enhance the facilitating effect of prucalopride on electrically induced cholinergic contractions in the functional assay. When rolipram was added before prucalopride, a clear tendency to enhanced facilitation of the cholinergic contractions by the 3 concentrations of prucalopride (0.01-01 µM) was indeed observed; this enhancement was also visible when rolipram was added after prucalopride. Surprisingly, in this series, rolipram per se enhanced cholinergic contractions mildly to moderately in some tissues. This might be related to a borderline role of PDE4 in the control of acetylcholine release per se from cholinergic nerves in pig gastric circular muscle. In equine trachea, IBMX per se in high concentrations enhanced acetylcholine release from cholinergic nerves induced by electrical field stimulation (Zhang et al., 1996). Although we did not observe a significant influence of IBMX and rolipram on electrically induced acetylcholine release, a tendency to enhancement of the S2/S1 ratio was seen (**Fig. 3.10B; 3.11**).

In conclusion, our results show that 5-HT_4 receptors are present on the cholinergic nerves towards the pig gastric circular muscle and their stimulation facilitates acetylcholine release. Although stimulation leads to a maintained response, the intracellular pathway of facilitation is under the influence of PDE4 illustrating that also in peripheral neurons PDEs can be involved in the regulation of neurotransmitter release. Combination of 5-HT_4 receptor activation with acetylcholinesterase inhibition has been shown to have synergistic gastrointestinal prokinetic effects and this combination was proposed as a possible therapeutic approach for conditions with slow gastrointestinal transit (Cellek et al., 2008; Campbell-Dittmeyer et al., 2009). Another way to increase the gastroprokinetic effect of 5-HT_4 receptor activation might be to combine a 5-HT_4 receptor agonist with selective inhibition of the PDE regulating the transmission of the 5-HT_4 receptors in peripheral cholinergic neurons. The actual study illustrates that in pig gastric circular muscle, this effect can be obtained with a PDE4-selective inhibitor. Extrapolation to humans will of course require to investigate the PDE subtypes active in cholinergic neurons versus those in smooth muscle cells in gastric tissue as well as colonic tissue, another human target tissue of 5-HT_4 receptor agonists.

3.VI. References

Alexander SPH, Mathie A, Peters JA. Guide to receptors and channels, 4th ed. Br. J. Pharmacol. 2009; 158: S1-S254

Barad M, Bourtchouladze R, Winder DG, Golan H, Kandel E. Rolipram, a type IV-specific phosphodiesterase inhibitor, facilitates the establishment of long-lasting long-term potentiation and improves memory. Proc. Natl. Acad. Sci. USA. 1998; 95: 15020-15025

Baraldi PG, Tabrizi MA, Gessi S, Borea PA. Adenosine receptor antagonists: Translating medicinal chemistry and pharmacology into clinical utility. Chem. Rev. 2008; 108: 238-263

Barbier AJ, Lefebvre RA. Relaxant influence of phosphodiesterase inhibitors in the cat gastric fundus. Eur. J. Pharmacol. 1995; 276: 41-47

Bouras EP, Camilleri M, Burton DD, McKinzie S. Selective stimulation of colonic transit by the benzofuran 5HT_4 agonist, prucalopride, in healthy humans. Gut 1999; 44: 682-686

Barnette MS, Grous M, Torphy TJ, Orsmbee HS. Activation of cyclic AMP-dependent protein kinase during canine lower esophageal sphincter relaxation. J. Pharmacol. Exp. Ther. 1990; 252: 1160-1166

Barnette MS, Manning CD, Price WJ, Barone FC. Initial biochemical and functional characterization of cyclic nucleotide phosphodiesterase isozymes in canine colonic smooth muscle. J. Pharmacol. Exp. Ther. 1993; 264: 801-812

Beavo JA, Reifsnyder DH. Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Tr. Pharmacol. Sci. 1990; 11: 150-155

Bouras EP, Camilleri M, Burton DD, Thomforde G, McKinzie S, Zinsmeister AR. Prucalopride accelerates gastrointestinal and colonic trnasit in patients with constipation without a rectal evacuation disorder. Gastroenterol. 2001; 120: 354-360

Camilleri M, Kerstens R, Rykx A, Vandeplassche L. A placebo-controlled trial of prucalopride for severe chronic constipation. N. Engl. J. Med. 2008; 358: 2344-2354

Campbell-Dittmeyer K, Hicks GA, Earnest DL, Greenwood-Van Meerveld B. Increased colonic transit in rats produced by a combination of a cholinesterase inhibitor with a 5-HT_4 receptor agonist. Neurogastroenterol. Motil. 2009; 21: 1197-1203

Cellek S, John AK, Thangiah R, Dass NB, Bassil AK, Jarvie EM, Lalude O, Vivekanandan S, Sanger GJ. 5-HT_4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. Neurogastroenterol. Motil. 2006; 18: 853-861

Cellek S, Thangiah R, Jarvie EM, Vivekanandan S, Lalude O, Sanger GJ. Synergy between 5-HT_4 receptor activation and acetylcholinesterase inhibition in human colon and rat forestomach. Neurogastroenterol. Motil. 2008; 20: 539-545

De Maeyer JH, Prins NH, Schuurkes JAJ, Lefebvre RA. Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors : an operational framework to explain and quantify organ-specific behavior. J. Pharmacol. Exp. Ther. 2006a; 317: 955-964

De Maeyer JH, Straetemans R, Schuurkes JAJ, Lefebvre RA. Porcine left and sinoatrial 5-HT_4 receptor-induced response: fading of the response and influence of development. Br. J. Pharmacol. 2006b; 147: 140-157

Duarte-Araujo M, Nascimento C, Timoteo MA, Magalhaes-Cardoso T, Correia-de-Sa P. Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A_{2A} and extrajunctional inhibitory A_1 receptors. Br. J. Pharmacol. 2004; 141: 925-934

Emmanuel AV, Kamm MA, Roy AJ, Antonelli K. Effect of a novel prokinetic drug, R093877, on gastrointestinal transit in healthy volunteers. Gut 1998; 42: 511-516

Gale JD, Grossman CJ, Whitehead JW, Oxford AW, Bunce KT, Humphrey PP. GR113808: a novel, selective antagonist with high affinity at the 5-HT_4 receptor. Br. J. Pharmacol. 1994; 111: 332-338

Galindo-Tovar A, Vargas ML, Escudero E, Kaumann AJ. Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT_4 receptors. Br. J. Pharmacol. 2009; 156: 237-249

Gershon MD, Tack J. The serotonin signaling system: From basic understanding to drug development for functional GI disorders. Gastroenterol. 2007; 132: 397-414

Irving HR, Tan YY, Tochon-Danguy N, Liu H, Chetty N, Desmond PV, Pouton CW, Coupar IM. Comparison of 5-HT_4 and 5-HT_7 receptor expression and function in the circular muscle of the human colon. Life Sci. 2007; 80: 1198-1205

Kilbinger H, Gebauer A, Haas J, Ladinsky H, Rizzi CA. Benzimidazoles and renzapride facilitate acetylcholine release from guinea-pig myenteric plexus via 5-HT_4 receptors. N.S. Arch. Pharmacol. 1995; 351: 229-236

Lakics V, Karran EH, Boess FG. Quantitative comparison of phosphodiesterase mRNA distribution in human brain and peripheral tissues. Neuropharmacol. 2010; 59: 367-374

Leclere PG, Lefebvre RA. Influence of nitric oxide donors and of the α_2-agonist UK-14,304 on acetylcholine release in the pig gastric fundus. Neuropharmacol. 2001; 40: 270-278

Leclere PG, Lefebvre RA. Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. Br. J. Pharmacol. 2002; 135: 135-142

Leclere PG, Prins NH, Schuurkes JAJ, Lefebvre RA. 5-HT_4 receptors located on cholinergic nerves in human colon circular muscle. Neurogastroenterol. Motil. 2005; 17: 8366-8375

Lefebvre RA, Smits GJM, Timmermans J-P. Study of NO and VIP as non-adrenergic and non-cholinergic neurotransmitters in the pig gastric fundus. Br. J. Pharmacol. 1995; 116: 2017-2026

Mandrek K, Milenov K. Responses of porcine gastric and duodenal smooth muscle to VIP. J. Autonom. Pharmacol. 1991; 11: 353-364

Maurice DH, Palmer D, Tilley DG, Dunkerley HA, Netherton SJ, Raymond DR, Elbatarny HS, Jimmo SL. Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. Mol. Pharmacol. 2003; 64: 533-546

McLean PG, Coupar IA. Further investigation into the signal transduction mechanism of 5-HT_4-like receptor in the circular smooth muscle of human colon. Br. J. Pharmacol. 1996; 118: 1058-1064

McLean PG, Coupar IA, Molenaar P. A comparative study of functional 5-HT_4 receptors in human colon, rat oesophagus and rat ileum. Br. J. Pharmacol. 1995; 115: 47-56

Menniti FS, Faraci WS, Schmidt CJ. Phosphodiesterases in the CNS: targets for drug development. Nat. Rev. Drug Discov. 2006; 5: 660-670

Miller ER, Ullrey DE. The pig as a model for human nutrition. Ann. Rev. Nutr. 1987; 7: 361-382

Park H, Clark E, Conklin JL. Effects of phosphodiesterase inhibitors on oesophageal neuromuscular functions. Neurogastroenterol. Motil. 2003; 15: 625-633

Prins NH, Akkermans LM, Lefebvre RA, Schuurkes JA. 5-HT(4) receptors on cholinergic nerves involved in contractility of canine and human large intestinal longitudinal muscle. Br. J. Pharmacol. 2000a; 131: 927-932

Prins NH, Akkermans LM, Lefebvre RA, Schuurkes JA. Characterization of the receptors involved in the 5-HT-induced excitation of canine antral longitudinal muscle. Br. J. Pharmacol. 2001a; 134: 1351-1359

Prins NH, Shankley NP, Welsh NJ, Briejer MR, Lefebvre RA, Akkermans LM, Schuurkes JAJ. An improved bioassay for the study of 5-HT(4) receptors in the human isolated large intestinal circular muscle. Br. J. Pharmacol. 2000b; 129: 1601-1608

Prins NH, van der Grijn A, Lefebvre RA, Akkermans LMA, Schuurkes JAJ. 5-HT_4 receptors mediating enhancement of contractility in canine stomach; an in vitro and in vivo study. Br. J. Pharmacol. 2001b; 132: 1941-1947

Ren J, Zhou X, Galligan JJ. 5-HT_4 receptor activation facilitates recovery from synaptic rundown and increases transmitter release from single varicosities of myenteric neurons. Am. J. Physiol. 2008; 294: G1376-G1383.

Reneerkens OAH, Rutten K, Steinbusch HWM, Blokland A. Selective phsophodiesterase inhibitors: a promising target for cognition enhancement. Psychopharmacol. 2009; 202: 419-443

Rivet-Bastide M, Vandecasteele G, Hatem S, Verde I, Bénardeau A, Mercadier J-J, Fischmeister R. cGMP-stimulated cyclic nucleotide phosphodiesterase regulates the basal calcium current in human atrial myocytes. J. Clin. Invest. 1997; 99: 2710-2718

Sarna SK. Gastrointestinal longitudinal muscle contractions. Am. J. Physiol. 1993; 265: G156-G164

Schmidt DT, Watson N, Dent G, Rühlman E, Branscheid D, Magnussen H, Rabe KF. The effect of selective and non-selective phosphodiesterase inhibitors on allergen- and leukotriene C4-induced contractions in passively sensitized human airways. Br. J. Pharmacol. 2000; 131: 1607-1618

Stephenson DT, Coskran TM, Wilhelms MB, Adamowicz WO, O'Donnell MM, Muravnick KB, Menniti FS, Kleiman RJ, Morton D. Immunohistochemical localization of phosphodiesterase 2A in multiple mammalian species. J. Histochem. Cytochem. 2009; 57: 933-949

Tack J, Van Outryve M, Beyens G, Kerstens R, Vandeplassche L. Prucalopride (Resolor) in the treatment of severe chronic constipation in patients dissatisfied with laxatives. Gut 2009; 58: 357-365.

Takada K, Sakurai-Yamashita Y, Yamashita K., Kaibara M, Hamada Y, Nakane Y, Hioki K, Taniyama K. Regional difference in correlation of 5-HT_4 receptor distribution with cholinergic transmission in the guinea pig stomach. Eur. J. Pharmacol. 1999; 374: 489-494

Tomkinson A, Raeburn D. The effect of isoenzyme-selective PDE inhibitors on metacholine-induced contraction of guinea-pig and rat ileum. Br. J. Pharmacol. 1996; 118: 2131-2139

Torphy TJ, Zhou H-L, Burman M, Huang LBF. Role of cyclic nucleotide phosphodiesterase isozymes in intact canine trachealis. Mol. Pharmacol. 1991; 39: 376-384

Wang P, Myers JC, Wu P. Cheewatrakoolpong, B., Egan, R.W., Billah, M.M.,. Expression, purification, and characterization of human cAMP-specific phosphodiesterase (PDE4) subtypes A, B, C, and D. Bioch. Biophys. Res. Comm. 1997; 234: 320-324

Wang S-J. An investigation into the effect of type IV phosphodiesterase inhibitor rolipram in the modulation of glutamate release from rat prefrontocortical nerve terminals. Synapse 2006; 59: 41-50

Yau WM, Dorsett J, Youther ML. Stimulation of acetylcholine release from myenteric neurons of guinea pig small intestine by forskolin and cyclic AMP. J. Pharmacol. Exp. Ther. 1987; 243: 507-510

Zhang X-Y, Robinson NE, Zhu F-X. Potentiation of acetylcholine release from tracheal parasympathetic nerves by cAMP. Am. J. Physiol. 1996; 270: L541-L546

Chapter 4

INVESTIGATION OF NEUROGENIC EXCITATORY AND INHIBITORY MOTOR RESPONSES AND THEIR CONTROL BY 5-HT$_4$ RECEPTORS IN CIRCULAR SMOOTH MUSCLE OF PIG DESCENDING COLON

Evelien K.V. Priem and Romain A. Lefebvre

Heymans Institute of Pharmacology, Ghent University, Gent, Belgium

Based on
European Journal of Pharmacology 2011; 667: 365-374

Chapter 4 Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT$_4$ receptors in circular smooth muscle of pig descending colon

4.I. Abstract

The aim of this study was to investigate whether the pig colon descendens might be a good model for the responses mediated via the different locations of human colonic 5-HT$_4$ receptors. The intrinsic excitatory and inhibitory motor neurotransmission in pig colon descendens was therefore first characterized. In circular smooth muscle strips, electrical field stimulation (EFS) at basal tone induced only in the combined presence of the NO synthase inhibitor N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) and the SK channel blocker apamin voltage-dependent on-contractions. These on-contractions were largely reduced by the neuronal conductance blocker tetrodotoxin (TTX) and by the muscarinic receptor antagonist atropine, illustrating activation of cholinergic neurons. The 5-HT$_4$ receptor agonist, prucalopride facilitated submaximal EFS-evoked cholinergic contractions and this effect was prevented by the 5-HT$_4$ receptor antagonist GR113808, supporting the presence of facilitating 5-HT$_4$ receptors on the cholinergic nerve endings innervating circular muscle in pig colon descendens.

Relaxations were induced by EFS in strips pre-contracted with substance P in the presence of atropine. The responses at lower stimulation voltages were abolished by TTX. L-NAME or apamin alone did not influence or only moderately reduced the relaxations, but L-NAME plus apamin abolished the relaxations at lower stimulation voltages, suggesting that NO and ATP act as inhibitory neurotransmitters in a redundant way. Prucalopride did not influence the EFS-induced relaxations at lower stimulation voltage, nor did it per se relax contracted circular muscle strips. No evidence for relaxing 5-HT$_4$ receptors, either on inhibitory neurons or on the muscle cells was thus obtained in pig colon descendens circular muscle.

4.II. Introduction

As in other parts of the gastrointestinal tract, the autonomic nervous control of human colon muscle is mediated through both excitatory and inhibitory motor neurons; the most important excitatory neurotransmitter is acetylcholine but also tachykinins are released (Stanton et al., 2003). For the inhibitory control, nitric oxide but also a purinergic neurotransmitter is involved (Boeckxstaens et al., 1993b; Gallego et al., 2006); no evidence for a contribution of vasoactive intestinal peptide (VIP) was obtained as the VIP receptor antagonist VIP6-28 and VIP desensitization did not influence nonadrenergic noncholinergic (NANC) relaxation in human colon (Mitolo-Chieppa et al., 1998; Benko et al., 2007). Recently, the selective 5-HT$_4$ receptor agonist prucalopride received European market authorization for treatment of chronic constipation in women, in whom laxatives fail to provide adequate relief (Manabe et al., 2010). Its principal site of action for accelerating colonic transit is stimulation of 5-HT$_4$ receptors on cholinergic nerves innervating circular smooth muscle enhancing acetylcholine release and contractility (Leclere et al., 2005). This mechanism is also present at the level of the human stomach (Leclere and Lefebvre, 2002). At the level of the human colon, 5-HT$_4$ receptors were reported to be also present on circular smooth muscle cells, inducing relaxation (McLean et al., 1995) and on nitrergic neurons, enhancing nitric oxide release and thus inhibiting contractility (Cellek et al., 2006). Coordinated activation of these receptors might contribute to the colonic peristaltic effect of prucalopride but this needs further investigation. In human jejunum as in rat, guinea-pig and mouse colon, 5-HT$_4$ receptors have been suggested to be present on the mucosal endings of intrinsic primary afferent neurons (IPANs; Grider et al., 1998; Grider, 2003). As far as we know, this mechanism has not experimentally been shown in human colon. The afferent location of 5-HT$_4$ receptors is under debate because there is also experimental evidence that the 5-HT$_4$ receptors on the intrinsic primary afferent neurons are not located at the mucosal endings but at the myenteric terminals, leading to increased release of calcitonin gene related peptide (CGRP) and acetylcholine (Liu et al., 2005; Gershon, 2005).

The pig is an omnivorous mammalian species with gastrointestinal morphology and physiology which shows similarity to that of humans (Miller and Ullrey, 1987). We have previously shown that the pig is a good model for the investigation of excitatory 5-HT$_4$ receptors on cholinergic neurons to gastric muscle (De Maeyer et al., 2006). Whether the pig might also be a good model for the different locations of human colonic 5-HT$_4$ receptors influencing motility and thus for the study of the potentially coordinated action of agonists at these receptors has not yet been investigated. In segments of descending colon in anesthetized pigs, it has been shown that nitrergic and cholinergic responses can be induced by electrical stimulation (Sevcencu et al.,

2005) but a careful *in vitro* investigation of contractile and relaxant responses induced by electrical field stimulation of motor neurons in pig descending colon is not available.

The primary aim of this study was therefore to assess the effect of electrical stimulation of intrinsic excitatory and inhibitory motor neurons in pig descending colon circular muscle. We found that pure cholinergic contractile responses can be obtained but that the combination of an NO synthesis inhibitor with the SK channel blocker apamin is required to inhibit NANC inhibitory responses. The secondary aim was to investigate the influence of the 5-HT_4 receptor agonist prucalopride on these electrically induced cholinergic contractions and NANC relaxations as well as its direct effect on circular smooth muscle.

4.III. Methods

4.III.1. Animals

Young male pigs (10-12 weeks, 15-25 kg – breed Line 36) were purchased from Rattlerow Seghers, Belgium. All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

4.III.2. Tissue preparation and tension recording

The pigs were anaesthetized with an intramuscular injection of 5 ml Zoletil 100 (100 mg/ml) (Virbac Belgium S.A., Belgium). After exsanguination, the entire colon descendens was removed, from 10 cm above the anus to the transverse colon and after the contents were rinsed out, the segment was placed in Krebs-Henseleit solution (composition in mM: glucose 11.1, $NaHCO_3$ 25, $KHPO_4$ 1.18, $CaCl_2$ 2.51, $MgSO_4$ 1.18, KCl 4.69, NaCl 118) which had been aerated with 5% CO_2/95% O_2. The colon descendens was opened along the mesenteric border and pinned with the mucosa side up. The mucosa was removed by sharp dissection and 8 full-thickness muscle strips (approx. 3 × 20 mm) were prepared along the circular axis, in pairs at the same level, starting 2 cm above the distal end. All strips were studied on the day of preparation. Strips were mounted in 5 ml organ baths between 2 platinum plate electrodes under a load of 2 g, to allow electrical field stimulation (EFS) performed by means of a Hugo Sachs D7806 stimulator (Hugo Sachs Elektronik-Harvard apparatus GmbH, Germany), a Grass S88 stimulator (Grass Technologies, U.S.A.) or a 4 channel custom-made stimulator. The organ baths contained aerated (5% CO_2 and 95% O_2) Krebs-Henseleit solution and temperature was maintained at 37 °C.

Changes in isometric tension were measured using MLT 050/D force transducers (ADInstruments, United Kingdom) and recorded on a PowerLab/8sp data recording system (ADInstruments, United Kingdom) with Chart v5.5.6 software.

4.III.3. Protocols

4.III.3.1. Characterization of EFS-induced contractions

The Krebs-Henseleit solution systematically contained guanethidine (4 µM) to avoid noradrenergic responses. After 60 min of stabilization with refreshing of the Krebs-Henseleit solution every 15 min, strips were contracted with the muscarinic receptor agonist carbachol (3 µM) to test their viability and responsiveness. This procedure was repeated with 20-min washout periods in between until the response was stable (maximally 4 expositions). Preliminary experiments learned that EFS of the tissues with 10 s trains only resulted in off-contractions, even when the NO synthase inhibitor N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; 0.3 mM) was added.

A first series of experiments was therefore performed as follows. Strips were stimulated with 10 s trains (0.25 ms pulse duration; frequency of 4 Hz; increasing voltage 10-50 V) at 3 min interval and these voltage-response curves were repeated in the presence of L-NAME (0.3 mM), the small conductance calcium-dependent potassium channel blocker apamin (0.5 µM) or L-NAME plus apamin. These experiments revealed the occurrence of clearcut on-contractions during EFS in the latter condition. Further experiments were therefore performed in the continuous presence of guanethidine (4 µM), L-NAME (0.3 mM) and apamin (0.5 µM).

In the second series, strips were stimulated with 10 s trains (0.25 ms; 4 Hz; increasing voltage 10-50 V) and the voltage-response curves were repeated under different conditions: in the presence of the neuronal conductance blocker tetrodotoxin (TTX; 3 µM), atropine (1 µM) or a combination of the tachykinin receptor antagonists (NK_1, 10 µM FK888; NK_2,1 µM MEN10627; NK_3, 0.3 µM SB222200). Parallel control strips were monitored in the absence of these antagonists.

4.III.3.2. Effect of prucalopride and 5-HT on EFS-induced cholinergic contractions

Muscle strips were studied in the continuous presence of guanethidine (4 µM), L-NAME (0.3 mM) and apamin (0.5 µM). Thirty minutes after the last administration of carbachol, the muscle strips were electrically stimulated at 35 V, 4 Hz, 0.25 ms in trains of 10 s at intervals of 3 min. The experiments under 2.3.1. had indeed shown that a maximal contractile response at 4 Hz was obtained from a stimulation voltage of 30 V onwards (see Results); therefore a voltage of 35 V was now used. Once the electrically induced contractions became reproducible, the voltage was adjusted to reduce the contraction force to approximately 50% (V50%) of the force evoked at 35 V and EFS was repeated until 5 reproducible contractions were obtained; prucalopride (0.3 µM) was then added and EFS was continued.

This protocol, but with an increased interval between stimulation trains of 5 min, was also performed in Krebs-Henseleit solution to which the tachykinin receptor antagonists (10 µM FK888, 1 µM MEN10627, 0.3 µM SB222200) or the prostaglandin synthesis inhibitor indomethacin (1 µM) or the combination of tachykinin receptor antagonists with indomethacin were added. In a new series, in the continuous presence of the tachykinin receptor antagonists, upon reaching a stable on-contraction at V50%, the EFS-induced response at V50% was obtained at least 5 times. GR113808 (0.3 µM) was then added after 1 h followed by addition of 1 µM prucalopride, or vice versa, and EFS-induced responses were continuously monitored till 1 h after the last addition. In parallel control tissues no GR113808 or prucalopride was added. The influence of 5-HT (1 µM) was tested in the same way. To avoid effects at other than 5-HT$_4$ receptors, methysergide, a 5-HT$_1$, 5-HT$_2$, 5-ht$_5$, 5-HT$_6$ and 5-HT$_7$ receptor antagonist (1 µM) and granisetron, a 5-HT$_3$ receptor antagonist (3 µM) were added in these experiments after the stabilization with carbachol.

4.III.3.3. Characterization of EFS-induced relaxations

To allow registration of relaxant responses, tissues had to be contracted. Several contractile agents were tested, but none was able to induce a sustained contraction. Also with substance P (SP), no sustained contraction could be obtained, but preliminary experiments showed that reproducible responses could be repetitively induced. The isolated concentration-response curve of substance P was evaluated, by exposing the tissues to increasing concentrations of substance P (0.001; 0.01; 0.1; 1; 3 µM) at 30 min interval. The contractions obtained were: 0.001 µM, 0.60; 0.01 µM, 1.83; 0.1 µM, 4.42; 1 µM, 6.52; 3 µM, 8.03 g/mm² (n=8).

Substance P (0.1 µM) was selected for further experiments to induce a degree of contraction that was certainly not maximal and could be functionally antagonized by relaxant neurotransmitters.

The Krebs-Henseleit solution was supplemented with guanethidine (4 µM) and atropine (1 µM). After a 60-min period of stabilization with refreshing of the solution every 15 min, the strips were contracted with substance P (0.1 µM) to test their viability and responsiveness. This procedure was repeated until two similar responses to substance P were obtained (maximum 4 expositions), with a 20-min washout period in between. Substance P (0.1 µM) was then added a further 10 times, at 40 min interval. One minute after its addition, one 10 s train of EFS (0.25 ms; 4 Hz) was applied at 50, 20, 15, 10 and 5 V during 5 consecutive substance P administrations. This was then repeated in the presence of L-NAME (0.3 mM), apamin (0.5 µM), L-NAME plus apamin, TTX (3 µM), the N-type Ca^{2+} channel blocker ω-conotoxin (1 µM), the $P2Y_1$ receptor antagonist MRS2179 (10 µM) or L-NAME plus MRS2179.

4.III.3.4. Effect of prucalopride on EFS-induced NANC relaxations

Experiments were performed in Krebs-Henseleit solution containing atropine (1 µM) and guanethidine (4 µM) and after a stabilization period as described in section 2.3.3., muscle strips were pre-contracted with substance P (0.1 µM) and EFS (0.25 ms; 4 Hz; 10 s; 50 V) was delivered on top of the substance P-induced contraction at a voltage of 50 V. During consecutive substance P-induced contractions, the voltage was adjusted to reduce the relaxation response to approximately 50 % of the response evoked at 50 V; this selected V50% was studied an additional 2 times. Prucalopride (1 µM) was then incubated for 30 min and the relaxant response to EFS at V50% was studied twice in its presence. The effect of prucalopride was also studied in Krebs-Henseleit solution containing L-NAME (0.3 mM), apamin (0.5 µM) or L-NAME plus apamin.

4.III.3.5. Influence of prucalopride and 5-HT on contracted tissue

The possible intrinsic relaxing effect of prucalopride and 5-HT was evaluated by administering prucalopride (1 µM) or 5-HT (1 µM) on top of substance P-induced contraction. Muscle strips used for observing the effect of 5-HT, were pre-incubated with methysergide (1 µM) and granisetron (3 µM) for 30 min. 5-HT was also evaluated on potassium chloride (40mM) pre-contracted muscle strips.

4.III.4. Drugs

Guanethidine sulphate, atropine sulphate, N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), carbachol, serotonin creatinine sulphate monohydrate (5-HT) and indomethacin were obtained from Sigma (Sigma, Belgium), apamin, ω-conotoxin GVIA and tetrodotoxin (TTX) from Alomone labs (Alomone labs, Israel), MEN10627 [(4R)-4-Hydroxy-1-[(1-methyl-1H-indol-3-yl)carbonyl]-L-prolyl-N-methyl-3-(2-naphthalenyl)-N-(phenylmethyl)-L-al aninamide], FK888, [cyclo(Dap-Leu-Met-Asp-Trp-Phe)(4β-1β lactam)], SB222200 [3-Methyl-2-phenyl-N-[(1S)-1-phenylpropyl]-4-quinolinecacarboxamide], GR113808 [1-methyl-1H-indole-3-carboxylicacid,[1-[2-[(methylsulfonyl)amino]ethyl]-4-piperidinyl] methy-l-ester], MRS2179 [2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt], substance P, granisetron hydrochloride, methysergide maleate from Tocris (Tocris Cookson, U.K.), prucalopride succinate (gift of Movetis, Belgium), potassium chloride (KCl) (Merck Belgolabo N.V., Belgium).

All compounds were dissolved and diluted in distilled water, except for GR113808, indomethacin, Substance P, FK888, MEN10627 and SB222200. GR113808, substance P and SB222200 were dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution and dilutions were made with distilled water. FK888 and MEN10627 were dissolved in DMSO. Indomethacin was dissolved and diluted in DMSO. The final concentration of DMSO in the organ bath medium was always < 0.1%. The stock solutions were stored at -20 °C.

4.III.5. Data analysis

Peak contractions versus baseline upon addition of carbachol, substance P and during (on-contraction) and after (off-contraction) EFS were measured. The contractions were expressed as gram per cross sectional area (g/mm²). Cross section area is weight per length x density. The tissue length was measured at the end of the equilibration period, the wet weight was measured at the end of the experiment after blotting the tissue; the density was assumed to be 1.05 mg/mm³ (Gordon and Siegman, 1971).

Contractile responses to EFS in the presence of prucalopride, 5-HT or GR113808 were determined as the mean of the response to 5 successive stimulations, starting 30 min after their addition (stimulation 6) and expressed as % of the mean of the response to 5 successive stimulations before their addition.

EFS-induced relaxations were expressed as % of the substance P-induced contraction. The influence of the agents per se on the substance P-induced contractions was assessed by expressing the mean of the 5 contractions evoked by substance P after the addition as % of the mean of the 5 contractions evoked by substance P before the addition of the involved agent. Relaxant responses to EFS in the presence of prucalopride were determined as the mean of the 2 relaxant responses obtained after the addition of prucalopride and expressed as % of the mean of the 2 relaxant responses obtained before its addition.

4.III.6. Statistics

All results are expressed as means ± S.E.M. *n* refers to tissues obtained from different animals except for one occasion which is indicated. Statistical analysis of responses within the same tissues, were performed by a paired *t*-test. When more than 2 sets of results within the same tissues had to be compared, repeated measures ANOVA followed by a Bonferroni corrected *t*-test was applied (GraphPad Prism v5.01, San Diego, CA, USA). $P < 0.05$ was considered to be statistically significant.

4.IV. Results

4.IV.1. Characterization of EFS-induced contractions

In circular muscle strips of pig descending colon, EFS (10-50 V) at basal tone evoked only off-contractions, after ending the stimulation train of 10 s (**Fig. 4.1A**). To investigate whether this might be due to functional antagonism by the simultaneously released neurotransmitters NO and ATP, experiments were performed in the presence of L-NAME or apamin and in the combined presence of both. In the presence of L-NAME (**Fig. 4.1B**) or apamin (**Fig. 4.1C**), little or no response was obtained during EFS, but the off-contractions were enhanced; this enhancement was more pronounced with L-NAME than with apamin. In the presence of L-NAME plus apamin (**Fig. 4.1D**), clear on-contractions were evoked during the stimulation train. Upon ending stimulation, the contractions still minimally increased and tissues then started to relax.

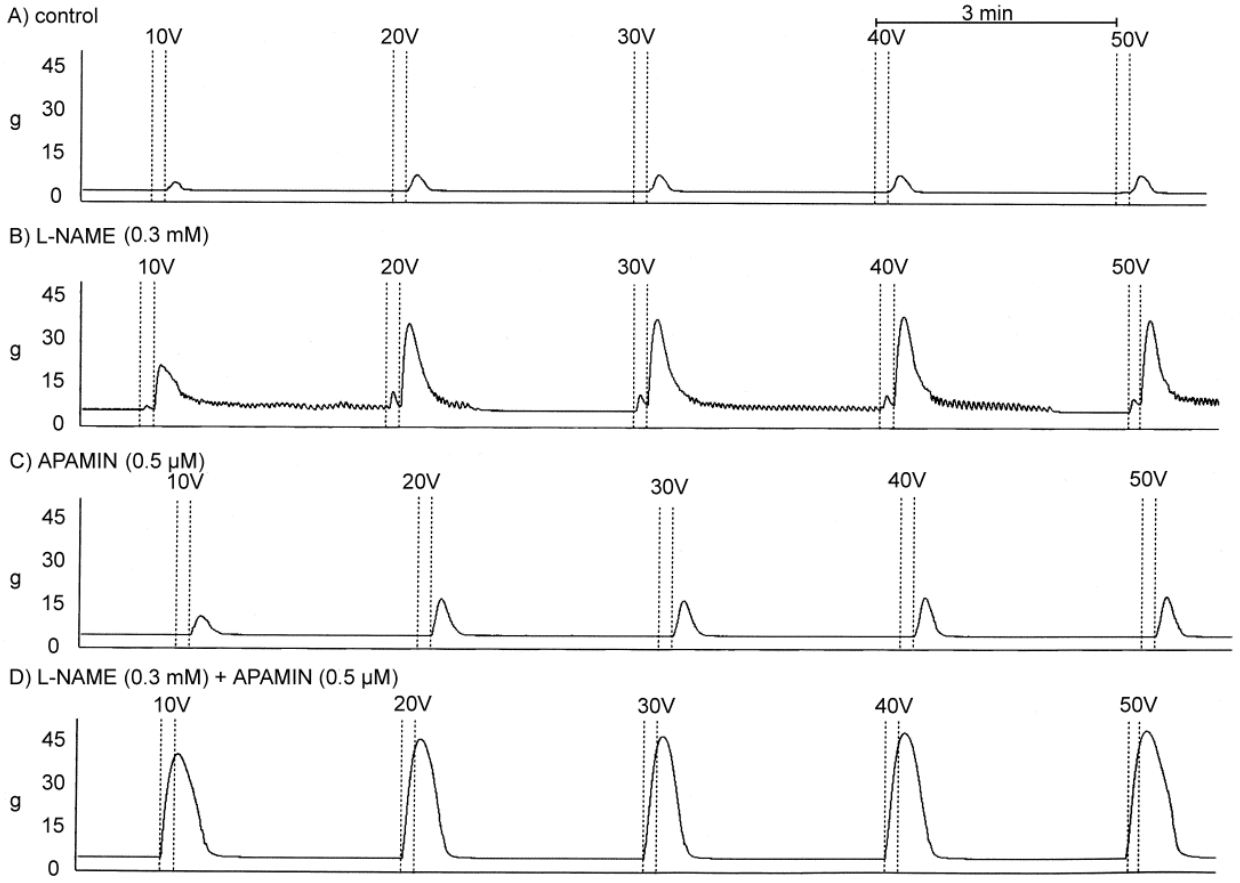


Figure 4.1

Representative traces showing contractile responses to EFS (10 s trains at 4 Hz; 0.25 ms; 10-50 V) at basal tone in (A) Krebs-Henseleit solution with 4 µM guanethidine (control) and in the additional presence of (B) 0.3 mM L-NAME, (C) 0.5 µM apamin or (D) L-NAME plus apamin. The dotted lines indicate the start and end of the 10 s stimulation train.

To further investigate EFS-induced contractions, they were from then on studied in the continuous presence of 0.3 mM L-NAME and 0.5 µM apamin; the contractions were measured at the end of the 10 s trains (on-contractions) of EFS. In these conditions, EFS (10 s trains at 4 Hz, 0.25 ms and 10-50 V) yielded voltage-dependent responses, the maximal response already being reached at a voltage of 30 V. In control tissues, these responses were reproducible. Atropine (1 µM; **Fig. 4.2, 4.3B**) and TTX (3 µM; **Fig. 4.3C**) largely reduced the EFS-induced responses illustrating that they are mainly neurogenic and cholinergic. Correspondingly, a combination of tachykinin NK$_1$, NK$_2$ and NK$_3$ receptor antagonists (10 µM FK888; 1 µM MEN10627; 0.3 µM SB222200; **Fig. 4.3D**) did not influence the EFS-induced contractions, neither during nor after the stimulation train.

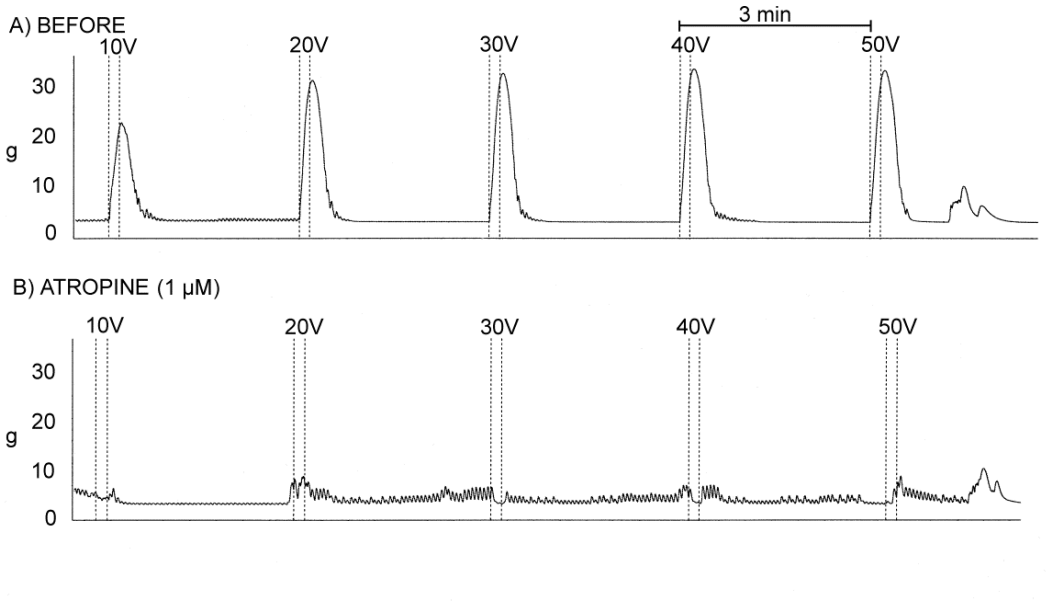


Figure 4.2

Actual traces showing contractile responses to EFS at basal tone (10 s trains at 4 Hz; 0.25 ms; 10-50 V) (A) before and (B) in the presence of atropine (1 µM). Experiment was performed in the continuous presence of 4 µM guanethidine, 0.3 mM L-NAME and 0.5 µM apamin.

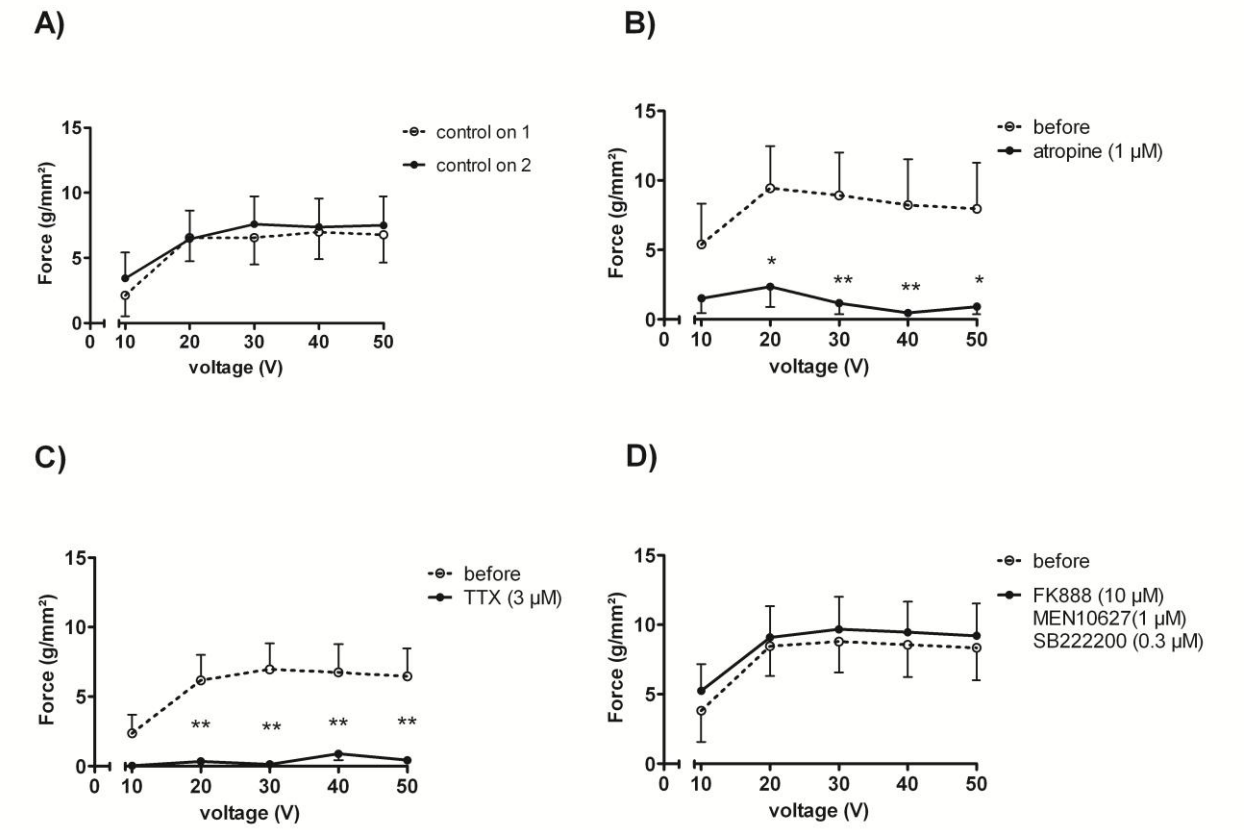


Figure 4.3

Mean ± S.E.M. (n=6) of EFS-induced on-contractions (10 s trains at 4 Hz; 0.25 ms; 10-50 V) in control strips (A) where the voltage-response curve was obtained twice (control 1 and 2) and before and in the presence of (B) atropine (1 µM), (C) tetrodotoxin (TTX, 3 µM) or (D) the tachykinin receptor antagonists FK888 (10 µM), MEN10627 (1 µM) and SB222200 (0.3 µM). Experiments were performed in the continuous presence of 4 µM guanethidine, 0.3 mM L-NAME and 0.5 µM apamin.

* $P < 0.05$; ** $P < 0.01$: significant difference versus before (paired t-test)

4.IV.2. Effect of prucalopride and 5-HT on EFS-induced cholinergic contractions

In the first series, prucalopride (0.3 µM) was tested on half maximal contractions induced by 10 s trains of EFS at 3 min interval in Krebs-Henseleit solution containing guanethidine, L-NAME and apamin. The concentration of prucalopride was selected as it had been used before in similar experiments to investigate the facilitatory influence of prucalopride on cholinergic neurotransmission in gastrointestinal smooth muscle preparations (Prins et al., 2000a, 2001); when a concentration-response curve for the facilitating effect in pig gastric longitudinal muscle was tested, the maximal effect was reached at 0.3 µM (De Maeyer et al., 2006).

Prucalopride tended to increase the electrically induced contractions but this could not systematically be shown as the basal tone of the strips became unstable and responses to EFS became variable. The interval between the stimulation trains was therefore increased to 5 min and the effect of prucalopride (0.3 µM) was tested on half maximal contractions in Krebs-Henseleit solution containing either indomethacin, or a combination of tachykinin 1, 2 or 3 receptor antagonists, or the combination of tachykinin receptor antagonists and indomethacin. In the presence of the tachykinin receptor antagonists, the stability of the electrically induced responses improved and prucalopride enhanced the contractions in 5 out of 6 strips, yielding a mean enhancement of 24 ± 3%. The condition with the tachykinin antagonists was therefore selected for the further series, but the concentration of prucalopride was increased to 1 µM in view of the moderate effect with 0.3 µM.

In the continuous presence of 4 µM guanethidine, 0.3 mM L-NAME, 0.5 µM apamin, 10 µM FK888, 1 µM MEN10627 and 0.3 µM SB222200, EFS at V50% in general induced stable contractions; tissues showing more than 30% difference in contraction amplitude were discarded (3 out of 12 control tissues). In the control tissues used, the contractile response by EFS at supramaximal voltage (35 V) was 8.7 ± 1.2 g/mm² (n=9, from 5 animals). Upon reducing the voltage to V50% the contractile response was decreased to 4.7 ±1.0 g/mm² (n=9, from 5 animals).

The response to EFS at V50% remained stable over the time course followed. The mean of the 5 contractions starting 30 min and 90 min after the basal series of5 contractions was 103 ± 9 % and 97 ± 13 % respectively (n=9, from 5 animals).

Prucalopride Prucalopride (1 µM) significantly enhanced the contractions induced by EFS at V50% (**Fig. 4.4A; 4.5A**); the maximal increase was reached at last after 30 min of incubation (6th contraction in the presence of prucalopride) and the effect remained stable from then on; the mean amplitude of contraction 6 to 10 in the presence of prucalopride was 127 ± 7 % (n=9, from 6 animals) of the mean response before. When 0.3 µM GR113808 was added 1 hour after incubation of prucalopride, the response decreased to 117 ± 9 %; this was not significantly different from the response in the presence of prucalopride or from that before adding prucalopride (**Fig. 4.5A**). When GR113808 (0.3 µM) was incubated 60 min before prucalopride (n=5, from 5 animals), it prevented the facilitating effect of prucalopride (**Fig. 4.4B, 4.5C**). GR113808 per se did not influence the EFS-induced contractions (**Fig. 4.5C**).

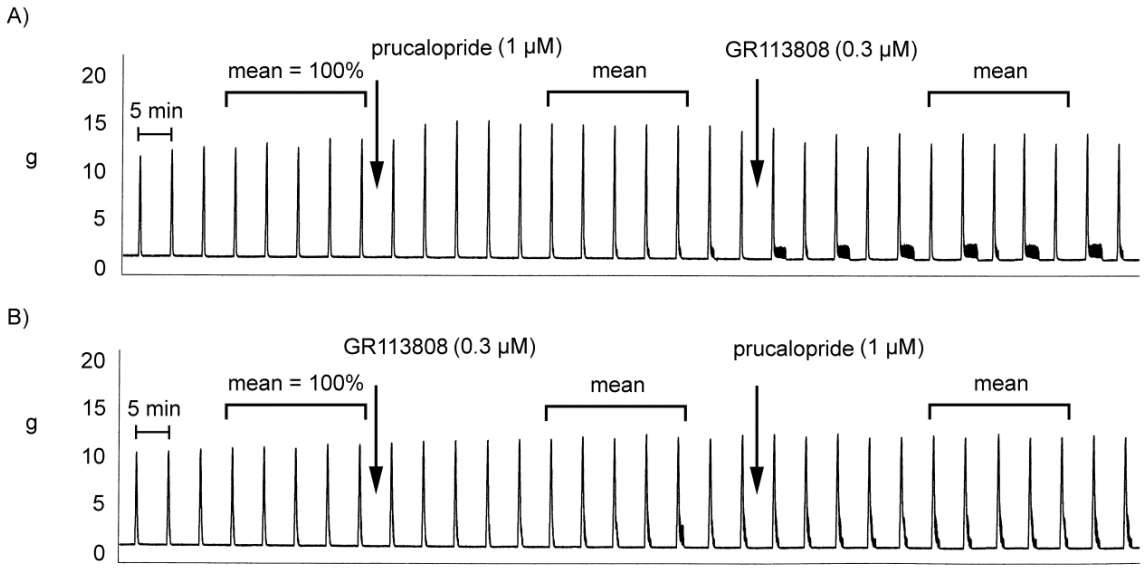


Figure 4.4

Actual traces showing contractile responses to EFS at basal tone (10 s trains at 4 Hz; 0.25 ms; V50%) in (A) a strip treated with prucalopride (1 µM) and after one hour with GR113808 (0.3 µM) and (B) a strip treated with GR113808 (0.3 µM) and after one hour with prucalopride (1 µM). Experiments performed in the continuous presence of 4 µM guanethidine, 0.3 mM L-NAME, 0.5 µM apamin, 10 µM FK888, 1 µM MEN10627 and 0.3 µM SB222200. Mean indicates the contractions that were measured to determine the mean response in the presence of prucalopride or GR1138708; this mean response was expressed as % of mean = 100% before their addition (mean results shown in fig. 5).

5HT In the presence of methysergide (1 µM) and granisetron (3 µM), 5-HT (1µM) enhanced the EFS-induced responses at V50%; the maximal increase was reached after 30 min of incubation with 5-HT and contractions attained 145 ± 14 % (n=8, from 5 animals) of those before addition of 5-HT (**Fig. 4.5B**). When GR113808 (0.3 µM) was added 60 min after 5-HT, the EFS-induced contractions decreased to 132 ± 15 % (n=5, from 5 animals), which was not significantly different from the responses in the presence of 5-HT nor those before adding 5-HT. When added before 5-HT, GR113808 prevented the facilitating effect of 5-HT (**Fig. 4.5D**).

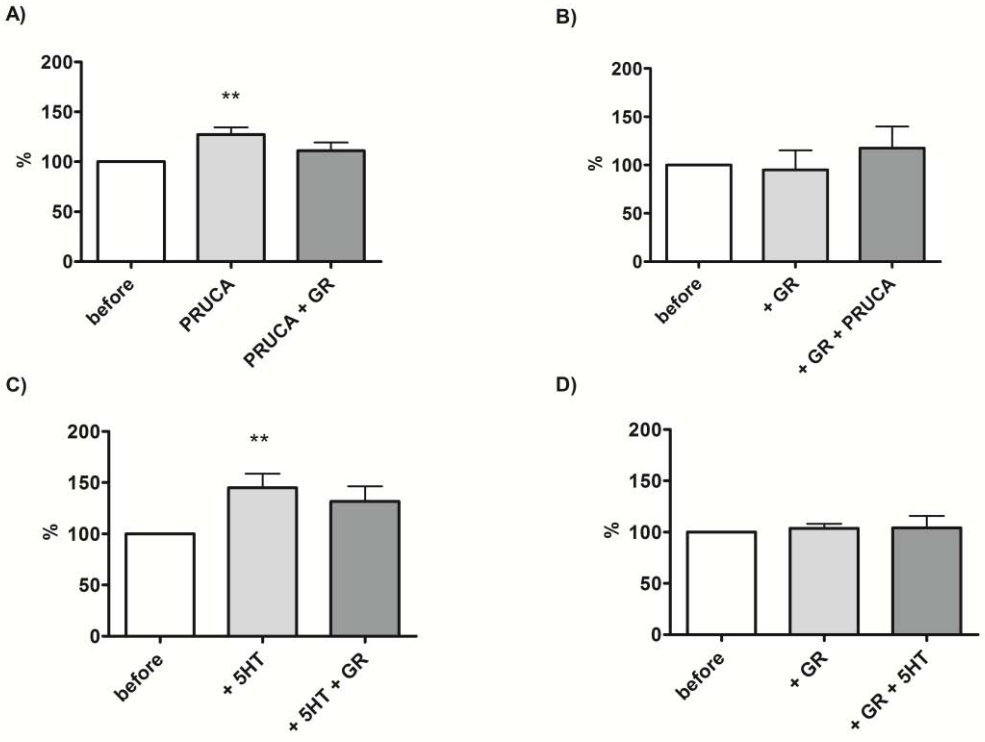


Figure 4.5

Mean ± S.E.M. of EFS-induced on-contractions (10 s trains at 4 Hz; 0.25 ms; V50%) before and (A) after adding prucalopride (PRUCA, 1 µM) and then GR113808 (GR, 0.3 µM) (n=9, from 6 animals); (B) after adding GR113808 (0.3 µM) and then prucalopride (1 µM) (n=5, from 5 animals); (C) after adding 5-HT (1 µM) and then GR113808 (0.3 µM) (n=8, from 5 animals); (D) after adding GR113808 (0.3 µM) and then 5-HT (1 µM) (n=5, from 5 animals). Responses are expressed as % of the on-contractions obtained before the addition of prucalopride (A), 5-HT (C) or GR113808 (B,D). Experiments were performed in the continuous presence of 4 µM guanethidine, 0.3 mM L-NAME, 0.5 µM apamin, FK888 (10 µM), MEN10627 (1 µM), SB222200 (0.3 µM). When 5-HT was studied (C, D), also methysergide (1 µM) and granisetron (3 µM) were continuously present.

** $P < 0.01$: significant difference versus before (repeated measures ANOVA followed by a Bonferroni corrected *t*-test)

4.IV.2. Characterization of EFS-induced relaxations

To investigate relaxant responses to EFS (10 s trains; 4 Hz; 0.25 ms; 50-5 V) the tone was increased with 0.1 µM substance P and EFS, at different stimulation voltages, was delivered on top of these substance P-induced contractions. These experiments were studied in the continuous presence of 4 µM guanethidine and 1 µM atropine, to block noradrenergic and cholinergic influences. In control tissues EFS induced clear-cut relaxations reaching a maximum amplitude already at EFS with 15 V (**Fig. 4.6A**); these responses were reproducible (**Fig. 4.7A**).

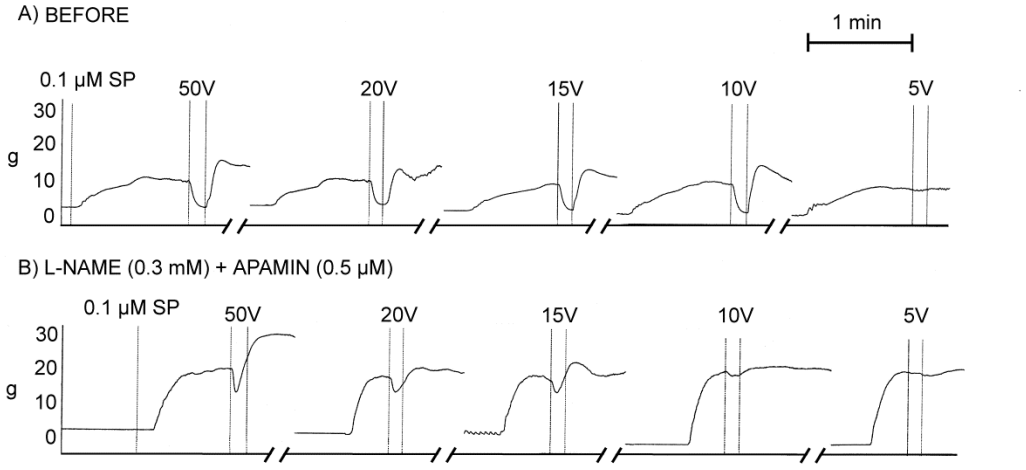


Figure 4.6

Representative traces showing relaxant responses to EFS (10 s trains at 4 Hz; 0.25 ms; 50-5 V) in tissues repetitively contracted with 0.1 µM substance P (SP) in Krebs-Henseleit solution with 4 µM guanethidine and 1 µM atropine before (A) and in the additional presence of 0.3 mM L-NAME plus 0.5 µM apamin (B). The dotted lines indicate the start and end of the 10 s stimulation train.

L-NAME (0.3 mM) did not influence the EFS-induced relaxations (**Fig. 4.7B**) while apamin (0.5 µM) reduced the relaxant response by EFS at 10 V (**Fig. 4.7C**). No relaxant responses at 5 and 10 V were obtained, in the combined presence of L-NAME plus apamin, while the relaxations at 15 to 50 V were clearly decreased (**Fig. 4.6B, 4.7D**). MRS2179 (10 µM) tended to reduce the relaxant responses with a significant result for the response by EFS at 10 V (**Fig. 4.7E**). Also in the presence of MRS2179 plus L-NAME, only the relaxant responses by EFS at 10 V were significantly reduced (**Fig. 4.7F**). The EFS-induced relaxations at 5 to 15 V were abolished by TTX (3 µM) and reduced at 20 and 50 V (**Fig. 4.7G**). ω-Conotoxin (1 µM) abolished the EFS-induced response at 5 V and reduced the EFS-induced responses at 10 to 20 V (**Fig. 4.7H**).

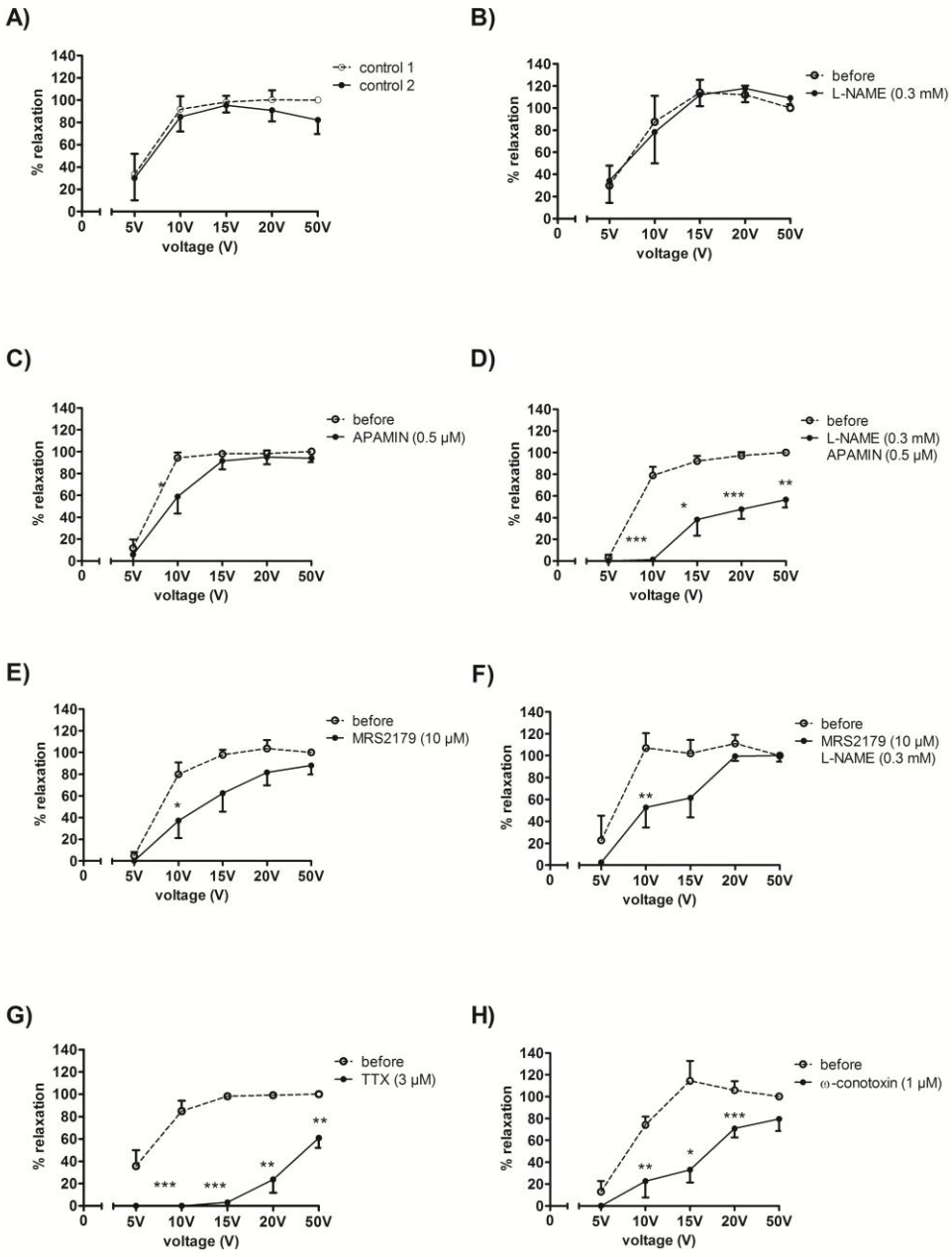


Figure 4.7

Mean ± S.E.M. (n=6) of EFS-induced relaxations (% of SP-induced contraction) (10 s trains at 4 Hz; 0.25 ms; 50-5 V) in control strips (A) where the voltage-response curve was obtained twice (control 1 and 2) and before and in the presence of 0.3 mM L-NAME (B), 0.5 µM apamin (C), 0.3 mM L-NAME plus 0.5 µM apamin (D), 10 µM MRS2179 (E), 10 µM MRS2179 plus 0.3 mM L-NAME (F), 3 µM tetrodotoxin (TTX) (G) and 1 µM ω-conotoxin (H). Experiments were performed in the continuous presence of 4 µM guanethidine and 1 µM atropine. Responses are expressed as % of substance P-induced contraction.

* P < 0.05; ** P < 0.01; *** P < 0.001: significant difference versus before (paired t-test)

In the above described experiments, L-NAME alone or in combination with apamin or MRS2179 significantly increased the amplitude of the substance P-induced contractions. (L-NAME: 170 ± 11 %, n=6, $p < 0.05$; L-NAME plus apamin: 201 ± 15 %, n=6, $p < 0.01$; L-NAME plus MRS2179: 151 ± 16 %, n=6, $p < 0.05$). The other agents did not significantly influence the amplitude of the substance P-induced contractions (apamin: 125 ± 15 %; MRS2179: 77 ± 10 %; TTX: 120 ± 12 %; ω-conotoxin: 84 ± 5 %; n=6 for each compound).

4.IV.3. Effect of prucalopride on EFS-induced NANC relaxations

The voltage required to induce relaxations by EFS with an amplitude of approximately 50 % of that by EFS at 50 V (V50%) varied from 11 to 5 V. This EFS-induced relaxation was not influenced by 1 µM prucalopride. The relaxant response by EFS at V50% in the presence of prucalopride was 102 ± 17 % of that before (n=4). The non-effect of prucalopride was maintained when 0.3 mM L-NAME (109 ± 10 %, n=4) or 0.5 µM apamin (98 ± 12 %, n=4) was present in the Krebs-Henseleit solution.

4.IV.3. Influence of prucalopride and 5-HT on contracted tissue

In the continuous presence of 4 µM guanethidine and 1 µM atropine, the intrinsic relaxant effect of 1 µM prucalopride was evaluated on top of substance P (0.1 µM)-induced contractions; prucalopride did not induce any response (n=4). The relaxant effect of 1 µM 5-HT was evaluated in the presence of 1 µM methysergide and 3 µM granisetron, on top of substance P (0.1 µM)-induced contractions (n=4) and also KCl (40mM)-induced contractions (n=4); 5-HT did not evoke any response (data not shown).

4.V. Discussion

4.V.1. Excitatory and inhibitory neurotransmitters

In the absence of the NO synthesis inhibitor L-NAME and the SK channel blocker apamin, classically used to block the effect of ATP as relaxant neurotransmitter, only off-contractions were obtained upon EFS. These off-contractions were enhanced by apamin and still more by L-NAME but only in the combined presence of L-NAME plus apamin, clear-cut on-contractions were obtained. These on-contractions were abolished by TTX and atropine, and not influenced by a combination of selective tachykinin receptor antagonists used in a concentration described

to antagonize their respective tachykinin receptor (FK888: Maggi et al., 1994b; MEN10627: Maggi et al., 1994a; SB222200: Onori et al., 2001). Although tachykinins have been shown to be present in the enteric nervous system of the porcine gastrointestinal tract (Schmidt et al., 1991), pure cholinergic excitatory neurotransmission was thus obtained in the experimental conditions used. The absence of on-contractions when L-NAME and apamin are not present, points to the release of NO and ATP, functionally antagonizing the concomitantly released acetylcholine. That off-contractions can be seen in the absence of L-NAME and apamin, is probably related to the faster inactivation of NO and ATP than of acetylcholine after ending stimulation. As highly reactive molecule, nitric oxide can be expected to disappear quickly by interaction with other molecules such as oxygen (Brookes, 1993). ATP is fastly degraded by multiple ecto-nucleotidases and soluble ATPases while the hydrolytic elimination of actylcholine from the synaptic cleft is performed by a single acetylcholinesterase (Zimmermann, 2008). Electrophysiological studies in colon circular muscle of different species including man (Hirst et al., 2004; Gallego et al., 2008) showed that stimulation of inhibitory nerves leads to a very fast purinergic inhibitory junction potential and a secondary more sustained nitrergic hyperpolarisation. NO might thus be more active than ATP towards the end and just after the 10 s stimulation trains used in this study. This might explain why L-NAME alone led to a greater enhancement of the off-contractions than apamin alone.

The experiments with EFS-induced relaxations, in the continuous presence of atropine, confirmed the concomitant release of ATP and NO during EFS. Within the experimental conditions used, this illustrates a redundant action of these inhibitory transmitters, as L-NAME had no influence on EFS-induced relaxations, apamin only reduced the response to EFS at 10 V, but L-NAME plus apamin abolished the responses by EFS at 10 V and reduced those at higher stimulation voltages. The involvement of both ATP and NO in inhibitory neurotransmission had also been shown in human colon circular muscle (Keef et al., 1993; Boeckstaens et al., 1993b; Benkó et al., 2007) and the pig colon descendens can thus be considered to be a good model for inhibitory neurotransmission in human colon. That ATP and NO do not act in an additive way but in a redundant way, was also shown in the colon of the rat (Van Crombruggen and Lefebvre, 2004) and man (Gallego et al., 2008). In human colon, the muscular purinergic receptor involved has been suggested to be the $P2Y_1$ receptor (Gallego et al., 2006). The $P2Y_1$ receptor antagonist MRS2179 was used to assess whether $P2Y_1$ receptors are also involved in purinergic transmission in pig colon. MRS2179 is comparatively more potent in humans, pigs or guinea-pigs than in rats and rodents (Gallego et al., 2011). Yet in the human (Gallego et al., 2011) and rat (Grasa et al., 2009) colon, MRS2179 is shown to be less potent

than the newest P2Y$_1$ receptor antagonists MRS2500 and MRS2279. However in the pig small intestine non-nitrergic relaxation is completely blocked by 10 µM MRS2179 (Gallego et al., 2008). 10 µM MRS2179 also reduced the relaxant response by EFS at 10 V in the actual study, similar to what was observed with apamin alone. This might suggest that at this stimulation voltage, ATP has some relaxant effect through P2Y$_1$ receptors, that cannot be taken over by NO. However, the overall inhibitory effect of MRS2179 plus L-NAME at the different stimulation voltages was clearly less pronounced than seen with the combination of apamin plus L-NAME. This suggests that besides P2Y$_1$ receptors, additional P2 receptors are involved in the relaxant effect of the purinergic neurotransmitter in pig colon descendens. Indeed, although purinergic neurotransmission in rat (Grasa et al., 2009) and mouse (Gallego et al., 2012; Hwang et al., 2012) colon was recently reported to also be due to an action at P2Y$_1$ receptors, several other relaxant muscular and neurogenic P2 receptors have been described for rat colon (Van Crombruggen et al., 2007). TTX abolished the EFS-induced relaxant responses to EFS at voltages of 5 to 15 V and reduced them at higher voltages. Whether the TTX-resistant relaxation at higher voltages indicates that high voltage stimulation can lead to action potential propagation breaking through the sodium channel blockade with TTX, or it can lead to transmitter generation and release at the nerve endings not requiring action potential propagation is not clear. As the generation of NO by the constitutive Ca^{2+}/calmodulin dependent nNOS as well as the exocytotic release of ATP from vesicles at the nerve endings is Ca^{2+} dependent (Bodin and Burnstock, 2001), the effect of the N-type calcium channel blocker ω-conotoxin was tested. It reduced but did not abolish the EFS-induced relaxations, as has also been observed in other tissues (Boeckxstaens et al., 1993a; Zygmunt et al., 1993; Van Geldre and Lefebvre, 2004); this points to the involvement of other voltage-operated calcium channels than N-channels in ATP release and NO synthase activation. As L-NAME and apamin did not abolish the relaxant responses by EFS at higher voltages, the contribution of another neurotransmitter than NO and ATP in inhibitory neurotransmission at the level of the pig colon descendens cannot be excluded. In the porcine ascending colon, VIP-immunoreactive nerve fibers towards the circular muscle layer have been described (Barbiers et al., 1995) but an electrophysiological study could not provide evidence for a role of VIP in NANC inhibitory neurotransmission (Jager and van der Schaar, 1988). Also in porcine jejunum, VIP was not involved in inhibitory neurotransmission, but pituitary adenylate cyclase activating peptide (PACAP) was suggested to partly contribute (Matsuda et al., 2004). In pig internal anal sphincter, NO and a purine acting via P2Y$_1$ receptors were found to be the major inhibitory neurotransmitters, with only a minor contribution of VIP/PACAP (Opazo et al., 2009).

The influence of prucalopride was studied on relaxant responses induced by EFS at V50%; V50% varied between 5 and 11 V i.e. in the voltage range where EFS-induced responses were abolished by L-NAME plus apamin. We therefore did not further search for a third neurotransmitter. Our results do also not allow to discriminate whether NO and ATP are released from the same nerve endings or from separate nerve endings. In rat colon, the coexistence of ATP with NAPDH-diaphorase activity as a marker for NO synthase has been demonstrated (Belai and Burnstock, 1994) but this cannot be automatically extrapolated to pig colon. Interestingly, L-NAME alone or in combination with apamin or MRS2179 but not apamin or MRS2179 alone significantly increased the amplitude of substance P-induced contractions. This suggests that substance P induces the release of NO, counteracting its contractile effect at muscular tachykinin receptors. Substance P preferentially activates tachykinin NK_1 receptors and tachykinin NK_1 receptors have been shown to be present on nitrergic neurons in guinea-pig and mouse colon (Bian et al., 2000; Mulé et al., 2007). In porcine colon descendens, substance P seems to be able to stimulate NO release at nerve endings without the need for action potential propagation as TTX did not mimic the effect of L-NAME on the substance P-induced contraction amplitude.

4.V.2. Influence of the 5-HT$_4$ receptor agonist prucalopride on excitatory cholinergic and inhibitory NANC neurotransmission.

Stimulation of 5-HT$_4$ receptors on cholinergic nerve endings, facilitating acetylcholine release and contractility, is a mechanism described at different levels of the gastrointestinal tract in several species (Gershon and Tack, 2007), including human colon longitudinal (Prins et al., 2000a) and circular (Leclere et al., 2005) and pig stomach longitudinal muscle (De Maeyer et al., 2006). In the actual study in circular muscle of pig colon descendens, we therefore first investigated the influence of the 5-HT$_4$ receptor agonist prucalopride on submaximal EFS-induced cholinergic contractions. Preliminary experiments had learned that EFS at V50% for longer periods was not feasible, as spontaneous contractions started to occur and EFS-induced responses became variable. Adding indomethacin to the bathing medium, which prevents spontaneous contractions to occur in similar experiments in pig stomach (De Maeyer et al., 2006) did not solve this methodological problem but the presence of the 3 tachykinin receptor antagonists prevented spontaneous contractions and allowed to register long term stable contractions by EFS at V50% in the majority of tissues. Apparently, although not involved in excitatory neurotransmission by EFS in the experimental conditions used (see 4.1), tachykinins

contribute to spontaneous contractility in circular muscle of pig colon descendens. In these conditions, 1 µM prucalopride enhanced the submaximal cholinergic contractions by only 27 % and 5-HT (1 µM) did so by 45 %. This effect was prevented by the 5-HT$_4$ receptor antagonist GR113808 (Gale et al., 1994) pointing to interaction of prucalopride and 5-HT with 5-HT$_4$ receptors. However, when GR113808 was added once the facilitating effect of prucalopride or 5-HT was stabilized, only a moderate non-significant reduction of the facilitation was obtained. This contrasts to pig stomach longitudinal muscle, where the facilitating effect of prucalopride was reversible with GR113808, although it was resistant to repetitive washing. Our observation in pig colon descendens might suggest that the maintained effect of prucalopride and 5-HT on cholinergic contractions in this tissue is at least partially related to intraneuronal mechanisms that do not require continuous stimulation of the 5-HT$_4$ receptors. E.g. in cultured murine colliculi neurons, short term stimulations of 5-HT$_4$ receptors with 5-HT led to a transient activation of cAMP-dependent protein kinase but a long-lasting inhibition of phosphatases (Ansanay et al., 1995). Alternatively, prucalopride might have a slow off-rate from the 5-HT$_4$ receptor on cholinergic neurons in pig colon descendens.

The facilitating effect of prucalopride (PRU: 27 %) and 5-HT (45 %) at 1 µM on cholinergic neurotransmission was less pronounced than their facilitating effect in similar conditions (half maximal cholinergic contractions; NO synthase inhibitor present) reported in other tissues: canine colon longitudinal muscle (PRU, 0.3µM: 58 %; 5-HT, 0.3µM: 65 %; Prins et al., 2000a), human colon longitudinal muscle (PRU, 0.3µM: 54%; Prins et al., 2000a), pig gastric longitudinal muscle (PRU, 0.3µM: 92 %; 5-HT, 0.3µM: 87 %; De Maeyer et al., 2006), canine gastric longitudinal (PRU, 1µM: 88 %; 5-HT, 0.3µM: 92 %) and circular (PRU, 0.3µM: 185%) muscle (Prins et al., 2001). Only the reported effect of 5-HT (0.3µM) in human colon longitudinal muscle (45 %; Prins et al., 2000a) was similar. The moderate effect of prucalopride and 5-HT in pig colon descendens circular muscle suggests either a limited 5-HT$_4$ receptor number on the cholinergic nerve endings and/or less effective coupling of the receptors. The possibility that the moderate facilitating effect of prucalopride and 5-HT is due to a counteracting relaxant effect of prucalopride and 5-HT via circular muscle 5-HT$_4$ receptors which have been described in human colon (McLean et al., 1995; Irving et al., 2007) can be excluded. In a previous study in circular muscle of human colon, we were not able to obtain a systematic enhancement of EFS-induced cholinergic contractions, while direct measurement of acetylcholine release confirmed that facilitating 5-HT$_4$ receptors are present on the cholinergic nerve endings (Leclere et al., 2005). But, in pig colon descendens we did not obtain any evidence for relaxant 5-HT$_4$ receptors on the circular smooth muscle cells as prucalopride and 5-HT were not able to relax contracted tissues,

even when KCl was used as contractile agent, which has been described to facilitate the analysis of relaxations by muscular 5-HT$_4$ receptor stimulation in human colon (Prins et al., 2000b). Cellek et al. (2006) reported the presence of 5-HT$_4$ receptors on neurons, facilitating nitric oxide release in human colon circular muscle. In circular muscle of pig colon descendens, prucalopride did not influence submaximal NANC relaxations induced by EFS in a voltage range, where the responses were fully due to release of NO and ATP. Even in the presence of apamin, when the EFS-induced relaxations are only due to the action of NO, prucalopride did not enhance them, and the same was true for purinergic responses in the presence of L-NAME. No evidence for facilitation of NO and/or ATP release by 5-HT$_4$ receptor stimulation was thus obtained. Our results thus suggest that the 5-HT$_4$ receptors found by RT-PCR in the myenteric plexus-muscle layer of pig colon (De Maeyer et al., 2008) at least partly correspond to those that were now functionally detected on the cholinergic nerves. RT-PCR of the myenteric plexus-muscle layer of pig colon might also detect 5-HT$_4$ receptors that are possibly present on mucosal or myenteric endings of intrinsic primary afferent neurons when the cell body of the intrinsic primary afferent neurons is located in the myenteric plexus.

4.VI. Conclusion

In circular muscle of pig colon descendens, on-contractions can be obtained by EFS only in the combined presence of L-NAME and apamin; these contractions are purely cholinergic indicating acetylcholine as the principal excitatory neurotransmitter within the experimental conditions used; in these conditions NO and ATP function as inhibitory neurotransmitters in a redundant way. The 5-HT$_4$ receptor agonist prucalopride facilitates cholinergic neurotransmission via 5-HT$_4$ receptors but the moderate level of effect suggests a limited 5-HT$_4$ receptor number on the cholinergic nerve ending and/or less effective coupling of these receptors than in other parts of the porcine gastrointestinal tract or in other species, where more pronounced effects were reported. No evidence for 5-HT$_4$ receptors facilitating the release of NO or inducing smooth muscle relaxation was obtained. The pig colon descendens can thus not be considered as a model for the 3 locations of 5-HT$_4$ receptors, that have been described in human colon.

4.VII. References

Ansanay H, Dumuis A, Sebben M, Bockaert J, Fagni L. cAMP-dependent, long-lasting inhibition of a K^+ current in mammalian neurons. Proc. Natl. Acad. Sci. 1995; 92: 6635-6639

Barbiers ., Timmermans J-P, Adriaensen D, De Groodt-Lasseel MHA, Scheuermann DW. Projections of neurochemically specified neurons in the porcine colon. Histochem. 1995; 103: 115-126

Belai A, Burnstock G. Evidence for coexistence of ATP and nitric oxide in non-adrenergic, non-cholinergic (NANC) inhibitory neurons in the rat ileum, colon and anococcygeus muscle. Cell Tissue Res. 1994; 278: 197-200

Benkó R, Undi S, Wolf M, Vereczkei A, Illényi L, Kassai M, Cseke L, Kelemen D, Horváth OP, Antal A, Magyar K, Barthó L. P2 purinoceptor antagonists inhibit the non-adrenergic, non-cholinergic relaxation of the human colon in vitro. Neuroscience 2007; 147: 146-152

Bian X-C, Bertrand PP, Furness JB, Bornstein JC. Evidence for functional NK_1-tachykinin receptors on motor neurons supplying the circular muscle of guinea-pig small and large intestine. Neurogastroenterol. Mot. 2000; 12: 307-315

Brookes SJH. Neuronal nitric oxide in the gut. Gastroenterol Hepatol. 1993; 8: 590-603

Bodin P, Burnstock G. Purinergic signalling: ATP release. Neurochem. Res. 2001; 26: 959-969

Boeckxstaens GE, De Man JG, Pelckmans PA, Cromheeke KM, Herman A.G., Van Maercke Y.M., 1993a. Ca^{2+} dependency of the release of nitric oxide from non-adrenergic non-cholinergic nerves. Br. J. Pharmacol. 110, 1329-1334

Boeckxstaens GE, Pelckmans PA, Herman AG, Van Maercke YM. Involvement of nitric oxide in the inhibitory innervation of the human isolated colon. Gastroenterology 1993b; 104: 690-697

Cellek S, John AK, Thangiah R, Daas NB, Bassil AK, Jarvie EM, Lalude O, Vivekanandan S, Sanger GJ. 5-HT_4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. Neurogastroenterol. Motil. 2006; 18: 853-861

De Maeyer JH, Aerssens J, Verhasselt P, Lefebvre RA. Alternative splicing and exon duplication generates 10 unique porcine 5-HT_4 receptor splice variants including a functional homofusion variant. Physiol. Genomics 2008; 34: 22-33

De Maeyer JH, Prins NH, Schuurkes JAJ, Lefebvre RA. Differential effects of 5-HT_4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ specific behavior. J. Pharmacol. Exp. Ther. 2006; 317: 955-964

De Maeyer JH, Schuurkes JAJ, Lefebvre RA. Selective desensitization of the 5-HT_4 receptor mediated response in pig atrium but not in stomach. Br. J. Pharmacol. 2009; 156: 362-376

Gale JD, Grossman CJ, Whitehead JW, Oxford AW, Bunce KT, Humphrey PP. GR113808: a novel, selective antagonist with high affinity at the 5-HT_4 receptor. Br. J. Pharmacol. 1994; 111: 332-338

Gallego D, Gil V, Aleu J, Martinez-Cutillas M, Clavé P, Jiménez M. Pharmacological characterization of purinergic inhibitory neuromuscular transmission in the human colon. Neurogastroenterol Motil. 2011; 23: 792-e338

Gallego D, Gil V, Martínez-Cutillas M, Mañé N, Martín MT, Jiménez M. Purinergic neuromuscular transmission is absent in the colon of P2Y(1) knocked out mice. J Physiol. 2012; 590: 1943-1956

Gallego D., Gil V., Aleu J., Aulí M., Clavé P., Jiménez, M. Purinergic and nitrergic junction potential in the human colon. Am. J. Physiol. Gastrointest. Liver Physiol. 2008; 295: G522-G533

Gallego D., Hernández P., Clavé P., Jiménez, M. $P2Y_1$ receptors mediate inhibitory purinergic neuromuscular transmission in the human colon. Am. J. Physiol. Gastrointest. Liver Physiol. 2006; 291: G584-G594

Gallego D, Vanden Berghe P, Farré R, Tack J, Jiménez M. $P2Y_1$ receptors mediate inhibitory neuromuscular transmission and enteric neuronal activation in small intestine. Neurogastroenterol. Motil. 2008; 20: 159-168

Gershon MD. Nerves, reflexes, and the enteric nervous system. J. Clin. Gastroenterol. 2005; 39: Suppl 3, S184-S193

Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 2007; 132: 397-414

Gordon AR, Siegman MJ. Mechanical properties of smooth muscle. I. Length-tension and force-velocity relations. Am. J. Physiol. 1971; 221: 1243–1249

Grasa L, Gil V, Gallego D, Martin MT, Jiménez M. P2Y(1) receptors mediate inhibitory neuromuscular transmission in the rat colon. Br. J. Pharmacol. 2009; 158: 1641-1652

Grider JR. Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. J. Pharmacol. Exp. Ther. 2003; 307: 460-467

Grider JR, Foxx-Orenstein AE, Jin JG. 5-Hydroxytryptamine4 receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. Gastroenterology 1998; 115: 370-380

Hirst GDS, Bywater RAR, Teramoto N, Edwards FR. An analysis of inhibitory junction potentials in the guinea-pig proximal colon. J. Physiol. 2004; 558: 841-855.

Hwang SJ, Blair PJ, Durnin L, Mutafova-Yambolieva V, Sanders KM, Ward SM. P2Y1 purinoreceptors are fundamental to inhibitory motor control of murine colonic excitability and transit. J Physiol. 2012; 590: 1957-1972

Irving HR, Tan YY, Tochon-Danguy N, Liu H, Chetty N, Desmond PV, Pouton CW, Coupar IM. Comparison of 5-HT_4 and 5-HT_7 receptor expression and function in the circular muscle of the human colon. Life Sci. 2007; 80: 1198-1205

Jager LP, van der Schaar MW. Characterization of inhibitory innervation in porcine colonic circular muscle. Can. J. Physiol. Pharmacol. 1988; 66: 1386-1397

Keef KD, Du C, Ward SM, McGrego B, Sanders KM. Enteric inhibitory neural regulation of human colonic circular muscle: role of nitric oxide. Gastroenterology 1993; 105: 1009-1016

Leclere PG, Lefebvre RA. Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. Br. J. Pharmacol. 2002; 135: 135-142

Leclere PG, Prins NH, Schuurkes JAJ, Lefebvre RA. 5-HT_4 receptors located on cholinergic nerves in human colon circular muscle. Neurogastroenterol. Motil. 2005; 17: 366-375

Liu M, Geddis MS, Wen Y, Setlik W, Gershon MD. Expression and function of 5-HT_4 receptors in the mouse enteric nervous system. Am. J. Physiol. Gastrointest. Liver Physiol. 2005; 289: G1148-G1163

Maggi CA, Astolfi M, Giuliani S, Goso C, Manzini S, Meini S, Patacchini S, Pavone V, Redone C, Quartara L. MEN10627, a novel polycyclic peptide antagonist of tachykinin NK_2 receptor. J. Pharmacol Exp. Ther. 1994a; 271: 1489-1500

Maggi CA, Holze P, Giuliani S. Effect of omega-conotoxin on cholinergic and tachykininergic excitatory neurotransmission to the circular muscle of the guinea-pig colon. Naunyn Schmiedebergs Arch Pharmacol. 1994b; 350: 529-536

Manabe N, Wong BS, Camilleri M. New-generation 5-HT_4 receptor agonists: potential for treatment of gastrointestinal motility disorders. Expert Opin. Investig. Drugs 2010; 19: 765-775

Matsuda NM, Miller SM, Sha L, Farrugia G, Szurszewski JH. Mediators of non-adrenergic non-cholinergic inhibitory neurotransmission in porcine jejunum. Neurogastroenterol. Motil. 2004; 16: 605-612

McLean PG, Coupar IM, Molenaar P. A comparative study of functional 5-HT_4 receptors in human colon, rat oesophagus and rat ileum. Br. J. Pharmacol. 1995; 115: 47-56

Miller ER, Ullrey DE. The pig as a model for human nutrition. Annu. Rev. Nutr. 1987; 7: 361-382

Mitolo-Chieppa D, Mansi G, Rinaldi R, Montagnani M, Potenza MA, Genualdo M, Serio M, Mitolo CI, Rinaldi M, Altomare DF, Memeo V. Cholinergic stimulation and nonadrenergic, noncholinergic relaxation of human colonic circular muscle in idiopathic chronic constipation. Dig. Dis. Sci. 1998; 43: 2719-2726

Mulè F, Amato A, Serio R. Role for NK(1) and NK(2) receptors in the motor activity in mouse colon. Eur. J. Pharmacol. 2007; 570: 196-202

Onori L, Aggio A, Taddei G, Ciccocioppo R, Severi C, Carnicelli V, Tonini M. Contribution of NK_3 receptors to propulsion in the rabbit isolated distal colon. Neurogastroenterol. Mot. 2001; 13: 211-219

Opazo A, Lecca B, Admella C, Fantova MJ, Jiménez M, Martí-Ragué J, Clavé P. A comparat4e study of structure and function of longitudinal muscle of the anal canal and the internal anal sphincter in pigs. Dis. Colon rectum 2009; 52: 1902-1911

Prins NH, Akkermans LMA, Lefebvre RA, Schuurkes JAJ. 5-HT_4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. Br. J. Pharmacol. 2000a; 131: 927-932

Prins NH, Shankley NP, Welsh NJ, Briejer MR, Lefebvre RA, Akkermans LMA, Schuurkes JAJ. An improved in vitro bioassay for the study of 5-HT_4 receptors in the human isolated large intestinal circular muscle. Br. J. Pharmacol. 2000b; 129: 1601-1608

Prins NH, van Der Grijn A, Lefebvre RA, Akkermans LM, Schuurkes JA. 5-HT(4) receptors mediating enhancement of contractility in canine stomach; an in vitro and in vivo study. Br. J. Pharmacol. 2001; 132: 1941-1947

Schmidt P, Poulsen SS, Rasmusse TN, Bersani M, Holst JJ. Substance P and neurokinin A are codistributed and colocalized in the porcine gastrointestinal tract. Peptides 1991; 12: 963-973

Sevcencu C, Rijkhoff NJ, Gregersen H, Sinkjaer T. Propulsive activity induced by sequential electrical stimulation in the descending colon of the pig. Neurogastroenterol. Motil. 2005; 17: 376-387

Stanton MP, Hengel PT, Southwell BR, Chow CW, Keck J, Hutson JM, Bornstein JC. Cholinergic transmissions to colonic circular muscle of children with slow-transit constipation is unimpaired but transmission via NK_2 receptors is lacking. Neurogastroenterol. Motil. 2003; 15: 669-678

Van Crombruggen K, Lefebvre RA. Nitrergic-purinergic interactions in rat distal colon motility. Neurogastroenterol. Motil. 2004; 16: 81-98

Van Crombruggen K, Van Nassauw L, Timmermans J-P, Lefebvre RA. Inhibitory purinergic P2 receptor characterization in rat distal colon. Neuropharmacology 2007; 53: 257-271

Van Geldre LA, Lefebvre RA. Nitrergic relaxation in rat gastric fundus: influence of mechanism of induced tone and possible role of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase. Life Sci. 2004; 74: 3259-3274

Zimmermann H. ATP and acetylcholine, equal brethren. Neurochem. Int. 2008; 52: 634-648

Zygmunt PM, Zygmunt PK, Högestätt ED, Andersson KE. Effects of omega-conotoxin on adrenergic, cholinergic and NANC neurotransmission in the rabbit urethra and detrusor. Br. J. Pharmacol. 1993; 110: 1285-1290

Chapter 5

INFLUENCE OF PHOSPHODIESTERASES ON BASAL AND 5-HT$_4$ RECEPTOR FACILITATED CHOLINERGIC CONTRACTILITY IN PIG DESCENDING COLON

Evelien K.V. Priem[a], Joris H. De Maeyer[b] and Romain A. Lefebvre[a]

[a]Heymans Institute of Pharmacology, Ghent University, Gent, Belgium,

[b]Shire-Movetis NV, Turnhout, Belgium

submitted to European Journal of Pharmacology

Chapter 5 Influence of phosphodiesterases on basal and 5-HT$_4$ receptor facilitated cholinergic contractility in pig descending colon

5.I. Abstract

This study in pig colon descendens circular muscle investigated the possible role of phosphodiesterases (PDEs) 1) in the control of smooth muscle activity and 2) in the signal transduction of the 5-HT$_4$ receptors located on the cholinergic neurons. Submaximal cholinergic contractions were electrically induced in colonic circular muscle strips and the influence of the non-selective PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) and selective inhibitors for the 5 classic PDE families (1-5) vinpocetine (PDE1), EHNA (PDE2), cilostamide (PDE3), rolipram (PDE4) and zaprinast (PDE5) was evaluated. IBMX and cilostamide concentration-dependently reduced the amplitude of the cholinergic contractions, as good as abolishing them at 30 and 0.3 µM respectively. EHNA only reduced the contractions significantly at the highest concentration tested (30 µM). Rolipram showed a biphasic effect, significantly increasing the contractions at 0.1 and 0.3 µM but decreasing them at 30 µM. vinpocetine did not influence the electrically induced contractions while zaprinast enhanced the responses at 3-30 µM. The 5-HT$_4$ receptor agonist prucalopride (1 µM) significantly enhanced the electrically induced cholinergic contractions. IBMX, vinpocetine and EHNA did not influence the facilitating effect of prucalopride but rolipram tended to enhance it. When rolipram was added after prucalopride, the facilitating effect of prucalopride was significantly enhanced. These results suggest that PDE3 is the main regulator of circular smooth muscle activity and that the signal transduction of 5-HT$_4$ receptors on the cholinergic nerves towards the circular muscle layer is regulated by PDE4 in pig colon descendens.

5.II. Introduction

Selective 5-HT_4 receptor agonists stimulate gastrointestinal motility through facilitated acetylcholine release from excitatory motor neurons (Gershon and Tack, 2007). Until it was withdrawn because of non-5-HT_4 receptor mediated side effects, the non-selective 5-HT_4 receptor agonist cisapride was used for stimulating gastric emptying in patients with gastroparesis (Kendall et al., 1997; Dutta et al., 1999). The stimulated gastric motility can be related to interaction at facilitatory 5-HT_4 receptors, that were shown on cholinergic nerve endings in human stomach circular muscle (Leclere and Lefebvre, 2002). The pig stomach is a good model to investigate gastric 5-HT_4 receptors, as they are present on cholinergic neurons controlling both longitudinal (De Maeyer et al., 2006a) and circular (Priem et al., 2012) gastric muscle and pronounced facilitation of cholinergic transmission can be obtained by stimulation with selective 5-HT_4 receptor agonists. 5-HT_4 receptors are coupled to adenylate cyclase and initiate their effect through generation of cAMP (Raymond et al., 2001). Phosphodiesterases (PDEs) are the sole family of isozymes, degrading the cyclic nucleotides cAMP and cGMP. PDEs are present in smooth muscle cells and are involved in regulating smooth muscle tone, as also shown for some regions and species in the gastrointestinal tract (Kaneda et al., 1997; Jones et al., 2002). In line with the intraneuronal expression and functions of PDEs in the brain (Kleppisch, 2009), interest has also developed for the peripheral neuronal distribution of PDEs (Coskran et al., 2006; Stephenson et al., 2009). We recently showed that the intraneuronal transduction pathway of the facilitatory 5-HT_4 receptors on cholinergic neurons in porcine gastric circular muscle is regulated by PDE4, as PDE4 inhibition enhanced cholinergic facilitation by 5-HT_4 receptor stimulation; this opens the possibility of combining a PDE4 inhibitor with 5-HT_4 receptor agonists to enhance their gastroprokinetic effect (Priem et al., 2012).

The highly selective 5-HT_4 receptor agonist prucalopride is used in patients with severe chronic constipation resistant to laxative treatment (Quigley, 2012). Its stimulatory effect on colonic transit (Bouras et al., 2001) can be related to activation of facilitatory 5-HT_4 receptors, that were shown on cholinergic nerve endings towards human colonic longitudinal (Prins et al., 2000) and circular (Leclere et al., 2005) muscle, enabling pronounced enhancement of cholinergic neurotransmission. In a previous study, we showed that 5-HT_4 receptors are also present on cholinergic nerves towards circular muscle in pig colon descendens (Priem and Lefebvre, 2011). The facilitating effect of prucalopride on cholinergic neurotransmission in pig colon descendens circular muscle was moderate in comparison to similar models in other species, which might be related to a limited 5-HT_4 receptor number on the cholinergic nerve endings and/or less effective coupling of the receptors. One possibility for the latter is a tight control by one or more PDEs.

The aim of the actual study was therefore to investigate the role of PDEs in porcine colonic circular muscle activity and in the signal transduction of the 5-HT_4 receptors located on the cholinergic nerve endings towards pig colon circular muscle. This was done by studying the influence of the non-selective PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) and selective inhibitors for the 5 classic PDE families (1-5) on electrically driven cholinergic smooth muscle activity and on the facilitating effect of prucalopride on cholinergic neurotransmission.

5.III. Methods

5.III.1. Tissue preparation and tension recording

Young male pigs (10-12 weeks, 15-25 kg – breed Line 36) were obtained from Rattlerow Seghers, Belgium. All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

On the morning of the experiment, pigs were anaesthetized with an intramuscular injection of 5 ml Zoletil 100 (containing 50 mg/ml tiletamine and 50 mg/ml zolazepam; virbac Belgium S.A., Belgium). After exsanguination, the colon descendens was prelevated 10 cm above the anus to the transverse colon and was placed in aerated (5% CO_2/95% O_2) Krebs-Henseleit solution (composition in mM: glucose 11.1, $NaHCO_3$ 25, $KHPO_4$ 1.18, $CaCl_2$ 2.51, $MgSO_4$ 1.18, KCl 4.69, NaCl 118). The colon descendens was opened along the mesenteric border and after removal of the mucosa, 8 full-thickness circular muscle strips (approx. 3 × 20 mm) were prepared in pairs at the same level, starting 2 cm above the distal end; the strips were mounted in 10 ml organ baths between 2 platinum plate electrodes under a load of 2 g to allow electrical field stimulation (EFS) performed by means of a 4 channel custom-made stimulator. The aerated (5% CO_2/95% O_2) Krebs-Henseleit solution in the organ baths systematically contained 4 µM of the noradrenergic neuron blocker guanethidine and 0.3 mM of the NO synthase inhibitor N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) to avoid noradrenergic and nitrergic responses respectively. After 60 min of stabilization with refreshing of the Krebs-Henseleit solution every 15 min, strips were contracted with the muscarinic receptor agonist carbachol (3 µM). This procedure was repeated with a 20-min washout period in between. After the second carbachol administration and washout period, the small conductance calcium-dependent potassium channel blocker apamin (0.5 µM) and a combination of the tachykinin receptor antagonists (NK_1, 10 µM FK888; NK_2, 1 µM MEN10627; NK_3, 0.3 µM SB222200) were added and incubated for 30 min before the first electrical stimulation.

We previously showed that the addition of the tachykinin receptor antagonists to the medium, also containing guanethidine, L-NAME and apamin allows to obtain reproducible cholinergic contractions by EFS (Priem and Lefebvre, 2011).

Strips were then stimulated for 1 hour (12 stimulations) with 5 min interval at supramaximal voltage (35 V) (10 s trains; 0.25 ms pulse duration; frequency of 4 Hz). After 1 hour, EFS was stopped, muscle strips were rinsed and apamin (0.5 µM) and the combination of the tachykinin receptor antagonists were again added and incubated for 30 min before the next stimulation. EFS (10 s; 0.25 ms; 4 Hz) was then applied with 5 min interval at an initial voltage of 15 v. The voltage was further adjusted to reduce the contraction force to approximately 50% (V50%) of the force evoked at 35 V and EFS was repeated until 5 reproducible contractions were obtained at V50%. The protocols described in 2.2 then started. Experiments where the EFS-induced submaximal contractions in time controls decreased by more than 25% in the course of the experiment, were not taken in account (14/48).

Changes in isometric tension were measured using MLT 050/D force transducers (ADInstruments, United Kingdom) and recorded on a PowerLab/8sp data recording system (ADInstruments, United Kingdom) with Chart v5.5.6 software.

5.III.2. Protocols

5.III.2.1. Influence of PDE inhibitors on EFS-induced submaximal cholinergic contractions

The influence of the non-selective PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) and the selective PDE inhibitors vinpocetine (PDE1 inhibitor), EHNA (PDE2 inhibitor), cilostamide (PDE3 inhibitor), rolipram (PDE4 inhibitor) and zaprinast (PDE5 inhibitor) was tested on EFS-evoked submaximal (V50%) cholinergic contractions. A cumulative concentration-response curve for the different PDE inhibitors was obtained by adding them in half log unit increasing concentrations, starting after 5 reproducible contractions at V50% had been obtained and registering the responses to 6 trains (30 min) after adding each concentration. Parallel to the cumulative concentration-response curve of rolipram, an isolated concentration-response curve was obtained by adding one single concentration per tissue in 3 animals. Control tissues did not receive any solvent nor PDE inhibitor. The solvents DMSO and ethanol were tested separately by adding them cumulatively in the matching dilutions as for the cumulative concentration series of the corresponding PDE inhibitor.

5.III.2.2. Influence of PDE inhibitors on the effect of prucalopride on EFS-induced submaximal cholinergic contractions

The facilitating effect of prucalopride on EFS-induced submaximal cholinergic contractions was tested in the absence and presence of different PDE inhibitors. After obtaining 5 reproducible contractions by EFS at V50%, one PDE inhibitor (IBMX 0.3 µM or 1 µM; vinpocetine 10 µM; EHNA 10 µM; rolipram 1 µM or 3 µM) was added per tissue. After 30 min (6 stimulations) of EFS, 1 µM prucalopride was then added in all parallel muscle strips and EFS-induced responses were further monitored till 1 h (12 stimulations) after the addition of prucalopride. Two sets of parallel control tissues were run, one receiving only prucalopride, one being a real time control not receiving any agent.

The interaction between the PDE4 inhibitor rolipram and prucalopride was also investigated by adding rolipram after prucalopride. Three tissues were studied in parallel. After obtaining 5 reproducible contractions by EFS (10 s; 0.25 ms; 4 Hz) at v50%, prucalopride (1 µM) was added in 2 tissues, that were followed for 2 hours (24 stimulations); in one of the 2 tissues, rolipram (3 µM) was added after 12 trains in the presence of prucalopride had been obtained. The third tissue was a time control not receiving prucalopride nor rolipram.

5.III.3. Drugs

Guanethidine sulphate, N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), carbachol, 3-isobutyl-1-methyl-xanthine (IBMX) and zaprinast were obtained from Sigma (Belgium). Apamin was obtained from Alomone labs (Israel). MEN10627 [(4R)-4-Hydroxy-1-[(1-methyl-1H-indol-3-yl)carbonyl]-L-prolyl-N-methyl-3-(2-naphthalenyl)-N(phenylmethyl)-L-alaninamide], FK888 [cyclo(Dap-Leu-Met-Asp-Trp-Phe)(4β-1β lactam)], SB222200 [3-Methyl-2-phenyl-N-[(1S)-1-phenylpropyl]-4-quinolinecacarboxamide], cilostamide, erythro-9-(2-hydroxyl-3nonyl)adenine hydrochloride (EHNA), rolipram and vinpocetine were from Tocris Cookson (U.K.). Prucalopride succinate was a gift of Shire-Movetis (Belgium). Compounds were dissolved and diluted in distilled water, except for FK888, MEN10627, SB222200, cilostamide, rolipram and vinpocetine (stock solution in dimethyl sulfoxide (DMSO), dilutions with distilled water), zaprinast (dissolved and diluted in DMSO) and IBMX (stock solution in 50% ethanol, dilutions with distilled water). The stock solutions were stored at -20 °C; fresh dilutions were made on experimental days.

5.III.4. Data analysis and statistics

Stimulation trains were numbered starting from the 5 consecutive stimulations at V50% with reproducible contractions just before adding substances (1, 2, 3, 4, 5,…). The mean contractile response to these 5 stimulations was taken as 100% reference for all the following responses.
Results are expressed as means ± S.E.M., n referring to tissues from different animals except when otherwise indicated. Statistical analysis was performed by use of Graphpad Prism v.5.01 (San Diego, U.S.A.); $P < 0.05$ was considered statistically significant. When adding PDE inhibitors cumulatively, the last contraction in the presence of each concentration was compared to the reference by repeated measures ANOVA followed by a Bonferroni corrected t-test. In experiments, where prucalopride was added after a PDE inhibitor, responses induced by stimulation 13, corresponding to the 2^{nd} stimulation after adding prucalopride, were compared between the time controls, the tissues with prucalopride alone and the tissues with addition of prucalopride after a PDE inhibitor was added, by ONE-WAY ANOVA followed by a Bonferroni corrected t-test. In the experiments, where rolipram was added after prucalopride, the response to stimulation 7 (i.e. the 2^{nd} stimulation after adding prucalopride) was compared to the mean response to stimulations 3 to 5 by a paired t-test; the response by stimulation 19 (i.e. the 2^{nd} stimulation after adding rolipram) was similarly compared to the mean response to stimulations 15 to 17.

5.IV. Results

5.IV.1. Influence of PDE inhibitors on EFS-induced submaximal cholinergic contractions

In the control tissues shown in **figure 1A**, the contractile response by EFS at supramaximal voltage (35 V) was 43 ± 5% (n = 7; 6 animals) of that induced by 3 µM carbachol at the beginning of the experiment. Once stimulation voltage was reduced to V50%, EFS-induced contractions in these control tissues attained an amplitude of 52 ± 3% (n=7; 6 animals) of that induced at supramaximal voltage of 35 V. In the control tissues, the amplitude of the contractile responses by EFS at V50% remained stable upon repetitive stimulation (amplitude of the contraction at the last stimulation was 94 ± 6% of the mean response to stimulation train 1 to 5 (n=7; 6 animals).

The non-selective PDE inhibitor IBMX induced a concentration-dependent reduction of the EFS-induced contractions reaching significance from 1 µM onwards; in the presence of 30 µM IBMX, the contractions were nearly abolished (**Fig. 5.1B**). Part of this inhibition can probably be ascribed to its solvent (maximal ethanol concentration in the organ bath 0.15%; **Fig. 5.2C**) that reduced the EFS-induced contractions by maximally 30 %, but even when subtracting this IBMX thus clearly reduces cholinergic contractions in pig descending colonic circular muscle. The PDE1 inhibitor vinpocetine (0.1 µM-30 µM) had no significant influence on the EFS-induced contractions (**Fig. 5.1C**). The PDE2 inhibitor EHNA had no significant influence on the submaximal cholinergic contractions till 10 µM, but the highest concentration (30 µM) induced a significant reduction of 33 ± 14% (n=6; **Fig. 5.1D**). The PDE3 inhibitor cilostamide (0.01 µM - 3 µM) reduced the contractions in a concentration-dependent manner from 0.01 µM onwards, as good as abolishing them from 0.3 µM on (**Fig. 5.1E**). The PDE5 inhibitor zaprinast (0.1 µM-30 µM) significantly increased the EFS-induced contractions with 35 ± 16% at 3 µM, 45 ± 19% at 10 µM and 39 ± 14% at 30 µM (n=6; **Fig. 5.1F**).

When added cumulatively, the PDE4 inhibitor rolipram showed a biphasic response as it increased the EFS-induced submaximal contractions at 0.1 and 0.3 µM (both with 11%, n=6) while it decreased the responses with 19 ± 5% (n=6) at the highest concentration tested (30 µM; **Fig. 5.2B**). The latter inhibition might to some extent be spontaneous as the parallel control series also showed some significant decline at the corresponding stimulation train (**Fig. 5.2A**). Still, in parallel tissues where the solvent of rolipram, DMSO, was added as for a cumulative concentration-response curve of rolipram, the EFS-induced responses were stable (**Fig. 5.2D**). Additionally, an isolated concentration-curve of rolipram, constructed by adding a single concentration of rolipram per tissue, was obtained in 3 animals and showed the same biphasic trend (results not shown).

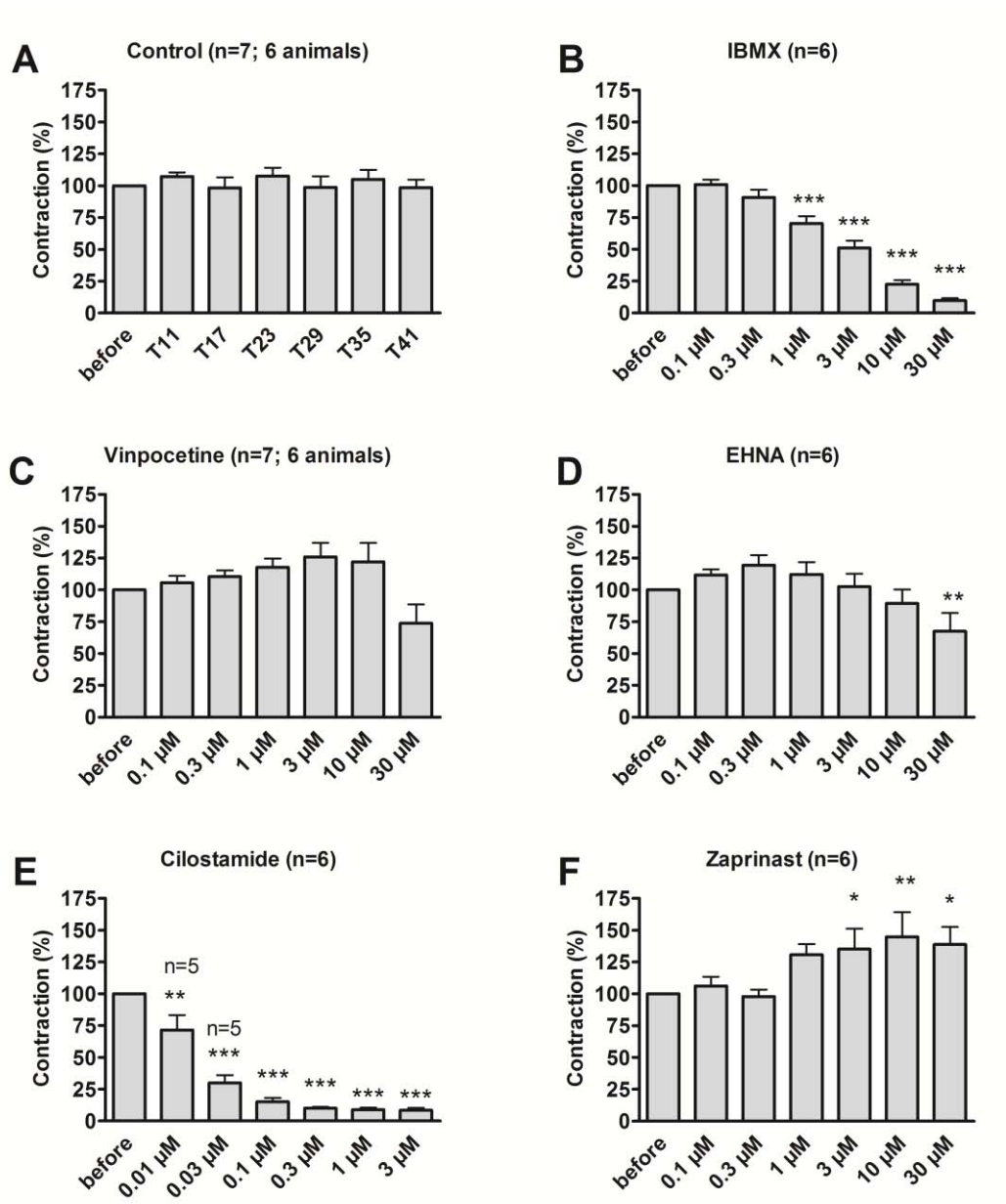


Figure 5.1

Influence of increasing concentrations of the PDE inhibitors IBMX (B), vinpocetine (C), EHNA (D), cilostamide (E) and zaprinast (F) on EFS (10 s trains at 4 Hz; 0.25 ms;V50%) induced submaximal contractions. Six trains of EFS were applied in the presence of each concentration of PDE-inhibitor and the response of the 6th train was expressed as percentage of the mean of the 5 contractions before adding the lowest concentration of the PDE inhibitor. Control tissues (A) were stimulated 41 times and the response was measured at each 6th train from train 11 (T11) on. Means ± S.E.M. of n=6-7. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$: significant difference versus before (repeated measures ANOVA followed by a Bonferroni corrected t-test)

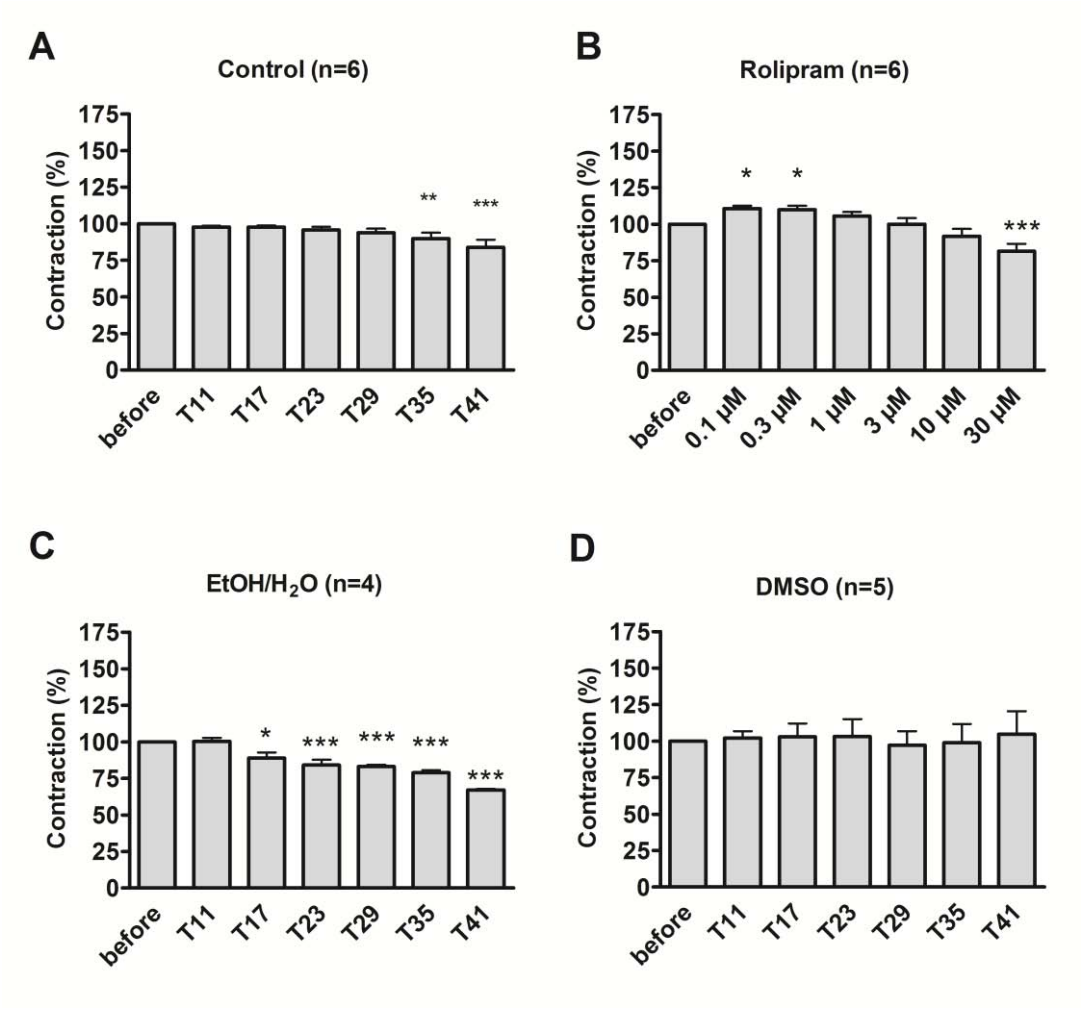


Figure 5.2

Influence of increasing concentrations of the PDE4 inhibitor rolipram (B) on EFS (10 trains at 4 Hz; 0.25 ms;V50%) induced submaximal contractions, expressed as described in the legend of figure 1. Parallel time controls, not receiving an agent (A), tissues receiving the 50% ethanol dilution series as for IBMX (C) and tissues receiving the DMSO dilution series as for rolipram, cilostamide and vinpocetine (D) are also shown. Means ± S.E.M. of n=4-6. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$: significant difference versus before (repeated measures ANOVA followed by a Bonferroni corrected t-test)

5.IV.2. Influence of PDE inhibitors on the effect of prucalopride on EFS-induced submaximal cholinergic contractions

In the different series of pig descending colon circular muscle strips, where prucalopride was added in the presence of a PDE-inhibitor (**Fig. 5.3**), the contractile responses by EFS at V50% remained stable upon repetitive stimulation for the course of the experiment in the control tissues. Prucalopride (1 µM) alone systematically enhanced the EFS-induced contractions reaching the maximal effect already at the 2nd stimulation in the presence of prucalopride (train 13; not significant in series with vinpocetine). IBMX (0.3 µM and 1 µM) and EHNA (10 µM) did not enhance the facilitating effect of prucalopride (**Fig. 5.3A, 5.3B and 5.3D**). vinpocetine (10 µM) per se enhanced the EFS-induced submaximal contractions. When adding prucalopride, a small additional effect was seen but the contractile response to train 13 in the presence of vinpocetine plus prucalopride was not significantly enhanced compared to that in the presence of prucalopride alone (**Fig. 5.3B**). Rolipram, 1 µM, enhanced now the EFS-induced contractions per se (to 132 ± 7%, n=6) while 3 µM did not. In the presence of both concentrations of rolipram, the EFS-induced contractions after adding prucalopride attained higher values than with prucalopride alone although this did not reach significance (response to train 13: 175 ± 19%, n=6, in the presence of 1 µM rolipram plus prucalopride versus 161 ± 7%, n=6, in the presence of prucalopride alone, **Fig. 5.3E**; 142 ± 5%, n=8, in the presence of 3 µM rolipram plus prucalopride versus 127 ± 7%, n=8, in the presence of prucalopride alone, **Fig. 5.3F**).

An additional series was performed where 3 µM rolipram was added after prucalopride (**Fig. 5.4**). The time control tissues showed a stable response. In the 2 sets of tissues where prucalopride was administered, it enhanced the EFS-induced submaximal contractions, the response at train 7 (2nd train in its presence) being significantly enhanced compared to the mean response by trains 3 to 5 just before its administration. In one of these 2 sets, rolipram was administered after train 17; this induced a clearcut and significant enhancement of the EFS-induced responses.

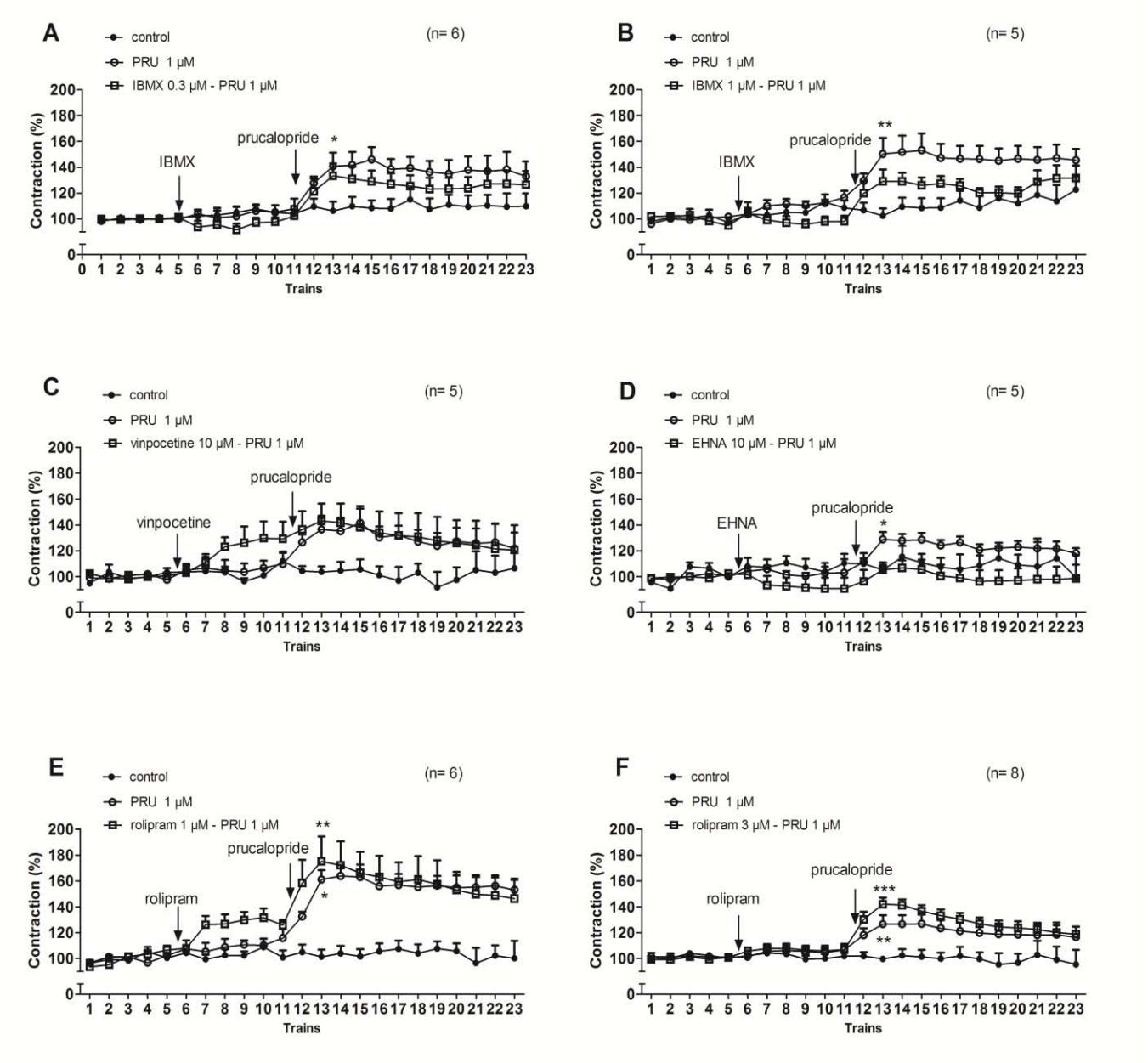


Figure 5.3

Facilitating effect of 1 µM prucalopride (PRU) on EFS-induced submaximal cholinergic contractions in the presence of PDE inhibitors IBMX 0.3 µM (A) or 1 µM (B), vinpocetine 10 µM (C), EHNA 10 µM (D), rolipram 1 µM (E) or 3 µM (F). Means ± S.E.M. of n = 5-8. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$: significant difference of the response at stimulation train 13 (2nd stimulation train after adding prucalopride) versus that in control tissues without prucalopride (one-way ANOVA followed by a Bonferroni corrected *t*-test)

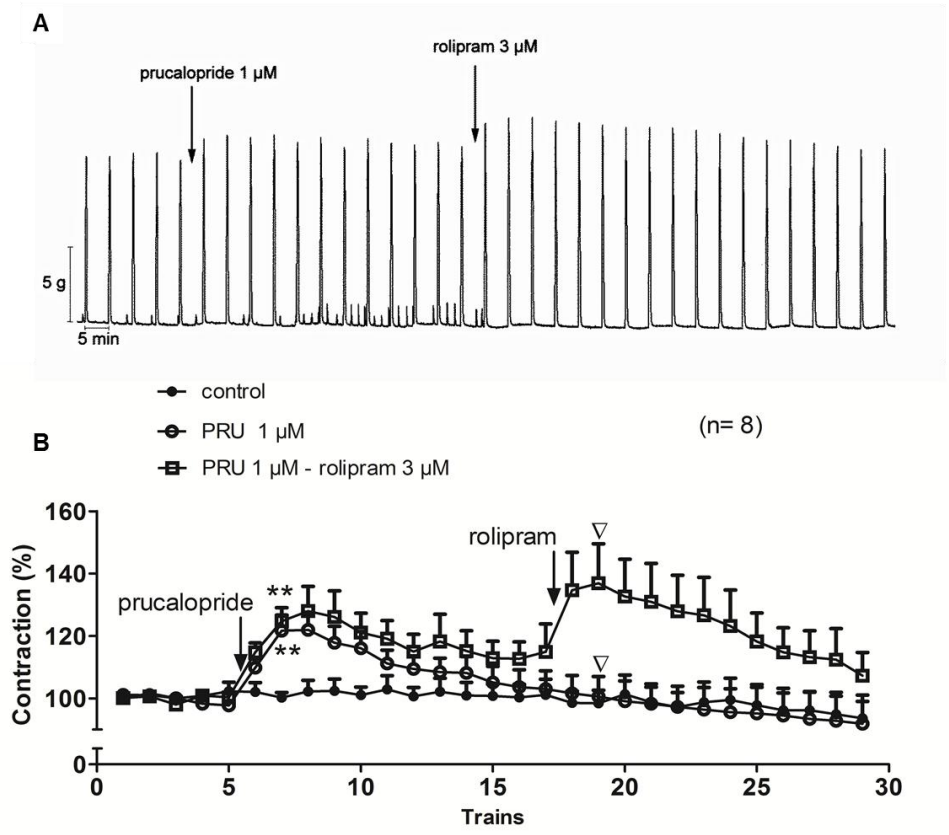


Figure 5.4

(A) Representative trace showing the influence on submaximal EFS-induced contractions of consecutive administration of 1 µM prucalopride and 3 µM rolipram.

(B) Mean (± S.E.M.; n=8) result of the experiment shown in panel A, and in parallel tissues only receiving prucalopride, or no substance at all (time control).

** P < 0.01: significant difference of the response to stimulation train 7 (2nd stimulation train after adding prucalopride) versus the mean response to stimulation train 3-5 just before adding prucalopride (paired t-test)

∇ P < 0.01: significant difference of the response to stimulation train 19 (2nd stimulation train after adding rolipram) versus the mean response to stimulation train 15-17 (paired t-test)

5.V. Discussion

5.V.1. Influence of PDE inhibitors on EFS-induced submaximal cholinergic contractions

Two main inhibitory neurotransmitters in the gastrointestinal tract, NO and VIP, induce gastrointestinal smooth muscle relaxation through generation of cGMP and cAMP respectively. The concentration of these cyclic nucleotides in the smooth muscle cells is determined by the balance between their synthesis by cyclases and their metabolism by phosphodiesterases (Murthy, 2001; Murthy et al., 2002). The classic PDE subtypes 1, 2 and 3 catalyse the breakdown of both cAMP and cGMP, while PDE 4 and 5 are respectively cAMP and cGMP specific (Maurice et al., 2003). By studying the relaxant effect of selective PDE inhibitors on basal or exogenous agonist-induced tone, one can investigate the functionally important PDEs in gastrointestinal smooth muscle (Barbier and Lefebvre, 1995; Tomkinson and Raeburn, 1996; Kandeda et al., 1997; Jones et al., 2002). In pig descending colon circular muscle, we have now investigated the inhibitory effect of selective inhibitors of PDE 1 to 5 and of the non-selective inhibitor IBMX versus contractions induced by endogenous acetylcholine, released by electrical stimulation of the cholinergic nerves. In this model, there should be taken in account that the PDE inhibitors might also act presynaptically on acetylcholine release from the cholinergic nerves, as stable analogues of cAMP as well as IBMX were shown to increase acetylcholine release from synaptosomes and from longitudinal muscle-myenteric plexus preparations of guinea pig small intestine (Reese and Cooper, 1984; Yau et al., 1987); correspondingly, neuronal cAMP was also shown to modulate acetylcholine release from airway cholinergic nerves (Zhang et al., 1996).

Two PDE inhibitors concentration-dependently inhibited EFS-induced cholinergic contractions in circular muscle of pig colon descendens: IBMX and the PDE3 selective inhibitor cilostamide. The concentration range where IBMX showed its concentration-dependent effect (1-30 µM) corresponds to the IC_{50} range of this non selective PDE inhibitor (2-50 µM; Beavo and Reifsnyder, 1990). None of the PDE subtype selective inhibitors mimicked the inhibitory effect of IBMX except for cilostamide, being about 100 times more potent than IBMX. Reported IC_{50} values for cilostamide at PDE3 include 0.005 and 0.064 µM (Elks and Manganiello, 1984; Beavo and Reifsnyder, 1990). In this concentration range (0.03 µM), cilostamide already inhibited EFS-induced cholinergic contractions by 75%.

These results illustrate that PDE3 is the main functionally important PDE in pig colon descendens circular muscle. The small inhibitory effect on cholinergic contractions, observed with the highest concentration of EHNA and rolipram tested (30 µM), which is 30 times higher than their reported K_i values at PDE2 and PDE4 respectively (Maurice et al., 2009), might indicate a very minor role of PDE2 and PDE4 in pig colonic muscle or might be related to a non-specific action at PDE3. The principal role of PDE3 in pig colon descendens circular muscle differs from our recent results in pig gastric circular muscle, where we observed a redundant role of PDE3 and PDE4 in controlling cyclic nucleotide levels with PDE3 being predominant (Priem et al., 2012). In dog colonic smooth muscle on the other hand, PDEs 1 to 5 were shown to be present using DEAE sepharose chromatography but functionally PDE4 was most important in regulating contractility in colonic smooth muscle, with a less pronounced role of PDE3 (Barnette et al., 1993). The selective PDE5 inhibitor zaprinast, which is able to concentration-dependently relax gastrointestinal smooth muscle (Barbier and Lefebvre, 1995; Kaneda et al., 2004), enhanced EFS-induced cholinergic contractions in pig colon descendens at concentrations of 3 to 30 µM. This result does not exclude that PDE5 might have some role in the control of colonic smooth muscle activity. In dog proximal colon, zaprinast abolished mechanical activity but in the presence of the NO synthesis inhibitor L-NAME, this inhibitory effect was greatly reduced (Ward et al., 1992). This shows that endogenous NO largely contributes to the basal cGMP levels in colonic smooth muscle. Our experiments performed in pig colon descendens were performed in the continuous presence of L-NAME, which will thus decrease the possible postsynaptic effects of zaprinast and facilitate the predominance of presynaptic effects of zaprinast in the cholinergic nerves. Indeed, both 8-bromo-cGMP and zaprinast were shown to enhance basal as well as electrically stimulated acetylcholine release in longitudinal muscle-myenteric plexus preparations in guinea pig ileum (Hebeiss and Kilbinger, 1996). A similar effect in pig colon descendens can thus explain the enhanced cholinergic contractions with zaprinast. A significant increase of the EFS-induced contractions in pig colon was also seen with 0.1 and 0.3 µM of the PDE4 inhibitor rolipram. Also in pig gastric muscle, rolipram tended to increase electrically induced acetylcholine release and cholinergic contraction, suggesting some basal control by PDE4 of acetylcholine release per se from cholinergic nerves (Priem et al., 2012).

5.V.2. Influence of PDE inhibitors on the effect of prucalopride on EFS-induced submaximal cholinergic contractions

The selective 5-HT$_4$ receptor agonist prucalopride (1 µM) systematically enhanced EFS-induced cholinergic submaximal contractions, confirming the presence of facilitating 5-HT$_4$ receptors on the cholinergic nerve endings in pig colon descendens circular muscle (Priem and Lefebvre, 2011). The maximal facilitating effect of prucalopride was generally reached at the 2nd stimulation train in its presence, and ranged between 20 to 40% (see **Fig. 5.3 and 5.4**), corresponding to the degree of the effect reported before (Priem and Lefebvre, 2011), except in 2 series where prucalopride alone enhanced the EFS-induced contractions with 50 and 61% (**Fig. 5.3B and 5.3E**). The degree of facilitation by prucalopride (20-40 %) in pig colon descendens circular muscle is less pronounced than its facilitating effect on half maximal cholinergic contractions in other species/GI regions where facilitation up to 185% has been reported (for figures and references, see Priem and Lefebvre, 2011); additionally in pig colon descendens the effect of prucalopride is less well maintained. One possible explanation is less effective coupling of the 5-HT$_4$ receptors. 5-HT$_4$ receptors are coupled to adenylate cyclase and we recently showed that even in pig gastric circular muscle, where prucalopride induced pronounced and sustained facilitation of cholinergic neurotransmission, the intracellular pathway of the 5-HT$_4$ receptors on the cholinergic nerves is regulated by PDE4 (Priem et al., 2012). In porcine left atrium, the 5-HT$_4$ receptor is under very tight control of PDE3 and PDE4, as prucalopride only has a very moderate and fading effect in the absence of both PDE3 and PDE4 inhibitors (De Maeyer et al., 2006b; Galindo-Tovar et al., 2009; Weninger et al., 2012). We therefore tested the influence of inhibitors of the PDEs that metabolize cAMP on the response to prucalopride in pig colon descendens, except for the PDE3 inhibitor cilostamide in view of its pronounced effect at the level of the muscle cells. Similar to pig gastric circular muscle, the PDE1 inhibitor vinpocetine and the PDE2 inhibitor EHNA did not influence the facilitating effect of prucalopride on cholinergic neurotransmission. Although the presence of PDE2A has been shown in enteric ganglia of stomach and colon (Stephenson et al., 2009), this PDE isoform is thus not involved in controlling 5-HT$_4$ receptors on enteric cholinergic nerves. Inhibition of PDE4 with rolipram (1 and 3 µM) tended to increase the effect of prucalopride, similar to what we reported in pig gastric circular muscle; in the latter tissue, the regulating role of PDE4 in the signal transduction of the 5-HT$_4$ receptors on the cholinergic nerves was definitely established by measuring acetylcholine release directly (Priem et al., 2012). In pig colon descendens, this was shown in the experiments where rolipram was added after prucalopride.

In a concentration (3 µM) that per se did not influence EFS-induced contractions (see **Fig. 5.2B and 5.3F**), rolipram added after prucalopride induced a clear-cut further increase in EFS-induced contractions (see **Fig. 5.4**). Although rolipram was not able to sustain the contractions at this enhanced level, responses in the presence of prucalopride and rolipram were enhanced versus those with prucalopride alone over the observed period. In pig gastric circular muscle, the PDE-mediated control of the 5-HT_4 receptors on the cholinergic nerves was also picked up with the non-selective PDE inhibitor IBMX. This was not the case in pig colon, which might be related to the fact that in this tissue the postsynaptic muscular effects of IBMX, which will counteract the presynaptic ones, started at 3 fold lower concentrations than in pig stomach.

5.VI. Conclusion

These results show that in pig colon descendens circular muscle, PDE3 is the main regulator of muscle activity at least in conditions where NO synthesis is inhibited. Similarly as in pig gastric circular muscle, the 5-HT_4 receptors on the colonic cholinergic nerves are under the regulatory influence of PDE4. Combination of a 5-HT_4 receptor agonist and a PDE4 inhibitor will thus lead to more pronounced kinetic effects at the level of pig stomach and colon. When confirmed in humans, this might form an alternative therapeutic possibility for conditions with slowed gastrointestinal transit to the combination of a 5-HT_4 receptor agonist with an acetylcholinesterase inhibitor, which was recently also proposed as a therapeutic possibility (Cellek at al., 2008; Campbell-Dittmeyer et al., 2009). PDE4 inhibitors are examined for chronic respiratory diseases and the PDE4 inhibitor roflumilast is approved for use in patients with severe COPD (Michalski et al., 2012). PDE4 inhibition will not increase the chance of cardiac effects by the 5-HT_4 receptor agonist as the 5-HT_4 receptor in human heart is under the sole control of PDE3 (Galindo-Tovar et al., 2009).

5.VII. References

Barbier AJ, Lefebvre RA. Relaxant influence of phosphodiesterase inhibitors in the cat gastric fundus. Eur. J. Pharmacol. 1995; 276: 41-47

Barnette MS, Manning CD, Price WJ, Barone FC. Initial biochemical and functional characterization of cyclic nucleotide phosphodiesterase isozymes in canine colonic smooth muscle. J Pharmacol Exp Ther. 1993; 264: 801-812

Beavo JA, Reifsnyder DH. Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Trends. Pharmacol. Sci. 1990; 11: 150-155

Bouras EP, Camilleri M, Burton DD, Thomforde G, McKinzie S, Zinsmeister AR. Prucalopride accelerates gastrointestinal and colonic transit in patients with constipation without a rectal evacuation disorder. Gastroenterology 2001; 120: 354-360

Campbell-Dittmeyer K, Hicks GA, Earnest DL, Greenwood-van Meerveld B. Increased colonic transit in rats produced by a combination of a cholinesterase inhibitor with a 5-HT4 receptor agonist. Neurogastroenterol Motil. 2009; 21: 1197-1108

Cellek S, Thangiah R, Jarvie EM, Vivekanandan S, Lalude O, Sanger GJ. Synergy between 5-HT4 receptor activation and acetylcholinesterase inhibition in human colon and rat forestomach. Neurogastroenterol. Motil. 2008; 20: 539-545

Coskran TM, Morton D, Menniti FS, Adamowicz WO, Kleiman RJ, Ryan AM, Strick CA, Schmidt CJ, Stephenson DT. Immunohistochemical localization of phosphodiesterase 10A in multiple mammalian species. J. Histochem. Cytochem. 2006; 54: 1205-1213

De Maeyer JH, Prins NH, Schuurkes JA, Lefebvre RA. Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. J. Pharmacol. Exp. Ther. 2006a; 317: 955-964

De Maeyer JH, Straetemans R, Schuurkes JA, Lefebvre RA. Porcine left atrial and sinoatrial 5-HT(4) receptor-induced responses: fading of the response and influence of development. Br J Pharmacol. 2006b; 147: 140-157

Dutta U, Padhy AK, Ahuja V, Sharma MP. Double blind controlled trial of effect of cisapride on gastric emptying in diabetics. Trop. Gastroenterol. 1999; 20: 116-119

Elks ML, Manganiello VC. Selective effects of phosphodiesterase inhibitors on different phosphodiesterases, adenosine 3',5'-monophosphate metabolism, and lipolysis in 3T3-L1 adipocytes. Endocrinology 1984; 115: 1262-1268

Galindo-Tovar A, Vargas ML, Escudero E, Kaumann AJ. Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT(4) receptors. Br J Pharmacol. 2009; 156: 237-249

Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 2007; 132: 397-414

Hebeiss K, Kilbinger H. Differential effects of nitric oxide donors on basal and electrically evoked release of acetylcholine from guinea-pig myenteric neurones. Br. J. Pharmacol. 1996; 118: 2073-2078

Jones OM, Brading AF, McC Mortensen NJ. Phosphodiesterase inhibitors cause relaxation of the internal anal sphincter in vitro. Dis. Colon Rectum. 2002; 45: 530-536

Kaneda T, Shimizu K, Nakajyo S, Urakawa N. Effects of various selective phosphodiesterase inhibitors on muscle contractility in guinea pig ileal longitudinal smooth muscle. Jpn. J. Pharmacol. 1997; 75: 77-85

Kaneda T, Shimizu K, Urakawa N, Nakajyo S. Effects of various selective phosphodiesterase inhibitors on carbachol-induced contraction and cyclic nucleotide contents in guinea pig taenia coli. J. Vet. Med. Sci. 2004; 66: 1047-1052

Kendall BJ, Kendall ET, Soykan I, McCallum RW. Cisapride in the long-term treatment of chronic gastroparesis: a 2-year open-label study. J. Int. Med. Res. 1997; 25: 182-189

Kleppisch T. Phosphodiesterases in the central nervous system. Handb. Exp. Pharmacol. 2009; 191: 71-92

Leclere PG, Lefebvre RA. Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. Br. J. Pharmacol. 2002; 135: 135-142

Leclere PG, Prins NH, Schuurkes JAJ, Lefebvre RA. 5-HT_4 receptors located on cholinergic nerves in human colon circular muscle. Neurogastroenterol. Motil. 2005; 17: 366-375

Maurice DH, Palmer D, Tilley DG, Dunkerley HA, Netherton SJ, Raymond DR, Elbatarny HS, Jimmo SL. Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. Mol. Pharmacol. 2003; 64: 533-546

Michalski JM, Golden G, Ikari J, Rennard SI. PDE4: a novel target in the treatment of chronic obstructive pulmonary disease. Clin. Pharmacol. Ther. 2012; 91: 134-142

Murthy KS. Activation of phosphodiesterase 5 and inhibition of guanylate cyclase by cGMP-dependent protein kinase in smooth muscle. Biochem. J. 2001; 360: 199-208

Murthy KS, Zhou H, Makhlouf GM. PKA-dependent activation of PDE3A and PDE4 and inhibition of adenylyl cyclase V/VI in smooth muscle. Am. J. Physiol. Cell. Physiol. 2002; 282: C508-C517

Raymond JR, Mukhin YV, Gelasco A, Turner J, Collinsworth G, Gettys TW, Grewal JS, Garnovskaya MN. Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacol. Ther. 2001; 92: 179-212

Priem EKV, Lefebvre RA. Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT4 receptors in circular smooth muscle of pig descending colon. Eur. J. Pharm. 2011; 667: 365-374

Priem E, Van Colen I, De Maeyer JH, Lefebvre RA. The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric muscle is regulated by phosphodiesterase 4. Neuropharmacology 2012; 62: 2126-2135

Prins NH, Akkermans LMA, Lefebvre RA, Schuurkes JAJ. 5-HT_4 receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. Br. J. Pharmacol. 2000; 131: 927-932

Quigley EM. Prucalopride: safety, efficacy and potential applications. Ther. Adv. Gastroenterol. 2012; 5: 23-30

Reese JH, Cooper JR. Stimulation of acetylcholine release from guinea-pig ileal synaptosomes by cyclic nucleotides and forskolin. Biochem. Pharmacol. 1984; 33: 3007-3011

Stephenson DT, Coskran TM, Wilhelms MB, Adamowicz WO, O'Donnell MM, Muravnick KB, Menniti FS, Kleiman RJ, Morton D. Immunohistochemical localization of phosphodiesterase 2A in multiple mammalian species. J. Histochem. Cytochem. 2009; 57: 933-949

Tomkinson A, Raeburn D. The effect of isoenzyme-selective PDE inhibitors on methacholine-induced contraction of guinea-pig and rat ileum. Br. J. Pharmacol. 1996; 118: 2131-2139

Ward SM, Dalziel HH, Bradley ME, Buxton IL, Keef K, Westfall DP, Sanders KM. Involvement of cyclic GMP in non-adrenergic, non-cholinergic inhibitory neurotransmission in dog proximal colon. Br. J. Pharmacol. 1992; 107: 1075-1082

Weninger S, De Maeyer JH, Lefebvre RA. Study of the regulation of the inotropic response to 5-HT(4) receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium. Naunyn Schmiedebergs Arch. Pharmacol. 2012; 385: 565-577

Yau WM, Dorsett JA, Youther ML. Stimulation of acetylcholine release from myenteric neurons of guinea pig small intestine by forskolin and cyclic AMP. J. Pharmacol. Exp. Ther. 1987; 243: 507-510

Zhang XY, Robinson NE, Zhu FX. Potentiation of acetylcholine release from tracheal parasympathetic nerves by cAMP. Am. J. Physiol. 1996; 270: L541-L546

Chapter 6

PREDOMINANT MUCOSAL EXPRESSION OF 5-HT$_{4(+h)}$ RECEPTOR SPLICE VARIANTS IN PIG STOMACH AND COLON

Evelien K.V. Priem[a], Joris H. De Maeyer[b], Mado Vandewoestyne[c], Dieter Deforce[c] and Romain A. Lefebvre[a]

[a]Heymans Institute of Pharmacology, Ghent University, Gent, Belgium

[b]Shire-Movetis NV, Turnhout, Belgium

[c]Laboratory for Pharmaceutical Biotechnology, Ghent University, Ghent, Belgium

Based on a manuscript, accepted in World Journal of Gastroenterology

Chapter 6 Predominant mucosal expression of 5-HT$_{4(+h)}$ receptor splice variants in pig stomach and colon

6.I. Abstract

AIM: Investigating cellular 5-HT$_{4(-h/+h)}$ receptor distribution , with attention to the epithelial layer, by laser microdissection and PCR in porcine gastrointestinal tissues.

METHODS: A stepwise approach was used to evaluate RNA quality and to study cell-specific 5-HT$_4$ receptor mRNA expression in porcine gastric fundus and colon descendens. After freezing, staining, laser microdissection and pressure catapulting (LMPC), RNA quality was evaluated by the Experion automated electrophoresis system. 5-HT$_4$ receptor and GAPDH expression was examined by endpoint RT-PCR in mucosal and muscle-myenteric plexus (MMP) tissue fractions, in mucosal and MMP parts of hematoxylin&eosin (H&E) stained tissue sections and in microdissected patches of the epithelial and circular smooth muscle cell layer in these sections. In pig gastric fundus tissue sections were also stained immunohistochemically (IHC) for enterochromaffin cells (EC cells; MAB352); these cells were isolated by LMPC and examined by endpoint RT-PCR.

RESULTS: After H&E staining, the epithelial and circular smooth muscle cell layer of pig colon descendens and the epithelial cell layer of gastric fundus were morphologically identified and isolated by LMPC. EC cells of pig gastric fundus were successfully stained by IHC and isolated by LMPC. Freezing, H&E and IHC staining, and LMPC had no influence on RNA quality. 5-HT$_4$ receptor and GAPDH mRNA expression was detected in mucosa and MMP tissue fractions, and in mucosal and MMP parts of H&E stained tissue sections of pig colon descendens and gastric fundus. In the mucosa tissue fractions of both gastrointestinal regions, the expression of h-exon containing receptor (5-HT$_{4(+h)}$ receptor) mRNA was significantly higher ($p < 0.01$) compared to 5-HT$_{4(-h)}$ receptor expression, and a similar trend was obtained in the mucosal part of H&E stained tissue sections. Large microdissected patches of the epithelial and circular smooth muscle cell layer of pig colon descendens and of the epithelial cell layer of pig gastric fundus, also showed 5-HT$_4$ receptor and GAPDH mRNA expression. No 5-HT$_4$ receptor mRNA expression was detected in gastric LMPC-isolated EC cells from IHC stained tissues, while the cells were positive for GAPDH.

CONCLUSION: Porcine gastrointestinal mucosa predominantly expresses 5-HT$_{4(+h)}$ receptor splice variants, suggesting their contribution to 5-HT$_4$ receptor-mediated mucosal effects of 5-HT.

6.II. Introduction

The 5-HT$_4$ receptor is a G-protein coupled receptor (GPCR) which activates the adenylyl cyclase/cyclic adenosine monophosphate/protein kinase A pathway in response to serotonin (5-HT). The 5-HT$_4$ receptor is expressed on excitatory motor neurons in the gut, facilitating acetylcholine release hereby stimulating gastrointestinal (GI) motility (Gershon and Tack, 2007; Liu et al., 2005; Ren et al., 2008). This presynaptic facilitation is thought to be the principal mechanism for the prokinetic action of 5-HT$_4$ receptor agonists, explaining their therapeutic use in GI dysmotility related disorders such as chronic constipation, gastroparesis and gastroesophageal reflux disease (Manabe et al., 2010). The selective 5-HT$_4$ receptor agonist prucalopride is now used in patients with chronic laxative-resistant constipation; it indeed facilitates acetylcholine release from cholinergic neurons towards human colonic circular (Leclere et al., 2005) as well as longitudinal (Prins et al., 2000) smooth muscle. The non-selective 5-HT$_4$ receptor agonist cisapride was, until it was withdrawn because of non-specific cardiac side effects, used for increasing gastric emptying in patients with gastroparesis (Dutta et al., 1999). Also prucalopride accelerates gastric emptying in man (Bouras et al., 2001), corresponding with its facilitating effect on acetylcholine release from cholinergic nerves towards human gastric circular muscle (Leclere and Lefebvre, 2002). Our group has previously shown that the pig is a good model for human 5-HT$_4$ receptors on GI cholinergic neurons, as the presence of facilitatory 5-HT$_4$ receptors on cholinergic neurons innervating pig gastric circular (Priem et al., 2012) and longitudinal (De Maeyer et al., 2006) muscle and colonic circular muscle (Priem and Lefebvre, 2011) was illustrated in functional assays.

However apart from cholinergic neurons, other locations for the 5-HT$_4$ receptor in colon and stomach have been proposed. In the human colon, 5-HT$_4$ receptors were reported to be present on circular smooth muscle cells, inducing relaxation (McLean et al., 1995). A functional study by Borman and Burleigh (1996) reported that 5-HT-induced secretion in human sigmoid colon is mediated via 5-HT$_{2A}$ receptors but that in the ascending colon a combination of 5-HT$_{2A}$ and 5-HT$_4$ receptors appears to be involved. Still a recent study showed the presence of mRNA of several 5-HT$_4$ receptor splice variants in the mucosal layer of human sigmoid colon (Chetty et al., 2009); 5-HT$_4$ receptor mRNA was also reported in pig colonic mucosa (De Maeyer et al., 2008). In rat colon, it has been suggested that 5-HT-induced mucosal ion transport and Cl^- secretion is mediated by 5-HT$_4$ receptors (Albuquerque et al., 1998; Budhoo and Kellum, 1994; Budhoo et al., 1996; Ning et al., 2004). Immunohistochemical and functional assays showed the presence of 5-HT$_4$ receptors in mouse colonic epithelial cells, including enterochromaffin cells and goblet cells, inducing mucosal 5-HT release and Cl^- secretion (Ning et al., 2004).

The presence of 5-HT$_4$ receptor transcripts detected by RT-PCR has also been reported in the mucosa of human (Hoffman et al., 2012; van Lelyveld et al., 2007) and pig (De Maeyer et al., 2008) stomach, but cellular distribution within the epithelial layer has not yet been investigated. More detailed information on the expression and localization of GPCRs, with special attention to the 5-HT$_4$ receptor, is needed in human enteric neuronal subpopulations, mast cells and epithelial cells leading to a better understanding of function and activity of 5-HT$_4$ receptors in the GI wall, which may offer new therapeutic perspectives (van Nassauw and Timmermans, 2010). To date, the majority of information on 5-HT$_4$ receptor distribution is based on functional studies (Priem and Lefebvre, 2011) or on 5-HT$_4$ receptor expression studies using homogenates of tissues (Chetty et al., 2009; De Maeyer et al., 2008; Hoffman et al., 2012; van Lelyveld et al., 2007). However, homogenates of tissues limit the potential of expression studies, since important cell-specific transcript information is lost because of the heterogeneity of tissues such as GI tissues. Techniques have been developed to enable collection of particular cells from mixed populations which generally involve either fluorescence activated cell sorting (FACS) purification of dissociated cells or laser-assisted microdissection. In contrast to FACS, microdissection can be applied to most tissues (Brown and Smith, 2009) and laser microdissection has already been used in previously reported gene expression studies to investigate site-specific gene expression: In laser microdissected enteric ganglia of the human intestine, 5-HT$_{3A}$ receptor mRNA expression was described (Böttner et al., 2010) and in microdissected human colonic mucosal epithelium, transcripts encoding for 5-HT$_{3A}$, 5-HT$_{3C}$, 5-HT$_{3D}$ and 5-HT$_{3E}$ subunits were detected (Kapeller et al., 2011). Therefore, the aim of the present study was to develop and validate an experimental protocol for the assessment of 5-HT$_4$ receptor distribution at the cellular level in laser microdissected porcine GI tissues with special attention for the mucosal layer of pig colon descendens and gastric fundus.

6.III. Methods

6.III.1. Tissue preparation and tissue processing

Young male pigs (10-12 weeks, 15-25 kg – breed Line 36) were obtained from Rattlerow Seghers, Belgium. All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.
The pigs were anaesthetized with an intramuscular injection of 5 ml Zoletil 100 (containing 50 mg/ml tiletamine and 50 mg/ml zolazepam; Virbac Belgium S.A., Belgium). After exsanguination, the stomach and the colon descendens, prelevated 10 cm above the anus to the transverse colon, were removed and thoroughly washed in ice cold aerated (5% CO_2/95% O_2) phosphate buffered saline (PBS) at pH 7.4 (Life Technologies Europe, Belgium). The gastric fundus was cut open along the lesser curvature and small pieces of tissue were cut in the direction of the circular muscle layer from the ventral side. The colon descendens was opened along the mesenteric border, fat tissue was removed and tissues were cut in the direction of the circular muscle layer.

6.III.1.1. Freezing tissue fractions for direct RNA processing

The gastrointestinal tissues were divided by blunt dissection into a mucosal-submucosal (mucosa) fraction and a muscular-myenteric plexus (MMP) fraction. The fractions were cut into small pieces, put in a RNase-free vial (Life Technologies Europe, Belgium), rapidly frozen in liquid N_2 and stored at -80°C. After frozen tissue homogenization and before RNA extraction, MMP samples were treated with proteinase K (Qiagen, Belgium) to increase the total RNA output. Proteinase K removes proteins such as the contractile proteins, connective tissue and collagen, which define a fibrous tissue such as the smooth muscle layer (Rneasy fibrous tissue handbook, Qiagen, Belgium). RNA from mucosa and MMP fractions was extracted using the RNeasy Mini Kit (Qiagen, Belgium) according to manufacturer's guidelines and RNA samples were stored at -80°C.

6.III.1.2. Freezing tissues for section preparation and laser microdissection

Whole tissues, containing the mucosal as well as the smooth muscle layer, were cut into full-thickness small pieces with a sterile scalpel, placed in tissue embedding medium PELCO CryO-Z-T (Pelco International, U.S.A.), rapidly frozen in liquid N_2 containing cold isopentane and stored at -80°C. The frozen tissue samples were cut into 8 µm-thick sections using a cryostat (Leica CM 1950; Leica Microsystems, Belgium) with disposable RNase-free knifes. Sections at 8 µM thickness are considered to represent a monolayer of cells (Bevilacqua et al., 2010; Pinzani et al., 2006). The sections were placed on chilled (-20°C) nuclease free polyethylene naphthalate (PEN)-covered membrane slides (Carl Zeiss, Germany) and immediately stored at -80°C until staining procedure. The membrane slides used for immunohistochemistry were extra coated with poly-L-Lysine (Sigma, Belgium), which was diluted with 0.1% diethylpyrocarbonate (DEPC)-treated water. All materials (pincets, brushes, etc.) were treated with RNase ZAP (Sigma, Belgium) and glassware and pincets were heated for 6 h at 200°C, to remove all exogenous RNases.

6.III.2. Staining

6.III.2.1. Hematoxylin-Eosin staining

To morphologically distinguish the different layers of the tissue sections for laser microdissection, the frozen tissue sections were stained with hematoxylin and eosin (Sigma, Belgium) in RNase-free conditions. Hematoxylin-eosin (H&E) staining started with fixing the slides in 70% ethanol for 1 min, followed by dipping the slides for 15 sec in DEPC-treated water to remove PELCO CryO-Z-T embedding medium. Hematoxylin staining was carried out by placing the slides for 1 min in the hematoxylin solution (0.1%), followed by dipping the slides for 15 sec in DEPC-treated water and 15 sec in 70% ethanol. Slides were then placed for 1 min in eosin solution (0.25%), followed by dehydrating the slides for 15 sec in the following order: DEPC-treated water, 70% ethanol, 100% ethanol. The staining procedure was finished with a 3 min xylene treatment and the slides were air dried for 10 min at room temperature before scraping off the whole tissue section, or the mucosal and MMP part of the tissue section separately, or applying laser microdissection. Staining solutions based on ethanol and xylene were pre-cooled at -20°C; aqueous solutions were pre-cooled at 4°C. All solutions were diluted with 0.1% DEPC-treated water, kept in 50 mL RNase-free conical tubes (Life Technologies Europe, Belgium) and kept on ice during the staining procedure.

6.III.2.2. Immunohistochemistry (IHC)

To distinguish and isolate enterochromaffin (EC) cells with the laser microdissection and pressure catapulting technique (LMPC), visualization with cell-specific antibodies of these cells is needed. To extract integer RNA of the cell samples, an IHC protocol under RNase-free conditions was developed according to the staining procedure reported by Brown and Smith (2009). Cryosections were 15 sec rinsed with cold (4°C) PBS (pH 7.4; Life Technologies Europe, Belgium) and then 5 min fixed in ice-cold (-20°C) aceton. Aceton was removed by a cold PBS rinse (15 s) and slides were incubated for 30 min at 4°C with blocking buffer (0.25% Triton X-100, 1% bovine serum albumin, 10% goat serum) supplemented with 1M NaCl. Then, sections were shortly rinsed with cold PBS and incubated overnight at 4°C with the rat anti-serotonin primary antibody MAB352 (Milipore, Belgium), which was diluted 1:200 in PBS supplemented with 1M NaCl. Unbound primary antibody was removed by rinsing 3 times with cold PBS supplemented with 1M NaCl. Sections were then incubated with chicken anti-rat secondary antibody Alexa Fluor 488 (Life Technologies, Belgium) diluted 1:100 in PBS with 1M NaCl for 2 h at 4°C. Unbound secondary antibody was removed by rinsing 3 times with cold PBS with 1M NaCl and excess NaCl was removed by a PBS rinse (5 sec). Sections were dehydrated in 70% and then 100% ethanol (3 min each) and air dried for 10 min at room temperature prior to laser microdissection.

6.III.3. Laser Microdissection and pressure catapulting (LMPC)

LMPC was performed using the laser microdissection system from PALM Technologies (Carl Zeiss, Germany) containing a PALM Microbeam, RoboStage and a PALM RoboMover [PALM RoboSoftware version 4]. Under direct microscopic visualization, LMPC permits procurement of histologically or immunohistologically defined tissue and cell samples (**Fig. 6.1**). Approximately 15 large patches of cells from the epithelium or circular smooth muscle layer in H&E stained sections, or 70 EC cells in IHC stained sections were laser-dissected and pressure-catapulted in 50 µL RLT lysis buffer (RNeasy kit, Qiagen, Belgium). The cell collecting time was limited to 2 h per slide and after 2 h of cell sampling, the remaining tissue on the membrane slide was scraped off and RNA was extracted to determine if RNA integrity was still preserved after 2 h. The samples were homogenized by vortexing, spinned down and then placed at -80°C for later use. 7 EC cell collections were pooled to 1 sample with a final volume of 350 µL, resulting in a collection of approximately 500 cells per sample. Total RNA from the cell samples was extracted using the RNeasy Micro kit (Qiagen, Belgium) according to the manufacturer's instructions.

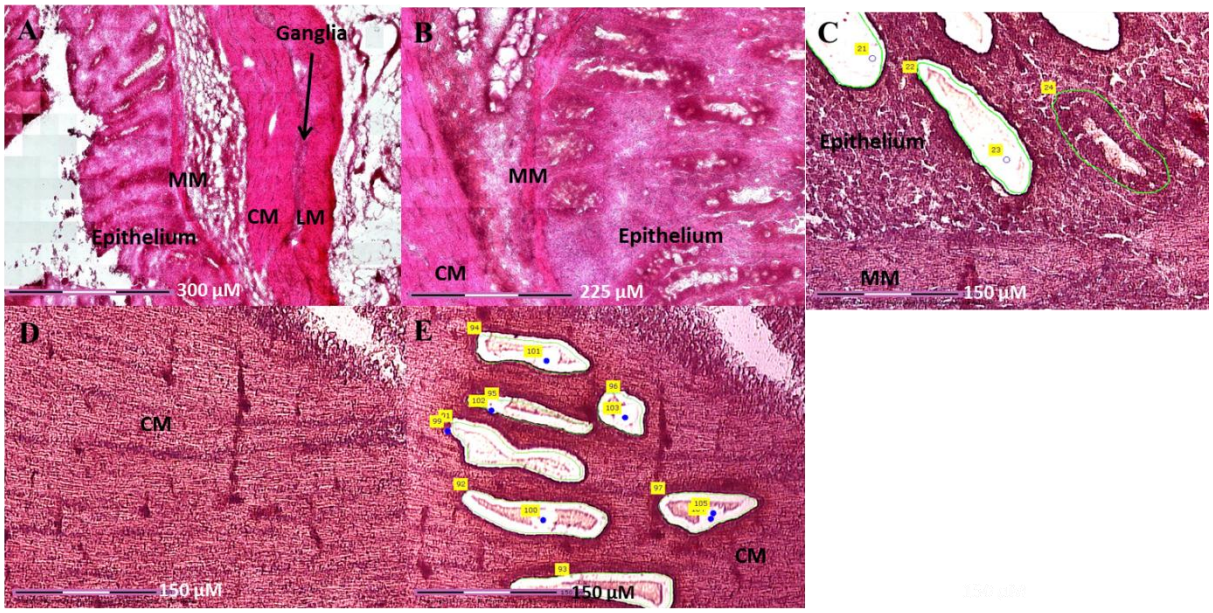


Figure 6.1

Photomicrographs of H&E stained tissue sections of colon descendens: epithelium, muscularis mucosae (MM), circular muscle layer (CM), longitudinal muscle layer (LM) and ganglion. (A) Overview of all layers in colon descendens; (B) Detail of the epithelium; (C) Epithelium with large patches microdissected by LMPC; (D) Details of CM; (E) CM with large patches of smooth muscle cells microdissected by LMPC.

6.III.4. Endpoint RT-PCR

Quantification of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Isogen Life Science, Belgium) and the quality of RNA extracted from tissue fractions and tissue sections was assessed by using the Experion automated electrophoresis system (BioRad, Belgium).

cDNA of tissue fractions was prepared from 1 µg total RNA, whereas cDNA of whole tissue sections, parts of tissue sections and LMPC samples was prepared from the maximal input of total RNA as possible, as the amount of total RNA was less than 1 µg. The transfer of sample RNA to cDNA by reverse transcriptase (RT) was carried out according to manufacturer's instructions using SuperScript III Reverse Transcriptase SuperMix (Life Technologies Europe, Belgium) containing random hexamers and oligo(dT)$_{20}$. The obtained cDNA was stored at -20°C before PCR. cDNA amplification reactions were carried out using the AccuPrime *Pfx* SuperMix (Life Technologies Europe, Belgium).

The template cDNA of mucosa and MMP tissue fractions for amplification was diluted 1:10. Expression of the 5-HT_4 receptor within the samples was analysed using 5-HT_4 receptor-specific primers spanning exon-intron-exon junctions: an exon 4-specific forward primer and an exon 5-specific reverse primer (**Fig. 6.2B**). These primers will detect alternative splicing of the h-exon because the h-exon is located between exon 4 and exon 5. The quality of cDNA produced was assessed by amplifying cDNA for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To amplify cDNA of tissue fractions and tissue sections, PCR reactions were performed using the following protocol: 5 min at 95°C followed by 36 cycles with annealing temperature of 54°C. Because of the low RNA output of LMPC samples, PCR reactions to amplify the cDNA for both 5-HT_4 receptor and GAPDH, were performed using 2 rounds of PCR (Chetty et al., 2009), according to the following protocol: a first reaction with 5 min at 95°C followed by 36 cycles at 54°C annealing temperature was performed, followed by a second reaction using 1.5 µL of the product of the first reaction as a template for a second round of PCR (5 min at 95°C followed by 36 cycles) with the same primers but with a higher annealing temperature of 56°C to increase specificity. RT and endpoint PCR reactions were run on a C1000 Thermal Cycler (BioRad, Belgium). PCR products were separated by 2% agarose gel electrophoresis and visualised by ethidium bromide staining. The primers (Eurogentec, Belgium) used were previously published by De Maeyer et al. (2008) and are listed as follows:

5-HT_4R forward primer (5'-ACAGGAACAAGATGACCCCT-3'),

5-HT_4R reverse primer (5'-AGGAGGAACGGGATGTAGAA-3'),

GAPDH forward primer (5'-ACCACAGTCCATGCCATCAC-3'),

GAPDH reverse primer (5'-TCCACCCTGTTGCTGTA-3').

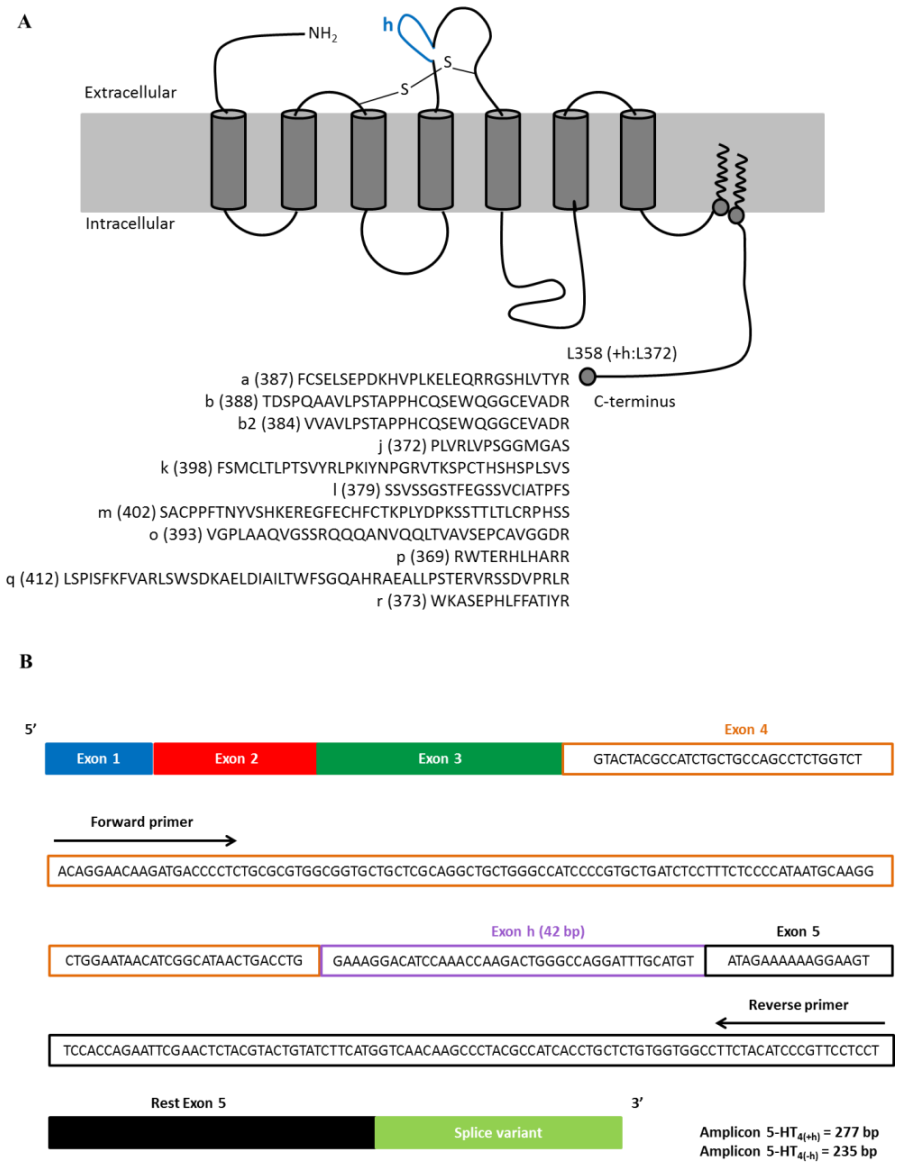


Figure 6.2

Schematic representation of:

A) the porcine 5-HT$_4$ receptor splice variants. The porcine 5-HT$_4$ receptor variants have identical sequences up to Leu^{358}, or Leu^{372} when exon h is included, and differ by the length and composition of their C-terminal domain. The presence of the h sequence of 14 amino acids in the second extracellular loop depends on the all or none inclusion of exon h between exon 4 and 5.

B) cDNA of the pig 5-HT$_{4(hb)}$ receptor based on gene browsing (transcript ID: ENSSSCT00000015770 on http://www.ensembl.org/Sus_scrofa/Gene). The positions of the boundaries in between exons and the positions of primers used in this study are indicated. The primers will detect all 5-HT$_4$ receptor splice variants. Amplification will result in a 277 bp amplicon, when containing the 42 bp h-exon or a 235 bp amplicon, not containing the h-exon.

6.III.5. Data analysis and statistics

Semi-quantification of PCR products was determined by the intensity of PCR bands on the agarose gels using Image J 1.45 software. Band intensity was expressed as relative absorbance units and the background of the image was determined and subtracted from the gel image. The ratio between the 5-HT_4 receptor and GAPDH RNA was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Data presented are means ± standard error of the mean for *n* animals. Statistical analyses were performed using Graphpad Prism software v.5.01 (U.S.A.). Differences in intensity were determined by an unpaired *t* test; $P < 0.05$ was considered statistically significant.

6.IV. Results

6.IV.1. Evaluating cell-specific visualization and RNA integrity

The main difficulties, when using LMPC to analyse gene expression of a specific cell type, are first to efficiently and selectively isolate the desired cells and second to obtain RNA of good quality. Therefore optimization of the LMPC experimental design was needed for the pig GI tissues, which are highly heterogeneous and rich in endogenous RNase and enzymes. First, to select the desired cells from the heterogeneous GI tissues, a good visualization of the tissue layers and cells under direct microscopy is necessary. This requires preserved morphology of the tissue, as fractures and air bubbles within the specimen will hamper the view. Tissues for section preparation and LMPC were therefore frozen in liquid N_2 containing isopentane. After H&E staining, the different layers of colon descendens (**Fig. 6.1**) and gastric fundus (not shown) could be identified based on their morphological characteristics. After cell-specific IHC staining, EC cells (MAB352; **Fig. 6.3**) could be identified in the gastric fundus and isolated by LMPC.

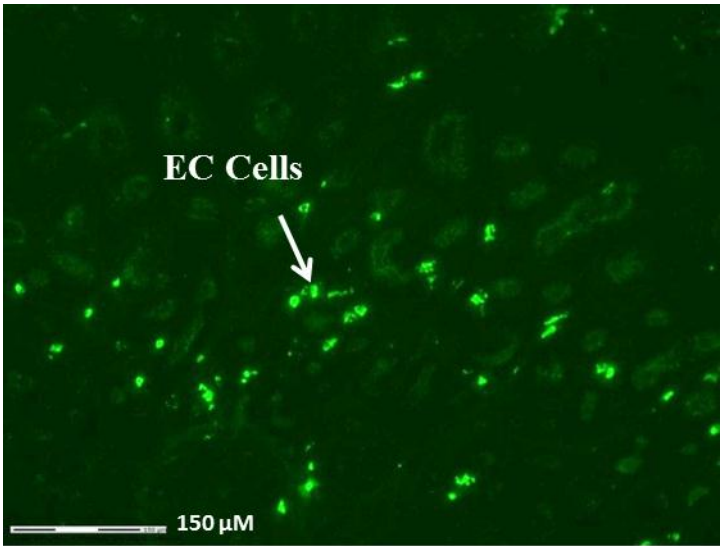


Figure 6.3

Photomicrographs of immunohistologically stained 8 µm sections of pig gastric fundus showingMAB352 (1:200) immunofluorescent enterochromaffin cells (EC) in the epithelium (20X).

To evaluate the impact of the different protocol steps on RNA integrity, a systematic approach was followed by evaluating RNA yield and quality after each protocol step. RNA quality was assessed by comparing 28S and 18 S and pre-18S ribosomal peaks to a set of degradation standards using the Experion automated electrophoresis system, the RNA quality indicator (RQI) returning a number between 10 (intact RNA) and 1 (highly degraded RNA) (Denisov et al., 2008). The analysis showed that RNA quality was not affected after tissue fraction collection (**Fig. 6.4A**), H&E staining (**Fig. 6.4B**), LMPC (**Fig. 6.4C**) or after IHC staining (**Fig. 6.4D**). However, electropherograms of RNA collected by LMPC could not systematically be analysed due to a too low amount of RNA collected. In **Fig. 6.4C**, RNA quality from large microdissected patches of either epithelial or smooth muscle cells is shown, indicating that RNA was still mainly intact but the small ribosomal RNA peaks (18S/28S) indicate a low amount of RNA.

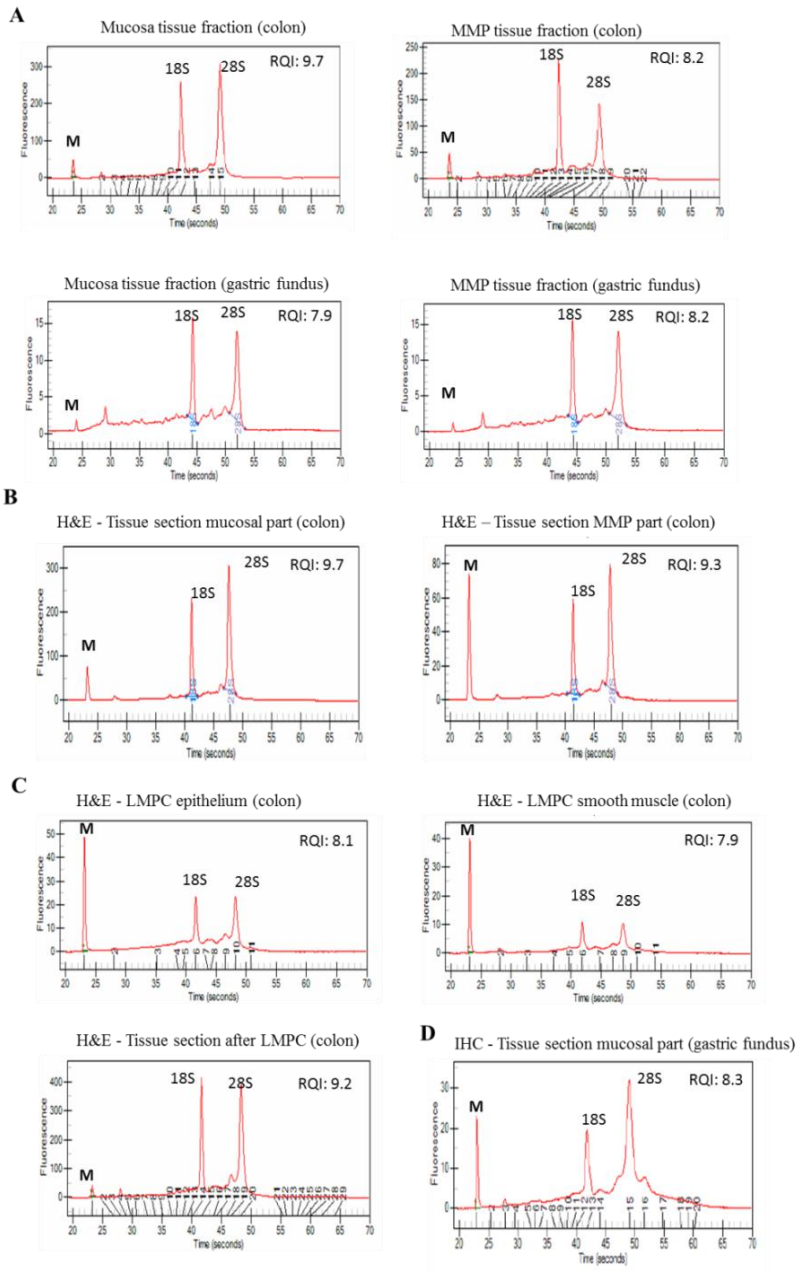


Figure 6.4

Representative Experion electropherograms of RNA collected from (A) mucosa and MMP tissue fractions of colon descendens and gastric fundus; (B) the mucosal part and the MMP part of H&E stained tissue sections of colon descendens; (C) large patches of epithelial cells and smooth muscle cells obtained by LMPC from H&E stained tissue sections and from the whole H&E stained tissue section scraped off after LMPC in colon descendens; (D) the mucosal part of an immunohistochemically (IHC) stained tissue section of gastric fundus. Electropherograms show fluorescence [ordinate] vs. time [abscissa] with RNA Quality Indicator (RQI) values. Positions of 18S and 28S ribosomal RNA and marker (M) peaks are indicated.

6.IV.2. Expression of the 5-HT$_4$ receptor in porcine tissue fractions

Tissue samples from pig colon descendens and gastric fundus were dissected into the mucosal-submucosal (mucosa) fraction and the muscular-myenteric plexus (MMP) fraction before freezing in liquid N_2. After RNA extraction and endpoint PCR analysis of these fractions, 5-HT$_4$ receptor expression was detected in mucosa as well as MMP fractions of colon descendens and gastric fundus (**Fig. 6.5**). All tissue samples were positive for the GAPDH housekeeping gene, confirming the integrity of the samples and completed PCR reactions. In all samples 5-HT$_4$ receptors containing the h-exon (277 bp; 5-HT$_{4(+h)}$ receptor) as well as 5-HT$_4$ receptors without h-exon (235 bp; 5-HT$_{4(-h)}$ receptor) were present (**Fig. 6.5**).

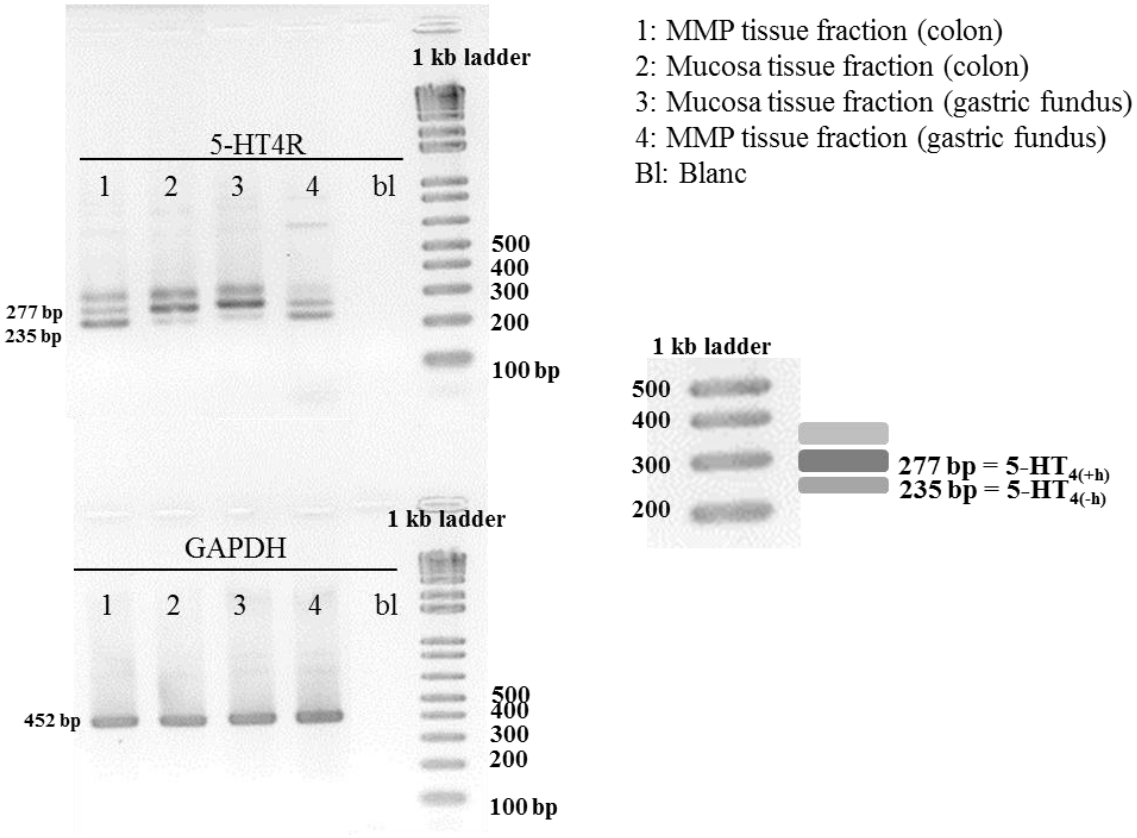


Figure 6.5

Invert color image of endpoint PCR analysis of the 5-HT$_4$ receptor and of the GAPDH housekeeping gene expressed in mucosa and MMP tissue fractions of pig colon descendens and gastric fundus. Dominant expression of 5-HT$_{4(+h)}$ receptor in the mucosa of the colon descendens and gastric fundus is observed. Part of the ladder is increased to indicate the size of the expected PCR products. A third unknown upper band is shown above the 277 bp band, due to dimerization of the PCR product with other PCR fragments after the PCR reaction.

However, a third extra band, corresponding to a fragment of more than 300 bp was also observed. Therefore, we isolated this unknown PCR band (QIAquick Gel extraction kit, Qiagen, Belgium) and determined the DNA sequence of this band (ABI3130XL sequencer, Life Technologies, Belgium). After sequence analysis, we aligned the unknown sequence with the 5-$HT_{4(+h)}$ and 5-$HT_{4(-h)}$ receptor sequence and observed that the unknown band contained the same sequence and length as the 5-$HT_{4(+h)}$ receptor, but chromatogram details suggested the presence of additional nucleotides in the tail of the sequence, possibly due to formation of a heteroduplex with a 5-$HT_{4(+h)}$ strand and a 5-$HT_{4(-h)}$ strand, or formation of a triplex with other PCR fragments, resulting in a different electrophoresis separation.

Mucosa fractions of colon descendens and gastric fundus contained relatively more h-exon containing 5-HT_4 receptor. Semi-quantification by expressing the intensity of the bands towards the housekeeping gene GAPDH and statistical analysis (**Fig. 6.6**), confirmed the significantly ($p < 0.01$) more pronounced expression of 5-$HT_{4(+h)}$ receptor within the mucosa fractions (the ratio versus GAPDH was in colon descendens: 0.55 ± 0.10; gastric fundus: 0.69 ± 0.13; n=4) compared to the expression of the 5-$HT_{4(-h)}$ receptor (colon descendens: 0.12 ± 0.03; gastric fundus: 0.21 ± 0.10; n=4). Within the MMP fraction of colon descendens and gastric fundus there was no difference in expression of 5-$HT_{4(+h)}$ receptor (colon descendens: 0.18 ± 0.06; and gastric fundus: 0.18 ± 0.05; n=4) and 5-$HT_{4(-h)}$ receptor (colon descendens: 0.26 ± 0.12; gastric fundus: 0.17 ± 0.09; n=4).

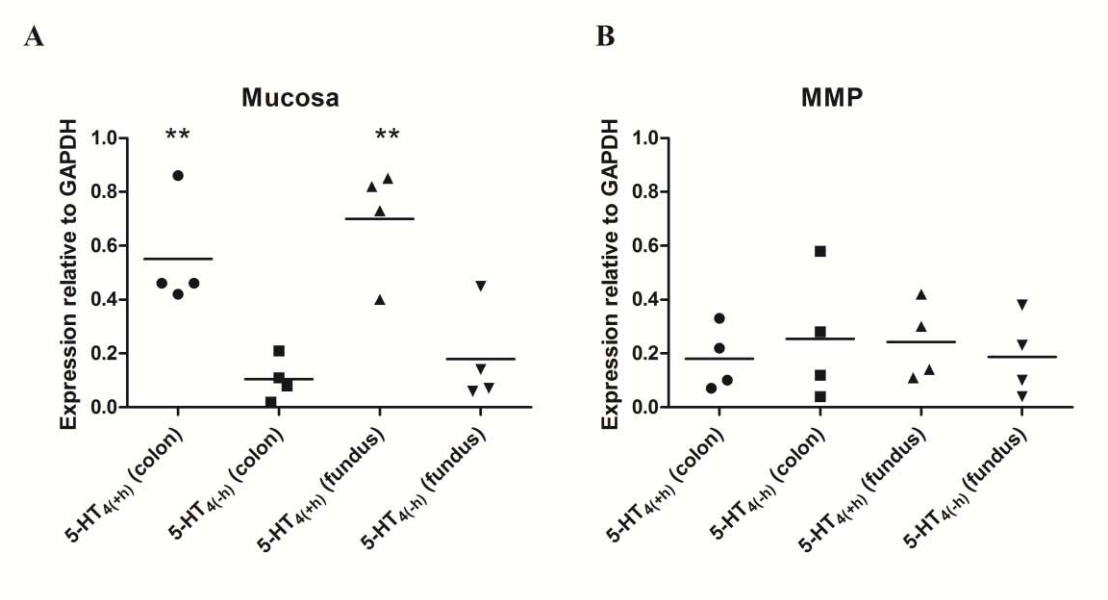


Figure 6.6

Expression of 5-HT$_{4(+h)}$ and 5-HT$_{4(-h)}$ receptors in the mucosa (A) and MMP (B) fractions of colon descendens and gastric fundus. Data are given as ratio relative to GAPDH expression. The line indicates the mean of n = 4 for each region-fraction. ** $p < 0.01$ versus values for 5-HT$_{4(-h)}$ receptors in colon descendens or gastric fundus.

6.IV.3. Expression of the 5-HT$_4$ receptor in porcine H&E stained tissue sections

Whole tissue sections, and the mucosal or MMP part of tissue sections of colon descendens and gastric fundus were scraped off a membrane slide and 5-HT$_4$ receptor expression was analyzed. *Colon descendens:* 5-HT$_4$ receptor and GAPDH expression was detected in the whole tissue sections, and in the mucosal and MMP parts of tissue sections of colon descendens (**Fig. 6.7A**). After semi-quantification, the values for 5-HT$_4$ receptor expression were within whole tissue sections (5-HT$_{4(+h)}$R: 1.21 ± 0.66 and 5-HT$_{4(-h)}$R: 0.63 ± 0.45; n=3), within the mucosal part of tissue sections (5-HT$_{4(+h)}$R: 0.86 ± 0.39, 5-HT$_{4(-h)}$R: 0.28 ± 0.14; n=3) and within the MMP part of tissue sections (5-HT$_{4(+h)}$R: 0.47 ± 0.05, 5-HT$_{4(-h)}$R: 0.24 ± 0.11; n=3). The tendency for more pronounced expression of the h-exon containing splice variant did not reach significance.

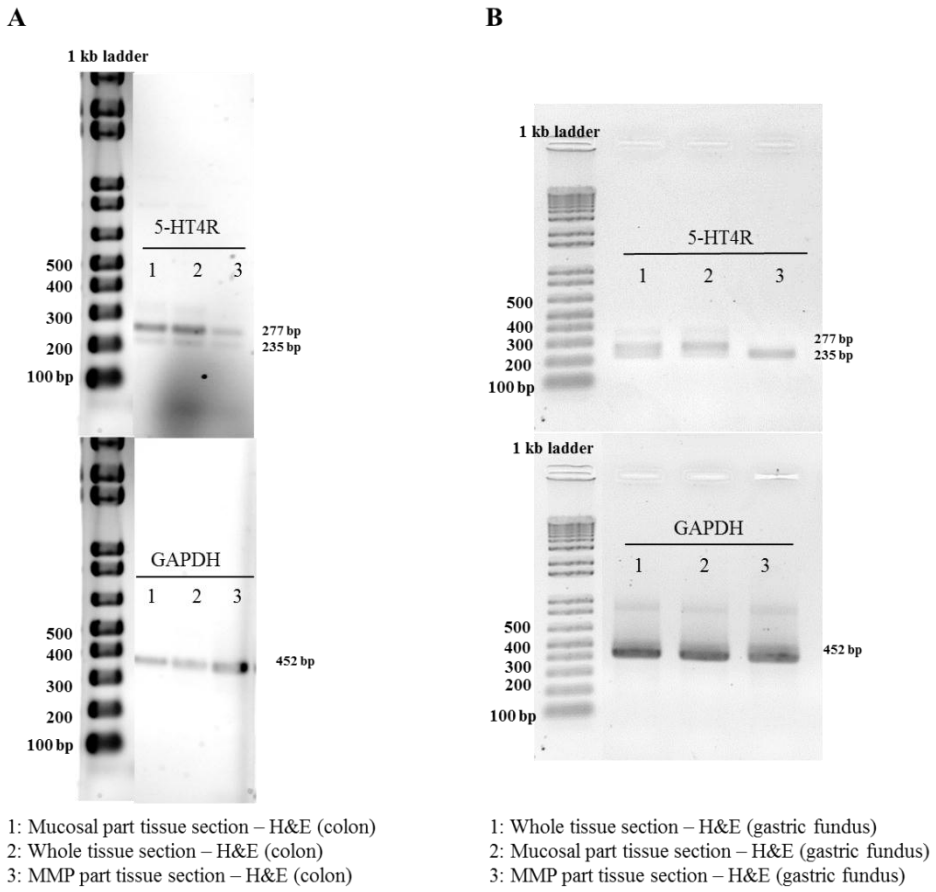


Figure 6.7

Invert color image of endpoint PCR analysis of 5-HT$_4$ receptor and GAPDH expression in a H&E stained whole tissue section, and in the mucosal as well as the MMP part of a H&E stained tissue section of colon descendens (A) and gastric fundus (B). The size of the expected PCR products is indicated.

Gastric Fundus: 5-HT$_4$ receptor and GAPDH expression was also detected in the whole tissue sections, and mucosal and MMP parts of tissue sections of gastric fundus (**Fig. 6.7B**). After semi-quantification, expression values were 0.51 and 0.19 for 5-HT$_{4(+h)}$R and 0.23 and 0.11 for 5-HT$_{4(-h)}$R in whole tissue sections, and 1.13 and 0.24 for 5-HT$_{4(+h)}$R and 0.31 and 0.05 for 5-HT$_{4(-h)}$R in the mucosal part of tissue sections. In the 2 samples with the MMP part of tissue sections, the 5-HT$_{4(-h)}$ receptor was found in both (0.24 and 0.45) while the 5-HT$_{4(+h)}$ receptor was only found in one of the samples (0.53) (**Fig. 6.7B** lane 3, showing the result with only the 5-HT$_{4(-h)}$ receptor detected).

6.IV.4. Expression of the 5-HT$_4$ receptor in LMPC-isolated cell populations from H&E stained porcine tissue sections

After H&E staining, large microdissected patches of cells were taken from the epithelium and the circular smooth muscle layer of the colon descendens and from the epithelium of the gastric fundus. Due to low RNA yield, only after a second round of PCR, with the same 5-HT$_4$ receptor specific-primers, 5-HT$_4$ receptor expression was detected in large microdissected patches of epithelial cells (**Fig. 6.8A and 6.8B**) or smooth muscle cells (**Fig. 6.8A**). Aspecific amplification occasionally occurred because of the high number of cycles (**Fig. 6.8A**). In *colon descendens* (**Fig. 6.8A**) 5-HT$_{4(+h)}$ receptor was more expressed compared to 5-HT$_{4(-h)}$ receptor within the LMPC-isolated epithelial cells (5-HT$_{4(+h)}$R: 1.03 and 0.85; 5-HT$_{4(-h)}$R: 0.24 and 0.11), while the opposite occurred for the smooth muscle cells (5-HT$_{4(+h)}$R: 1.14 and 0.91; 5-HT$_{4(-h)}$R: 5.07 and 4.03). In *gastric fundus* epithelial cells (**Fig. 6.8B**) the expression appeared similar for the 5-HT$_{4(+h)}$R (0.45 and 0.20) and 5-HT$_{4(-h)}$R (0.67 and 0.24).

6.IV.5. Expression of the 5-HT$_4$ receptor in LMPC-isolated EC cells from IHC stained porcine tissue sections

After IHC staining, EC cells (MAB352; **Fig 6.8C**), were isolated by LMPC from gastric fundus tissue sections. After a second round of PCR with the same 5-HT$_4$ receptor specific-primers, 5-HT$_4$ receptor expression was not detected in LMPC-isolated EC cells although cells were positive for the GAPDH housekeeping gene, confirming the integrity of the samples and completed PCR reactions at least for GAPDH. Additionally, in the mucosal part (**Fig. 6.8C**) of IHC stained tissue sections, scraped off after LMPC of EC cells, 5-HT$_4$ receptor and GAPDH expression was detected.

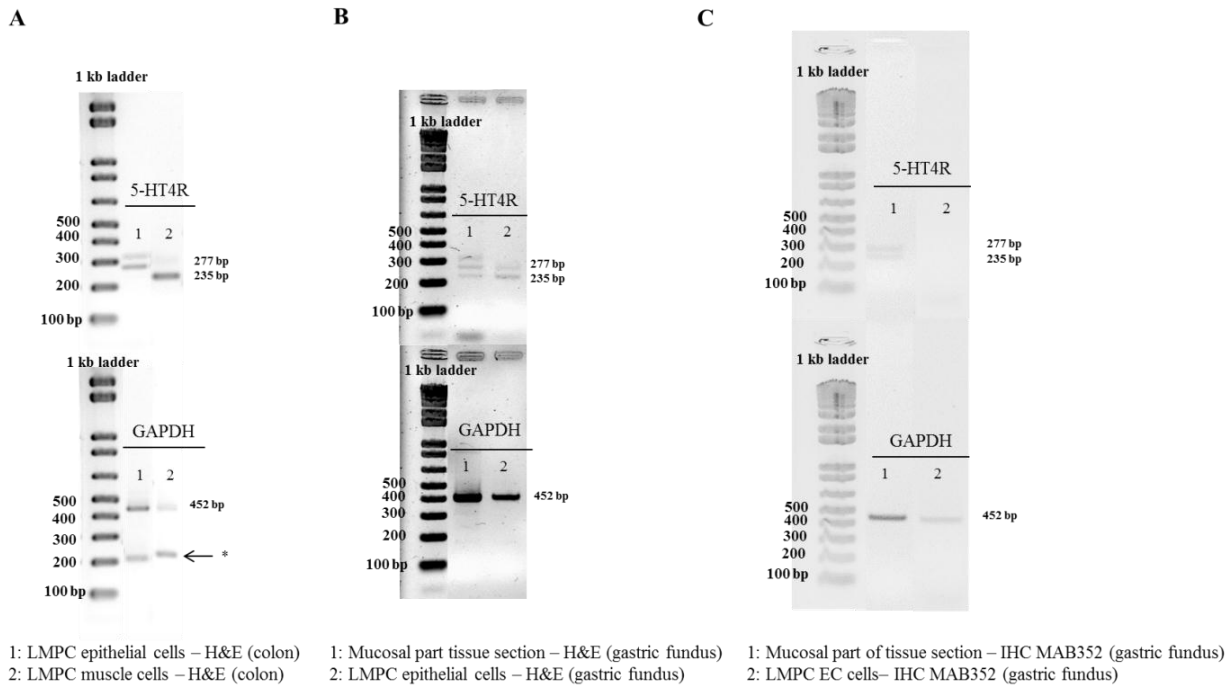


Figure 6.8

Invert color representation of the double round of endpoint PCR analysis of 5-HT$_4$ receptor and GAPDH expression in large patches of epithelium cells and smooth muscle cells obtained by LMPC from H&E stained tissue sections of colon descendens (A) and gastric fundus (B, only epithelial cells were obtained), and in EC cells obtained by LMPC from MAB352 IHC stained tissue sections of gastric fundus (C). For comparison, the result obtained in the mucosal part of the H&E or IHC stained tissue section of gastric fundus is also shown in B and C. The size of the expected PCR products and presence of aspecific amplification are indicated (*).

6.V. Discussion

The aim of this study was to investigate the 5-HT$_4$ receptor distribution in pig GI tract by confining gene expression analysis to site-specific regions of interest, with special attention to the mucosal layer of the pig colon descendens and gastric fundus by isolating epithelial cells using the LMPC technique. A stepwise approach was used by first studying mucosal and MMP tissue fractions, then tissue sections where different cell layers were discerned morphologically by H&E staining, and finally tissue sections stained for a particular cell type by IHC. The impact of the freezing method and staining method on the RNA quality was evaluated in mucosa and MMP tissue fractions (**Fig. 6.4A**) and mucosal and MMP parts of H&E stained tissue sections (**Fig. 6.4B**) of the pig colon descendens and gastric fundus.

The major advantages of LMPC are the isolation of biological material without direct user contact, hereby avoiding contamination, and the preserved cellular integrity (Kuhn et al., 2007). The main obstacles, when using LMPC to analyse 5-HT$_4$ receptor mRNA expression in different cell types, are first to recognize the cells of interest and second to obtain RNA of good quality. To recognize the cells of interest while preserving RNA integrity, a H&E protocol and a cell-specific IHC protocol was developed in RNase-free conditions. Developing a suitable IHC staining procedure was more complex in contrast to the H&E staining, because a standardized IHC procedure requires a long overnight antibody incubation to obtain good antibody labeling, but long incubation in aqueous buffers activates endogenous RNases, resulting in RNA degradation (Fend et al., 1999). Our attempts to develop a fast IHC protocol resulted in diminished visualization due to the short antibody labeling time. Therefore, our IHC staining protocol was based on the report published by Brown and Smith (2009), where overnight antibody incubation and RNA integrity could be maintained with addition of 1M NaCl in all aqueous solutions, resulting in superior protection of RNA. It is not yet clear why a saline solution preserves RNA, although we can speculate that normal saline protects the integrity of cell membranes, and hence, prevents the release of intracellular RNases (Vincek et al., 2003). Our results confirm the preservation of RNA after H&E staining (**Fig. 6.4B**) as well as after overnight IHC staining (**Fig. 6.4D**). EC cells in the mucosal layer of the pig gastric fundus were visualized by IHC staining with MAB352 antibody against 5-HT (**Fig. 6.3**), which has been shown to be selective for EC cells in the human gastric mucosa (Penkova et al., 2010).

In this study, 5-HT$_4$ receptor expression was detected in both mucosa and MMP tissue fractions (**Fig. 6.5 and 6.6**) and mucosal and MMP parts of H&E stained tissue sections (**Fig. 6.7**) of the colon descendens as well as of the gastric fundus, confirming previously reported data by De Maeyer et al. (2008); expression of both a variant with ($5HT_{4(+h)}$ receptor, 277 bp) and one without the h-exon ($5HT_{4(-h)}$ receptor, 235 bp) was observed. This is possible by analysis of the 5-HT$_4$ receptor mRNA expression profile by using forward and reverse primers in exon 4 and 5, respectively (**Fig. 6.2B**). Because exon h is located in between exon 4 and 5, this primer combination allows the detection of alternatively spliced 5-HT$_{4h}$ variants. As in other species, porcine 5-HT$_4$ receptors have a common amino acid structure until L358 followed by an alternatively spliced C-terminus. These C-terminal splice variants seem to be different compared to other species with at least 9 different variants that have not been described in other species (**Fig. 6.2A**). In addition, unique splicing variation has been described with variants composed of duplicated exons (De Maeyer et al., 2008).

Splice variants in the extracellular loops of G-protein coupled receptors are rare (Kilpatrick et al., 1999). One of these exceptions is the 5-HT_4 receptor, which can have an extra insertion of 14 amino acids in the second extracellular loop, encoded by the h-exon (**Fig. 6.2**). In human, this h variant has only been described in combination with the b-terminal exon (5-$HT_{4(hb)}$) (Bender et al., 2000). However, De Maeyer et al. (2008) showed that the porcine 5-$HT_{4(h)}$ splice variant also exists in combination with other C-terminal exons than 5-$HT_{4(b)}$, namely 5-$HT_{4(ha)}$, 5-$HT_{4(hm)}$ and 5-$HT_{4(hr)}$. As we used primers designed to amplify part of the common receptor region encoded by exon 4 and 5, exons that flank exon h (**Fig. 6.2B**), the $5HT_{4(+h)}$ receptor detected might thus include several C-terminal splice variants. Semi-quantitative analysis revealed that in the mucosa tissue fractions of colon descendens and gastric fundus, the expression level of the 5-$HT_{4(+h)}$ receptor was significantly higher compared to the 5-$HT_{4(-h)}$ receptor, and a similar trend was obtained in the mucosal part of H&E stained tissue sections. While evaluation of 5-HT_4 receptor RNA expression in human GI full-thickness tissue samples, showed similar levels in the stomach compared to more distal levels (Mader et al., 2006), expression in human gastric mucosal specimens was much less pronounced than in mucosal specimens of more distal regions of the GI tract (Hoffman et al., 2012; van Lelyveld et al., 2007). In pig gastric fundus mucosa however, the expression of the 5-HT_4 receptor was similar as in the mucosa of the colon descendens with clear-cut preponderance of the h-exon containing receptor. The predominant mucosal location of h-exon containing 5-HT_4 receptor splice variants might correspond to the preferential involvement of this type of 5-HT_4 receptor splice variants in mucosal effects of 5-HT_4 receptor activation such as goblet cell degranulation, chloride secretion and control of 5-HT release (Hoffman et al., 2012).

Both the mucosal and the MMP part of the GI tract contain several cell types on which the presence of 5-HT_4 receptors has been suggested at least in some regions in some species such as enterochromaffin cells, smooth muscle cells of the muscularis mucosae and submucosal intrinsic neurons in the mucosal part; myenteric cholinergic neurons, smooth muscle cells and interstitial cells of Cajal in the MMP part (Grider et al., 1998; Poole et al., 2006; Sakurai-Yamashita et al., 1999; 2000; Wouters et al., 2007). To obtain more information on the cell specific distribution of the 5-HT_4 receptors, cell layers or particular cell types were isolated by LMPC. In colon descendens, patches of the epithelial cell layer obtained by LMPC showed expression of 5-HT_4 receptors, predominantly the $5HT_{4(+h)}$ receptor. Possible cell types involved might be enterochromaffin cells and goblet cells, which were recently shown to express 5-HT_4 receptors in mouse intestine (Hoffman et al., 2012).

In mouse, application of 5-HT$_4$ receptor agonists led to mucosal 5-HT release and mucus secretion in a tetrodotoxin-insensitive way indicating direct activation of stimulatory 5-HT$_4$ receptors on the enterochromaffin cells and goblet cells respectively (Hoffman et al., 2012); in porcine and human small intestine however, analysis of 5-HT release suggested the presence of inhibitory 5-HT$_4$ receptors on the enterochromaffin cells (Schwörer and Ramadori, 1998). As relaxant 5-HT$_4$ receptors have been proposed on circular smooth muscle in human colon on the basis of functional data (McLean et al., 1995), patches of cells were also obtained from the circular muscle layer of the pig colon descendens indeed revealing 5-HT$_4$ receptor expression. Still, some authors were not able to confirm the presence of relaxant 5-HT$_4$ receptors in human colonic circular muscle strips (Cellek et al., 2006) and we did not obtain evidence for muscular 5-HT$_4$ receptors in pig colonic circular muscle strips (Priem and Lefebvre, 2011).

We can thus not exclude that the 5-HT$_4$ receptor expression observed in LMPC-isolated cell patches from the pig colonic circular muscle layer represents the 5-HT$_4$ receptors on intercalated interstitial cells of Cajal. Also in pig gastric fundus, LMPC-isolated cell patches of the epithelial cell layer showed 5-HT$_4$ receptor expression. As the human gastric mucosa was shown to contain a considerable number of enterochromaffin cells scattered within the lining epithelium (Penkova et al., 2010), we stained enterochromaffin cells in porcine gastric mucosa immunohistochemically and isolated them by LMPC. Although the 5-HT$_4$ receptor was still detected in the full mucosal part of these IHC stained sections, 5-HT$_4$ receptor expression was not detected in the isolated enterochromaffin cells. As the enterochromaffin cells showed expression of GAPDH, this might be related to the small amount of 5-HT$_4$ receptor RNA even when pooling 500 LMPC-isolated cells. In gastric fundus, LMPC was not applied to obtain cell patches from the muscle layer, as there are no functional data suggesting 5-HT$_4$ receptors to be present on muscle cells in the stomach.

6.VI. Conclusion

This study using endpoint RT-PCR confirms the presence of 5-HT_4 receptors in the mucosal and in the MMP part of porcine gastric fundus and colon descendens, and shows that the mucosa predominantly expresses h-exon containing 5-HT_4 receptors. The mucosal h-exon containing 5-HT_4 receptors might form additional sites of action for 5-HT_4 receptor agonists. 5-HT_4 receptors were detected in LMPC-isolated epithelial cell patches in gastric fundus and colon descendens, and circular muscle cell patches in the colon descendens. No 5-HT_4 receptor expression was detected in gastric LMPC-isolated enterochromaffin cells stained by immunohistochemistry, the expression of 5-HT_4 receptors in individual cell types might be too low to pick them up by LMPC and endpoint PCR.

6.VII. References

Albuquerque FC Jr, Smith EH, Kellum JM. 5-HT induces cAMP production in crypt colonocytes at a 5-HT4 receptor. J Surg Res. 1998; 77: 137-140

Bender E, Pindon A, van Oers I, Zhang YB, Gommeren W, Verhasselt P, Jurzak M, Leysen J, Luyten W. Structure of the human serotonin 5-HT4 receptor gene and cloning of a novel 5-HT4 splice variant. J Neurochem. 2000; 74: 478-489

Bevilacqua C, Makhzami S, Helbling JC, Defrenaix P, Martin P. Maintaining RNA integrity in a homogeneous population of mammary epithelial cells isolated by Laser Capture Microdissection. BMC Cell Biol 2010; 11: 95

Borman RA, Burleigh DE. Human colonic mucosa possesses a mixed population of 5-HT receptors. Eur J Pharmacol 1996; 309: 271-274

Böttner M, Bär F, Von Koschitzky H, Tafazzoli K, Roblick UJ, Bruch HP, Wedel T. Laser microdissection as a new tool to investigate site-specific gene expression in enteric ganglia of the human intestine. Neurogastroenterol Motil 2010; 22: 168-172

Bouras EP, Camilleri M, Burton DD, Thomforde G, McKinzie S, Zinsmeister AR. Prucalopride accelerates gastrointestinal and colonic transit in patients with constipation without a rectal evacuation disorder. Gastroenterology. 2001; 120: 354-360

Brown AL, Smith DW. Improved RNA preservation for immunolabeling and laser microdissection. RNA 2009; 15: 2364-2374

Budhoo MR, Harris RP, Kellum JM. 5-Hydroxytryptamine-induced Cl- transport is mediated by 5-HT3 and 5-HT4 receptors in the rat distal colon. Eur J Pharmacol 1996; 298: 137-144

Budhoo MR, Kellum JM. Evidence for a 5-HT4 receptor pathway mediating chloride secretion in the rat distal colon. J Surg Res 1994; 57: 44-48

Cellek S, John AK, Thangiah R, Dass NB, Bassil AK, Jarvie EM, Lalude O, Vivekanandan S, Sanger GJ. 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. Neurogastroenterol Motil. 2006; 18: 853-861

Chetty N, Coupar IM, Tan YY, Desmond PV, Irving HR. Distribution of serotonin receptors and interacting proteins in the human sigmoid colon. Neurogastroenterol Motil 2009; 21: 551-558, e14-5

De Maeyer JH, Aerssens J, Verhasselt P, Lefebvre RA. Alternative splicing and exon duplication generates 10 unique porcine 5-HT4 receptor splice variants including a functional homofusion variant. Physiol Genomics 2008; 34: 22-33

De Maeyer JH, Prins NH, Schuurkes JA, Lefebvre RA. Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. J Pharmacol Exp Ther 2006; 317: 955-964

Denisov V, Strong W, Walder M, Gingrich J, Wintz H. Development and validation of RQI: an RNA quality indicator for the Experion automated electrophoresis system. Bio-rad Technical note 2008; bulletin 5761

Dutta U, Padhy AK, Ahuja V, Sharma MP. Double blind controlled trial of effect of cisapride on gastric emptying in diabetics. Trop. Gastroenterol. 1999; 20: 116-119

Fend F, Emmert-Buck MR, Chuaqui R, Cole K, Lee J, Liotta LA, Raffeld M. Immuno-LCM: laser capture microdissection of immunostained frozen sections for mRNA analysis. Am J Pathol 1999;154: 61-66

Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 2007; 132: 397-414

Grider JR, Foxx-Orenstein AE, Jin JG. 5-Hydroxytryptamine4 receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. Gastroenterology 1998; 115: 370-380

Hoffman JM, Tyler K, MacEachern SJ, Balemba OB, Johnson AC, Brooks EM, Zhao H, Swain GM, Moses PL, Galligan JJ, Sharkey KA, Greenwood-Van Meerveld B, Mawe GM. Activation of colonic mucosal 5-HT(4) receptors accelerates propulsive motility and inhibits visceral hypersensitivity. Gastroenterology 2012; 142: 844-854

Kapeller J, Möller D, Lasitschka F, Autschbach F, Hovius R, Rappold G, Brüss M, Gershon MD, Niesler B. Serotonin receptor diversity in the human colon: Expression of serotonin type 3 receptor subunits 5-HT3C, 5-HT3D, and 5-HT3E. J Comp Neurol. 2011 519: 420-432

Kilpatrick GJ, Dautzenberg FM, Martin GR, Eglen RM. 7TM receptors: the splicing on the cake. Trends Pharmacol Sci. 1999; 20: 294-301

Kuhn DE, Roy S, Radtke J, Khanna S, Sen CK. Laser microdissection and capture of pure cardiomyocytes and fibroblasts from infarcted heart regions: perceived hyperoxia induces p21 in peri-infarct myocytes. Am J Physiol Heart Circ Physiol. 2007; 292: H1245-H1253

Leclere PG, Lefebvre RA. Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. Br J Pharmacol 2002; 135: 135-142

Leclere PG, Prins NH, Schuurkes JA, Lefebvre RA. 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. Neurogastroenterol Motil. 2005; 17: 366-375

Liu M, Geddis MS, Wen Y, Setlik W, Gershon MD. Expression and function of 5-HT4 receptors in the mouse enteric nervous system. Am J Physiol Gastrointest Liver Physiol. 2005; 289: G1148-G1163

Mader R, Kocher T, Haier J, Wieczorek G, Pfannkuche HJ, Ito M. Investigation of serotonin type 4 receptor expression in human and non-human primate gastrointestinal samples. Eur J Gastroenterol Hepatol. 2006; 18: 945-950

Manabe N, Wong BS, Camilleri M. New-generation 5-HT4 receptor agonists: potential for treatment of gastrointestinal motility disorders. Expert Opin Investig Drugs 2010; 19: 765-775

McLean PG, Coupar IM, Molenaar P. A comparative study of functional 5-HT4 receptors in human colon, rat oesophagus and rat ileum. Br J Pharmacol. 1995 May;115(1):47-56

Ning Y, Zhu JX, Chan HC. Regulation of ion transport by 5-hydroxytryptamine in rat colon. Clin Exp Pharmacol Physiol. 2004; 31: 424-428

Penkova NI, Baltadjiev GA, Koeva YA, Atanassova PK, Andonov VN, Trichkova VA. Serotonin-producing cells in human gastric mucosa--immunohistochemical and electron microscopic study. Folia Med 2010; 52: 31-37

Pinzani P, Orlando C, Pazzagli M. Laser-assisted microdissection for real-time PCR sample preparation. Mol Aspects Med. 2006; 27: 140-159

Poole DP, Xu B, Koh SL, Hunne B, Coupar IM, Irving HR, Shinjo K, Furness JB. Identification of neurons that express 5-hydroxytryptamine4 receptors in intestine. Cell Tissue Res. 2006; 325: 413-422

Priem E, Van Colen I, De Maeyer JH, Lefebvre RA. The facilitating effect of prucalopride on cholinergic neurotransmission in pig gastric circular muscle is regulated by phosphodiesterase 4. Neuropharmacology 2012; 62: 2126-2135

Priem EK, Lefebvre RA. Investigation of neurogenic excitatory and inhibitory motor responses and their control by 5-HT(4) receptors in circular smooth muscle of pig descending colon. Eur J Pharmacol 2011; 667: 365-374

Prins NH, Akkermans LM, Lefebvre RA, Schuurkes JA. 5-HT(4) receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. Br J Pharmacol. 2000; 131: 927-932

Ren J, Zhou X, Galligan JJ. 5-HT4 receptor activation facilitates recovery from synaptic rundown and increases transmitter release from single varicosities of myenteric neurons. Am J Physiol Gastrointest Liver Physiol. 2008; 294: G1376-G1383

Sakurai-Yamashita Y, Yamashita K, Kanematsu T, Taniyama K. Localization of the 5-HT(4) receptor in the human and the guinea pig colon. Eur J Pharmacol 1999; 383: 281-285

Sakurai-Yamashita Y, Yamashita K, Yoshimura M, Taniyama K. Differential localization of 5-hydroxytryptamine3 and 5-hydroxytryptamine4 receptors in the human rectum. Life Sci 2000; 66: 31-34

Schwörer H, Ramadori G. Autoreceptors can modulate 5-hydroxytryptamine release from porcine and human small intestine in vitro. Naunyn Schmiedebergs Arch Pharmacol. 1998; 357:548-552

van Lelyveld N, Ter Linde J, Schipper ME, Samsom M. Regional differences in expression of TPH-1, SERT, 5-HT(3) and 5-HT(4) receptors in the human stomach and duodenum. Neurogastroenterol Motil 2007; 19: 342-348

van Nassauw L, Timmermans JP. Detailed knowledge of cellular expression of G protein-coupled receptors in the human enteric nervous system is essential for understanding their diverse actions. Neurogastroenterol Motil 2010; 22: 959-694

Vincek V, Nassiri M, Knowles J, Nadji M, Morales AR. Preservation of tissue RNA in normal saline. Lab Invest 2003; 83: 137-138

Wouters MM, Farrugia G, Schemann M. 5-HT receptors on interstitial cells of Cajal, smooth muscle and enteric nerves. Neurogastroenterol Motil 2007; 19 Suppl 2: 5-12

Chapter 7

GENERAL DISCUSSION AND PERSPECTIVES

Chapter 7 General discussion and Perspectives

GI dysmotility is one of the underlying factors in GI disorders such as gastro-esophagal reflux, gastroparesis, functional dyspepsia, irritable bowel syndrome, diarrhea and constipation. The pharmaceutical industry has invested in the search for adequate therapies for patients suffering from GI motility disorders by aiming at mimicking a subset of the actions of 5-HT. GI prokinetic drugs such as the selective 5-HT_4 receptor agonist prucalopride, stimulate GI motility acting on 5-HT_4 receptors expressed on excitatory cholinergic neurons (Gershon and Tack, 2007; Manabe et al., 2010). ACh release is hereby facilitated towards the smooth muscle in the human stomach (Leclere and Lefebvre, 2002) and colon (Cellek et al., 2006; Leclere et al., 2005; Prins et al., 2000). However, in the human colon, 5-HT_4 receptors were also reported to be present on circular smooth muscle cells, inducing relaxation (McLean et al., 1995). A coordinated activation of the 5-HT_4 receptors located on cholinergic neurons and smooth muscle cells within the human colon, might play a role in colonic motility but this needs to be further investigated. A good animal model representing the different human colonic locations of 5-HT_4 receptors would be useful. The pig is considered a good model for investigating human GI functions in view of the similar morphology and physiology of the GI tract. Still, some regions of the porcine GI tract have not been studied in detail with regard to the nervous control of motility, one of these regions being the descending colon. A first goal of this thesis was therefore to investigate the nervous control of motility and the locations of 5-HT_4 receptors in the colon descendens of the pig.

When concentrating on 5-HT_4 receptors, the pig additionally allows to study possible cardiac effects of 5-HT_4 receptor agonists, as it expresses functional cardiac 5-HT_4 receptors under physiological conditions similar to man and monkey. In the porcine atrium, prucalopride was shown to activate the G-protein coupled 5-HT_4 receptors via a cAMP-mediated activation of PKA leading to positive inotropic, chronotropic and lusitropic responses (Galindo-Tovar et al., 2009; Weninger et al., 2012). However, the effect of prucalopride became only prominent and sustained upon PDE inhibition (De Maeyer et al., 2006a; 2006b). This has recently been related to PDE3 and PDE4 activity (Galindo-Tovar et al., 2009; Weninger et al., 2012) and shows the importance of PDEs in cardiac 5-HT_4 receptor signaling. The role of PDEs in the signal transduction of 5-HT_4 receptors on GI cholinergic nerves had not yet been investigated. The second goal of this study was therefore to investigate the possible regulation by PDEs of the influence of the selective 5-HT_4 receptor agonist prucalopride on cholinergic neurotransmission towards porcine gastric and colonic circular smooth muscle.

5-HT, as a neurotransmitter and mucosal messenger, has a wide range of effects in the intestine largely due to the presence of multiple receptor subtypes on myenteric neurons, smooth muscle cells, EC cells, enterocytes and possibly ICCs. The expression profile of 5-HT_4 receptors in the normal and abnormal GI tract helps to define the more precise role of these receptors in GI homeostasis (Mader, 2006). 5-HT_4 receptors are known to affect GI motor function via the 5-HT_4 receptors on both myenteric neurons as well as smooth muscle cells. However, apart from the role of the 5-HT_4 receptors in GI motility, also a role in GI secretion has been suggested (Beattie and Smith, 2008; De Ponti, 2004; Gershon and Tack, 2007), but the cellular distribution of the 5-HT_4 receptor within the epithelial layer has not yet been investigated. Therefore, the third goal of this thesis was to investigate the mucosal expression of 5-HT_4 receptors in pig stomach and colon by laser microdissection.

7.I. The pig colon descendens as a model for human colonic 5-HT_4 receptors

The pig has been shown to be a good animal model for GI functions (De Maeyer et al., 2006b; Hens et al., 2000; Leclere and Lefebvre, 1998; Schwörer and Ramadori, 1998; Sevencu et al., 2005) and for 5-HT_4 receptor mRNA distribution within the GI tract (De Maeyer et al., 2008). The distribution of human colonic 5-HT_4 receptors influencing motility is intriguing as they have been shown to be present on the cholinergic nerves towards both longitudinal and circular muscle (Leclere et al., 2005; Prins et al., 2000), facilitating contraction, but also on circular smooth muscle cells, inducing relaxation (McLean et al., 1995). Furthermore, one report suggests that they are also present on human colonic nitrergic nerves, facilitating nitrergic relaxation (Cellek et al. 2006). When studying the pig colon descendens, we did not obtain evidence for the presence of relaxant 5-HT_4 receptors on the circular smooth muscle cells, as neither 5-HT nor prucalopride induced any relaxation of circular smooth muscle strips contracted with exogenous agents. When delineating the inhibitory NANC neurotransmission towards the pig colonic circular muscle, no pure nitrergic relaxant responses could be obtained. The results pointed to a redundant action of the inhibitory neurotransmitters NO and ATP (**Fig. 7.1**). NANC relaxations induced by electrical field stimulation of the inhibitory nerves were not influenced by prucalopride so that also no evidence was obtained for 5-HT_4 receptors on porcine colonic inhibitory neurons. In the presence of the NO synthesis inhibitor L-NAME and of the SK channel blocker apamin, pure cholinergic contractions were obtained upon electrical field stimulation illustrating activation of cholinergic nerves. These cholinergic contractions could be enhanced by prucalopride, illustrating the presence of 5-HT_4 receptors on porcine colonic cholinergic nerves innervating the circular muscle (**Fig. 7.1**). The effect of prucalopride was however less pronounced than

generally reported for its effect on cholinergic neurotransmission in the colon and stomach of man and dog, and also than in the stomach of the pig. Our group had shown before a pronounced effect of prucalopride on cholinergic nerves towards porcine gastric longitudinal muscle (De Maeyer et al., 2006b) and in the actual study we confirmed this for the cholinergic nerves to porcine circular muscle, by measuring its effect on electrically induced cholinergic contractions as well as on electrically induced ACh release.

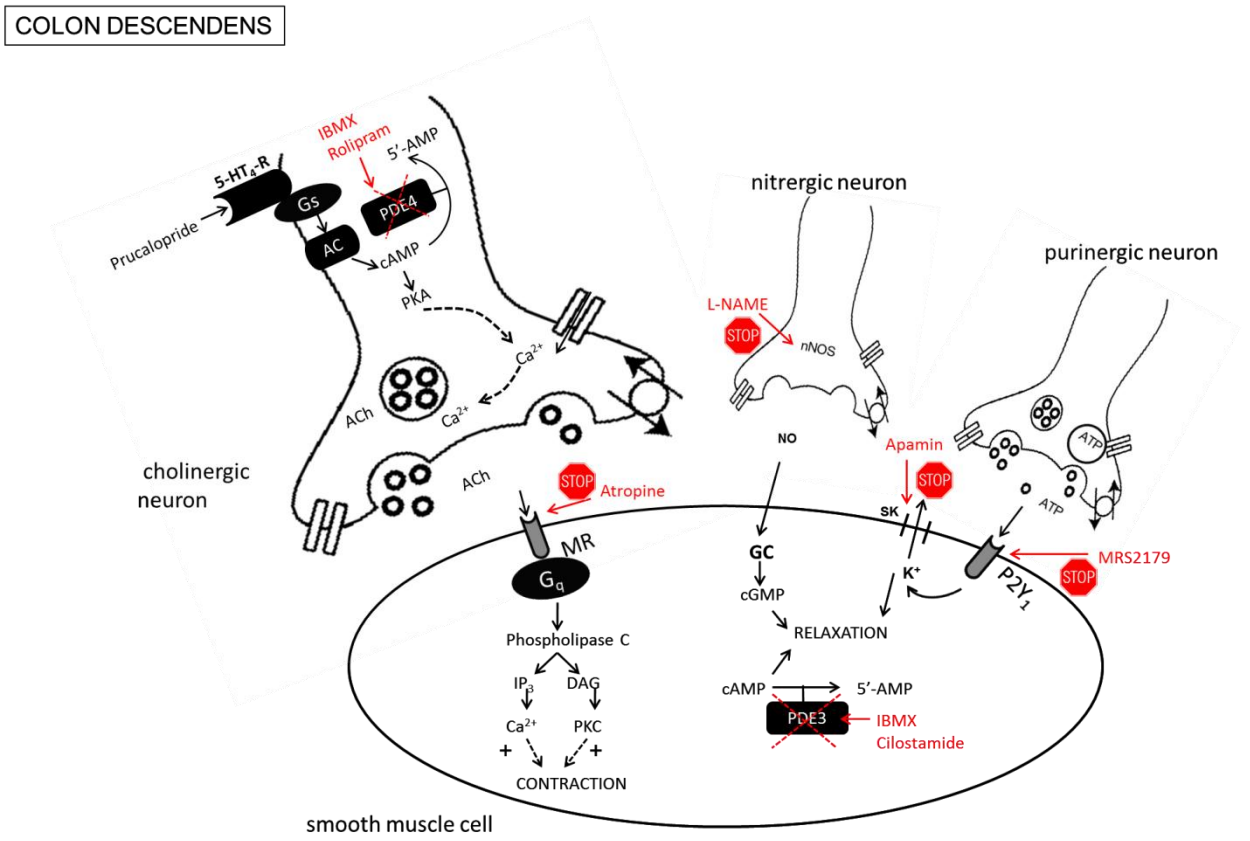


Figure 7.1. Schematic overview of the results obtained in the pig colon descendens: The enteric neurons studied, the action mechanism of the 5-HT_4 receptor located on the cholinergic neurons and the signal transduction of the neurotransmitters within the smooth muscle cells are represented. 5-HT_4-receptor (5-HT_4-R); acetylcholine (ACh); adenosine triphosphate (ATP); adenylate cyclase (AC); guanylate cyclase (GC); cyclic adenosine monophosphate (cAMP); cyclic guanosine monophosphate (cGMP); diacylglycerol (DAG); Gs protein (Gs); Gq protein (Gq); inositol 1,4,5-triphosphate (IP_3); 3-isobutyl-1-methylxanthine (IBMX); N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME); muscarinic receptor (MR); neuronal nitric oxide synthase (nNOS); nitric oxide (NO); phosphodiesterase (PDE); protein kinase A (PKA); protein kinase C (PKC).

Perspectives: The pig colon descendens can thus not be used as a model for the 3 locations of 5-HT_4 receptors, reported to be involved in human colonic motility (on cholinergic nerves, on nitrergic nerves and on smooth muscle cells). Still it can be used as a model for the colonic receptors located on the cholinergic nerves innervating the smooth muscle which is considered the principal site of action of gastroprokinetic 5-HT_4 receptor agonists. It should be kept in mind that the number of the porcine colonic 5-HT_4 receptors on cholinergic nerves might be less pronounced and/or their coupling less effective than in the human colon and in the porcine stomach. The latter can be considered as a very good model for human gastric 5-HT_4 receptors on cholinergic nerves and could be used for screening of the gastroprokinetic action of new 5-HT_4 receptor agonists. Although prucalopride is now clinically used in female patients with chronic laxative-resistant constipation, it also accelerates gastric emptying in man (Bouras et al., 2001); it still needs to be further elucidated in man as a possible drug treatment for dysmotility disorders of the stomach. Before being withdrawn for non-specific cardiac side effects, the non-selective 5-HT_4 receptor agonist cisapride was indeed frequently used for increasing gastric emptying in patients with gastroparesis. Our study of the NANC neurotransmission in pig colon descendens also illustrates that as in rat and man, a redundant action of NO and ATP are involved (Benkó et al., 2007; Boeckxstaens et al., 1993; Gallego et al., 2008; Gil et al., 2010). This means that the pig colon descendens can also be used in future studies as a model for human colonic NANC neurotransmission.

7.II. The role of PDEs in signaling of 5-HT_4 receptors facilitating cholinergic neurotransmission

5-HT_4 receptors are G-protein coupled receptors which activate AC generating cAMP. Cellular levels of cAMP are regulated by PDEs which catalyze their breakdown. In the porcine left atrium, the inotropic response upon stimulation of the 5-HT_4 receptors with prucalopride is very weak unless both PDE3 and PDE4 are inhibited, illustrating the tight control of the cAMP response by these PDEs (De Maeyer et al., 2006b; Galindo-Tovar et al., 2009; Weninger et al., 2012).

cAMP has been shown to depolarize myenteric cholinergic neurons by activation of cAMP-dependent PKA, acting downstream to enhance ACh release (Hegde and Eglen, 1996; Taniyama et al., 2000). In addition, Ren et al. (2008) showed that the facilitating effect of the 5-HT_4 receptor agonist renzapride on ACh release in the guinea pig small intestine is related to the AC/cAMP/PKA pathway. In contrast to its effect in the atrium, the facilitating effect of prucalopride on electrically induced cholinergic contractions in pig longitudinal (De Maeyer et al.,

2006b) and circular (Chapter 3) gastric muscle is well maintained. This does not exclude a regulatory role of PDEs in the AC/cAMP/PKA pathway of the 5-HT_4 receptors in the cholinergic neurons. Recent investigations showed mRNA distribution of PDEs in human stomach, small intestine and colon (Lakics et al., 2010). In addition, immunohistochemistry studies of PDE2A and PDE10A distribution showed peripheral neuronal including myenteric distribution of PDEs (Coskran et al., 2006; Stephenson et al., 2009), corroborating a possible regulatory role of PDEs in neuronal pathways.

Our results show that in circular muscle of porcine gastric fundus, the non-selective PDE inhibitor IBMX, in a concentration that per se only minimally reduced electrically induced cholinergic contractions, was able to enhance the facilitating effect of prucalopride on electrically induced contractions. When measuring ACh release directly, the enhancement of the facilitating effect of prucalopride by IBMX was confirmed and IBMX was even able to induce a facilitating effect of a non-effective concentration of prucalopride. These results suggest that the cAMP response in cholinergic neurons, when activating 5-HT_4 receptors, is somehow weakened by PDE activity (**Fig. 7.2**). To determine what PDE isoforms are involved, inhibitors for the 5 classic PDE isoforms (1-5) were tested versus the facilitating effect of prucalopride on ACh release. Only the PDE4 inhibitor rolipram enhanced the facilitating effect of prucalopride to the same extent as IBMX. When rolipram was tested in the assay with electrically induced cholinergic contractions, it did not reduce these contractions per se, but it clearly tended to enhance the facilitating effect of prucalopride. This set of results illustrates that the intraneuronal transduction pathway of the facilitatory 5-HT_4 receptors on cholinergic neurons in the porcine gastric circular muscle is regulated by PDE4 (**Fig. 7.2**). Similar results were obtained in the circular muscle of the porcine colon descendens although the regulatory role of PDE4 might be less pronounced (**Fig. 7.1**). Indeed, neither IBMX nor rolipram added before prucalopride were able to increase its facilitating effect on electrically induced contractions. In the porcine colon, the facilitating effect of prucalopride on electrically induced cholinergic contractions is less well maintained than in the stomach. When rolipram was added once the effect of prucalopride was declining, it induced a clearcut enhancement of electrically induced contractions.

These results suggest that the facilitating effect of prucalopride on GI ACh release and thus its prokinetic action can be enhanced by combining it with a PDE4 inhibitor. When considering this possibility, we must take into account that also in GI smooth muscle cells, cyclic nucleotides (cAMP and also cGMP) are essential second messengers which act downstream to relax the smooth muscle, their intracellular concentration being regulated by PDEs (Murthy, 2006). The increase in cAMP/cGMP content by PDE inhibition will here lead to relaxation, which will of

course counteract prokinetic effects. Indeed, in our experiments, the non-selective PDE inhibitor IBMX reduced the amplitude of the electrically induced cholinergic contractions in a concentration-dependent manner with near abolition at the highest concentration tested, both in porcine gastric as well as in colonic circular smooth muscle, confirming that PDEs control the cyclic nucleotide content and thus regulate tone within the GI smooth muscle (**Fig. 7.1 and Fig. 7.2**).

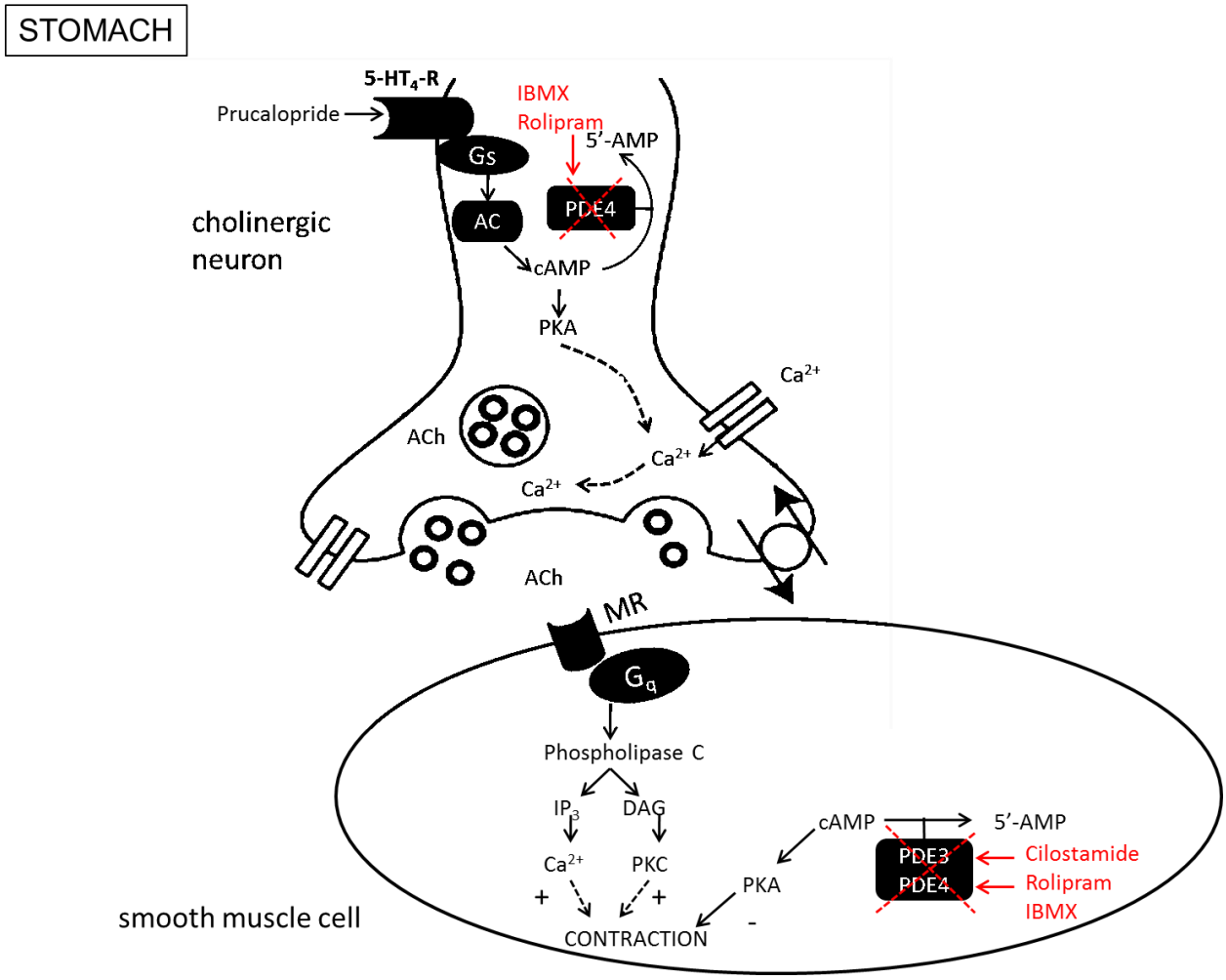


Figure 7.2. Schematic overview of the results obtained in the pig gastric fundus: Role of phosphodiesterases in the signal transduction control of the 5-HT_4 receptor located on the cholinergic neurons and in the control of cAMP levels within the smooth muscle cells are represented.
5-HT_4-receptor (5-HT_4-R); acetylcholine (ACh); adenylate cyclase (AC); cyclic adenosine monophosphate (cAMP); diacylglycerol (DAG); Gs protein (Gs); Gq protein (Gq); inositol 1,4,5-triphosphate (IP_3); 3-isobutyl-1-methylxanthine (IBMX); muscarinic receptor (MR); phosphodiesterase (PDE); protein kinase A (PKA); protein kinase C (PKC).

A thorough investigation of the PDE isoforms involved in porcine gastric smooth muscle, showed that none of the selective PDE inhibitors (PDE1: vinpocetine; PDE2: EHNA; PDE3: cilostamide; PDE4: rolipram; PDE5: zaprinast) was able to mimic the full abolition of electrically induced contractions as seen with IBMX. Cilostamide was the only inhibitor with some effect, inhibiting the contractions by maximally 32%. Only the combination of cilostamide with rolipram was able to mimic the inhibiting effect of IBMX illustrating a redundant action of PDE3 and PDE4 in controlling cyclic nucleotide levels in pig gastric circular muscle with some predominance of PDE4 (**Fig. 7.2**). Investigation of the PDE isoforms in the smooth muscle cells of the porcine colon descendens, showed that none of the selective PDE inhibitors, except the PDE3 inhibitor cilostamide, was able to fully mimic the inhibiting effect of IBMX on electrically induced cholinergic contractions. This points to a predominant role of PDE3 in porcine colonic circular smooth muscle (**Fig. 7.1**).

Perspectives: These results in pig stomach and colon suggest that the prokinetic action of prucalopride can be enhanced by combination with PDE4 inhibitors such as rolipram, as rolipram will increase the facilitating effect of prucalopride on Ach release without inducing counteracting relaxation because the latter requires combination of PDE3 and PDE4 inhibition in porcine stomach circular muscle and PDE3 inhibition in porcine colonic circular muscle. The gain in prokinetic action by combining prucalopride with a PDE4 inhibitor will probably be most pronounced at the level of the stomach as the regulatory role of PDE4 in the signal transduction of the 5-HT_4 receptors on cholinergic neurons is more pronounced in the stomach than in the colon. A similar investigation in human tissues will allow to decide whether the porcine data can be extrapolated to humans.

The AC/cAMP/PKA pathway is not the only one influencing ACh release from cholinergic neurons. Indeed, a synergistic action of activated PKC and Ca^{2+} mobilization has also been shown to potentiate the electrically induced ACh release in neurons of the guinea pig small intestine (Hashimoto et al., 1988) and an immunohistochemical study confirmed the presence of PKC in guinea pig myenteric neurons (Poole et al., 2003). The possible role of PKC in the control of ACh release from myenteric cholinergic neurons and in the facilitation of ACh release by 5-HT_4 receptor agonists can be further investigated by studying the influence of PKC inhibitors, in comparison to PKA inhibitors.

7.III. Gastric and colonic mucosal distribution of 5-HT_4 receptors

Next to the 5-HT_4 receptors located on cholinergic neurons and smooth muscle cells regulating GI motility, recent studies have also demonstrated 5-HT_4 receptor mRNA expression in the mucosa of the colon (Chetty et al., 2009; De Maeyer et al., 2008; van Lelyveld et al., 2007) and stomach (De Maeyer et al., 2008; Hoffman et al., 2012), suggesting a potential role in GI secretion. In mouse colon, an immunohistochemical study showed the presence of 5-HT_4 receptors in enterochromaffin (EC) cells and goblet cells in the epithelial layer (Hoffman et al., 2012). Functional 5-HT_4 receptors regulating secretion have already been shown in the colon of several species, but not yet in the stomach. In rat colon, 5-HT-induced secretion is mediated by 5-HT_4 receptors (Albuquerque et al., 1998; Budhoo and Kellum, 1994; Budhoo et al., 1996a; Ning et al., 2004), whereas in the human ascending sigmoid colon, 5-HT-induced secretion is mediated by a combination of 5-HT_{2A} and 5-HT_4 receptors (Borman and Burleigh, 1996). In addition, Hoffman et al. (2012) showed in the colon of rodents that 5-HT_4 receptors induced 5-HT release and Cl^- secretion. To investigate the mucosal distribution, an attempt was done to assess the 5-HT_4 receptor distribution in porcine stomach and colon at the cellular level by LMPC and endpoint RT-PCR. In comparison, the muscular layer was also investigated.

Our results showed the presence of 5-HT_4 receptor mRNA in the smooth muscle layer of both gastric fundus and colon descendens of the pig. Even when isolating large patches of smooth muscle cells outside the ganglia by LMPC, thereby excluding 5-HT_4 receptors in cholinergic neurons, 5-HT_4 receptor mRNA expression was obtained in colonic samples. This result is in contrast with our previously shown functional data in the colon descendens where no evidence of muscular 5-HT_4 receptors was obtained. 5-HT_4 receptor mRNA expression in the LMPC-isolated patches of colonic smooth muscle could indicate the presence of 5-HT_4 receptors on the ICCs. Our study consistently showed the presence of 5-HT_4 receptor mRNA in the mucosa and in LMPC-isolated samples of the epithelial layer of both gastric fundus and colon descendens of the pig. Semi-quantitative analysis of the expression of the 5-HT_4 receptor variant with and without the h-exon, coding for a 14 amino acid segment in the second extracellular loop of the 5-HT_4 receptor showed a significant predominant expression of the 5-$HT_{4(+h)}$ receptor in the mucosa tissue fractions of both colon and stomach. The same tendency was seen in the mucosal part of tissue sections of both stomach and colon. When LMPC-isolated patches of epithelium were studied, the tendency of a more pronounced expression of the 5-$HT_{4(+h)}$ receptor was still seen for the colon but no longer for the stomach. As the EC cells are an obvious possible source of the mucosal 5-$HT_{4(+h)}$ receptors, they were stained

immunohistochemically and isolated by LMPC but this material did not yield a positive signal for 5-$HT_{4(+h)}$ receptor mRNA.

Perspectives: Recent evidence of 5-HT_4 receptor expression on rodent colonic epithelial cells with the results from our study encourage further investigation of the cellular distribution of 5-HT_4 receptors in the epithelial layer of both colon and stomach. Our results suggest that laser microdissection may not be the ideal approach to obtain high cellular RNA yields. However, fluorescence activated cell sorting (FACS) purification could be an alternative technique, using mucosal tissue lysates and cell-specific antibodies to select and isolate specific epithelial cells (Brenmoehl et al., 2008; Terehara et al., 2008). RNA can then be obtained from the specific cell lysates and RT-PCR performed with 5-HT_4 receptor specific primers. Besides further investigation of EC cells, interesting target cells to investigate in human (gastric or colonic biopsy tissue) and/or porcine tissues would be the parietal cells, goblet cells and enterocytes.

The differential 5-$HT_{4(+h)}$ receptor expression between mucosa and smooth muscle layer should be further evaluated by quantitative PCR using primers designed with a forward primer developed in the h-exon area and a reverse primer in a neighboring exon. Further investigation of the predominant expression of the h-exon containing receptor could be interesting for drug targeting research, as this h-exon is translated in the second extracellular loop of the 5-HT_4 receptor. The ligand binding site of the 5-HT_4 receptor is mainly determined by the extracellular loops, which are the same for all 5-HT_4 receptor splice variants, except for 5-$HT_{4(+h)}$ receptor, so that development of selective agonists for the 5-$HT_{4(+h)}$ receptor containing the h-exon might be attempted. All other 5-HT_4 receptor splice variants differ at their C-terminus, which is directed intracellularly and makes splice variant specific targeting difficult. Further investigation towards distribution of splice variants remains interesting. However, immunohistochemical localization and functional studies are yet not an option as antibodies and agonists/antagonists specific for splice variants are not available.

Effects of 5-HT on Cl^- and HCO_3^- secretion mediated by 5-HT_4 receptors have already been described in human and rodent small intestine (Budhoo et al., 1996b; Kellum et al., 1994; Säfsten et al., 2006; Tuo et al., 2004) and the presence of 5-HT_4 autoreceptors on the EC cells of human and porcine small intestine has been shown (Schwörer and Ramadori, 1998). However, functional studies on the effect of 5-HT and 5-HT_4 receptor agonists on secretion in the colon and stomach are scarce. The effect of exogenous 5-HT and 5-HT_4 receptor agonist in the absence and presence of the neuronal conductance blocker tetrodotoxin (TTX), to eliminate neuronal involvement, could be evaluated in both mucosal and full-thickness preparations of stomach and colon by measuring ion transport and Cl^- secretion in Ussing chambers. However,

in rat and human colonic mucosa, evidence has been provided for involvement of 5-HT_4 receptors but also 5-HT_2 and 5-HT_3 receptors in 5-HT-induced Cl^- secretion (Borman and Burleigh, 1996; Budhoo et al., 1996a; Day et al., 2005). Also in the rat stomach evidence has been provided that a 5-HT_3 and/or 5-HT_1-like receptor might be involved in gastric secretion (Lai et al., 2009; LePard 1994; LePard et al., 1996). The possibility of the involvement of other 5-HT receptor types than 5-HT_4 receptors should thus be kept in mind.

In conclusion, our findings indicate that 5-HT_4 receptors are present on the cholinergic neurons innervating the muscle layer of porcine stomach and colon. The signal transduction pathway of these receptors is regulated by PDE4 so that concomitant treatment with a PDE4 inhibitor enhances the facilitating effect of the 5-HT_4 receptor agonist prucalopride on Ach release and thus on cholinergic contractions. If further investigation confirms this mechanism in humans, this opens the possibility of prokinetic combination therapy of 5-HT_4 receptor agonists with PDE4 inhibitors. Our results also show the mucosal expression of 5-HT_4 receptors in pig stomach and colon. As the information on 5-HT_4 receptor-mediated effects on GI secretion in mammals is limited, the pig can also be used as a model to further unravel the cellular distribution of 5-HT_4 receptors and the effects of 5-HT_4 receptor activation in the mucosa.

7.IV. REFERENCES

Albuquerque FC Jr, Smith EH, Kellum JM. 5-HT induces cAMP production in crypt colonocytes at a 5-HT4 receptor. J Surg Res. 1998; 77: 137-140

Beattie DT, Smith JA. Serotonin pharmacology in the gastrointestinal tract: a review. Naunyn Schmiedebergs Arch Pharmacol. 2008; 377(3): 181-203

Benkó R, Undi S, Wolf M, Vereczkei A, Illényi L, Kassai M, Cseke L, Kelemen D, Horváth OP, Antal A, Magyar K, Barthó L. P2 purinoceptor antagonists inhibit the non-adrenergic, non-cholinergic relaxation of the human colon in vitro. Neuroscience. 2007; 147(1): 146-152

Boeckxstaens GE, Pelckmans PA, Herman AG, Van Maercke YM. Involvement of nitric oxide in the inhibitory innervation of the human isolated colon. Gastroenterology. 1993; 104 (3): 690-697

Borman RA, Burleigh DE. Human colonic mucosa possesses a mixed population of 5-HT receptors. Eur J Pharmacol. 1996; 309(3): 271-274

Bouras EP, Camilleri M, Burton DD, Thomforde G, McKinzie S, Zinsmeister AR. Prucalopride accelerates gastrointestinal and colonic transit in patients with constipation without a rectal evacuation disorder. Gastroenterology. 2001; 120(2): 354-360

Brenmoehl J, Falk W, Göke M, Schölmerich J, Rogler G. Inflammation modulates fibronectin isoform expression in colonic lamina propria fibroblasts (CLPF). Int J Colorectal Dis. 2008; 23(10): 947-955

Budhoo MR, Harris RP, Kellum JM. 5-Hydroxytryptamine-induced Cl- transport is mediated by 5-HT3 and 5-HT4 receptors in the rat distal colon. Eur J Pharmacol 1996a; 298: 137-144

Budhoo MR, Harris RP, Kellum JM. The role of the 5-HT4 receptor in Cl- secretion in human jejunal mucosa. Eur J Pharmacol. 1996b; 314(1-2): 109-114

Budhoo MR, Kellum JM. Evidence for a 5-HT4 receptor pathway mediating chloride secretion in the rat distal colon. J Surg Res 1994; 57: 44-48

Chetty N, Coupar IM, Tan YY, Desmond PV, Irving HR. Distribution of serotonin receptors and interacting proteins in the human sigmoid colon. Neurogastroenterol Motil. 2009; 21(5): 551-558

Cellek S, John AK, Thangiah R, Dass NB, Bassil AK, Jarvie EM, Lalude O, Vivekanandan S, Sanger GJ. 5-HT4 receptor agonists enhance both cholinergic and nitrergic activities in human isolated colon circular muscle. Neurogastroenterol Motil. 2006; 18(9): 853-861

Coskran TM, Morton D, Menniti FS, Adamowicz WO, Kleiman RJ, Ryan AM, Strick CA, Schmidt CJ, Stephenson DT. Immunohistochemical localization of phosphodiesterase 10A in multiple mammalian species. J Histochem Cytochem. 2006; 54(11): 1205-1213

Day J, King B, Haque SM, Kellum JM. A nonneuronal 5-hydroxytryptamine receptor 3 induces chloride secretion in the rat distal colonic mucosa. Am J Surg. 2005; 190(5): 736-738

De Ponti F. Pharmacology of serotonin: what a clinician should know. Gut. 2004; 53(10): 1520-1535

De Maeyer JH, Aerssens J, Verhasselt P, Lefebvre RA. Alternative splicing and exon duplication generates 10 unique porcine 5-HT 4 receptor splice variants including a functional homofusion variant. Physiol Genomics. 2008; 34(1): 22-33

De Maeyer JH, Straetemans R, Schuurkes JA, Lefebvre RA. Porcine left atrial and sinoatrial 5-HT(4) receptor-induced responses: fading of the response and influence of development. Br J Pharmacol. 2006a; 147(2): 140-157

De Maeyer JH, Prins NH, Schuurkes JA, Lefebvre RA. Differential effects of 5-hydroxytryptamine4 receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ-specific behavior. J Pharmacol Exp Ther. 2006b; 317(3): 955-964

Galindo-Tovar A, Vargas ML, Escudero E, Kaumann AJ. Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT(4) receptors. Br J Pharmacol. 2009; 156(2): 237-249

Gallego D, Gil V, Aleu J, Aulí M, Clavé P, Jiménez M. Purinergic and nitrergic junction potential in the human colon. Am J Physiol Gastrointest Liver Physiol. 2008; 295(3): G522-G533

Gershon MD and Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology. 2007; 132(1): 397-414

Gil V, Gallego D, Grasa L, Martín MT, Jiménez M. Purinergic and nitrergic neuromuscular transmission mediates spontaneous neuronal activity in the rat colon. Am J Physiol Gastrointest Liver Physiol. 2010; 299(1): G158-G169

Hashimoto S, Shuntoh H, Taniyama K, Tanaka C. Role of protein kinase C in the vesicular release of acetylcholine and norepinephrine from enteric neurons of the guinea pig small intestine. Jpn J Pharmacol. 1988; 48(3): 377-385

Hegde SS, Eglen RM. Peripheral 5-HT4 receptors. FASEB J. 1996; 10(12): 1398-1407

Hens J, Schrödl F, Brehmer A, Adriaensen D, Neuhuber W, Scheuermann DW, Schemann M, Timmermans JP. Mucosal projections of enteric neurons in the porcine small intestine. J Comp Neurol. 2000; 421(3): 429-436

Hoffman JM, Tyler K, MacEachern SJ, Balemba OB, Johnson AC, Brooks EM, Zhao H, Swain GM, Moses PL, Galligan JJ, Sharkey KA, Greenwood-Van Meerveld B, Mawe GM. Activation of colonic mucosal 5-HT(4) receptors accelerates propulsive motility and inhibits visceral hypersensitivity. Gastroenterology 2012; 142: 844-854

Kellum JM, Budhoo MR, Siriwardena AK, Smith EP, Jebraili SA. Serotonin induces Cl- secretion in human jejunal mucosa in vitro via a nonneural pathway at a 5-HT4 receptor. Am J Physiol. 1994; 267(3 Pt 1): G357-G363

Lai YC, Ho Y, Huang KH, Tsai LH. Effects of serotonin on acid secretion in isolated rat stomach: the role of 5-HT3 receptors .Chin J Physiol. 2009; 52(5 Suppl): 395-405

Lakics V, Karran EH, Boess FG. Quantitative comparison of phosphodiesterase mRNA distribution in human brain and peripheral tissues. Neuropharmacology. 2010; 59(6): 367-374

Leclere PG, Lefebvre RA. Investigation of the interaction between cholinergic and nitrergic neurotransmission in the pig gastric fundus. Br J Pharmacol. 1998; 125(8): 1779-1787

Leclere PG, Lefebvre RA. Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. Br J Pharmacol 2002; 135: 135-142

Leclere PG, Prins NH, Schuurkes JA, Lefebvre RA. 5-HT4 receptors located on cholinergic nerves in human colon circular muscle. Neurogastroenterol Motil. 2005; 17: 366-375

Lepard KJ, Chi J, Mohammed JR, Gidener S, Stephens RL Jr. Gastric antisecretory effect of serotonin: quantitation of release and site of action. Am J Physiol. 1996; 271(4 Pt 1): E669-E677

LePard KJ, Stephens RL Jr. Serotonin inhibits gastric acid secretion through a 5-hydroxytryptamine1-like receptor in the rat. J Pharmacol Exp Ther. 1994; 270(3): 1139-1144

Mader R, Kocher T, Haier J, Wieczorek G, Pfannkuche HJ, Ito M. Investigation of serotonin type 4 receptor expression in human and non-human primate gastrointestinal samples. Eur. J. Gastroenterol. Hepatol. 2006; 18(9): 945-950

Manabe N, Wong BS, Camilleri M. New-generation 5-HT4 receptor agonists: potential for treatment of gastrointestinal motility disorders. Expert Opin. Investig. Drugs 2010; 19(6): 765-775

McLean PG, Coupar IM, Molenaar P. A comparative study of functional 5-HT4 receptors in human colon, rat oesophagus and rat ileum. Br J Pharmacol. 1995; 115(1): 47-56

Murthy KS. Signaling for contraction and relaxation in smooth muscle of the gut. Annu. Rev. Physiol. 2006; 68: 345-374

Ning Y, Zhu JX, Chan HC. Regulation of ion transport by 5-hydroxytryptamine in rat colon. Clin Exp Pharmacol Physiol. 2004; 31: 424-428

Poole DP, Hunne B, Robbins HL, Furness JB. Protein kinase C isoforms in the enteric nervous system. Histochem Cell Biol. 2003; 120(1): 51-61

Prins NH, Akkermans LM, Lefebvre RA, Schuurkes JA. 5-HT(4) receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. Br J Pharmacol. 2000; 131: 927-932

Ren J, Zhou X, Galligan JJ. 5-HT4 receptor activation facilitates recovery from synaptic rundown and increases transmitter release from single varicosities of myenteric neurons. Am J Physiol Gastrointest Liver Physiol. 2008; 294(6): G1376-G1383

Säfsten B, Sjöblom M, Flemström G. Serotonin increases protective duodenal bicarbonate secretion via enteric ganglia and a 5-HT4-dependent pathway. Scand J Gastroenterol. 2006; 41(11): 1279-1289

Schwörer H, Ramadori G. Autoreceptors can modulate 5-hydroxytryptamine release from porcine and human small intestine in vitro. Naunyn Schmiedebergs Arch Pharmacol. 1998; 357: 548-552

Sevcencu C, Rijkhoff NJ, Gregersen H, Sinkjaer T. Propulsive activity induced by sequential electrical stimulation in the descending colon of the pig. Neurogastroenterol Motil. 2005; 17(3): 376-387

Stephenson DT, Coskran TM, Wilhelms MB, Adamowicz WO, O'Donnell MM, Muravnick KB, Menniti FS, Kleiman RJ, Morton D. Immunohistochemical localization of phosphodiesterase 2A in multiple mammalian species. J Histochem Cytochem. 2009; 57(10): 933-949

Taniyama K, Makimoto N, Furuichi A, Sakurai-Yamashita Y, Nagase Y, Kaibara M, Kanematsu T. Functions of peripheral 5-hydroxytryptamine receptors, especially 5-hydroxytryptamine4 receptor, in gastrointestinal motility. J Gastroenterol. 2000; 35(8): 575-582

Terahara K, Yoshida M, Igarashi O, Nochi T, Pontes GS, Hase K, Ohno H, Kurokawa S, Mejima M, Takayama N, Yuki Y, Lowe AW, Kiyono H. Comprehensive gene expression profiling of Peyer's patch M cells, villous M-like cells, and intestinal epithelial cells. J Immunol. 2008; 180(12): 7840-7846

Tuo BG, Sellers Z, Paulus P, Barrett KE, Isenberg JI. 5-HT induces duodenal mucosal bicarbonate secretion via cAMP- and Ca2+-dependent signaling pathways and 5-HT4 receptors in mice. Am J Physiol Gastrointest Liver Physiol. 2004; 286(3): G444-G451

van Lelyveld N, Ter Linde J, Schipper ME, Samsom M. Regional differences in expression of TPH-1, SERT, 5-HT(3) and 5-HT(4) receptors in the human stomach and duodenum. Neurogastroenterol Motil 2007; 19: 342-348

Weninger S, De Maeyer JH, Lefebvre RA. Study of the regulation of the inotropic response to 5-HT4 receptor activation via phosphodiesterases and its cross-talk with C-type natriuretic peptide in porcine left atrium. Naunyn Schmiedebergs Arch Pharmacol. 2012; 385(6): 565-577

Chapter 8

SUMMARY

Chapter 8 Summary

Chapter I - Literature Survey. Serotonin (5-hydroytryptamine, 5-HT) is an essential messenger for the coordinated movement of food along the gastrointestinal (GI) tract, by interacting with a wide array of 5-HT receptors. Within the GI wall, 5-HT activates 5-HT receptors located on smooth muscle cells or on intrinsic and extrinsic sensory neurons, contributing to the regulation of peristalsis and communication between the brain and the GI tract. The 5-HT receptors are classified into seven receptor classes; in the gut, a role has been proposed for 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7 receptors. The 5-HT_4 receptor is expressed on excitatory cholinergic motor neurons in the GI tract, facilitating acetylcholine release hereby stimulating GI motility. This presynaptic facilitation is thought to be the principal mechanism of the prokinetic action of 5-HT_4 receptor agonists such as prucalopride, explaining their therapeutic use in GI dysmotility related disorders such as chronic constipation, gastroparesis and gastroesophagal reflux disease. The pharmaceutical industry has invested in the search for adequate therapies for patients suffering from GI motility disorders by aiming at mimicking a subset of the actions of 5-HT. Aside the presence of 5-HT_4 receptors on cholinergic neurons in the GI tract, their localization has also been proposed on other cell types within the colon and stomach. In the human colon 5-HT_4 receptor agonists lead to smooth muscle relaxation by stimulating 5-HT_4 receptors on smooth muscle cells and possibly 5-HT_4 receptors located on nitrergic neurons. 5-HT_4 receptor mRNA expression has also been reported in the human and pig gastric and colonic mucosa, and it has been suggested that 5-HT-induced mucosal secretion is mediated by 5-HT_4 receptors. But the cellular 5-HT_4 receptor distribution and its functional implications within the epithelial layer have not yet been investigated.More detailed information on the expression and localization of 5-HT_4 receptors and their signaling pathways would lead to a better understanding of the role of 5-HT_4 receptors in GI motility and secretion.

The **aim** of this study was to investigate whether the porcine colon descendens could function as a model for the role and location of 5-HT_4 receptors in the control of human colonic motility. Secondly, the influence of phosphodiesterases (PDEs) in the signal transduction pathway upon activation of 5-HT_4 receptors on excitatory cholinergic motor neurons was investigated in pig gastric and colonic circular muscle. Finally, the mucosal expression of 5-HT_4 receptors was studied in the stomach and colon of the pig.

In **Chapter III**, the influence of the selective 5-HT_4 receptor agonist prucalopride on electrically induced acetylcholine (Ach) release from cholinergic nerve endings innervating pig gastric circular muscle and on the contractions induced by this release was investigated; the possible regulation of this effect by PDEs was explored. Prucalopride concentration-dependently increased the amplitude of submaximal cholinergic contractions and of ACh release induced by electrical field stimulation. The effect of prucalopride on electrically induced cholinergic contractions was antagonized by the selective 5-HT_4 receptor antagonist GR113808 and this antagonism was confirmed in the release assay. The non-selective PDE inhibitor IBMX concentration-dependently reduced the amplitude of the cholinergic contractions, but, in a concentration that only mildly reduced these contractions, it enhanced the facilitating effect of prucalopride on both cholinergic contractions; IBMX was able to induce and enhance the facilitating effect of prucalopride on electrically induced ACh release. The selective inhibitors vinpocetine (PDE1), EHNA (PDE2) and cilostamide (PDE3) did not influence the effect of prucalopride on acetylcholine release but the PDE4-inhibitor rolipram enhanced the facilitating effect of prucalopride to the same extent as IBMX. These results demonstrate that 5-HT_4 receptors are present on the cholinergic nerves towards the pig gastric circular muscle, facilitating acetylcholine release and the intracellular transduction pathway of this facilitation is regulated by PDE4. As for the role of PDEs in the control of the cyclic nucleotide content in the porcine gastric circular muscle cells, our results point to a redundant role of PDE3 and PDE4 with slight predominance of PDE3.

In **Chapter IV** the pig colon descendens was first evaluated as possible model for the different locations of human colonic 5-HT_4 receptors. To evaluate the nervous control in porcine GI motility, the intrinsic excitatory and inhibitory motor neurotransmission was first characterized. In colonic circular smooth muscle strips, electrical field stimulation (EFS) was only able to voltage-dependently induce on-contractions in the combined presence of the NO synthase inhibitor L-NAME and the SK channel blocker apamin. The on-contractions were largely reduced by the neuronal conductance blocker tetrodotoxin and by the muscarinic receptor antagonist atropine, illustrating activation of cholinergic neurons. Prucalopride facilitated submaximal EFS-evoked cholinergic contractions and this effect was prevented by the 5-HT_4 receptor antagonist GR113808, supporting the presence of facilitating 5-HT_4 receptors on the cholinergic nerve endings innervating circular muscle in pig colon descendens. However, the facilitating effect of prucalopride was less pronounced than has been reported for canine and human stomach or colon, and for porcine stomach. This suggests a more limited number of 5-HT_4 receptors on the

cholinergic nerve endings and/or less effective signalling of these receptors in pig colon descendens. Relaxations were induced by EFS in strips pre-contracted with substance P in the presence of atropine. The responses at lower stimulation voltages were abolished by tetrodotoxin. L-NAME or apamin alone did not influence or only moderately reduced the relaxations, but L-NAME plus apamin abolished the relaxations at lower stimulation voltages, suggesting that NO and ATP act as inhibitory neurotransmitters in a redundant way. Prucalopride did not influence the EFS-induced relaxations at lower stimulation voltage, nor did it per se relax contracted circular muscle strips. No evidence for relaxing 5-HT_4 receptors, either on inhibitory neurons or on the muscle cells was thus obtained in pig colon descendens circular muscle. The pig colon descendens can thus only be used as a model for the 5-HT_4 receptors on cholinergic neurons in the human colon.

In **Chapter V** the role of PDEs in the control of the facilitating effect of prucalopride on cholinergic contractions and of smooth muscle tone in pig colon descendens was studied. The influence of the non-selective PDE inhibitor IBMX and selective inhibitors vinpocetine (PDE1), EHNA (PDE2), cilostamide (PDE3), rolipram (PDE4) and zaprinast (PDE5) was evaluated. IBMX and cilostamide concentration-dependently reduced the amplitude of the cholinergic contractions, as good as abolishing them at their highest concentrations. EHNA only reduced the contractions significantly at the highest concentration tested. Rolipram showed a biphasic effect, significantly increasing the contractions at 0.1 and 0.3 µM but decreasing them at 30 µM. Vinpocetine did not influence the electrically induced contractions while zaprinast enhanced the responses at 3-30 µM. IBMX, vinpocetine and EHNA did not influence the facilitating effect of prucalopride on electrically induced cholinergic contractions but rolipram tended to enhance it. When rolipram was added after prucalopride, the facilitating effect of prucalopride was significantly enhanced. These results suggest that PDE3 is the main regulator of the cyclic nucleotides regulating circular smooth muscle activity and that the signal transduction of 5-HT_4 receptors on the cholinergic nerves towards the circular muscle layer is regulated by PDE4 in pig colon descendens.

Finally the cellular 5-HT_4 receptor distribution, with attention to the epithelial layer was investigated in porcine stomach and colon in **Chapter VI** by laser microdissection (LMPC) and PCR. 5-HT_4 receptor and GAPDH mRNA expression was detected in mucosa and muscle-myenteric plexus tissue fractions, and in mucosal and muscle-myenteric plexus parts of hematoxylin&eosin (H&E) stained tissue sections of pig colon descendens and gastric fundus. In

the mucosal tissue fractions of both GI tissues, the expression of 5-$HT_{4(+h)}$ receptor mRNA, a splice variant containing an additional 14 amino acid segment in the second extracellular loop of the receptor, was significantly higher compared to 5-$HT_{4(-h)}$ receptor expression, and a similar trend was obtained in the mucosal part of H&E stained tissue sections. Large microdissected patches of the epithelial and circular smooth muscle cell layer of pig colon descendens and of the epithelial cell layer of pig gastric fundus, also showed 5-HT_4 receptor and GAPDH mRNA expression. No 5-HT_4 receptor mRNA expression was detected in gastric LMPC-isolated EC cells from IHC stained tissues, while the cells were positive for GAPDH. The expression of 5-HT_4 receptors in individual cell types might be too low to pick them up by LPCM and PCR. Porcine GI mucosa thus predominantly expresses 5-$HT_{4(+h)}$ receptor splice variants suggesting a preferential involvement of this type of 5-HT_4 receptor in mucosal effects of 5-HT.

In conclusion, our findings indicate that 5-HT_4 receptors are present on the cholinergic neurons innervating the muscle layer of porcine stomach and colon. The signal transduction pathway of these receptors is regulated by PDE4 so that concomitant treatment with a PDE4 inhibitor enhances the facilitating effect of the 5-HT_4 receptor agonist prucalopride on Ach release and thus on cholinergic contractions. If further investigation confirms this mechanism in humans, this opens the possibility of prokinetic combination therapy of 5-HT_4 receptor agonists with PDE4 inhibitors. Our results also show the mucosal expression of 5-HT_4 receptors in pig stomach and colon. As the information on 5-HT_4 receptor-mediated effects on GI secretion in mammals is limited, the pig can also be used as a model to further unravel the cellular distribution of 5-HT_4 receptors and the effects of 5-HT_4 receptor activation in the mucosa.

Chapter 9

SAMENVATTING

Chapter 9 Samenvatting

Hoofdstuk I - Literatuuroverzicht. Serotonine (5-hydroxytryptamine, 5-HT) is een essentiële boodschapper die instaat voor het gecoördineerde transport van voedsel in de gastro-intestinale (GI) tractus via interactie met verschillende 5-HT receptoren. 5-HT activeert binnen de GI tractus 5-HT receptoren, dewelke gelokaliseerd zijn op de gladde spiercellen of op de intrinsieke en extrinsieke sensorische zenuwen. Bovendien dragen deze 5-HT receptoren bij tot de regulatie van de GI peristaltiek en de communicatie tussen de hersenen en de GI tractus. De 5-HT receptoren zijn onderverdeeld in zeven klassen waarvan de 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 en 5-HT_7 receptoren een potentiële rol hebben in de GI tractus. De 5-HT_4 receptor, aanwezig op de exciterende cholinerge motorische zenuwen in de GI tractus, faciliteert vrijstelling van acetylcholine waarbij de motiliteit in de GI tractus wordt bevorderd. Deze verhoogde presynaptische vrijstelling ligt aan de basis van de prokinetische werking van 5-HT_4 receptor agonisten zoals prucalopride en verklaart het gebruik van deze 5-HT_4 receptor agonisten in de behandeling van motiliteitsstoornissen zoals chronische constipatie, gastroparese en gastro-oesophageale refluxziekte. De farmaceutische industrie voert onderzoek naar doeltreffende en gepaste behandelingen voor patiënten met motiliteitsstoornissen door zich te richten op de werking van 5-HT en zijn receptoren. Naast de aanwezigheid van de 5-HT_4 receptoren op de cholinerge zenuwen in de GI tractus, is hun aanwezigheid ook voorgesteld op andere celtypes binnen het colon en de maag. In het humaan colon, induceren 5-HT_4 receptor agonisten relaxatie van de gladde spier door 5-HT_4 receptoren te activeren, die gelegen zijn op de gladde spiercellen zelf en mogelijk ook deze op de nitrerge zenuwen. Daarbuiten is expressie van mRNA voor 5-HT_4 receptoren ook aangetoond in de mucosa van de maag en colon van de mens en het varken en wordt er gesuggereerd dat 5-HT_4 receptoren betrokken zijn bij de mucosale secretie geïnduceerd door 5-HT. Maar de cellulaire distributie van de 5-HT_4 receptor en de functionele gevolgen daarvan zijn nog niet onderzocht binnen de epitheliale laag. Nochtans zou meer gedetailleerde informatie over de expressie, lokalisatie en signaaltransductie van de 5-HT_4 receptor een beter inzicht geven over de rol van 5-HT_4 receptoren in de motiliteit en secretie binnen de GI tractus.

Het **doel** van dit proefschrift was te onderzoeken of het colon descendens van het varken kon dienen als een model voor de functie en de locaties van de 5-HT_4 receptoren betrokken bij de controle van de motiliteit van het humaan colon. Vervolgens, werd bij activatie van de 5-HT_4 receptoren op de exciterende cholinerge zenuwen de invloed van fosfodiësterasen (PDEs) in de signaaltransductie onderzocht in de circulaire spierlaag van de maag en het colon van het varken. Tenslotte, werd de expressie van de 5-HT_4 receptoren bestudeerd in de mucosa van de maag en het colon van het varken.

In **hoofdstuk III**, werd initieel de invloed van de selectieve 5-HT_4 receptor agonist prucalopride onderzocht in de circulaire spierlaag van de varkensmaag zowel rechtstreeks op acetylcholine (ACh) vrijstelling uit de cholinerge zenuwuiteinden uitgelokt door elektrische stimulatie als onrechtstreeks op de contracties uitgelokt door de vrijgestelde ACh. Hierbij werd de mogelijke regulerende rol van PDEs op dit effect verder onderzocht. Prucalopride verhoogde zowel de amplitudes van submaximale cholinerge contracties als de ACh vrijstelling geïnduceerd door elektrische veld stimulatie, dit op concentratieafhankelijke wijze. Het effect van prucalopride op elektrisch uitgelokte cholinerge contracties werd geblokkeerd door the selectieve 5-HT_4 receptor antagonist GR113808 en deze antagonistisch werking werd bevestigd in de studie met ACh vrijstelling. De niet-selectieve PDE inhibitor IBMX verminderde de amplitude van de cholinerge contracties. Vervolgens werd het faciliterend effect van prucalopride getest in de aanwezigheid van IBMX, dat werd toegediend in een concentratie die de contracties slechts gematigd reduceerde. Hierbij was IBMX in staat om het faciliterend effect van prucalopride op elektrisch uitgelokte ACh vrijstelling verder te verhogen. Behalve de PDE4 inhibitor rolipram, die hetzelfde verhoogde effect uitlokte als IBMX op het faciliterende effect van prucalopride op de elektrisch uitgelokte ACh vrijstelling, hadden de andere selectieve PDE inhibitoren vinpocetine (PDE1), EHNA (PDE2) en cilostamide (PDE3) geen invloed op het effect van prucalopride. Deze resultaten bevestigen dat 5-HT_4 receptoren aanwezig zijn op de cholinerge zenuwen die de circulaire spierlaag van de varkensmaag innerveren en bij activatie van deze receptoren wordt ACh vrijstelling bevorderd. Verder werd aangetoond dat de intracellulaire signaaltransductie bij receptor activatie en facilitatie gereguleerd wordt door PDE4. PDEs houden ook de niveaus van de cyclische nucleotiden onder controle in de gladde spiercellen van de varkensmaag; onze resultaten toonden aan dat PDE3 en PDE4 hier talrijk aanwezig zijn, met een lichte voorkeur voor PDE3.

In **Hoofdstuk IV** werd het colon descendens van het varken eerst geëvalueerd als een potentieel model voor de verschillende locaties van de 5-HT_4 receptoren in het humaan colon. Om na te gaan hoe de GI motiliteit van het varken onder zenuwcontrole staat, werd zowel de intrinsieke exciterende als de inhiberende motor neurotransmissie gekarakteriseerd. In de circulaire spierstrips van het colon werden enkel voltage-afhankelijke on-contracties uitgelokt door elektrische veld stimulatie (EFS) in de aanwezigheid van de NO synthase inhibitor L-NAME in combinatie met apamine, een SK kanaal blokker. Deze on-contracties waren cholinerg doordat deze grotendeels werden gereduceerd door het neurotoxine tetrodotoxine, dat de doorstroom van zenuwsignalen blokkeert en door de muscarine receptor antagonist atropine. Wij toonden aan dat prucalopride in staat was om de submaximale EFS-uitgelokte cholinerge contracties te verhogen, waarbij dit effect verhinderd werd door toedieningen van de 5-HT_4 receptor antagonist GR113808. Deze resultaten bevestigen de aanwezigheid van faciliterende 5-HT_4 receptoren op de cholinerge zenuwuiteinden, die de circulaire spierlaag van het varkenscolon innerveren. Toch was dit faciliterend effect van prucalopride minder uitgesproken dan het effect gerapporteerd in de maag of colon van de hond en mens, en in de varkensmaag. Dit zou mogelijks kunnen wijzen op een geringere aanwezigheid van 5-HT_4 receptoren op de cholinerge zenuwenuiteinden en/of een minder efficiënte koppeling van deze receptoren in het colon descendens van het varken. In de aanwezigheid van atropine werden de spierstrips in contractie gebracht met substantie P, een tachykinine 1 receptor agonist en hierop werden relaxaties uitgelokt door EFS. Bij lage voltages, werden deze antwoorden geblokkeerd door tetrodotoxine. L-NAME of apamine waren op zichzelf niet in staat, of slechts in geringe mate, om de relaxaties te reduceren, maar L-NAME in combinatie met apamine blokkeerden de relaxaties uitgelokt bij de laagste voltages. Dit suggereert dat NO en ATP op redundante wijze als inhiberende neurotransmitters fungeren. Prucalopride had noch een effect op de EFS-uitgelokte relaxaties bij lage voltages, noch een effect per se op de in contractie gebrachte spierstrips. Als gevolg konden we dus geen evidentie vinden voor de aanwezigheid van relaxerende 5-HT_4 receptoren in de circulaire spierlaag van het colon descendens van het varken, niet op de inhiberende zenuwen noch op de gladde spiercellen zelf. Hieruit konden we besluiten dat het colon descendens van het varken enkel kan gebruikt worden als een model voor de 5-HT_4 receptoren aanwezig op de cholinerge neuronen in het humaan colon.

In **Hoofdstuk V** werd de rol van PDEs in de controle van het faciliterende effect van prucalopride op cholinerge contracties en op de tonus van de gladde spier, bestudeerd. De invloed van zowel de niet-selectieve PDE inhibitor IBMX als de selectieve inhibitoren vinpocetine (PDE1), EHNA (PDE2), cilostamide (PDE3), rolipram (PDE4) en zaprinast (PDE5) werd hierbij geëvalueerd. IBMX en cilostamide waren beiden in staat om de amplitude van de cholinerge contracties op concentratie-afhankelijke wijze te reduceren en bij toediening in hun hoogste concentraties waren ze in staat om de cholinerge contracties zo goed als volledig te blokkeren. EHNA reduceerde de contracties enkel significant bij de hoogste concentratie. Rolipram vertoonde een bifasisch effect, waarbij de contracties eerst significant verhoogden bij 0.1 µM en 0.3 µM, maar werden daarna vanaf 30 µM gereduceerd. Vinpocetine had geen enkel effect op de elektrisch uitgelokte contracties, zaprinast daarentegen verhoogde de antwoorden vanaf 3-30 µM. IBMX, vinpocetine en EHNA hadden geen invloed op het faciliterende effect van prucalopride op de elektrisch uitgelokte cholinerge contracties, maar rolipram vertoonde een lichte tendens om dit effect te verhogen. Maar, wanneer rolipram werd toegediend na het effect van prucalopride, werd het faciliterend effect van prucalopride wel significant verhoogd. Deze resultaten suggereren dat voornamelijk PDE3 verantwoordelijk is voor de regulatie van de cyclische nucleotiden in de circulaire spier en dat de signaaltransductie van de 5-HT_4 receptoren, aanwezig op de cholinerge zenuwen naar de circulaire spierlaag van het colon van het varken toe, gereguleerd is door de PDE4.

Tenslotte werd in **Hoofdstuk VI** de cellulaire distributie van de 5-HT_4 receptor in de epitheliale laag van de maag en het colon van het varken onderzocht via laser microdissectie (LMPC) en PCR. 5-HT_4 receptor en GAPDH mRNA expressie werd gedetecteerd in de mucosa en in de spier-myenterische plexus van weefselfracties, en in mucosale en spier-myenterische plexus afgeschraapte delen van hematoxyline en eosine (H&E)-gekleurde weefselcoupes van de fundus en colon descendens van het varken. In de mucosale weefselfracties van beide weefsels, werden aangetoond dat er een significant hogere expressie was van het 5-$HT_{4(+h)}$ receptor mRNA ten opzichte van de 5-$HT_{4(-h)}$ receptor en een gelijkaardige trend werd aangetoond in de mucosale afgeschraapte delen van H&E-gekleurde weefselcoupes. De 5-$HT_{4(+h)}$ receptor is een splice variant die een extra segment van 14 aminozuren bevat in de tweede extracellulaire lus van de receptor. Grote fragmenten uit de epitheliale laag en uit de spierlaag van het colon descendens en uit de epitheliale laag van de fundus werden geïsoleerd aan de hand van LMPC; in deze LMPC-geïsoleerde fragmenten werden zowel 5-HT_4 receptor en GAPDH mRNA expressie aangetoond.

EC cellen, geïsoleerd door LMPC uit de epitheliale laag van de fundus, vertoonden wel GAPDH mRNA expressie, maar geen expressie voor de 5-HT_4 receptor. De expressie van de 5-HT_4 receptor door individuele celtypes is mogelijks te laag om gedetecteerd te worden via LMPC en PCR. Toch kunnen we uit deze resultaten besluiten dat de mucosa van GI weefsels van het varken de 5-$HT_{4(+h)}$ receptor splice variant predominant expresseert, waarbij deze variant een potentiële rol zou kunnen spelen in de respons op 5-HT in de mucosa.

Uit onze resultaten kunnen we besluiten dat de 5-HT_4 receptoren aanwezig zijn op de cholinerge zenuwen die de spierlaag van zowel de maag als het colon van het varken innerveren. Verder werd vastgesteld dat de signaaltransductie van deze 5-HT_4 receptoren gereguleerd is door PDE4. Hierdoor zal toediening van een PDE4 inhibitor het faciliterende effect van de 5-HT_4 receptor agonist prucalopride op ACh vrijstelling en dus cholinerge contracties, verder verhogen. Indien dit mechanisme kan bevestigd worden binnen de mens, kan dit nieuwe mogelijkheden bieden in het klinisch gebruik van prokinetische 5-HT_4 receptor agonisten in combinatie met PDE4 inhibitors. Verder konden we ook de mucosale expressie van de 5-HT_4 receptoren bevestigen in de maag en het colon van het varken. Hoewel de informatie over het effect op GI secretie via 5-HT_4 receptoren beperkt is, kan het varken ook gebruikt worden als model om de cellulaire distributie van de 5-HT_4 receptoren en de effecten van 5-HT_4 receptor activatie in de mucosa verder te onderzoeken.

DANKWOORD

Dankwoord

Veel mensen zullen onmiddellijk doorgebladerd hebben naar het dankwoord, want dat is het plezantste en iedereen wil weten of hij/zij erin voorkomt. Ik heb zelf ook het meeste uitgekeken om dit deel te schrijven. Toch heb ik er even over moeten nadenken hoe ik nu in een paar zinnen, zo origineel mogelijk, iedereen kan bedanken die zijn steentje heeft bijgedragen tot het voltooien van dit proefschrift. Want een proefschrift schrijf je niet alleen, dat is zeker.

Een doctoraatsproefschrift is niet alleen een samenvatting van de resultaten van je onderzoek, maar is het hoogtepunt van jarenlange noeste arbeid, waarbij je je laat adviseren door een heleboel mensen, zonder wiens advies en steun ik deze opdracht niet tot een goed einde had kunnen brengen. Velen hebben ooit wel eens gevraagd: “Over wat gaat je doctoraat? Vordert het een beetje? Hoe is het nog met de varkens?”. Vaak konden de meesten enkel het deel, waar ik varkens mocht opsnijden, onthouden. En ook al was het niet altijd eenvoudig om uit te leggen hoe het nu werkelijk zat, deze blijk van interesse door velen, zorgde voor nieuwe brandstof om door te zetten! Op deze laatste bladzijden wil ik graag iedereen bedanken die op één of andere manier heeft geholpen bij de uitvoering van mijn project, het schrijven van dit proefschrift en/of heeft bijgedragen om de omstandigheden, waarin ik de afgelopen jaren aan mijn project heb gewerkt, plezierig en luchtig te maken.

In de eerste plaats wil ik mijn promotor, Prof. Dr. Romain Lefebvre, bedanken voor zijn keuze om mij enkele jaren geleden aan te nemen als assistent bij de vakgroep Farmacologie en zo te kunnen werken in zijn labo om dit onderwerp mij eigen te maken. Zijn advies, vertrouwen, eindeloos geduld, tijd en hulp hebben een belangrijke bijdrage geleverd aan het resultaat en hebben ertoe geleid dat dit project succesvol werd afgerond en dat uiteindelijk een proefschrift werd neergepend. Daarnaast wil ik ook Joris De Maeyer bedanken met wie regelmatig werd overlegd en de finale manuscripten met toewijding heeft nagekeken. Mijn collega’s wil ik natuurlijk ook bedanken om een luisterend oor te zijn, te hulp te schieten waar nodig en vooral vrijwillig proefkonijnen waren voor al mijn experimentele lekkernijen. Bedankt Sarah Cosyns, Filip De Vin, Sze Men Choi, Dinesh Babu, Sabine Weninger, Ole De Backer, Inge Dhaese, Valère Geers, Annie De Smet, Tom Vanthuyne, Bart Blanckaert, Diego De Baere, Danielle Woods, Sam Tshitenge, Kelly Decaluwé, Bart Pauwels, Charlotte Boydens en Lies Vancraeynest voor de prettige sfeer. Maar ik wil mijn dank aan Els Van Deynse en Inge Van Colen extra in de verf zetten voor hun bijdrage en technische ondersteuning in het labowerk, maar ook om mijn loopconditie op peil te houden.

Ik wil Prof. Dieter Deforce en een aantal mensen van zijn onderzoeksgroep, zoals Mado, Trees en Sandra bedanken om gebruik te mogen maken van het PALM laser microdissection systeem en mij te helpen om deze techniek onder de knie te krijgen.

I would like to thank the exam committee, Prof. Dr. M. Jiménez, Prof. Dr. C. Cuvelier, Prof. Dr. J. Plum, Prof. Dr. J.-P. Timmermans, Prof. Dr. D. De Looze, Prof. Dr. I. Colle and Prof. Dr. G. Joos for their time to read this thesis and constructive feedback.

Uiteraard wil ik ook mijn vrienden en familie bedanken. Papa, mama, Kristof, Frederik, Katleen, Lukas en Karolien, ik neem aan dat jullie nog steeds niet goed begrijpen wat ik eigenlijk de voorbije vier jaar en half allemaal heb geleerd en bestudeerd en mij mee bezig heb gehouden. En niettegenstaande hebben jullie dat alles nauwlettend op de voet gevolgd en steeds geïnteresseerd geluisterd naar wat ik te vertellen had. Karolien, je deur stond altijd open voor mij, je telefoon altijd binnen handbereik om mij te woord te staan en als klankbord te fungeren of gewoon om leuke uitstapjes, verkleedpartijtjes of reizen te maken. Ik wil ook mijn grootste fan, mijn meter, extra bedanken voor haar lieve mailtjes, kaartjes en telefoontjes met wijze raad en mentale ondersteuning.

Ik wil in dit dankwoord ook een speciale bedanking plaatsen voor Jos Piederiet, die mij al die jaren mentaal heeft ondersteund om mijn hersenkronkels in toom te houden en ervoor gezorgd heeft dat ik de vrouw kan zijn, wie ik ben, graag wil zijn en graag wil blijven.

Tussen en na al die uren van zwoegen en zweten was er ook nood aan ontspanning en gerstenat. Daar zorgden al mijn backing vocals voor: Vrienden, bedankt voor de toffe sfeer en oppeppers gedurende al die jaren! Riskavonden, feestjes, koffietjes gaan drinken, sporten…. Sporten in de fitness zorgde voor de nodige uitlaatklep voor frustraties, maar ook voor het ontmoeten van talrijke leuke en sympathieke mensen, die mij ook tijdens al die jaren gesteund hebben en interesse toonden.

Jonas, Jan, Hans en Brecht DM, jullie hebben er jarenlang voor gezorgd om mij op tijd en stond mee te nemen voor een deugddoende frisse pint die de hyperactiviteit van de neuronale banen in mijn hersenen voldoende verdoofde. Cleome en Wouter wil ik in de bloemetjes zetten voor de gezellige koffie-namiddagen, ontspannende weekends, leuke werkavonden, onvergetelijke Gentse Feesten, maar ook voor de nodige peptalk. Daarnaast wil ik ook de biomedische meiden: Adelien, Laura, Machteld, Sylvie en Linde, bedanken voor de leuke studentenjaren vol

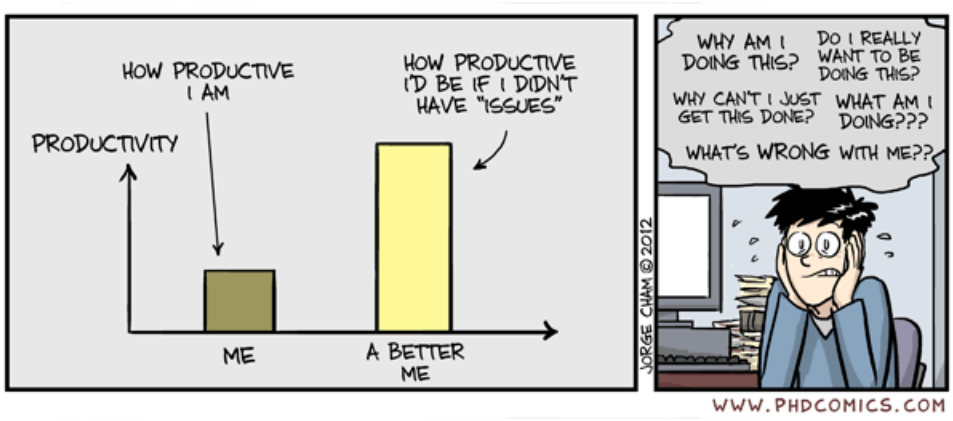
dolle pret, maar ook voor onze maandelijkse etentjes om bij te kletsen en doctorale frustraties te delen.

Last but not least. Brecht, een jaar geleden heeft onze passie voor dessert ons samengebracht en dit in het begin van mijn strijd. Het was niet gemakkelijk. Ik moest nog ten velde trekken met zwaard en schild om al die draken te temmen, maar je hebt voor me gezorgd, me gesteund en getroost in alle moeilijke momenten. Ik heb ook dankzij jou al die draken de kop ingedrukt en deze strijd heeft ons gebonden tot een stevige Italiaanse merengue. We go together like milk and cookies ☺.

“Dream the impossible dream, fight the unbeatable foe, strive with your last once of courage to reach the unreachable star.”

Evelien Priem (*Even a small star shines in the darkness)*

Uit het leven gegrepen cartoons van een doctorandus (Met dank aan Koen Mertens)



Figuur 1

Productivity curve during writing: “The Ugly Truth”

Figuur 2

Disappointment after writing.