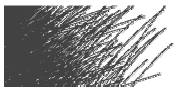



# Role of soluble guanylate cyclase isoforms and of hydrogen sulfide in gastrointestinal motility

**Ingeborg Dhaese**

**Promotor: Prof. Dr. R.A. Lefebvre**

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**Vakgroep Farmacologie – Heymans Instituut**

# Promotor

**Prof. Dr. R.A. Lefebvre**
Universiteit Gent, Belgium

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# List of Abbreviations

| | |
|---|---|
| 2-APB | 2-aminoethyl diphenylborinate |
| 4-AP | 4-aminopyridine |
| 8-br-cGMP | 8-bromo-cGMP |
| AC | adenylate cyclase |
| ANOVA | analysis of variance |
| ATP | adenosine triphosphate |
| AUC | area under the curve |
| $BH_4$ | tetrahydrobiopterin |
| $BK_{Ca}$ channel | large-conductance $Ca^{2+}$-dependent $K^+$ channel |
| cAMP | adenosine 3',5'-cyclic monophosphate |
| CBS | cystathionine β-synthase |
| cDNA | complementary deoxyribonucleic acid |
| cGK | cGMP-dependent protein kinase |
| cGMP | guanosine 3', 5'-cyclic monophosphate |
| CO | carbon monoxide |
| CSE | cystathionine γ-lyase |
| Ct | threshold cycle |
| DETA-NO | diethylenetriamine NONOate |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DSS | dextrane sulphate sodium |
| EAS | external anal sphincter |
| $EC_{50}$ | half maximal effective concentration |
| EDRF | endothelial-derived relaxant factor |
| EFS | electrical field stimulation |
| EIA | enzyme immunoassay |
| Emax | maximum effect |
| eNOS | endothelial NOS |
| ENS | enteric nervous system |
| FAD | flavin adenine dinucleotide |
| FAM | 6-carboxylfluorescein |
| FD70 | fluorescein-labelled dextran (70 kDa) |
| FMN | flavin mononucleotide |
| GC | geometric center |
| GI | gastrointestinal |
| GMCs | giant migrating contractions |
| GTP | guanosine triphosphate |
| HO | heme oxygenase |
| HPRT | hypoxanthine-guanine phosphoribosyltransferase |
| HRP | horseradish peroxidase |
| $H_2S$ | hydrogen sulfide |
| IAS | internal anal sphincter |

| | |
|---|---|
| $IC_{50}$ | half maximal inhibition concentration |
| ICC | interstitial cells of Cajal |
| ICS | ileocaecal sphincter |
| $IK_{Ca}$ channel | intermediate conductance $Ca^{2+}$-dependent $K^+$ channel |
| iNOS | inducible NOS |
| $IP_3$ | inositol triphosphate |
| IPAN | intrinsic primary afferent neuron |
| IRAG | Ins(1,4,5)*P*3-receptor associated cGMP kinase substrate |
| $K_{ATP}$ channel | ATP-dependent $K^+$ channel |
| KI | knock-in |
| KO | knock-out |
| $L_0$ | optimal load |
| LES | lower esophagal sphincter |
| L-NAME | $N^{\omega}$-nitro-L-arginine methyl ester |
| MLC | myosin light chain |
| MLCK | myosin light chain kinase |
| MLCP | myosin light chain phosphatase |
| MMC | migrating motor complex |
| mRNA | messenger ribonucleic acid |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NaHS | sodium hydrogen sulfide |
| NANC | non-adrenergic non-cholinergic |
| nNOS | neuronal NOS |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| OD | optical density |
| ODQ | 1H-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one |
| PACAP | pituitary adenylate cyclase activating peptide |
| PBS | phosphate buffered saline |
| PDE | phosphodiesterase |
| pGC | particulate guanylate cyclase |
| PGF2α | prostaglandin F2α |
| PKC | protein kinase C |
| PKG | protein kinase G |
| PLC | phospholipase C |
| PSD-95 | postsynaptic density 95 |
| RNA | ribonucleic acid |
| RNS | reactive nitrogen species |
| RT-PCR | reverse transcriptase –polymerase chain reaction |
| S.E.M. | standard error of the mean |
| SERCA | sarcoendoplasmic reticulum $Ca^{2+}$ ATPase |
| sGC | soluble guanylate cyclase |
| $SK_{Ca}$ channel | small conductance $Ca^{2+}$-dependent $K^+$ channel |
| SMC | smooth muscle cell |

| | |
|---|---|
| SNP | sodium nitroprusside |
| SQ 22536 | 9-(tetrahydro-2-furanyl)-9H-purin-6-amine |
| SR | sarcoplasmatic reticulum |
| TRPV1 | transient receptor potential vanilloid type 1 |
| UES | upper esophagal sphincter |
| VIP | vasoactive intestinal peptide |
| WT | wild-type |
| Y-2762 | (R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide |

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# Chapter I

# LITERATURE SURVEY

# Chapter I Literature Survey

## *I.1 General aspects of the gastrointestinal tract*

### I.1.1 Function

The human gastrointestinal (GI) tract consists of the alimentary canal from the mouth to the anus and the associated glandular organs that empty their contents in the canal. The overall function of the GI tract is to take in nutrients and eliminate wastes. To fulfil this role, the major physiological processes that occur in the GI tract are motility, secretion, digestion, absorption and elimination. Food is taken into the mouth as large particles containing macromolecules that are not absorbable. The breaking down of food into absorbable material occurs by grinding and mixing the food (motility) with various secretions containing enzymes, ions and water that enter the GI canal. The enzymes convert the macromolecules into absorbable molecules in a process termed digestion. The products of digestion are then transported across the epithelium to enter the blood or lymph by a process termed absorption. Also most of the added secretions are absorbed for reuse. Finally, undigested waste products are eliminated by GI motility (Raybould et al., 2003).

### I.1.2 Motility along the gastrointestinal canal

The GI canal, while functioning as whole in the transport of ingested material, is physiologically partitioned into subdivisions by a series of sphincters. The esophagus begins at the upper esophagal sphincter (UES) and extends to the lower esophagal sphincter (LES), while the stomach is defined proximally by the LES and distally by the pylorus. The small intestine is bordered by the pylorus and the ileocaecal sphincter (ICS). Finally, the most distal portion of the GI canal, the large intestine, begins at the ICS and continues to the exit of the GI canal at the internal (IAS) and external anal sphincter (EAS). The structure of the gastrointestinal wall is similar throughout the canal, consisting of the inner mucosal layer, the submucosa, the muscle layer and the serosa. GI motility is achieved by coordinated activity of the smooth muscle cells of the muscle layer. The muscle layer is organized in a thick inner circular and a thinner outer longitudinal smooth muscle layer. In the large intestine, the latter consists of three separate longitudinal ribbons of smooth muscle, the taenia coli. Sphincters

are thickened extensions of the circular smooth muscle layer, except for the UES and the EAS which are striated muscle.

#### I.1.2.1 The esophagus

The upper one third of the esophagus also makes up an exception in that it contains striated muscle instead of smooth muscle, whereas the lower two thirds of the esophagus contain smooth muscle, just as the rest of the GI canal. When food is taken in, relaxation of the UES allows the food bolus to enter from the pharynx into the esophagus. This produces esophagal distension, which initiates a peristaltic wave i.e. a ring of contraction that moves aborally over variable distances and thereby transports the food bolus further towards the stomach. Finally, relaxation of the LES allows the food bolus to pass into the stomach (Raybould et al., 2003).

#### I.1.2.2 The stomach

In a consideration of its motor functions, the stomach can be divided into three functional regions: the proximal stomach (cardia, fundus, proximal body), the distal stomach (distal body and antrum) and the pylorus. Coordinated actions of these regions regulate the emptying of gastric contents.

Upon arrival of the ingested food bolus in the stomach, the proximal stomach serves to accommodate and store the ingested food without a significant increase in intragastric pressure. Maintenance of intragastric pressure is controlled by two proximal gastric reflexes (receptive relaxation and gastric accommodation). Receptive relaxation is the reduction in proximal gastric tone that is initiated by the act of swallowing. Gastric accommodation or adaptive relaxation is the relaxation of the proximal stomach in response to gastric distension. Finally, the proximal stomach propels the chyme into the distal stomach by generating a compressive tonic force. The distal stomach exhibits phasic contractions of the antrum that serve to grind solid food and to regulate gastric emptying of solid and, to a lesser extent, liquid meals. Finally, in order to enter into the duodenum, the digesting food has to pass the pyloric sphincter. Prolonged periods of closure of the pylorus are interrupted by brief intervals during which the antral contents can pass into the intestine. The resistance to flow is provided by tonic and phasic pyloric motor activity. During the fed state, the pylorus acts as a sieve, not allowing large particles to traverse to the intestine. Instead, the latter are propelled back into the stomach for further grinding. Taken together, the tonic contractions of the proximal stomach, the propulsive contractions of the distal stomach and the pyloric activity are thus three important mechanisms for the regulation of gastric emptying.

Under fasting conditions, the distal stomach exhibits distinct phasic motor patterns than under postprandial conditions. The migrating motor complex (MMC) is a stereotypical pattern

that clears the stomach of undigested debris (food particles, mucus and sloughed epithelial cells) during fasting and has been termed the GI housekeeper. The MMC consists of four phases: Phase I is a period of motor quiescence, phase II exhibits irregular contractions and phase III is a period of intense and rhythmic contractions that begin in the gastric body and propagate to the pylorus. Phase IV is a transition period of irregular contractions between between phase III and phase I. Phase II is believed to represent a period in which fasting gastric contents are mixed, phase III contractions are highly propagative. During phase III, the pylorus is open and allows intestinal delivery of large particles (Hasler, 2003b).

#### I.1.2.3 The small intestine

The small intestine is divided into three functional regions: duodenum, jejunum and ileum. Through relaxation and thus opening of the sphincter of Oddi, bile, produced in the liver and stored in the gallbladder, and digestive enzymes, produced and stored in the pancreas, enter the GI canal at the level of the duodenum. The jejunum is the major organ for digestion and absorption of meal nutrients. The ileum has a specific role in the absorption of cobalamin (vitamin $B_{12}$). Following ingestion of a meal, the small intestine exhibits two motor patterns: segmentation and peristalsis. Segmentation is the process by which rings of contraction develop at uniform intervals, dividing the lumen into segments. Segmentation is the primary mechanism by which contents of the intestine are mixed with secretions and moved across the mucosa to enhance absorption. Peristalsis in the intestine consists of two phases: an excitatory response proximal to the food bolus (ascending contraction) and a distal inhibition (descending relaxation). The ascending contraction is characterized by simultaneous circular muscle shortening and longitudinal muscle relaxation, whereas the descending relaxation involves simultaneous longitudinal muscle contraction and circular muscle relaxation. Peristalsis is the primary mechanism by which contents are moved along the intestine in the digestive period. Under fasting conditions, the small intestine exhibits the same motor pattern as the stomach, called the MMC or intestinal housekeeper. The small intestinal MMC consist of the same four phases as the gastric MMC. As such, MMCs start in the stomach and pass along the intestine to the ileum, thereby propelling undigested food residue and sloughed enterocytes from the proximal gut into the large intestine. Every MMC cycle lasts about ninety minutes (Hasler, 2003a; Raybould et al., 2003).

#### I.1.2.4 The large intestine

The large intestine consists of the caecum, colon and rectum. The caecum receives the chyme from the small intestine, through the ICS. The caecum and ascending colon receive about 2 liters of ileal affluent daily. Further re-absorption of water and ions in the colon reduce the volume to about 200 milliliters per day. Prominent mixing patterns, such as short

and long duration contractions that are stationary or that propagate only for short distances in oral or aboral direction, promote the process of faecal desiccation. Due to the dehydration process, the faecal material is semisolid to solid after passage through the colon. The transverse, descending and sigmoid portions of the colon store faecal material and propel the material to the rectum. To this purpose, the colon exhibits both storage motor patterns (i.e. changes in tone) and propulsive motor patterns (i.e. phasic contractions). The latter include peristaltic motor patterns and giant migrating contractions (GMCs). GMCs, also termed high-amplitude propagated contractions, propagate aborally over extended distances and evoke mass movements of faeces. GMCs are most prominently after awakening, after eating, or in association with defecation. In the colon, an organized fasting motor pattern such as the MMC is not observed in humans (Hasler et al., 2003a).

The rectum exhibits a compliant wall that allows it to serve as a reservoir for faecal material until it can be expelled through the anal canal. Rectal motor complexes that do not propagate orally or aborally facilitate the storage function of the rectum. The anal canal is surrounded by two sphincters: the internal anal sphincter is a modified extension of the circular muscle of the rectum; the external anal sphincter is striated muscle. Distension of the rectum activates the rectoanal reflex, which relaxes the internal anal sphincter and simultaneously contracts the external anal sphincter. The latter preserves continence. When rectal pressure further increases, both the internal and the external anal sphincter will relax, resulting in a reflex expulsion of the faecal material in the rectum. Defecation can however be voluntarily inhibited by keeping the external anal sphincter contracted. In order to defecate, the individual allows the external anal sphincter to relax (Grundy et al., 2006; Hasler et al., 2003a).

## *I.2 Control of GI motility*

The different functions of the GI tract, including the motor patterns that are implicated in the generation of GI motility, are regulated by hormonal and neuronal control mechanisms. Hormonal control occurs via paracrine and endocrine regulation mechanisms. Paracrine regulation describes the process whereby a chemical released from a sensing cell diffuses through the interstitial space to influence the function of neighbouring cells. An example of a paracrine mechanism is the inhibition of gastrin release by somatostatin. Somatostatin is released from cells in the gastric antral mucosa in response to low intra-gastric pH. Endocrine regulation describes the process whereby the sensing cells respond to a stimulus by releasing their contents into the circulation to act on distant target cells. A particular target cell responds because it possesses high-affinity receptors specific for the hormone (Raybould et al., 2003). An example of endocrine regulation is the release of motilin from the

endocrine cells of the duodenal mucosa during fasting, which initiates MMC phase III contractions (De Smet et al., 2009). As in this thesis the role of the neurotransmitter nitric oxide (NO) in GI motility is investigated, the next sections of the introduction focus mainly on the neuronal control of GI motility.

Neuronal control is mediated via the extrinsic nervous system and the intrinsic nervous system (enteric nervous system; ENS). The presence of extensive intrinsic neuronal circuits make the GI tract unique among mammalian organs. The ENS contains reflex pathways capable of functioning independently of central control, although the extrinsic nervous system modifies activity within the gut wall. The ENS is most commonly the medium through which extrinsic neurons control GI function (Furness et al., 2003).

### I.2.1 Extrinsic innervation

The extrinsic nervous system consists of a parasympathetic and a sympathetic component (Fig. I.1). The parasympathetic nervous system can be anatomically subdivided into two nerves: the vagal nerve and the pelvic nerve. The vagal nerve fibers contain the axons of neurons whose cell bodies lie within the brain stem and innervate the upper GI tract. The pelvic nerve fibers originate from cell bodies lying in the sacral part of the spinal cord and innervate the distal colon and rectum. The vagal and pelvic neurons do not act directly on GI smooth muscle, but form synaptic connections with enteric neurons; the transmission of the neuronal input is mediated by acetylcholine acting on nicotinic receptors. The primary functions of the efferent vagal and pelvic nerves are stimulation of motility, secretion and blood flow.

The sympathetic nervous system originates in the thoraco-lumbar part of the spinal cord, where the neuronal cell bodies are located. The preganglionic sympathetic nerves release acetylcholine as neurotransmitter and form nicotinic synapses in the paravertebral ganglia or one of the three prevertebral ganglia, named the celiac ganglion, the superior mesenteric ganglion and the inferior mesenteric ganglion. From these ganglia, postganglionic sympathetic nerves, that release noradrenaline as neurotransmitter, originate and innervate respectively the upper, the middle and the lower part of the GI tract. The primary functions of the efferent sympathetic nerves are regulation of blood flow by constricting arterioles, reduction of water and electrolyte secretion and inhibition of motility. The regulation of motility is not mediated via a direct action of the adrenergic postganglionic nerves on the GI smooth muscle, except for sphincter regions, but via inhibition of the release of excitatory neurotransmitters from parasympathetic neurons or enteric neurons.

Besides the above mentioned efferent parasympathetic and sympathetic nerve fibers that mediate the control of the effector systems of the GI tract, also afferent or sensory nerve fibers run along both subdivisions of the extrinsic nervous system. The afferent neurons

transmit sensory information from the GI tract to the central nervous system and are subdivided in vagal and spinal afferent nerves. Vagal afferents are sparse in the colon but more numerous proximally (especially the stomach and esophagus), have cell bodies in the nodose ganglia and enter the brainstem. They are associated with upper GI sensations such as satiety, nausea and hunger. Spinal afferents are distributed throughout the GI tract, and travel in the splanchnic (sympathetic) nerves and in the pelvic (parasympathetic) nerves to the respectively thoraco-lumbar and sacral part of the spinal cord. Their cell bodies are located in dorsal root ganglia. Spinal afferents are associated with sensations of discomfort and pain (Blackshaw & Gebhart, 2002; Furness, 2003; Grundy et al., 2006; Lundgren, 2000).

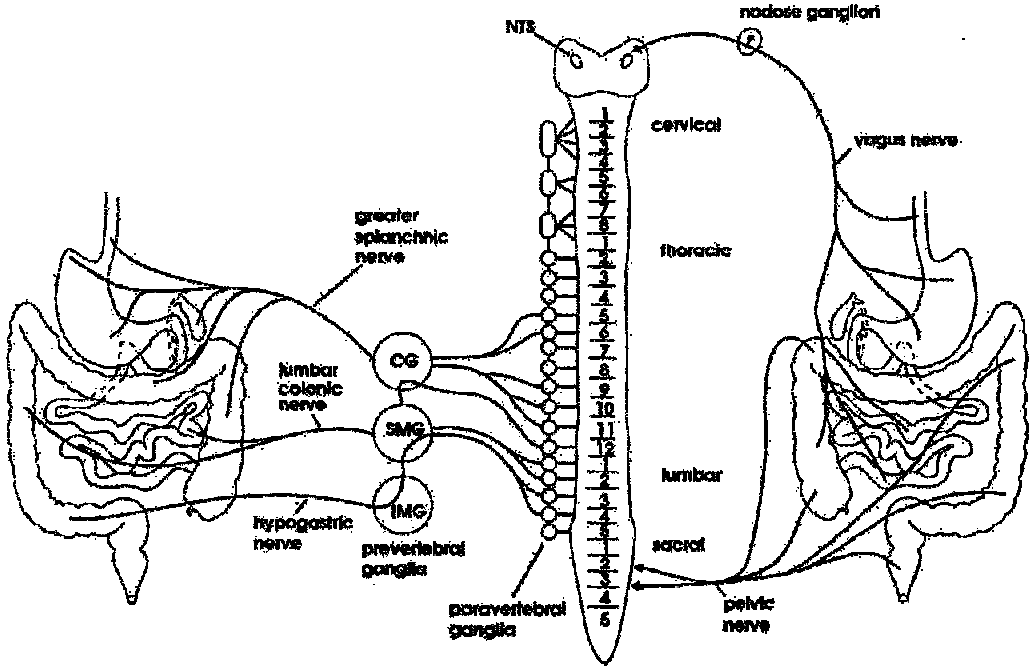


**Figure I.1**

The extrinsic innervation of the GI tract. The parasympathetic branch is shown on the right side, the sympathetic branch on the left side. The parasympathetic nervous system is subdivided into the vagal nerve and the pelvic nerve. The vagal nerve fibers contain the axons of neurons whose cell bodies lie within the brain stem and innervate the GI tract till the transverse colon. The pelvic nerve fibers originate from cell bodies lying in the sacral part of the spinal cord and innervate the distal colon and rectum. The sympathetic nervous system originates in the thoraco-lumbar part of the spinal cord, where the neuronal cell bodies are located. The preganglionic sympathetic nerves form synapses in the paravertebral ganglia or one of the three prevertebral ganglia. From these ganglia, postganglionic sympathetic nerves innervate respectively the upper, the middle and the lower part of the GI tract. CG: celiac ganglion, SMG: superior mesenteric ganglion, IMG: inferior mesenteric ganglion (adapted from Blackshaw & Gebhart, 2002).

## I.2.2 Intrinsic innervation

The enteric nervous system (ENS) consists of nerve cell bodies embedded in the wall of the gut. The nerve cell bodies are grouped in small aggregates, the enteric ganglia, which are connected to form two major ganglionated plexuses in the GI tract: the myenteric plexus, also called the Auerbach plexus, and the submucosal plexus, which is often referred to as the Meissner plexus. The myenteric plexus lies between the longitudinal and circular smooth muscle layers and forms a continuous network around the circumference of the GI tract from the upper esophagus to the IAS. In the parts of the large intestine where the longitudinal muscle is gathered into taenia, the myenteric plexus is prominent underneath the taenia and is sparser over the rest of the colonic surface. The submucosal plexus of ganglia is significant only in the small and large intestines; extensive networks of linked ganglia are not found in the submucosa of the esophagus and stomach, although isolated ganglia are sometimes encountered in these regions. In humans, an additional deep muscular plexus innervates the interface of the inner and outer circular muscle layers of the small intestine and colon (Furness et al., 2003) (Fig. I.2).

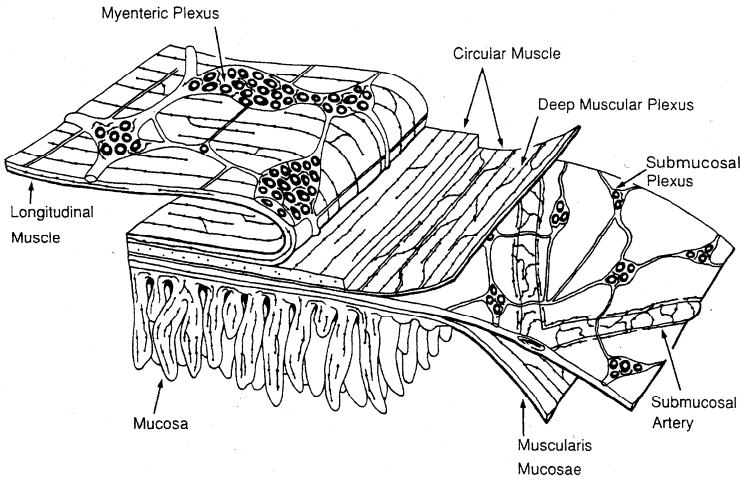


**Figure I.2**

Schematic representation of the enteric plexuses (Furness et al., 2003). The submucosal plexus lies within the submucosa and is only significant in the small and large intestines. The myenteric plexus is located between the longitudinal and circular smooth muscle layers of the upper esophagus to the IAS. In humans, an additional deep muscular plexus is found in the interface of the inner and outer circular muscle layers of the small intestine and colon.

The myenteric plexus primarily controls contraction and relaxation of GI smooth muscle (motility) (Kunze & Furness, 1999), whereas the submucosal plexus controls the secretory and absorptive functions of the GI epithelium, local blood flow and neuro-immune function.

Both plexuses receive synaptic inputs from efferent and afferent nerve fibers of the extrinsic nervous system. However, most of the synaptic inputs to enteric neurons come from other enteric neurons (Galligan, 2002).

The neurons in both plexuses can be classified as: 1) sensory neurons, 2) interneurons and 3) motor neurons. The sensory neurons, also called “intrinsic primary afferent neurons” (IPANs) become activated by three types of stimuli (distension, mechanical distortion of the mucosa and change in luminal chemistry), and initiate motility changes as well as secretory and blood flow changes. IPANs have their sensory endings in the mucosa and their cell bodies in the myenteric or submucosal plexus. The IPANs synapse with interneurons, which in turn make connections with other orally (ascending) or anally (descending) directed interneurons and finally with motor neurons. The motor neurons are the final effectors and are involved in the regulation of secretion (secretoneurons) or motility. The motor neurons that innervate the longitudinal and circular smooth muscle layers can be classified into excitatory and inhibitory neurons. The primary neurotransmitter released by activation of the excitatory motor neurons is acetylcholine. Also tachykinins, which represent excitatory non-cholinergic non-adrenergic or NANC neurotransmitters, are released albeit playing a lesser role in the contraction of smooth muscle cells than acetylcholine. The activation of the inhibitory motor neurons results in the release of inhibitory NANC neurotransmitters (see I.2.3) causing relaxation of the smooth muscle cells (Furness, 2003; Kunze & Furness, 1999).

To study which transmitters mediate the contractile and relaxing responses of GI smooth muscle, experiments are designed using an organ bath equipped with two platinum electrodes and force transducers to record contraction/relaxation of GI smooth muscle preparations. The latter contain both muscle layers and the myenteric plexus which is lying between the circular and longitudinal muscle. The neurons of the myenteric plexus can be activated by electrical field stimulation. Electrical field stimulation is achieved by applying current through two platinum electrodes which are positioned close to the GI smooth muscle preparation. By use of particular stimulation parameters the applied electrical pulses depolarize the cell membranes of the enteric neurons and generate action potentials. These action potentials activate enteric synapses to release their inhibitory and/or excitatory neurotransmitters. Thereby, a cocktail of differently acting transmitters is released and causes relaxation as well as contraction of the GI smooth muscle preparation. The transmitters involved can be identified by adding antagonists to the organ bath. For example, as the excitatory neurotransmitter acetylcholine acts on muscarinic receptors on the smooth muscle, it is possible to block acetylcholine-induced contraction by using the muscarinic antagonist atropine.

In between the motor neurons of the ENS and the smooth muscle cells, a network of interstitial cells of Cajal (ICC) is located. The ICCs are referred to as the pacemaking cells of the gut, generating and propagating slow wave activity and potential oscillations. The ICCs are closely associated with the varicose nerve terminals via synaptic-like structures and express receptors for the neurotransmitters utilized by the enteric motor neurons. This enables the ICCs to receive neuronal input, which leads to the generation of electrical signals. The electrical responses elicited in the ICCs are conducted to the smooth muscle cells via gap junctions, which lead to excitatory depolarization or inhibitory hyperpolarization responses and thus respectively contraction or relaxation of the smooth muscle cells. The ICCs are as such suggested to play an important role in the cholinergic excitatory and nitrergic inhibitory neurotransmission (Sanders, 1996; Ward & Sanders, 2001).

## I.2.3 Inhibitory NANC neurotransmission

The first functional evidence for the presence of non-cholinergic non-adrenergic or NANC neurotransmitters in the GI tract was provided by Burnstock et al. (1963), based on the observation that stimulation of intrinsic nerves in guinea-pig taenia coli resulted in a smooth muscle inhibitory junction potential that was not influenced by the muscarinic receptor antagonist atropine, with muscarinic receptors being the effectors of acetylcholine, and the adrenergic neuron blocker bretylium.

NANC neurotransmitters can either be excitatory or inhibitory. Tachykinins elicit excitatory smooth muscle responses, whereas ATP, vasoactive intestinal peptide (VIP), nitric oxide (NO), carbon monoxide (CO) and recently hydrogen sulfide ($H_2S$) are reported to elicit inhibitory smooth muscle responses. This section focuses on the inhibitory NANC neurotransmitters. ATP and VIP represent “classical” or “typical” neurotransmitters in the sense that they are synthesized and stored in vesicles in the neurons, released by exocytosis into the synaptic cleft, and act upon receptors in the plasma membrane of the adjacent target cell. NO, CO and $H_2S$ are however atypical neural messengers, being small gaseous molecules that are enzymatically synthesized only on demand, capable of diffusing freely across the plasma membrane and acting directly on (intra)cellular targets in the adjacent target cells (Barañano et al., 2001; Burnstock, 2006; Kasparek et al., 2008; Wang, 2002). The term “gasotransmitter” was introduced to characterize these three gases (Wang, 2002). The evidence that these three substances act as a GI neurotransmitter is very strong for NO, much weaker for CO and weakest for $H_2S$.

### I.2.3.1 ATP, VIP/PACAP

The role of the purine nucleotide ATP as a NANC neurotransmitter was proposed for the first time by Burnstock and colleagues and is now generally accepted. In addition to the presence

of high concentrations of ATP in subpopulations of myenteric neurons in different regions of the gut (Belai & Burnstock, 1994; Crowe & Burnstock, 1981) also the release of ATP was reported (Burnstock et al., 1978), providing evidence for the purinergic neurotransmission. Moreover, the role of ATP as a co-neurotransmitter in NANC relaxation was demonstrated or suggested for different regions of the GI tract in several species, such as the mouse stomach (Mulè & Serio, 2003), the rat pyloric sphincter (Soediono & Burnstock, 1994), the mouse (De Man et al., 2003) and human (Xue et al., 1999) jejunum, the rat ileum (Benko et al., 2006), the mouse (Serio et al., 2003a), rat (Pluja et al., 1999; Van Crombruggen & Lefebvre, 2004), hamster (El Mahmoudy et al., 2006) and human (Benko et al., 2007; Boeckxstaens et al., 1993; Keef et al., 1993) colon and the guinea pig IAS (Rae & Muir, 1996).

ATP is a ligand for P2 purinoceptors existing in two main subtypes: (1) the P2X receptors that are ligand-gated ion channels and (2) the P2Y receptors that are coupled to G proteins (Burnstock, 2006). Inhibitory responses are assumed to be mediated mainly by P2Y receptors although some reports suggest the involvement of P2X receptor subtypes in relaxant responses (De Man et al., 2003; Van Crombruggen et al., 2007). The main transduction pathway activated by binding of ATP to P2Y receptors involves the activation of phospholipase C (PLC), increased production of inositol triphosphate ($IP_3$) (Boyer et al., 1989) and release of $Ca^{2+}$ from internal stores via $IP_3$ receptors ($Ca^{2+}$ puffs) and to a lesser extent ryanodine receptors ($Ca^{2+}$ sparks). It is suggested that this $Ca^{2+}$ release is highly directional and causes local $Ca^{2+}$ transients near the plasma membrane without significant changes in global cytoplasmatic $Ca^{2+}$ concentration, leading to the activation of small conductance $Ca^{2+}$-dependent $K^+$ channels ($SK_{Ca}$ channels), hyperpolarization and subsequently relaxation of the smooth muscle cell (Bayguinov et al., 2000; Koh et al., 1997; Kong et al., 2000). Also other, PLC-independent, pathways activated by P2Y receptors have been proposed (von Kügelgen & Wetter, 2000), including the activation of adenylate cyclase (Qi et al., 2001; Zizzo et al., 2006). In addition, ATP was suggested to induce relaxation via the production of NO (Giaroni et al., 2002; Xue et al., 2000b).

A role of neurotransmitter in NANC relaxation was also proposed for the peptide VIP and the closely related pituitary adenylate cyclase activating peptide (PACAP) as both were found to be localized in the myenteric plexus and in varicose nerve terminals running along the smooth muscle layers (McConalogue et al., 1995; Portbury et al., 1995; Sundler et al., 1992; Suzuki et al., 1996). An involvement of VIP in NANC relaxation was suggested in different GI regions such as the mouse stomach (Mulè & Serio, 2003), the human (Tonini et al., 2000), rat (D'Amato et al., 1992; Li & Rand, 1990), cat (Barbier & Lefebvre, 1993) and pig (Lefebvre et al., 1995) gastric fundus, the mouse jejunum (Satoh et al., 1999), the hamster (El-Mahmoudy et al., 2006) and mouse (Satoh et al., 1999) colon and the mouse IAS (Rattan et

al., 2005). In the above mentioned gastric fundus tissues, the authors proposed a joint role for NO and VIP in the NANC relaxation with NO being released during the initial fast but also during sustained relaxation, whereas VIP was only released during the sustained relaxation; an exception is Tonini et al. (2000) who reported the release of VIP during short-lasting electrical stimulation. PACAP was reported to be involved in the NANC relaxation in for example the mouse colon (Satoh et al., 1999) and the guinea pig caecum (McConalogue et al., 1995).

Three G protein-coupled receptors for VIP/PACAP have been identified: $VPAC_1$ and $VPAC_2$ receptors with a similar affinity for both VIP and PACAP and the $PAC_1$ receptors with a much higher affinity for PACAP than for VIP (Harmar et al., 1998). The principal transduction pathway following the peptidergic activation of these receptors starts with the activation of adenylate cyclase and subsequent stimulation of cAMP, although also cAMP-independent such as the activation of phospholipase C or the stimulation of tyrosine kinase, the latter leading to the activation of $SK_{Ca}$ channels, have been reported (Kishi et al., 2000; Laburthe & Couvineau, 2002; MacKenzie et al., 2001; Takeuchi et al., 1999).

#### I.2.3.2 NO

In 1980, Furchgott & Zawadski observed that a substance released by vascular endothelial cells induced relaxation of the smooth muscle cells beneath. The substance was called the endothelium-derived relaxing factor (EDRF). It was only at the end of the 1980s that the identity of EDRF was unravelled and was reported to be NO by the work of two groups, one led by Ignarro (Ignarro et al., 1987) and one by Moncada (Palmer et al., 1987).

NO is a small gaseous molecule that is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). The synthesis of NO involves the formation of the intermediate N-hydroxy-L-arginine after which further oxidation leads to the production of NO and L-citrulline. L-citrulline can then be reconverted to L-arginine. In order to exert its action, NOS requires the presence of two co-substrates i.e. adenine dinucleotide phosphate (NADPH) and $O_2$ and several co-factors such as flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin ($BH_4$), heme protoporphyrin IX and calmodulin (CaM) (Fig. I.3).

NOS exists in three isoforms that are all active as homo-dimers. Two isoforms can be classified as constitutive enzymes i.e. neuronal NOS (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3), and one as an inducible enzyme i.e. inducible NOS (iNOS or NOS-2).

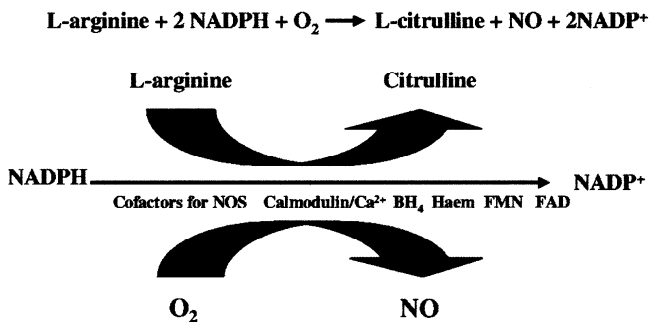


**Figure I.3**

Schematic representation of the biosynthesis of NO (Bruckdorfer, 2005). The enzyme NOS catalyses the synthesis of NO from L-arginine. The reaction is the result of a complete series of oxido/reductive events, involving five co-factors for which there are specific binding sites on the enzyme. Besides NO also L-citrulline is generated.

The constitutive isoforms are $Ca^{2+}$ dependent as association with co-factor CaM only occurs in the presence of $Ca^{2+}$. The inducible isoform however is active once expressed as it contains tightly bound CaM even in the absence of $Ca^{2+}$ (Bruckdorfer et al., 2005; Hobbs et al., 1999). In response to cytokines and bacterial products, iNOS is expressed in a variety of mainly immune-related cell types but the most important ones are the macrophages (Xie et al., 1992). However, Mancinelli et al. (2001) suggested that iNOS is also constitutively expressed in mouse colon smooth muscle cells where it would be involved in maintaining coordinated peristalsis. The constitutive isoforms nNOS and eNOS are predominantly located in respectively neurons of the central and peripheral nervous system and endothelial cells, platelets and mesangial renal cells (Martin et al., 2001). However, nNOS as well as eNOS were also reported to be present in GI smooth muscle cells of some species (Chakder et al., 1997; Mulè et al., 2001; Teng et al., 1998) and ICCs in mouse colon (Vannucchi et al., 2002). Finally, besides nNOS, also eNOS and even iNOS were shown to be present in the myenteric neurons of mouse colon (Vannucchi et al., 2002). Still, it is the nNOS isoform localised in the myenteric neurons that accounts for the synthesis of NO as an inhibitory NANC neurotransmitter in the GI tract (Bredt et al., 1990; Chakder et al., 1997; Kim et al., 1999). The fact that the release of NO was demonstrated in several GI tissues such as in the canine ileocolonic junction (Boeckxstaens et al., 1991b; Bult et al., 1990), in the rat gastric fundus (Boeckxstaens et al., 1991a) and more recently in the guinea pig ileum (Patel *et al*., 2008) further supports the role of NO as an inhibitory NANC neurotransmitter.

The first results indicating that NO is a mediator of NANC relaxation in the GI tract were obtained in the rat anococcygeus by Li & Rand (1989). Since then, based on in vitro studies using NOS inhibitors, NO is reported to be involved, as main or co-neurotransmitter, in the NANC relaxation in different regions of the GI tract such as the mouse LES (Kim et al.,

1999), the guinea pig (Lefebvre et al., 1992) and pig (Lefebvre et al., 1995) gastric fundus, the rat pyloric sphincter (Soediono et al., 1994), the mouse duodenum (Serio et al., 2003b), the rat (Vanneste et al., 2004), equine (Rakestraw et al., 1996) and human (Murr et al., 1999) jejunum, the rat ileum (Kanada et al., 1992), the guinea pig (Shuttleworth et al., 1999), rat (Van Crombruggen & Lefebvre, 2004) and human (Boeckxstaens et al., 1993; Keef et al., 1997) colon, the rat rectum (Takeuchi et al., 1998) and the mouse (Rattan et al., 2005) and human (O'Kelly et al., 1993) IAS. The important role of NO in NANC relaxation and hence GI motility becomes also evident from the study of nNOS knock-out (KO) mice and in vivo studies in various species. nNOS KO mice, who lack the nNOS gene and are therefore chronically deprived of nNOS generated NO, show an enlarged stomach with hypertrophy of the gastric circular muscle layer and the pyloric sphincter (Huang et al., 1993; Mashimo et al., 2000). The NANC relaxation is reduced in the gastric fundus strips of these mice (Dick et al., 2002) and gastric emptying of solids and liquids is delayed (Mashimo et al., 2000), all pointing to an essential role for NO in gastric motility.

An influence of NO on gastric emptying is reported in various studies, however, conflicting results are obtained. Inhibition of NOS was reported to delay (semi-)solid gastric emptying in dogs (Orihata & Sarna, 1994) and pigs (Lefebvre et al., 2005), to increase semi-liquid gastric emptying in humans (Konturek et al., 1999), while having no effect on solid gastric emptying in humans (Hirsch et al., 2000). These discrepancies might be related to the fact that NO was reported to not only influence the gastric accommodation of the proximal stomach (Desai et al., 1991; Tack et al., 2002), but also the antral motor activity (Konturek et al., 1999) and the pyloric sphincter relaxation (Sivarao et al., 2008). The consequences of NOS inhibition on these three important mechanisms in the regulation of gastric emptying (see I.1.2.2) might inter- and counteract with each other leading to the various implications on gastric emptying. In this regard, the delayed gastric emptying observed in the nNOS KO mice by Mashimo et al. (2000), despite the accelerating effect due to decreased gastric fundus relaxation (Dick et al., 2002), could be explained by a counteraction resulting from the impairment of the pyloric sphincter relaxation (Sivarao et al., 2008).

The nNOS KO mice not only revealed a role of NO in the gastric motility, but also in the jejunal inhibitory neurotransmission as NANC relaxations were found to be reduced in the jejunal strips of these mice (Xue et al., 2000a). Furthermore, the use of NOS inhibitors resulted in a delayed small intestinal transit in rats (Karmeli et al., 1997), dogs (Chiba et al., 2002) and humans (Fraser et al., 2005), which might be related to the conversion of regular peristalsis to irregular non-propulsive contractions when NOS is inhibited (Bogeski et al., 2005). NO was also reported to play a role in the interdigestive MMC which migrates along

the small intestine, as NOS inhibition was associated with stimulation of phase III activity (Russo et al., 1999).

Finally, studies using NOS inhibitors showed a role for NO in the descending relaxation in rat colon (Hata *et al*., 1990) and the colonic propulsive activity in guinea pigs (Foxx-Orenstein & Grider, 1996). NOS inhibitors delayed the colonic transit in rats, suggesting that NO enhances transit in the rat colon by mediating descending relaxation which, in turn, facilitates the propulsion of the colonic contents (Mizuta et al., 1999). NO was also reported to modulate the direction and frequency of the spontaneous colonic MMCs which were recorded along the mouse colon (Powel & Bywater, 2001).

The principal intracellular target of NO is the heme–containing protein soluble guanylate cyclase (sGC). By binding to the prosthetic heme group of sGC, NO activates sGC, which leads to the production of the second messenger cyclic guanosine 3'-5'-monophosphate (cGMP) (Lucas et al., 2000). The mechanisms downstream of cGMP leading to smooth muscle relaxation are discussed in I.3.3.2. However, effects of NO not involving the sGC-cGMP pathway have also been reported. Bolotina et al. (1994) provided evidence that NO is able to directly activate $Ca^{2+}$-dependent $K^+$ channels in vascular smooth muscle, whereas Koh et al. (1995) and Lang & Watson (1998) proposed a direct activation by NO of respectively voltage-activated and $Ca^{2+}$-dependent $K^+$ channels in colonic smooth muscle. The direct (i.e. sGC-independent) action of NO on these channels is suggested to involve chemical modification of the sulfhydryl groups present on the channels (Bolotina et al., 1994). The latter is called S-nitrosylation and results from the interaction of NO with $O_2$ or $O_2^{\cdot-}$ leading to the formation of reactive nitrogen species (RNS) such as $NO^+$, which is then added to the sulfhydryl group of a cysteine residue. RNS can also lead to oxidation (when one or two electrons are removed) or nitration (when $NO_2^+$ is added) of substrates. These RNS-mediated effects of NO were initially proposed to be mainly involved in pathological conditions, as they only prevail at higher concentrations of NO (> 1 µM), such as these produced when iNOS is induced. In contrast, the direct effects of NO, such as the physiologically relevant interaction with heme-containing proteins as sGC and also cytochrome P450, predominate at low concentrations of NO (< 1 µM), which are produced by the constitutive isoforms. However, there is increasing evidence that nitration of proteins may also occur in normal cells, albeit at a much lower level. It is possible that these nitrations may play a role in signal transduction processes but this is still a matter for speculation (Davis et al., 2001; Bruckdorfer et al., 2005). The identification of a population of endogenously S-nitrosylated proteins, including metabolic, structural and signalling proteins that may be effectors for nNOS-generated NO points to protein S-nitrosylation as a physiological signalling mechanism for NO (Ahern et al., 2002; Jaffrey et al., 2001).

### I.2.3.3 CO

CO was suggested as a second gaseous messenger based on its marked similarities to NO (Schmidt et al., 1992). Two heme oxygenase enzymes, heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2) catalyse the synthesis of CO from Fe protoporhyrin IX (heme) (Fig. I.4).

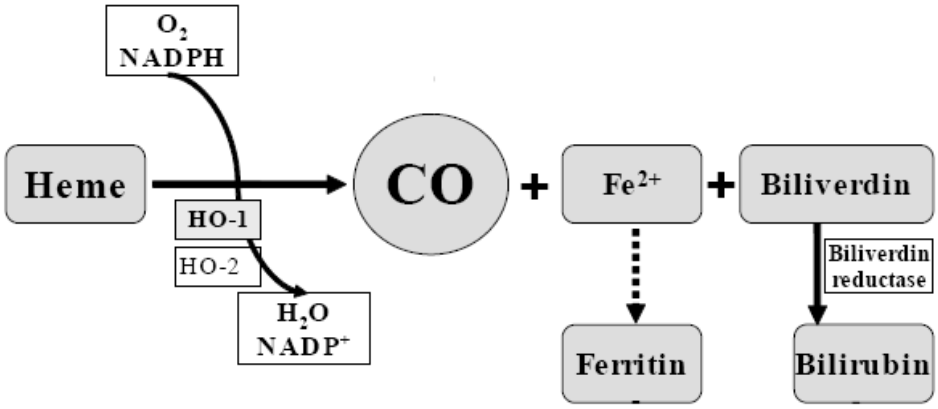


**Figure I.4**

Schematic representation of the biosynthesis of CO (adapted from Babusikova et al., 2008). Two heme oxygenase enzymes, HO-1 and HO-2, catalyse the synthesis of CO from heme. The reaction involves the oxidation of NADPH and $O_2$ and generates not only CO but also $Fe^{2+}$ and biliverdin. The released $Fe^{2+}$ ions induce the synthesis of ferritin, an iron-sequestering protein, while biliverdin is converted to bilirubin by biliverdin reductase.

Whereas HO-1 is only induced under conditions of stress or injury, HO-2 is constitutively expressed (Gibbons & Farrugia, 2004). The presence of HO-2 was reported in the myenteric plexus in different GI regions in a number of species (Battish et al., 2000; Miller et al., 2001; Ny et al., 1996, Rattan et al., 2005). A role of CO in the inhibitory NANC neurotransmission has been suggested in mouse ileum (Zakhary et al., 1997) and mouse jejunum (Xue et al., 2000a), using mice with a targeted deletion of HO-2.

However, more recently, Rattan et al. (2005) could not show a significant role of CO in the NANC relaxation in the mouse IAS, despite the fact that HO-2 was present in the myenteric neurons and exogenous CO induced relaxation in this tissue. Farrugia et al. (2003) observed a hyperpolarizing effect of CO in the mouse small intestine and colon and hypothesized that ICCs might be the source of the hyperpolarizing CO. Thus, CO may function as a simple messenger molecule between ICCs and smooth muscle cells. HO-2 was indeed detected in ICCs of the mouse small intestine and colon (Farrugia et al., 2003) and also in these of the opossum anorectum (Battish et al., 2000). De Backer & Lefebvre (2007) reported that the relaxant properties of CO in the mouse jejunum are reduced upon NOS inhibition, and suggested that CO signalling might be amplified by the production of NO in the

ICCs. As a result of these observations, the role for CO as a neurotransmitter is still under debate.

The most important target of CO is sGC. Indeed, CO can activate sGC (Stone & Marletta, 1994), resulting in an increased production of second messenger cGMP. The mechanisms downstream of cGMP leading to smooth muscle relaxation are discussed in I.3.3.2. The involvement of sGC in CO-induced relaxation was shown by Rattan et al. (2004) in the internal anal sphincter. CO was also reported to directly activate large conductance $Ca^{2+}$-dependent $K^+$ channels in vascular smooth muscle (Wang & Wu, 1997).

#### I.2.3.4 $H_2S$

More recently, it was hypothesized that $H_2S$ represents a third gaseous neurotransmitter or gasotransmitter, besides NO and CO. $H_2S$ can indeed be produced endogenously from L-cysteine by two enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Chen et al., 2004; Stipanuk & Beck, 1982) (Fig.I.5). Research regarding the role of $H_2S$ as neurotransmitter and more specifically its role in the control of GI motility is still in its infancy. This is further discussed under I.4.

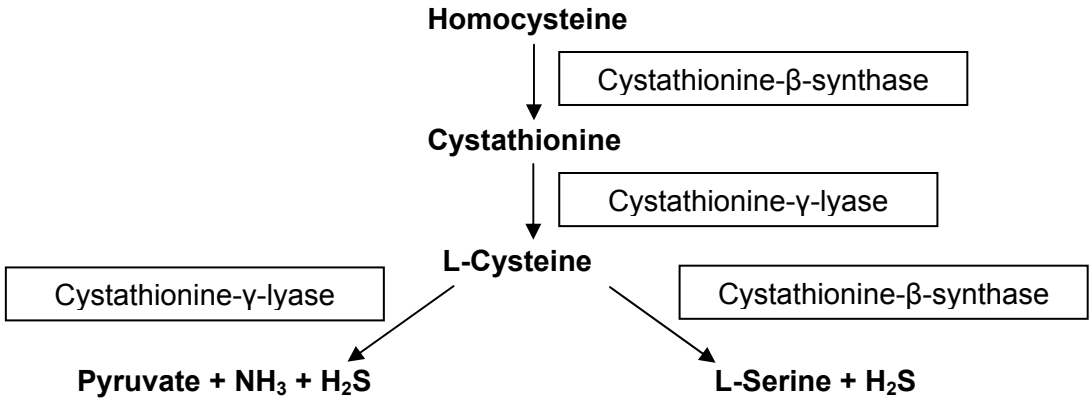


**Figure I.5**

Schematic representation of the biosynthesis of $H_2S$ (adapted from Ebrahimkhani et al., 2005). $H_2S$ is produced endogenously from desulphydration of L-cysteine. The reaction is catalysed by two enzymes, CBS and CSE.

## *I.3 Soluble guanylate cyclase*

In 1963, cGMP was discovered in urine (Ashman et al., 1963). By 1969, an enzyme capable of synthesizing cGMP, i.e. guanine nucleotidyl (guanylyl; guanylate) cyclase (GC), was found to be present in all tissues studied (Hardman et al., 1969). However, progress in the characterization and purification of this enzyme and in understanding the physiological

significance of cGMP was delayed until the 1980s when two important discoveries were made. The first was the discovery that a peptide made in the heart, atrial natriuretic peptide (ANP), could increase cGMP by binding to the transmembrane form of GC (particulate guanylate cyclase; pGC). The second was the discovery that the endogenous molecule EDRF could activate the soluble form of guanylate cyclase (soluble guanylate cyclase; sGC). EDRF was later identified as NO, which established NO as an endogenous activator of sGC. Hence, the NO-sGC-cGMP transduction pathway was born. Extensive research has shown this signalling pathway to be widespread in mammalian tissues and important in mediating numerous physiological processes including peripheral and central neurotransmission and smooth muscle relaxation (Hobbs, 1997; Beavo & Brunton, 2002; Pyriochou & Papapetropoulos, 2005).

### I.3.1 Structure

sGC is a heterodimeric heme-containing protein that is composed of a larger α subunit and a smaller β subunit (Harteneck et al., 1991). Each subunit can be divided into three functional domains: an N-terminal regulatory heme-binding domain, a central dimerization domain and a C-terminal catalytic domain (Lucas et al., 2000) (Fig. I.6A).

The N-terminal domain of the β subunit is the most important for the heme-binding, with histidine at position 105 as the essential amino acid required for the binding of the heme moiety (Wedel et al., 1994; Zhao et al., 1998). The prosthetic heme group is as such a five-membered ring wherein four nitrogen atoms are coordinated with a central iron ($Fe^{2+}$; reduced or ferrous form) and with as fifth member of the ring histidine 105 as axial ligand (Lucas et al., 2000). More recently, other residues from the β subunit, i.e. tyrosine 135, arginine 139 and serine 137, were reported to be involved in stabilizing the binding of the heme moiety and to form a unique heme binding motif (Schmidt et al., 2005).

The central dimerization domain is involved in the formation of heterodimers, which is a pre-requisite for sGC to exhibit catalytic activity (Pyriochou & Papapetropoulos, 2005). Indeed, both subunits are necessary for catalytic activity (Harteneck et al., 1990).

The C-terminal catalytic domains of the sGC subunits are the most conserved regions that also exhibit a substantial degree of similarity with adenylyl cyclase. sGC converts guanosine-5'-triphosphate (GTP) to cGMP (Pyriochou & Papapetropoulos, 2005).

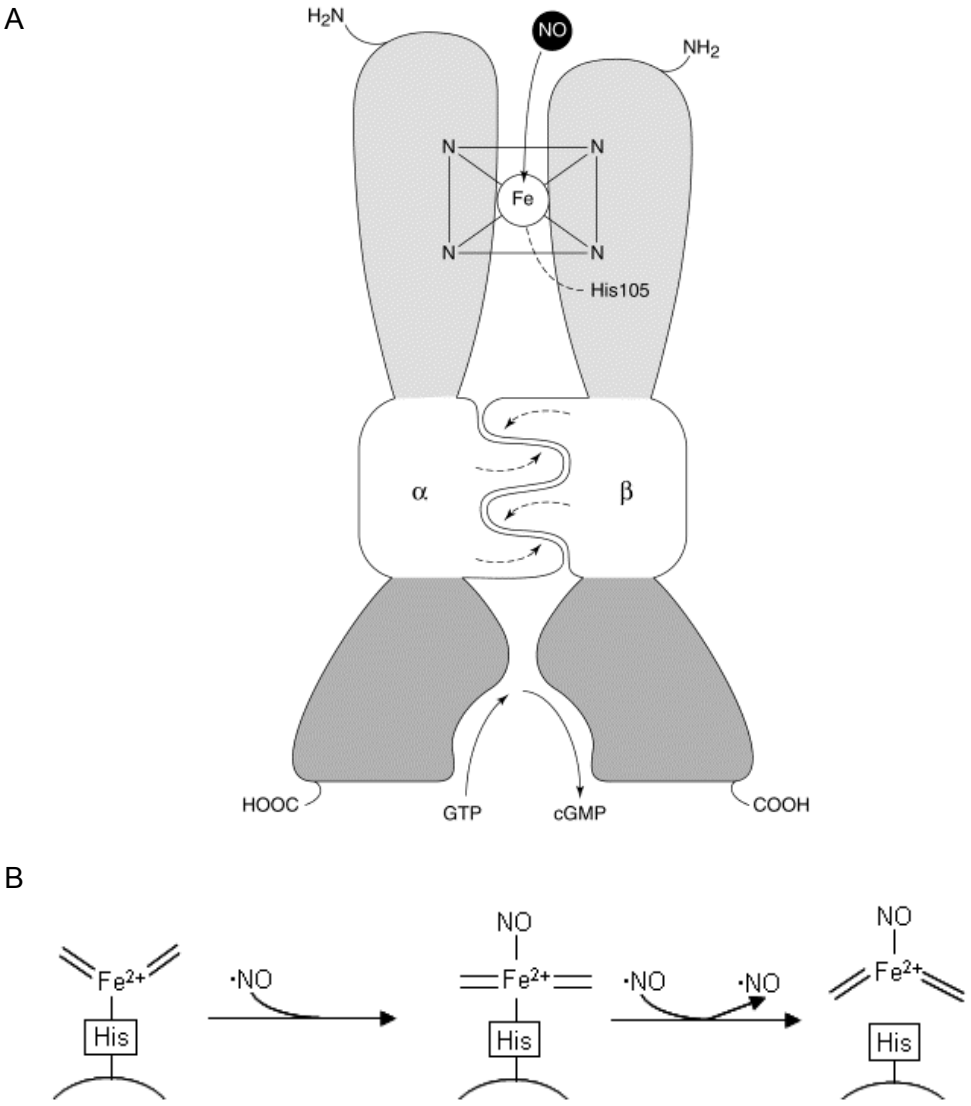


**Figure I.6**

A) Schematic representation of a sGC α/β heterodimer (Hobbs, 1997). The N-terminal region constitutes a heme-binding domain with histidine 105 in the β subunit providing the axial ligand to the fifth coordinate of the heme-iron. The central portion of each subunit contains sequences which mediate dimerization of the monomers, a pre-requisite for catalytic activity. The C-terminal region forms the catalytic domain, responsible for substrate binding (GTP) and conversion to cGMP.

B) Model for the sGC activation mechanism by NO (adapted from Koesling et al., 2004). NO first forms an intermediate six-coordinate nitrosyl-heme complex, after which a NO concentration-dependent conversion into a five-coordinate complex takes place, resulting in rupture of the iron–histidine bond.

## I.3.2 Expression

The α subunit as well as the β subunit exists in 2 isoforms: $\alpha_1$ and $\alpha_2$ (Harteneck et al., 1991), and $\beta_1$ and $\beta_2$ (Yuen et al., 1990). Alternative splicing of $\alpha_1$, $\alpha_2$ and $\beta_2$ also gives rise to additional subunits (Behrends et al., 1995; Okamoto, 2004; Ritter et al., 2000; Sharina et al.,

2008). However, these additional subunits as well as the $\beta_2$ subunit are generally deemed not active or even dominant negative (Behrends et al., 1995; Sharina et al., 2008), although the formation of an active $\beta_2$ homodimer (Koglin et al., 2001) and an $\alpha_1\beta_2$ heterodimer with reduced sensitivity to NO (Gupta et al., 1997) have been reported. But the fact that the $\beta_2$ subunit was almost undetectable in tissue argues against a relevant physiological role of this subunit (Mergia et al., 2003). This leaves the $\alpha_1\beta_1$ and the $\alpha_2\beta_1$ as the only physiological active isoforms, with no differences in kinetic properties and sensitivity towards NO between the two isoforms (Russwurm et al., 1998). The $\alpha_1$ and the $\beta_1$ subunit were detected in all tested human (Budworth et al., 1999) and mouse (Mergia et al., 2003) tissues whereas the $\alpha_2$ subunit showed a more restricted expression pattern with high levels in brain, placenta, spleen and uterus only (Budworth et al., 1999). In brain, the amount of $\alpha_1\beta_1$ was quantitatively similar to that of $\alpha_2\beta_1$; in all other tissues however, including ileum and colon, $\alpha_1\beta_1$ was the predominant isoform (Mergia et al., 2003).

Although the enzyme is originally called 'soluble' guanylate cyclase, it was demonstrated in brain that the $\alpha_2\beta_1$ isoform is associated with the PDZ-containing post-synaptic density protein-95 (PSD-95), this via the C-terminal PDZ domain of the $\alpha_2$ subunit. As a consequence of this interaction, the sGC$\alpha_2\beta_1$ isoform is recruited to the membrane of synaptosomes, where it would then be co-localized with the PSD-95 interacting neuronal NO synthase (Nedvetsky et al., 2002; Russwurm et al., 2001). Also the translocation of the $\alpha_1\beta_1$ isoform to the plasma membrane in response to elevated $Ca^{2+}$ concentrations has been reported, rendering the enzyme more sensitive to NO (Zabel et al., 2002).

## I.3.3 Smooth muscle relaxation by sGC

### I.3.3.1 Activation of sGC

To induce relaxation via sGC, NO and CO first need to activate sGC by binding to the enzyme. NO binds hereby to the heme moiety of sGC, following a two-step mechanism (Fig. I.6B). First, a six-coordinate intermediate NO-$Fe^{2+}$-histidine complex is formed. Next, the breakage of the $Fe^{2+}$-histidine bond leads to the formation of a five-coordinate nitrosyl-heme complex and concomitant conformational changes of the enzyme. This results in activation of sGC, as these rearrangements move the C-terminal domain of the β subunit closer to the C-terminal domain of the α subunit, thus forming a more closed, circular catalytic domain (Kosarikov et al., 2001). Besides the first step, also the second step was reported to be dependent of the NO concentration (Zhao et al., 1999). This implies that this second step involved additional reactions of NO with sGC. The requirement of the binding of a second NO molecule to the proximal face of the heme, thereby effectively displacing the histidine was suggested by Lawson et al. (2003). The second NO than repels the first, distally bound, NO

to end up with the five-coordinate nitrosyl-heme complex. A modified version of this hypothesis claims the existence of two possible NO-bound states: a fully active state formed in the presence of the products of the enzyme (cGMP, PPi, $Mg^{2+}$) and a low-activity state with NO bound on the proximal side of the heme (Russwurm & Koesling, 2004). Besides the all-heme site model of Lawson and colleagues, Cary et al. (2005) proposed a non-heme site model. At high (acute) concentrations of NO, a second NO molecule binds to an non-heme site, giving rise to a high-activity state. When the NO concentration drops to tonic (low) levels, the second NO molecule dissociates from the non-heme-site leaving only one NO molecule at the heme, which leads to the transition to a low-activity state. This model should explain the discrepancy between the slow dissociation of NO from the heme observed in vitro and the in vivo data showing that sGC rapidly deactivates as soon as NO levels drop. The two two-sites models (all-heme and non-heme) indeed can serve as an explanation for the behaviour of sGC in vitro, especially the presence of a low-activity (NO-bound) form that is spectrally indistinguishable from the fully active form. However, according to Roy & Garthwaite (2006), the non-heme site model for the regulation of sGC activity is of doubtful relevance to cells. Earlier on, Bellamy et al. (2002) reported that the data available still can be interpreted using the simple two-step single-site model and that the proposition of additional NO-binding sites is unwarranted at this stage.

Besides NO, also CO is able to activate sGC in a heme-dependent manner forming a six-coordinate CO-$Fe^{2+}$-histidine complex in which the $Fe^{2+}$-histidine bond remains intact. Unlike NO, which leads to a 100- to 200 fold increase in catalytic sGC activity, CO only activates the enzyme by about 4-fold (Stone & Marletta, 1994).

In the last ten years, novel compounds were described that are able to activate sGC in an NO-independent manner. These compounds can be classified in two groups: the NO-independent but heme-dependent sGC stimulators and the NO- and heme-independent sGC activators. Examples of sGC stimulators are YC-1, BAY 41-2272, BAY 41-8543, CFM-1571 and A-350619. These compounds show a strong synergy with NO and, like NO, a loss of activation after oxidation of the heme moiety. sGC stimulators might offer an increase in the efficacy of current therapies in modulating NO-sGC-cGMP signalling, due to their ability to enhance the sensitivity of sGC to NO. This effect is based, at least partially, on the strong stabilization of the nitrosyl-heme complex and the postulated transformation of the NO-activated sGC from a low to a fully active state. The sGC activators include BAY 58-2667 and HMR-1766 which require neither NO nor heme, and demonstrate even more pronounced action on the heme-deficient sGC or the oxidized form of sGC, in which the ferrous ($Fe^{2+}$) heme moiety is oxidized to its ferric ($Fe^{3+}$) state. BAY 58-2667 and the heme moiety compete for the unique heme-binding motif (see I.3.1). Because of the strongly

reduced affinity of the oxidized heme for the sGC heme-binding pocket in comparison to the reduced heme, only the oxidized heme can be effectively replaced by BAY 58-2267. sGC activators might offer an advantage over current therapies using NO-donors in the treatment of various pathophysiological conditions associated with oxidative stress. Indeed, oxidative stress leads to a disequilibrium between the oxidized and the reduced form of sGC, in favour of oxidized form. sGC activators, unlike NO, are able to activate oxidized sGC (Evgenov et al., 2006).

### I.3.3.2 Mechanisms downstream of cGMP

Activation of sGC results in increased intracellular cGMP levels. The resultant accumulated cGMP exerts its physiological effects through three intracellular effectors: cGMP-dependent protein kinases (cGKs or PKGs), cyclic nucleotide-gated ion channels (CNGs) and cGMP-regulated phosphodiesterases (PDEs). The most important mediators of GI signalling are PKGs and PDEs. The PKGs belong to the serine/threonine kinase family and exist in two subtypes: the cytosolic PKG I, which is present in high concentrations in smooth muscle, and the membrane-bound PKG II, which is expressed in the intestinal mucosa. The hypothesis is that PKG I plays an important role in intestinal smooth muscle relaxation, whereas PKG II regulates intestinal secretion. Due to alternative splicing, PKG I has two isoforms i.e. PKG Iα and PKG Iβ (Hofmann et al., 2000; Lucas et al., 2000).

PKG I leads to smooth muscle relaxation by lowering the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and/or by desensitization of the contractile apparatus to $[Ca^{2+}]_i$ (Lucas et al., 2000; Toda & Herman, 2005) (Fig. I.7).

PKG I can reduce $[Ca^{2+}]_i$ by several mechanisms. One mechanism is the inhibition of the release of $Ca^{2+}$ from the sarcoplasmatic reticulum (SR), either by inhibition of agonist-induced inositol 1,4,5-triphosphate ($IP_3$)-generation and/or by inhibition of the $IP_3$-receptor. The first likely involves phosphorylation of a member of the G protein family or an isozyme of the phospholipase C family (Ruth et al., 1993); the latter is dependent of the interaction of PKG Iβ with the $IP_3$ receptor-associated cGMP kinase substrate (IRAG) (Ammendola et al., 2001). Secondly, PKG I can promote $Ca^{2+}$ sequestration through activation of the sarcoplasmatic reticulum $Ca^{2+}$ pumping ATP-ase (SERCA) by phosphorylating and thus inactivating phospholamban and thereby relieving SERCA from its inhibitory effect (Cornwell et al., 1991). A third mechanism to reduce $[Ca^{2+}]_i$ is inhibiting $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels by both direct impairment of the channel activity (Liu et al., 1997) and indirect hyperpolarization via an increase in the open probability of the $Ca^{2+}$-dependent $K^+$ channels (Schubert & Nelson, 2001).

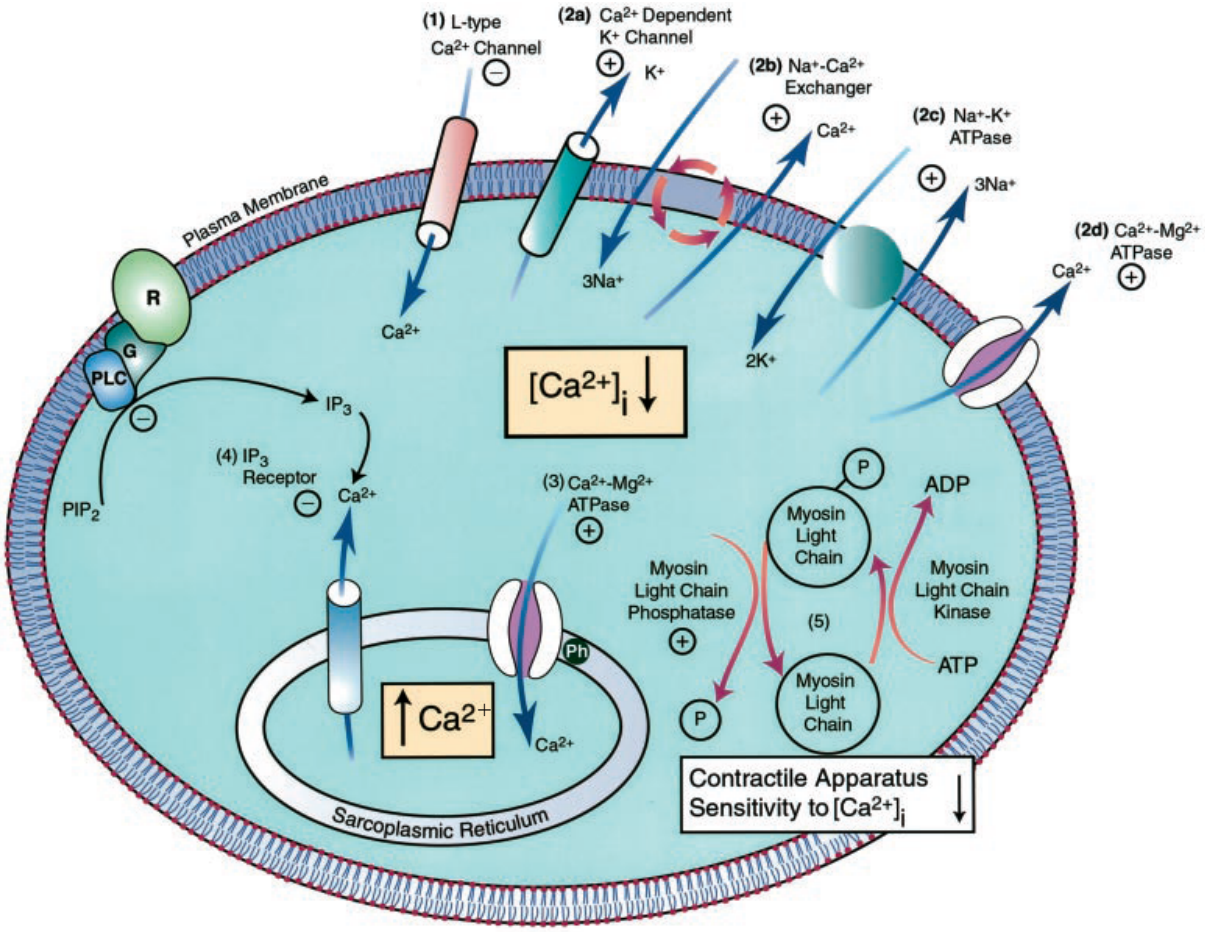


**Figure I.7**

Mechanisms underlying smooth muscle relaxation mediated by cGMP (adapted from Lucas et al., 2000). cGMP induces smooth muscle relaxation by lowering ($[Ca^{2+}]_i$) and/or by desensitization of the contractile apparatus to $[Ca^{2+}]_i$. cGMP can reduce $[Ca^{2+}]_i$ by (1) inhibiting $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels; (2) increasing $Ca^{2+}$ efflux through activation of (2d) the $Ca^{2+}$-pumping ATPase and (2b) the $Na^+/Ca^{2+}$ exchanger; also, cGMP may produce membrane hyperpolarization through activation of (2c) the $Na^+/K^+$ ATPase and (2a) $K^+$ channels, thereby increasing $Ca^{2+}$ extrusion by the $Na^+/Ca^{2+}$ exchanger; (3) increasing of $Ca^{2+}$ sequestration through activation of the sarcoplasmatic reticulum $Ca^{2+}$-pumping ATPase by inactivating phospholamban [Ph] and thereby relieving the sarcoplasmatic reticulum $Ca^{2+}$-pumping ATPase from its inhibitory effect; (4) decreasing of $Ca^{2+}$ mobilization through inhibition of agonist-induced $IP_3$ formation or inhibition of the $IP_3$ receptor in the sarcoplasmatic reticulum. cGMP desensitizes the contractile apparatus to $Ca^{2+}$ (5) probably by activating the myosin light chain phosphatase, resulting in dephoshorylation of the myosin light chain. ⊖: inhibitory effect; ⊕: stimulatory effect. R, receptor; G, G protein; PLC, phospholipase C.

Finally, PKG I can increase the $Ca^{2+}$ efflux through activation of the $Ca^{2+}$-pumping ATPase, the $Na^+/Ca^{2+}$ exchanger or induce hyperpolarization via activation of the $Na^+/K^+$ ATPase, thereby again increasing $Ca^{2+}$ extrusion by the $Na^+/Ca^{2+}$ exchanger (Lucas et al., 2000).

Taken together, the resulting decrease in $[Ca^{2+}]_i$ will attenuate the activity of the $Ca^{2+}$/calmodulin-dependent myosin light chain kinase (MLCK), leading to a reduced phosphorylation of the myosin light chain (MLC). This will subsequently reduce myosin's ATPase activity and cross-bridge cycling with relaxation of the smooth muscle cell as a result. In contrast to the activation by MLCK, myosin's ATPase activity can be inhibited by the myosin light chain phosphatase (MLCP), which dephosphorylates the MLC. It is in fact the MLCK to MLCP activity ratio which determines the phosphorylation state of the MLC and thus the myosin's ATPase activity and the contraction level of the smooth muscle.

PKG I can activate MLCP without inducing a decrease in $[Ca^{2+}]_i$; this mechanism of action is called $Ca^{2+}$ desensitization as relaxation of the contractile apparatus then occurs in the presence of an unaltered/high $[Ca^{2+}]_i$ (Somlyo & Somlyo, 2003). A direct stimulation of MLCP through interaction with PKG Iα was reported by Surks et al. (1999). In addition, PKG I was shown to relieve MLCP from the inhibitory actions of the Rho-A/Rho-kinase pathway (Sauzeau et al., 2000) or protein CPI-17 (Bonnevier & Arner, 2004). Finally, PKG I was reported to phosphorylate telokin, thereby up-regulating its enhancing effect on MLCP (Wu et al., 1998).

PDEs cleave the phosphodiester bond of cGMP and adenosine 3'-5' cyclic monophosphate (cAMP), hydrolysing the second messengers to their corresponding nucleotide, 5' monophosphate (Lucas et al., 2000). The most important cGMP-regulated PDE in smooth muscle is PDE-5 and inhibition of this PDE induces relaxation by means of cGMP accumulation (Rybalkin et al., 2003).

### I.3.4 Role of sGC in GI motility

Up to date, investigators approached the study of the role of sGC in GI motility mainly by inhibiting sGC by means of the sGC inhibitor 1H[1,2,4,]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ; Schrammel et al., 1996). In vitro experiments with ODQ revealed for example a role for sGC in the NANC relaxation of rat gastric fundus (Lefebvre, 1998), rat (Vanneste et al., 2004) and human (Zyromski et al., 2001) jejunum and rat distal colon (Van Crombruggen & Lefebvre, 2004). Similar findings were reported for mouse duodenum (Serio et al., 2003b), jejunum and ileum (Ueno et al., 2004).

Knocking out cGMP's effector PKG I induced dilation of the stomach and signs of pyloric stenosis. The electrically induced relaxation of gastric fundus strips was impaired (Ny et al., 2000) as well as gastric emptying of a barium suspension (Pfeifer et al., 1998). The interruption of the NO-sGC-cGMP-PKG I pathway also delayed small intestinal transit of a barium suspension. Instead of regular peristalsis, spastic contractions of long intestinal segments followed by scarce and slow relaxations were observed in the PKG I KO mice

(Pfeifer et al., 1998). Taken together, these findings suggest an important role for sGC in the GI tract.

## *I.4 Hydrogen sulfide*

$H_2S$, just as NO and CO, was formerly considered solely as an environmental toxicant and metabolic poison. Indeed, all of these gases potently interact with metals/metalloproteins, notably the heme moiety. For example, all are potent inhibitors of mitochondrial cytochrome c oxidase, thereby inhibiting the respiratory system (Li & Moore, 2007). The turning point in 'gas biology' came with the realization that these gases are generated naturally within mammalian cells (Li & Moore, 2007).

### I.4.1 Identification of $H_2S$ as neurotransmitter

After NO and CO, $H_2S$ became the third possible gaseous neurotransmitter, based on its endogenous production by CSE/CBS (see I.2.3.4) and its biological effects such as smooth muscle relaxation. Both $H_2S$-synthetizing enzymes were shown in human and guinea-pig myenteric neurons (Schicho et al., 2006). CSE but not CBS was shown to be present in mouse colon myenteric neurons, and the intact colonic muscle layer containing the myenteric plexus generated detectable levels of $H_2S$ (Linden et al., 2008). Also, $H_2S$ was shown to inhibit spontaneous or induced contractions in the guinea-pig ileum (Hosoki et al., 1997; Teague et al., 2002), the rat and rabbit ileum (Teague et al., 2002), the human and rat colon and the mouse colon and jejunum (Gallego et al., 2008). Moreover, brain CBS (like NOS) activity was shown to be both $Ca^{2+}$- and calmodulin-dependent (Eto et al., 2002), suggesting that 'short-term' control of neuronal $H_2S$ (such as NO) production might be achieved by influx of $Ca^{2+}$ into neurons following depolarization (Li & Moore, 2007). Designating a molecule as a neurotransmitter presumes however also that it possesses a mechanism of inactivation. Indeed, $H_2S$ is metabolized to sulfate and thiosulfate via the oxidation metabolism in mitochondria or is scavenged by methemoglobin or metallo- or disulfide-containing molecules such as oxidized glutathione. $H_2S$ can also be converted into lower toxic compounds of methylmercaptan and dimethylsulfate via the methylation metabolism in cytosol. The metabolic product exhausts from the kidney and intestinal tract and lungs within 24 hours, so the endogenously-generated $H_2S$ under physiological condition is hardly accumulated or toxic to cells due to the balanced metabolism of the gas (Chen et al., 2007; Wang, 2002).

### I.4.2 Influence of $H_2S$ on smooth muscle contractility

$H_2S$-induced smooth muscle relaxation was observed not only in the GI tract (Gallego et al., 2008; Hosoki et al., 1997; Teague et al., 2002) but also in the vascular system (Cheng et al., 2004; Webb et al., 2008; Zhao et al., 2001), the respiratory system (Kubo et al., 2007) and the reproductive system (d'Emmanuele di Villa Bianca et al., 2009; Sidhu et al., 2001; Srilatha et al., 2007).

The mechanism by which $H_2S$ brings about smooth muscle relaxation is however not fully understood. The most complete body of evidence comes from work based on vascular smooth muscle. According to studies in this tissue, the $H_2S$-induced smooth muscle relaxation appears to be mediated in large part by the opening of $K^+$ channels on the surface of smooth muscle cells. The most important participant in $H_2S$-induced vasodilatation seems to be the ATP-dependent $K^+$ channel ($K_{ATP}$ channel), this based on studies where the effect of $H_2S$ was shown to be mimicked by the $K_{ATP}$ channel opener pinacidil and inhibited by the $K_{ATP}$ channel blocker glibenclamide (Cheng et al., 2004; Webb et al., 2008; Zhao et al., 2001). The stimulatory effect of $H_2S$ on $K_{ATP}$ channel was further demonstrated by directly measuring the $K_{ATP}$ channel current (Cheng et al., 2004; Tang et al., 2005; Zhao et al., 2001). The activation of $K_{ATP}$ channels by $H_2S$ leads to membrane hyperpolarization, which in turn closes the voltage-dependent $Ca^{2+}$ channels with relaxation of the smooth muscle as a result.

Although most reports indicate $K_{ATP}$ channels as mediator of $H_2S$-induced vasorelaxation, there are some reports that suggest alternative mechanisms for $H_2S$ to induce relaxation. $H_2S$ may directly inhibit the voltage-dependent $Ca^{2+}$ channels as suggested by Zhao & Wang (2002). A study by Kiss et al. (2008) hypothesize that $H_2S$-induced vasorelaxation is induced by the inhibition of cytochrome c oxidase, leading to loss of mitochondrial ATP generation and thus energy deficit and intracellular acidosis, which in turn activates the $Cl^-/HCO_3^-$ exchanger. Similarly, it is shown that $H_2S$ regulates intracellular pH in vascular smooth muscle cells via activation of the $Cl^-/HCO_3^-$ exchanger and that this is, at least partially, responsible for $H_2S$-mediated vasorelaxation (Lee et al., 2007).

Instead of a direct effect on the smooth muscle, Zhao & Wang (2002) suggested that $H_2S$ may also affect vasocontractility by facilitating the release of endothelium-derived relaxant factors such as NO. However, Kiss et al. (2008) could not show a role of NO in the $H_2S$-induced vasorelaxation. Also, as opposed to NO and CO, $H_2S$-induced relaxation seems not to be mediated by the sGC-cGMP signalling pathway, as the sGC inhibitor ODQ did not reduce the $H_2S$-mediated relaxation in rat aortic rings (Zhao & Wang, 2002) and mouse bronchial rings (Kubo et al., 2007).

Similar to results in the vascular system, $K^+$ channels were also reported to be possibly involved in the $H_2S$-induced relaxation of GI smooth muscle. Indeed, Gallego et al. (2008) showed that the $H_2S$-induced inhibition of spontaneous motor complexes in mouse colon and jejunum was mediated by $SK_{Ca}$ channels, whereas both $SK_{Ca}$ channels and $K_{ATP}$ channels seemed to be involved in the $H_2S$-induced inhibition of spontaneous motility in rat and human colon. Also in a rat model using colorectal distension, the $H_2S$-induced antinociceptive and relaxant effects were mediated by $K_{ATP}$ channels (Distrutti et al., 2006). However, Teague et al. (2002) could not show a role for $K_{ATP}$ channels in the $H_2S$-induced relaxation in the guinea-pig ileum. Also about the possible role of NO in the $H_2S$-induced relaxation, conflicting data are reported: Teague et al. (2002) and Gallego et al. (2008) showed no influence of NOS-inhibitors on the $H_2S$-induced relaxation in the guinea pig ileum and the mouse colon and jejunum respectively, whereas Distrutti et al. (2006) observed that NOS inhibition almost completely reversed the antinociceptive and relaxant effects of $H_2S$ in rat colon. These discrepant results together with the to date only limited number of reports concerning the $H_2S$-induced smooth muscle relaxation in the GI tract make the latter an interesting territory for future experiments.

There should also be mentioned that another potential source of $H_2S$, besides neuronal endogenous production, is $H_2S$ produced by bacteria within the bowel lumen. However, a physiological role of $H_2S$ originating from this source has not yet been described (Kasparek et al., 2008). Colonic mucosa possesses specialized detoxification systems involving the enzymes thiol methyltransferase (Weisiger et al., 1980) or rhodanese (Picton et al., 2002), protecting the colon from what otherwise might be injurious concentrations of $H_2S$.

## *I.5 References*

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# Chapter II

## AIMS

# Chapter II Aims

Hormonal and neuronal control mechanisms regulate gastrointestinal (GI) motility. NO is considered to be an important inhibitory NANC neurotransmitter in the GI tract, and hence to play an important role in the regulation of GI smooth muscle relaxation. However, also other inhibitory NANC neurotransmitters such as ATP, VIP/PACAP and possibly CO are reported to be involved in smooth muscle relaxation depending upon species and region of the GI tract.

The principal intracellular target of NO to induce relaxation is sGC. For years, it was a paradigm to equal NO synthesis with the activation of sGC, cGMP synthesis and activation of PKGs and PDEs. A number of observations, however, indicate that this assumption is not correct. In the first place, there is the growing notion of the importance of sGC-independent activities of NO, acting through nitr(osyl)ation of proteins. Previously, the latter was considered to be “collateral damage” without specific physiological regulatory meaning, but more recent studies point to nitr(osyl)ation as a physiological signalling mechanism for NO (Ahern et al., 2002; Jaffrey et al., 2001). Secondly, there is the recognition that NO is not the only activator of sGC. Indeed, also CO can activate sGC in a heme-dependent manner (Stone & Marletta, 1994).

sGC exists in two isoforms i.e., $\alpha_1\beta_1$ and $\alpha_2\beta_1$, of which the $\alpha_1\beta_1$-isoform predominates in the GI tract (Mergia et al., 2003). Research on sGC has been seriously hampered by the fact that reliable specific inhibitors with in vivo usefulness are lacking and that there are no isoform-specific inhibitors. Using a mouse transgenic approach, our first aim was to investigate the relative importance of the two isoforms of sGC in the NO-mediated action on GI motility. Our group previously studied the consequences of knocking out the $\alpha_1$-subunit of sGC at the level of the stomach. Results showed that sGC$\alpha_1\beta_1$ plays an important role in gastric nitrergic relaxation in vitro, but that some nitrergic relaxation is maintained via activation of sGC$\alpha_2\beta_1$, contributing to a moderate in-vivo consequence on gastric emptying (Vanneste et al., 2007). We now investigated the consequences of knocking out the $\alpha_1$-subunit of sGC in the jejunum and the distal colon, tissues with tonic as well as phasic activity. The results of these experiments are described in **Chapter III** (jejunum) and **Chapter IV** (distal colon).

By means of sGC$\beta_1$his105phe knock in (KI) mice, where neither sGC$\alpha_1\beta_1$ nor sGC$\alpha_2\beta_1$ can be activated by heme-dependent sGC activators such as NO and CO, our second aim was to

investigate to what extent 1) nitrergic relaxation of stomach, jejunum and colon is sGC-dependent and 2) in vivo GI motility is influenced when heme-dependent sGC activation is lacking. The results of this study are summarized in **Chapter V**.

Recently, a role for $H_2S$ as a neurotransmitter was suggested (Wang, 2002). $H_2S$ is as such a third possible gaseous neurotransmitter (or "gasotransmitter") besides NO and CO, with the evidence for their role as a neurotransmitter being very strong for NO, weaker for CO and still very limited for $H_2S$. The effect of $H_2S$ on smooth muscle contractility and its mechanism of action is mainly investigated in the vascular tree. In contrast to NO and CO, $H_2S$ does not relax vascular smooth muscle through activation of sGC but mainly by activation of ATP-dependent $K^+$ channels. Up to date, there are only a few reports concerning the influence of $H_2S$ on GI smooth muscle. Our third aim was therefore to investigate the influence of $H_2S$ on contractility in mouse gastric fundus (**Chapter VI**) and distal colon (**Chapter VII**) and to explore its mechanism of action in these two tissues.

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# Chapter III

# SMALL INTESTINAL MOTILITY IN SOLUBLE GUANYLATE CYCLASE $\alpha_1$ KNOCKOUT MICE

**Ingeborg Dhaese [1], Gwen Vanneste [1], Patrick Sips [2,3], Emmanuel S. Buys [2,3,4], Peter Brouckaert [2,3], Romain A. Lefebvre [1]**

[1] Heymans Institute of Pharmacology, Ghent University, Ghent, Belgium

[2] Department of Molecular Biomedical Research, VIB, Ghent, Belgium

[3] Department of Molecular Biology, Ghent University, Ghent, Belgium

[4] Anesthesia Center for Critical Care Research, Department of Anesthesia & Critical Care, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

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# Chapter III Small intestinal motility in soluble guanylate cyclase $\alpha_1$ knockout mice

## *III.1 Abstract*

Nitric oxide (NO) activates soluble guanylate cyclase (sGC) to produce guanosine-3',5'-cyclic-monophosphate (cGMP). The aim of this study was to investigate the nitrergic regulation of jejunal motility in sGC$\alpha_1$ knock-out (KO) mice.

Functional responses to nitrergic stimuli and cGMP levels in response to nitrergic stimuli were determined in circular muscle strips. Intestinal transit was determined.

Nitrergic relaxations induced by electrical field stimulation and exogenous NO were almost abolished in male KO strips, but only minimally reduced and sensitive to ODQ in female KO strips. Basal cGMP levels were decreased in KO strips but NO still induced an increase in cGMP levels. Transit was not attenuated in male nor female KO mice.

In vitro, sGC$\alpha_1\beta_1$ is the most important isoform in nitrergic relaxation of jejunum but nitrergic relaxation can also occur via sGC$\alpha_2\beta_1$-activation. The latter mechanism is more pronounced in female than in male KO mice. In vivo, no important implications on intestinal motility were observed in male and female KO mice.

## *III.2 Introduction*

Nitric oxide (NO) is an important mediator in jejunal inhibitory neurotransmission as electrical field stimulation (EFS)-induced smooth muscle relaxations are reduced in jejunum strips of neuronal NO synthase-1 (NOS-1) knock-out (KO) mice (Xue et al., 2000). The importance of NO in jejunal non-adrenergic, non-cholinergic (NANC) neurotransmission was further established by reports that NOS inhibitors reduced smooth muscle relaxations induced by EFS in longitudinal muscle of mouse and rat jejunum and circular muscle of canine, equine and human jejunum (Niioka et al., 1997; Satoh et al., 1999; Stark et al., 1993; Rakestraw et al., 1996; Murr et al., 1999). NO was also suggested to play a role in in-vivo small intestinal motility as the use of NOS inhibitors revealed a delay in small intestinal transit of rats (Karmeli et al., 1997), dogs (Chiba et al., 2002) and humans (Fraser et al., 2005).

Endogenous NO, released during EFS, activates soluble guanylate cyclase (sGC), as the sGC inhibitor ODQ reduced the EFS-induced relaxations in longitudinal muscle of human and mouse jejunum (Zyromski et al., 2001; Ueno et al., 2004) and circular muscle of rat jejunum (Vanneste et al., 2004). sGC activation was also reported to play a role in in-vivo intestinal motility as sildenafil, an inhibitor of the guanosine-3',5'-cyclic-monophosphate (cGMP) degrading enzyme phosphodiesterase (PDE) 5, was found to delay intestinal transit of mice and the sGC inhibitor methylene blue blocked this inhibitory effect of sildenafil on intestinal transit (Patil et al., 2005).

The intestinal relaxing effects of cGMP, raised by activation of sGC, are mediated by cGMP-dependent protein kinase (cGK) type I as a cGKI inhibitor reduced relaxations evoked by a membrane-permeable cGMP analog and a NO-donor in longitudinal muscle of rat small intestine (Huber et al., 1998). In addition, the use of a cGKI KO model revealed that interruption of the NO-sGC-cGMP-cGKI signaling pathway delayed small intestinal transit. Instead of regular peristalsis, spastic contractions of long intestinal segments followed by scarce and slow relaxations were observed (Pfeifer et al., 1998). NO has also sGC-independent effects (Jaffrey et al., 2001); in both rat and mouse duodenum cGMP-independent relaxant effects of NO were reported (Martins et al., 1995; Serio et al., 2003). Also, sGC can be stimulated by other stimuli than NO (Behrends, 2003).

$sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ are the physiologically active sGC isoforms with $sGC\alpha_1\beta_1$ being the predominant isoform in the gastrointestinal (GI) tract (Mergia et al., 2003). To further investigate the role of sGC in nitrergic inhibition of small intestinal motility, the consequence of knocking-out $sGC\alpha_1$ on in-vitro relaxation by endogenous and exogenous NO and on in-vivo intestinal transit was now investigated. We recently reported that proximal gastric nitrergic relaxation in these $sGC\alpha_1$ KO mice is clearly reduced, but some nitrergic relaxation is maintained via activation of $sGC\alpha_2\beta_1$ (Vanneste et al., 2007).

## *III.3 Materials and Methods*

### III.3.1 Animals

sGC$\alpha_1$ KO mice were generated as described by Buys et al. (2008). Briefly, sGC$\alpha_1$ KO mice were generated by targeting exon 6 of the sGC$\alpha_1$ gene which codes for an essential part of the catalytic domain. Chimeras were generated by aggregating R1 embryonic stem cells carrying the mutant sGC$\alpha_1$ allele with Swiss morula-stage embryos.

Wild-type (WT) and KO Swiss/129 mice of both sexes (WT male: 9-34 weeks, 23-52 g; KO male: 9-37 weeks, 32-48 g; WT female: 10-34 weeks, 24-41 g; KO female: 9-34 weeks, 24-43 g) and male heterozygous Swiss/129 mice (19-25 weeks, 37-42 g) had free access to water and commercially available chow. However, when investigating transit using the phenol red or fluorescein-labelled dextran method (see below), food was withheld for 16 hours overnight with free access to water in a cage with a grid floor to prevent coprophagy. All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

### III.3.2 Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

A fragment of jejunum starting approximately 10 cm distal to the pylorus was harvested, rinsed in Krebs solution and transferred to RNA*later* stabilization reagent (QIAGEN; The Netherlands) until RNA was prepared using the RNeasy® Protect Mini kit (QIAGEN, The Netherlands), according to the manufacturer's instructions. One µg of RNA was used in the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, USA) to produce copy deoxyribonucleic acid (cDNA) using random hexameric primers. cDNA was subsequently used for relative expression quantitation using real-time fluorescence detection. To this end, specific primer-probe sets were synthesized for sGC$\alpha_1$ (detecting exon 5), $\alpha_2$, $\beta_1$ and the household gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) for normalization (for sequence, see Vanneste et al., 2007). The probe for $\alpha_1$ was directed to exon 5, which is intact in the sGC$\alpha_1$ KO mice, as the latter were generated by targeting exon 6 of the sGC$\alpha_1$ gene. Quantitative real-time PCR was performed using the Taqman universal PCR master mix (Applied Biosystems; CA; USA) in an ABI Prism 7700 Sequence Detector. The cycling parameters were: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Relative changes in gene expression were determined using the comparative threshold cycle (Ct) method described in Applied Biosystems User Bulletin #2 (1997) in which the amount of target, normalised to an endogenous reference and relative to a calibrator is given by $2^{-\Delta\Delta Ct}$. In brief, the Ct, or threshold cycle, represents the PCR cycle at which an increase in reporter

fluorescence above a baseline signal can first be detected, ΔCt refers to the difference between the threshold cycles of the target (sGC subunits) and the endogenous reference (HPRT); and ΔΔCt indicates the difference between the ΔCt values obtained in the KO animals to the mean ΔCt value obtained in the calibrator group (WT mice).

## III.3.3 Muscle tension experiments

### III.3.3.1 Tissue preparation

Animals were killed by cervical dislocation; the GI tract was removed and put in aerated Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). A ± 5 cm long fragment of small bowel starting approximately 10 cm distal to the pylorus was isolated and opened along the mesenteric border. The fragment was pinned mucosa side up in physiological salt solution. The mucosa was removed by sharp dissection under a microscope and 2 (WT and KO mice) or 4 (heterozygous mice) full-thickness muscle strips (4 × 5 mm) were cut along the circular axis.

### III.3.3.2 Isometric tension recording

After a silk thread (USP 4/0) was attached to both ends, strips were mounted in 5 ml organ baths between 2 platinum plate electrodes (6 mm apart). The organ baths contained aerated (5% $CO_2$ in $O_2$) Krebs solution, maintained at 37°C. Changes in isometric tension were measured using MLT 050/D force transducers (ADInstruments; UK) and recorded on a PowerLab/8sp data recording system (ADInstruments; UK) with Chart software.

After an equilibration period of 30 min with flushing every 10 min at a load of 0.125 g the length-tension relationship was determined. Muscle tissues were stretched by load increments of 0.125 g and at each load level exposed to 0.1 μM carbachol to determine the optimal load ($L_o$; the load at which maximal response to the contractile agent occurred). Tissues were then allowed to equilibrate for 60 min at $L_o$ with flushing every 15 min in Krebs solution.

### III.3.3.3 Protocol

In a first series, cumulative contractile responses to carbachol (1 nM – 30 μM) or prostaglandin F2α (PGF2α; 1 nM – 3 μM) were obtained in Krebs solution without atropine and guanethidine. Each concentration was left in contact with the tissue for 2 min. The organ bath medium was then switched to Krebs solution containing 1 μM atropine and 4 μM guanethidine to block cholinergic and noradrenergic responses, respectively. Ten min after adding 300 nM PGF2α to induce contraction, relaxations were induced by application of EFS (40V, 0.1 ms, 1-2-4-8 Hz for 10 s at 5 min interval) via the platinum plate electrodes by

means of a Grass S88 Stimulator (Grass, W. Warwick, RI, USA), followed by the application of exogenous NO (1-10-100 μM with an interval of at least 5 min during which the effect of a given concentration of NO had disappeared) and finally vasoactive intestinal polypeptide (VIP, 100 nM). Strips were washed for 30 min, and were subsequently incubated with the sGC inhibitor 1H[1,2,4,]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ; 10 μM) for 30 min. PGF2α was then applied again and the responses to EFS, NO and VIP were studied again in the presence of ODQ. The reproducibility of the responses to EFS, NO and VIP was evaluated by running time-control strips in parallel, that received the solvent of ODQ (ethanol).

A second series immediately started in Krebs solution containing atropine and guanethidine. In this series, the influence of the NOS inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 300 μM; 30 min incubation) on the responses to EFS, exogenous NO and VIP was tested. Again, time-control strips were run in parallel to evaluate the reproducibility of the responses to EFS, NO and VIP.

A third series was performed in the presence of Krebs solution containing 300 μM L-NAME. Under basal conditions, contractile responses to EFS (40V, 0.1 ms, 1-2-4-8 Hz for 10 s at 2 min interval) were investigated in the absence and in the presence of atropine (1 μM; 30 min incubation). Time-control strips were run in parallel to evaluate the reproducibility of the EFS-induced responses.

Immediately after the experiment, tissue wet weight was determined.

All three series were performed in WT and KO mice of both sexes. However, in male heterozygous mice, only EFS- and NO-induced responses were investigated.

#### III.3.3.4 Data analysis

The contractile responses to carbachol or PGF2α were measured as area under the curve (AUC) above baseline to construct concentration-response curves. The duration of these responses was set at 2 min (i.e., the contact time with the tissue). $EC_{50}$ values were calculated by linear interpolation.

The duration of the inhibitory responses was determined as 10 s for EFS (i.e., the length of the stimulus train applied) and 5 min for the long acting peptide VIP. The duration of the inhibitory responses to NO was calculated as the difference between the time of administration and the time point, where the peaks of resuming phasic activity reached 50 % of the amplitude of the NO-induced inhibitory response; the latter was calculated as the difference of the mean peak level of phasic activity during the 2 min before administration of NO and the lowest tone level during NO exposure. The inhibitory response to EFS, NO and VIP was quantified by calculating the AUC of the registered activity during the determined

duration, and subtracting it from the AUC of the registered activity during a corresponding period before applying the relaxant stimulus or drug.
The duration of the contractile responses to EFS was determined as 10 s (i.e., the length of the stimulus train applied); the contractile responses to EFS were quantified by subtracting the AUC of the registered activity during the 10 s before applying EFS from the AUC of the registered activity during the stimulus.
The contractile as well as the inhibitory responses are expressed as (g.s)/mg wet weight.

## III.3.4 cGMP analysis

Circular jejunum muscle strips were prepared and weighed to obtain the tissue wet weight before they were mounted as described above, under a load approximating the mean optimal load determined in the tension experiments (0.125 g for jejunal WT and 0.25 g for KO strips). Pre-contracted strips were snap-frozen in liquid nitrogen as such (control), or at maximal relaxation by NO (10 μM), or upon EFS (40 V, 0.1 ms at 4 Hz for 10 s). Snap-frozen tissues were stored at -80°C until further processing. They were pulverized by a Mikro-dismembrator U (B-Braun Biotech International, Germany) and dissolved in cold 6 % trichloroacetic acid to give a 10 % (w/v) homogenate. The homogenate was centrifuged at 2000 *g* for 15 min at 4 °C; the supernatant was recovered and washed 4 times with 5 volumes of water saturated diethyl ether. The aqueous extract was then dried under a stream of nitrogen at 60 °C and dissolved in a 10 or 50 times volume of assay buffer depending on the cGMP content detected in preliminary experiments. cGMP concentrations were determined using an enzyme immunoassay kit (EIA Biotrak System; Amersham Biosciences; UK) after acetylation of the samples and according to the manufacturer's instructions. The optical density was measured with a 96-well plate reader (Biotrak II, Amersham Biosciences; UK) at 450 nm. The tissue cGMP concentration was expressed as pmol per gram tissue wet weight.

## III.3.5 Transit

### III.3.5.1 Small intestinal transit (phenol red method)

As modified from de Rosalmeida et al. (2003), mice (age: 20 ± 3 weeks) were, after food was withheld overnight, administered 250 μl of a phenol red meal (0.1 % w $v^{-1}$ dissolved in water) by gavage with a feeding needle. Sixty minutes later, mice were killed by cervical dislocation and the stomach and small bowel were clamped at both sides. The GI tract from stomach to colon was resected and put on the bench to measure the length of the small intestine. The bowel was divided into 12 parts, 1 part stomach, 10 equal parts of the small bowel and 1 part caecum. Every part was cut into small fragments and placed into 20 ml of 0.1 N NaOH in a 50 ml Falcon tube. This mixture was homogenised for approximately 30 s and allowed to

stand for 20 min at room temperature. Ten ml of supernatant was placed into a 15 ml Falcon tube and centrifuged for 10 min at 2800 rpm. Proteins in 5 ml supernatant were precipitated with 0.5 ml of 20 % (w/v) trichloroacetic acid and the solution was centrifuged for 20 min at 2800 rpm. 0.5 ml of supernatant was added to 0.667 ml of 0.5 N NaOH and the absorbance of 300 µl of this mixture was spectrophotometrically determined at 540 nm in a Biotrak II plate reader (Amersham Biosciences; UK).

The amount of phenol red recovered in each segment of the small bowel was then expressed as % of total recovery (stomach not included) and the geometric centre of the phenol red migration pattern was then calculated as (Σ (% phenol red per segment x segment number))/100. Phenol red recovery throughout the GI tract was determined as the amount of phenol red recovered in all 12 segments, expressed as % of the amount of phenol red administered.

#### III.3.5.2 Intestinal transit (fluorescein-labelled dextran method)

Mice (age: 20 ± 3 weeks) were, after food was withheld overnight, administered 200 µl of non-absorbable fluorescein-labelled dextran (FD70; 70 kDa, 2.5 % w $v^{-1}$ dissolved in water) by gavage with a feeding needle. Sixty minutes later, mice were killed by cervical dislocation. For a full description of the technical details of this method, we refer to De Backer et al. (2008). Briefly, the entire GI tract was excised and the mesenterium was removed. The GI tract was then pinned down in a custom-made Petri dish filled with Krebs solution. Immediately after, FD70 was visualized using the Syngene Geneflash system (Syngene, Cambridge, UK). Two full-field images- one in normal illumination mode and another in fluorescent mode- were taken and matched for analysis. The fluorescent intensity throughout the intestinal tract was calculated and data were expressed as the percentage of fluorescence intensity per segment (sb, small bowel segments 1-10; caecum; col, colon segments 1-2). The geometric centre was calculated as (Σ (% FD70 per segment x segment number))/100.

#### III.3.5.3 Whole gut transit (carmine method)

As adapted from Friebe et al. (2007), mice (age: 20 ± 3 weeks) were administered 200 µl carmine (6 % w $v^{-1}$ dissolved in 0.5 % methylcellulose) by gavage with a feeding needle. Mice were then returned to individual cages, without food deprivation. The time taken for excretion of the first red coloured faeces was determined at 30 minute intervals.

### III.3.6 Statistics

All results are expressed as means ± S.E.M. n refers to tissues obtained from different animals unless otherwise indicated (n =…, out of … animals). Comparison between KO and

WT tissues or between parallel tissues of either WT or KO was done with an unpaired Student *t*-test. Comparison within tissues of either WT or KO was done by a paired Student *t*-test. When relaxant responses in time-control strips showed a significant decline when studied a second time, the change in relaxant response by an interfering substance in the parallel tissue was compared to the spontaneous change in the control strips by an unpaired Student *t*-test. When more than 2 groups of tissues had to be compared, one-way analysis of variance (ANOVA) followed by a Bonferroni corrected *t*-test was applied. A P-value less than or equal to 0.05 was considered to be statistically significant (GRAPHPAD, San Diego, CA, USA).

### III.3.7 Drugs Used

Atropine, carmine, guanethidine, L-NAME, phenol red, PGF2$\alpha$, VIP (all from Sigma-Aldrich, USA), carbachol (Fluka AG, Switzerland), ODQ (Tocris Cookson, UK), fluorescein-labelled dextran (70 kDa, FD70) (Invitrogen, Belgium). All drugs were dissolved in de-ionized water except carmine and ODQ. Carmine was dissolved in 0.5 % methylcellulose and ODQ was dissolved in 100 % ethanol. Saturated NO solution was prepared from gas (Air Liquide, Belgium) as described by Kelm and Schrader (1990).

## *III.4 Results*

All experiments were performed in WT and KO mice of both sexes because gender-related differences in the cardiovascular phenotype were observed with male but not female sGC$\alpha_1$ KO mice developing hypertension (Buys et al., 2008). In addition, the responses to EFS and NO were investigated in male heterozygous mice. No gross morphological GI differences between WT, heterozygous and KO animals were observed.

### III.4.1 Real-time quantitative RT-PCR

The relative levels of $\alpha_1$, $\alpha_2$ and $\beta_1$ subunit mRNA in male KO tissues versus male WT tissues were 3.5 ± 1.6, 1.9 ± 0.6 and 2.8 ± 1.4, respectively (n = 4); in female KO versus female WT tissues, these levels were 4.0 ± 2.4, 1.7 ± 1.4 and 2.9 ± 2.4, respectively (n = 4). In male as well as female mice, the levels in KO tissues did not significantly differ from those in WT tissues.

## III.4.2 Muscle tension experiments

### III.4.2.1 General observations in WT and KO mice

The length of the strips (measured after determination of optimal load) and the tissue wet weight (measured immediately after the experiment) were not different between WT and KO in both male and female mice; $L_o$ was increased in both male and female KO compared to WT strips (Table III.1). ODQ or L-NAME did not influence basal or PGF2$\alpha$-induced tone.

**Table III.1: Optimal load, length and wet weight of smooth muscle strips.**

| | MALE | | FEMALE | |
|---|---|---|---|---|
| | WT | KO | WT | KO |
| Optimal load (g) | 0.16 ± 0.01 | 0.22 ± 0.01 (***) | 0.16 ± 0.01 | 0.25 ± 0.02 (***) |
| Length (mm) | 4.9 ± 0.2 | 5.0 ± 0.2 | 5.1 ± 0.2 | 5.0 ± 0.2 |
| Wet weight (mg) | 0.66 ± 0.04 | 0.75 ± 0.03 | 0.69 ± 0.05 | 0.81 ± 0.05 |

Values are mean ± S.E.M. of n = 64-67 out of 25-29 animals (male), n = 47-52 out of 24-26 animals (female);
*** $P < 0.001$: unpaired student *t*-test (KO *vs.* WT)

### III.4.2.2 Contractile responses to carbachol and PGF2$\alpha$ in WT and KO mice

Contractile responses to carbachol and PGF2$\alpha$ did not significantly differ in male KO mice compared to male WT mice (Table III.2). In female mice, the $EC_{50}$ value of the contractile responses to carbachol was significantly lower in KO mice compared to WT mice while the $E_{max}$ for carbachol and $E_{max}$ and $EC_{50}$ for PGF2$\alpha$ were not significantly different (Table III.2).

**Table III.2: $E_{max}$ and $EC_{50}$ of the contractions to carbachol and PGF2$\alpha$.**

| | | MALE | | FEMALE | |
|---|---|---|---|---|---|
| | | WT | KO | WT | KO |
| Carbachol | $E_{max}$ ((g.s)/mg wet weight) | 46.0 ± 8.1 | 65.6 ± 19.2 | 48.2 ± 4.5 | 38.2 ± 5.5 |
| | $EC_{50}$ (nM) | 101 ± 20 | 58 ± 6 | 66 ± 7 | 27 ± 5 (***) |
| PGF2$\alpha$ | $E_{max}$ ((g.s)/mg wet weight) | 31.7 ± 6.9 | 23.9 ± 5.4 | 16.3 ± 3.4 | 26.9 ± 6.3 |
| | $EC_{50}$ (nM) | 28 ± 7 | 50 ± 14 | 86 ± 41 | 17 ± 4 |

Values are mean ± S.E.M. of n = 8-9 experiments; *** $P < 0.001$: unpaired student *t*-test (KO *vs.* WT)

### III.4.2.3 Contractile responses to electrical field stimulation in WT and KO mice

When studied under basal conditions and in the presence of L-NAME, EFS at 1-8 Hz induced concentration-dependent contractions. In male KO strips, the EFS-evoked contractions were reduced when compared to the WT strips; no differences between WT and KO strips were observed in female mice (Fig. III.1A). After application of atropine, the EFS-induced contractions were nearly abolished in WT and KO strips of male and female mice (Fig. III.1B).

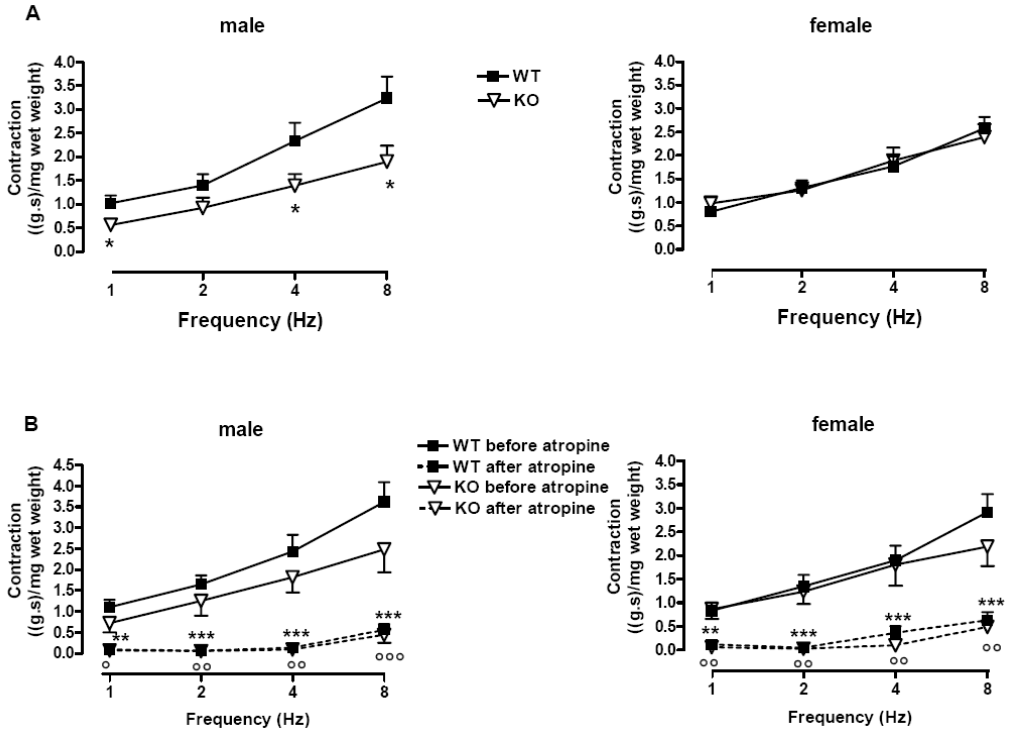



**Figure III.1**

Frequency-response curves of contractions induced by electrical field stimulation (EFS, 40 V, 0.1 ms, 1-8 Hz, 10 s trains) in jejunum circular muscle strips of male (left) and female (right) wild-type (WT) and knock-out (KO) mice (A). In B, responses before and after incubation with atropine (1 μM) are shown. The experiments were performed in the continuous presence of L-NAME (300 μM). Data are expressed as (g.s)/mg wet weight.

A) Values are means ± S.E.M. of n = 14-18 strips out of 7-9 animals; * P < 0.05: unpaired Student *t*-test (KO *vs*. WT).

B) Values are means ± S.E.M. of n = 7-9 strips; * * *P* < 0.01, * * * *P*< 0.001: paired Student *t*-test (WT: after *vs*. before) or ° P < 0.05, ° ° *P* < 0.01, ° ° ° *P* < 0.001: paired Student *t*-test (KO: after *vs*. before).

### III.4.2.4 Inhibitory responses to electrical field stimulation in WT and KO mice

PGF2α (300 nM) induced a tonic contractile response with superimposed phasic activity; the response stabilised after a few min of contact with PGF2α (Fig. III.2). The contractile response to PGF2α was not significantly different between WT and KO strips and between male and female strips.

In male WT strips, EFS-induced relaxations (mainly consisting of an interruption of phasic activity) were very similar at the different stimulation frequencies (1–8 Hz); the inhibition of phasic activity abruptly stopped at the end of the 10 s stimulation train, and at 4 and 8 Hz some frequency-dependent contractile activity was seen after stopping EFS. These responses were reproducible. In male KO strips, the EFS-induced relaxations were greatly reduced (Fig. III.2A and Fig. III.3A) and 9 of the 33 male KO strips displayed an increase in contractile activity during EFS at 8 Hz (Fig. III.2A).

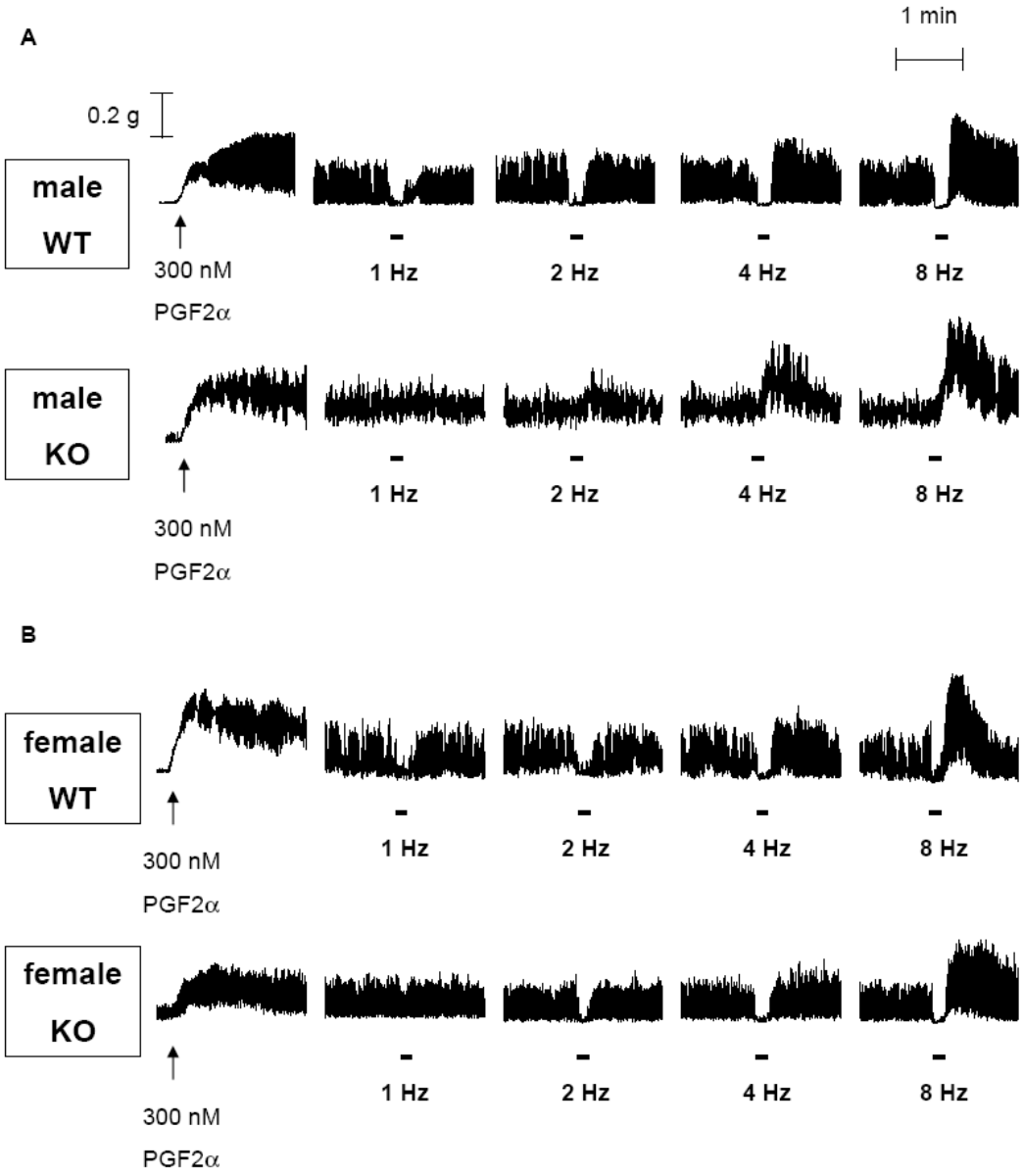
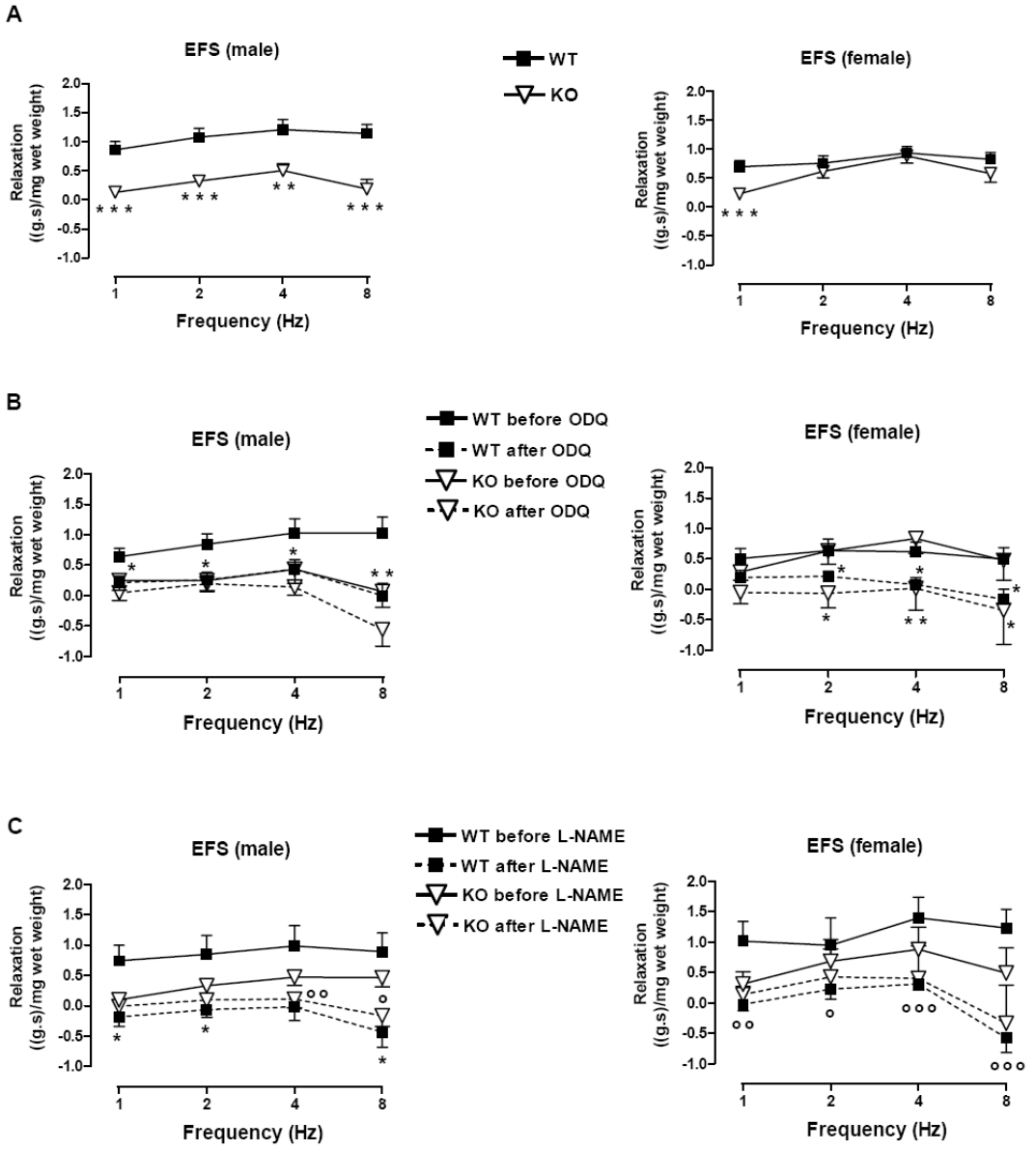



**Figure III.2**

Representative traces showing the inhibitory responses of pre-contracted (PGF2α; 300 nM) circular muscle strips of male (A) and female (B) WT and KO mouse jejunum to electrical field stimulation (EFS; 40 V, 0.1 ms, 1-8 Hz, 10 s trains). The experiments were performed in the continuous presence of atropine (1 μM) and guanethidine (4 μM). Arrows indicate the moment of PGF2α administration. Bars indicate EFS.




## Figure III.3

Frequency-response curves of relaxations induced by electrical field stimulation (EFS, 40 V, 0.1 ms, 1-8 Hz, 10 s trains) in jejunum circular muscle strips of male (left) and female (right) wild-type (WT) and knock-out (KO) mice (A). In B and C, responses before and after incubation with ODQ (10 μM, B) and L-NAME (300 μM, C) are shown. The experiments were performed in the continuous presence of atropine (1 μM) and guanethidine (4 μM). Data are expressed as (g.s)/mg wet weight. Negative values indicate contractile responses.

A) Values are means ± S.E.M. of n = 31-34 strips out of 16 -17 animals; * * P < 0.01, * * * P < 0.001: unpaired Student *t*-test (KO *vs*. WT).

B) and C) Values are means ± S.E.M. of n = 7-9 strips; * P < 0.05, * * P < 0.01, * * * P < 0.001: paired Student t-test (after vs. before) or ° P < 0.05, ° ° P < 0.01, ° ° ° P < 0.001: unpaired Student *t*-test (after *vs*. before) The EFS-induced responses in the WT (female only) and KO (male and female) time-control strips (results not shown) for the tissues where L-NAME was administered, were reduced when studied a second time. Therefore the change in relaxant response by L-NAME was compared to the spontaneous change in the control strips by an unpaired *t*-test.

In male WT strips, the EFS-evoked responses were reduced at 1, 2 and 4 Hz and abolished at 8 Hz by ODQ (Fig. III.3B), while L-NAME abolished the EFS-evoked responses at all stimulation frequencies; small contractions occurred upon EFS after application of L-NAME (Fig. III.3C). In male KO strips, the small relaxations to EFS were significantly reduced at 4 Hz and abolished at 8 Hz by L-NAME (Fig. III.3C); the reduction of the EFS-induced relaxation by ODQ at 4 Hz and the abolishment at 8 Hz did not reach significance (Fig. III.3B).

In female WT strips, EFS evoked similar reproducible relaxations as in male WT tissues. The response by EFS at 1 Hz was significantly reduced in female KO strips as compared to female WT strips but the responses by EFS at 2-8 Hz were not changed (Fig. III.2B and Fig. III.3A). In female WT strips, application of ODQ reduced the EFS-induced relaxations at 2 and 4 Hz and abolished them at 8 Hz; in female KO strips, ODQ abolished the EFS-induced relaxations at 2, 4 and 8 Hz (Fig. III.3B). Application of L-NAME decreased the EFS-induced relaxations at 2 and 4 Hz and abolished them at 1 and 8 Hz in female WT strips; in female KO strips, the reduction of the EFS-induced relaxation by L-NAME at 1, 2 and 4 Hz and the abolishment at 8 Hz did not reach significance (Fig. III.3C).

#### III.4.2.5 Inhibitory responses to exogenously applied NO in WT and KO mice

In male WT strips, application of exogenous NO interrupted phasic activity; the duration of the response was concentration-dependent. In male KO strips, the relaxant responses to NO were almost abolished in comparison to the relaxation obtained in WT strips (Fig. III.4A and Fig. III.5A). In male WT strips, ODQ reduced the NO-induced relaxations (Fig. III.5B) but application of L-NAME increased the responses to 1 and 100 μM NO (Fig. III.5C). The small relaxations to exogenous NO in male KO strips were not influenced by L-NAME (Fig. III.5C) but abolished by ODQ (Fig. III.5B).

Female WT strips showed similar NO-induced responses as in male WT strips, but in female KO strips, NO-induced relaxations were only moderately reduced compared to female WT strips (Fig. III.4B and Fig. III.5A). In female WT strips, the NO-induced relaxations were not influenced by L-NAME (Fig. III.5C) but were reduced by ODQ, reaching significance at 1 and 10 μM NO (Fig. III.5B). In female KO strips, relaxations induced by NO were reduced by ODQ again reaching significance at 1 and 10 μM NO (Fig. III.5B); the responses were nearly not influenced by L-NAME (Fig. III.5C).

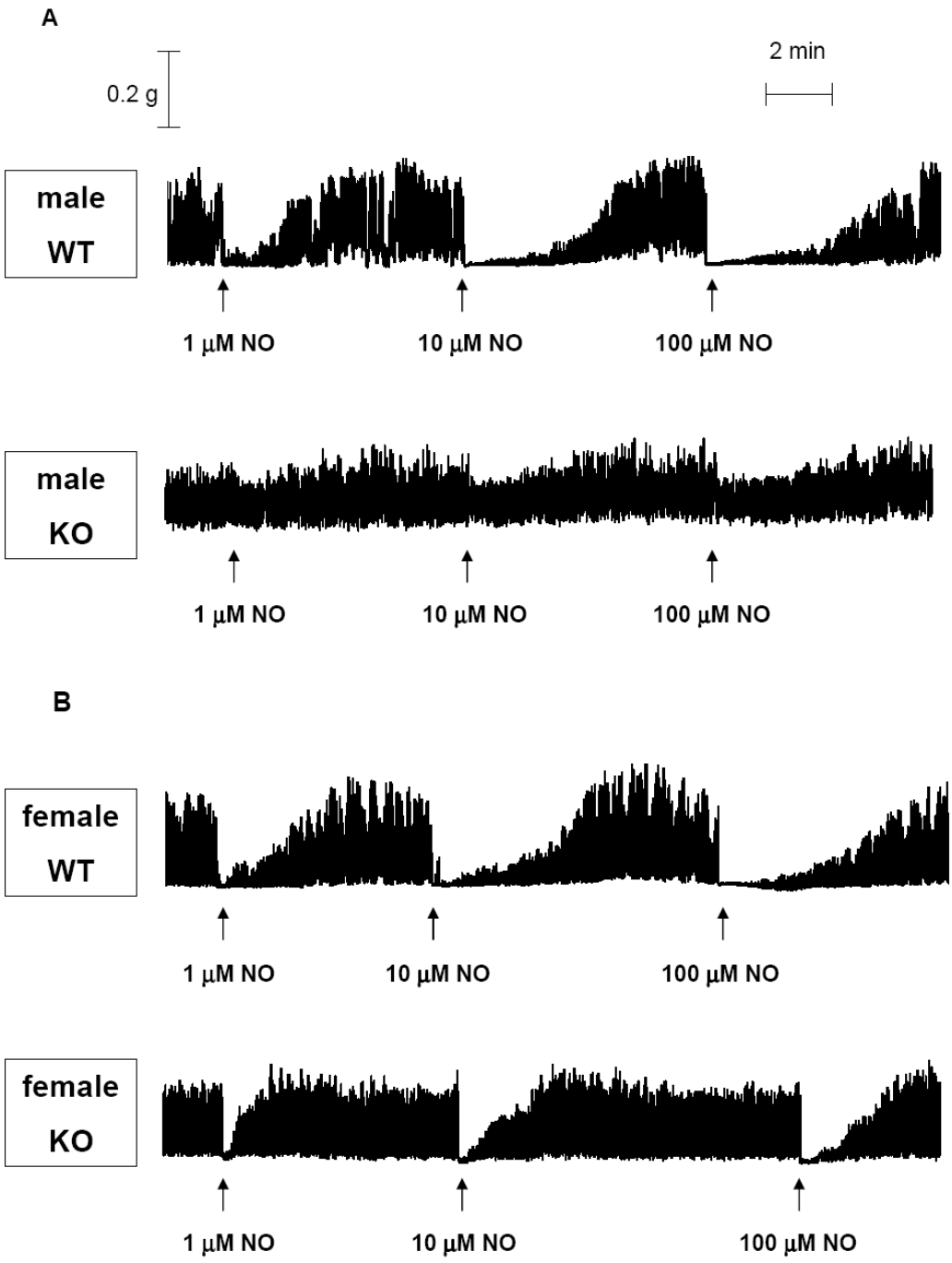
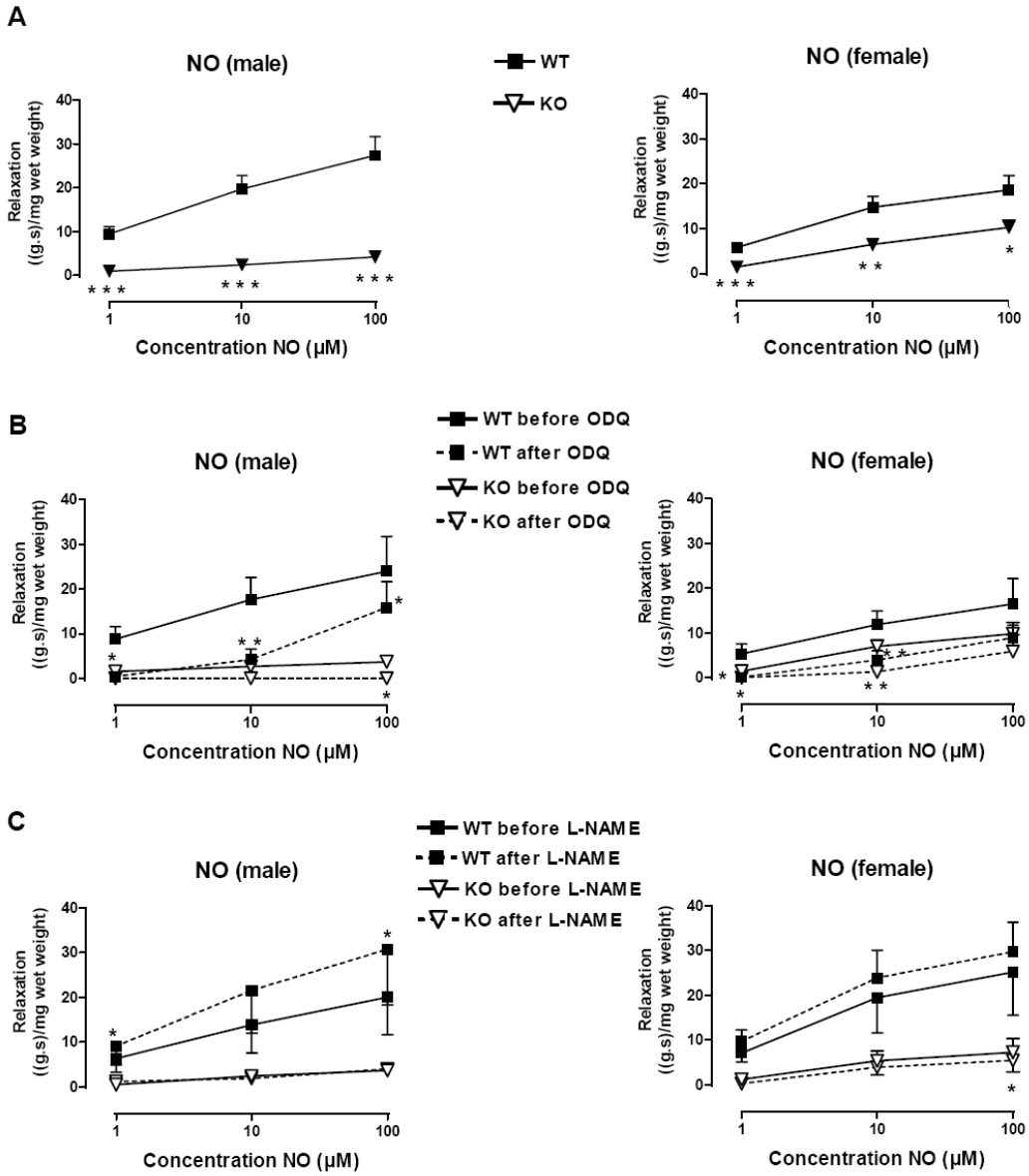



**Figure III.4**

Representative traces showing the inhibitory responses of pre-contracted (PGF2$\alpha$; 300 nM) circular muscle strips of male (A) and female (B) mouse WT and KO jejunum to NO (1-100 $\mu$M). The experiments were performed in the continuous presence of atropine (1 $\mu$M) and guanethidine (4 $\mu$M). Arrows indicate the moment of NO administration.




## Figure III.5

Concentration-response curves of NO (1-100 µM) in jejunum circular muscle strips of male (left) and female (right) wild-type (WT) and knock-out (KO) mice (A). In B and C, responses before and after incubation with ODQ (10 µM, B) and L-NAME (300 µM, C) are shown. The experiments were performed in the continuous presence of atropine (1 µM) and guanethidine (4 µM). Data are expressed as (g.s)/mg wet weight.

A) Values are means ± S.E.M. of n = 31-34 strips out of 16 -17 animals; * P < 0.05, * * P < 0.01, * * * P < 0.001: unpaired Student *t*-test (KO *vs*. WT).

B) and C) Values are means ± S.E.M. of n = 7-9 strips; * *P* < 0.05, * * P < 0.01: paired Student *t*-test (after *vs*. before).

#### III.4.2.6 Inhibitory responses to exogenously applied VIP in WT and KO mice

Exogenously applied VIP (100 nM) induced a sustained relaxation that was reproducible. In male WT strips, the VIP-induced relaxation measured 42.1 ± 5.3 (g.s)/mg wet weight; this was not significantly changed in male KO strips (29.2 ± 4.1 (g.s)/mg wet weight; n = 30-34 out of 16-17 animals). Similar results were obtained in tissues of female mice (WT: 33.0 ± 4.3; KO: 40.1 ± 4.2 (g.s)/mg wet weight; n = 30-34 out of 16-17 animals).

#### III.4.2.7 Inhibitory responses to electrical field stimulation and exogenously applied NO in male heterozygous mice

The responses to EFS and exogenous NO were also studied in male heterozygous mice, in parallel with a new set of WT and KO mice born in the same period. The difference in responses to EFS and NO between male WT and KO strips was confirmed (compare Fig. III.6 to Fig. III.3A and Fig. III.5A).

The EFS-evoked relaxations in heterozygous strips were decreased compared to WT strips, although this only reached significance at 1 Hz, and they were not significantly different from the responses in KO strips (Fig. III.6A). The NO-induced relaxations in heterozygous strips were not altered compared to those observed in WT strips. The NO-induced relaxations in KO strips were significantly decreased compared to the responses in both WT and heterozygous strips (Fig. III.6B).

### III.4.3 cGMP analysis

In male WT strips, basal cGMP levels were increased 10-fold by NO but were not changed by EFS (40 V, 0.1 ms at 4 Hz for 10 s) (Table III.3). In male KO strips, the basal cGMP levels were significantly lower than in WT strips and NO induced a moderate but significant 2.5-fold increase in cGMP levels (Table III.3).

In female WT and KO strips, similar results as in male tissues were obtained (Table III.3).

**Table III.3: cGMP levels**

| | MALE | | FEMALE | |
|---|---|---|---|---|
| | WT | KO | WT | KO |
| basal | 13.8 ± 2.7 | 3.4 ± 0.6 (**) | 18.5 ± 3.5 | 3.0 ± 0.3 (**) |
| after NO | 139.6 ± 27.2 (°°) | 8.5 ± 2.4 (**, °) | 191.4 ± 34.6 (°°°) | 7.5 ± 2.0 (***, °) |
| after EFS | 8.8 ± 1.2 | 2.2 ± 0.5 (**) | 16.0 ± 3.3 | 2.7 ± 0.6 (**) |

cGMP values are given in pmol /(g tissue). Values are mean ± S.E.M. of n = 5-6; * * P < 0.01, * * * P < 0.001: unpaired student t-test (KO *vs.* WT); ° P < 0.05, ° ° P < 0.01, ° ° ° P < 0.001: ANOVA + Bonferroni corrected t-test (stimulus vs. basal)

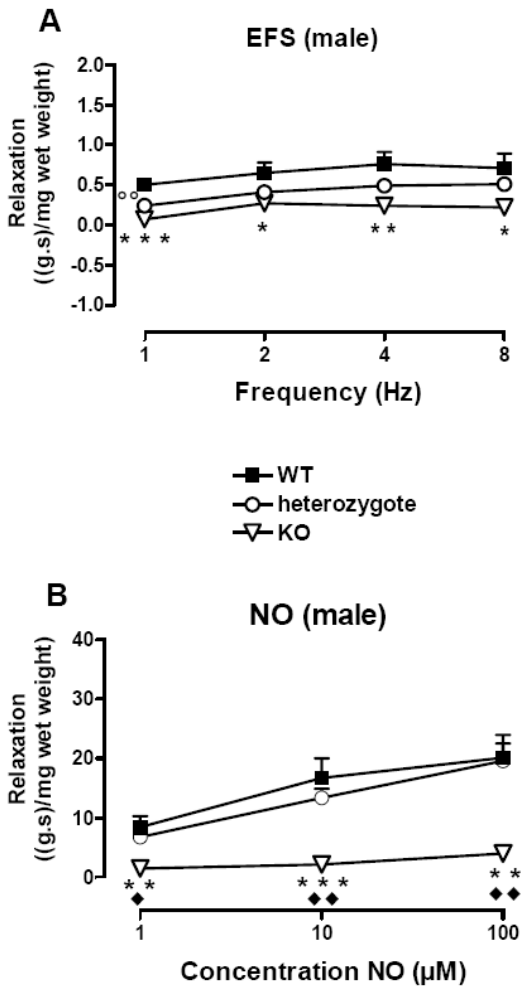



**Figure III.6**

Frequency-response curves of electrical field stimulation (EFS, 40 V, 0.1 ms, 1-8 Hz, 10 s trains) (A) and concentration-response curves of NO (1-100 µM) (B) in jejunum circular muscle strips of male wild-type (WT), heterozygous and knock-out (KO) mice. The experiments were performed in the continuous presence of atropine (1 µM) and guanethidine (4 µM). Data are expressed as (g.s)/mg wet weight.

Values are means ± S.E.M. of n = 14-22 strips out of 6-11 animals; * P < 0.05, * * P < 0.01, * * * P < 0.001: ANOVA + Bonferroni corrected t-test (KO *vs*. WT); ♦ P < 0.05, ♦♦ P < 0.01: ANOVA + Bonferroni corrected t-test (KO *vs*. heterozygous) or ° ° P < 0.01: ANOVA + Bonferroni corrected t-test (heterozygous *vs*. WT).

## III.4.4 Transit

### III.4.4.1 Small intestinal transit

The phenol red recovery was 78.6 ± 1.0% and 77.4 ± 2.0% in respectively male WT and KO mice and 72.3 ± 3.4% and 73.0 ± 2.9% in respectively female WT and KO mice (n = 8).

The small bowel length was significantly increased in male KO mice compared to male WT (from 30.9 ± 0.9 cm to 36.0 ± 0.9 cm, n = 8, $P < 0.01$). Sixty min after gavage, small intestinal transit tended to be delayed, but the shift in geometric centre did not reach significance (geometric centre: male WT: 6.0 ± 0.5; male KO: 4.6 ± 0.6, n = 8) (Fig. III.7A).
The small bowel length was not different between female WT and KO mice (WT: 29.9 ± 1.5; KO: 33.6 ± 1.2 cm; n = 8). Small intestinal transit was delayed in female KO mice (Fig. III.7A) as also manifest from the significant decrease in geometric centre (WT: 5.8 ± 0.3; KO: 4.8 ± 0.3, n = 8, $P < 0.05$) (Fig. III.7A).

#### III.4.4.2 Intestinal transit

The distribution of fluorescein-labelled dextran in the intestinal tract, sixty min after gavage, was not different between male WT and KO mice (geometric centre: male WT: 6.1 ± 1.0; male KO: 6.7 ± 0.4, n = 7-8) and female WT and KO mice (geometric centre: female WT: 6.6 ± 0.3; female KO: 6.5 ± 0.7, n = 7-8) (Fig. III.7B).

#### III.4.4.3 Whole gut transit time

The whole gut transit time, determined as the time taken for excretion of the first red coloured faeces after gavage with carmine, was not significantly different between male WT and KO mice (mean value: male WT: 159 ± 16 min; male KO: 188 ± 21, n = 7-8) nor between female WT and KO mice (mean value: female WT: 153 ± 16 min; female KO: 154 ± 19, n = 8-9) (Fig. III.7C).

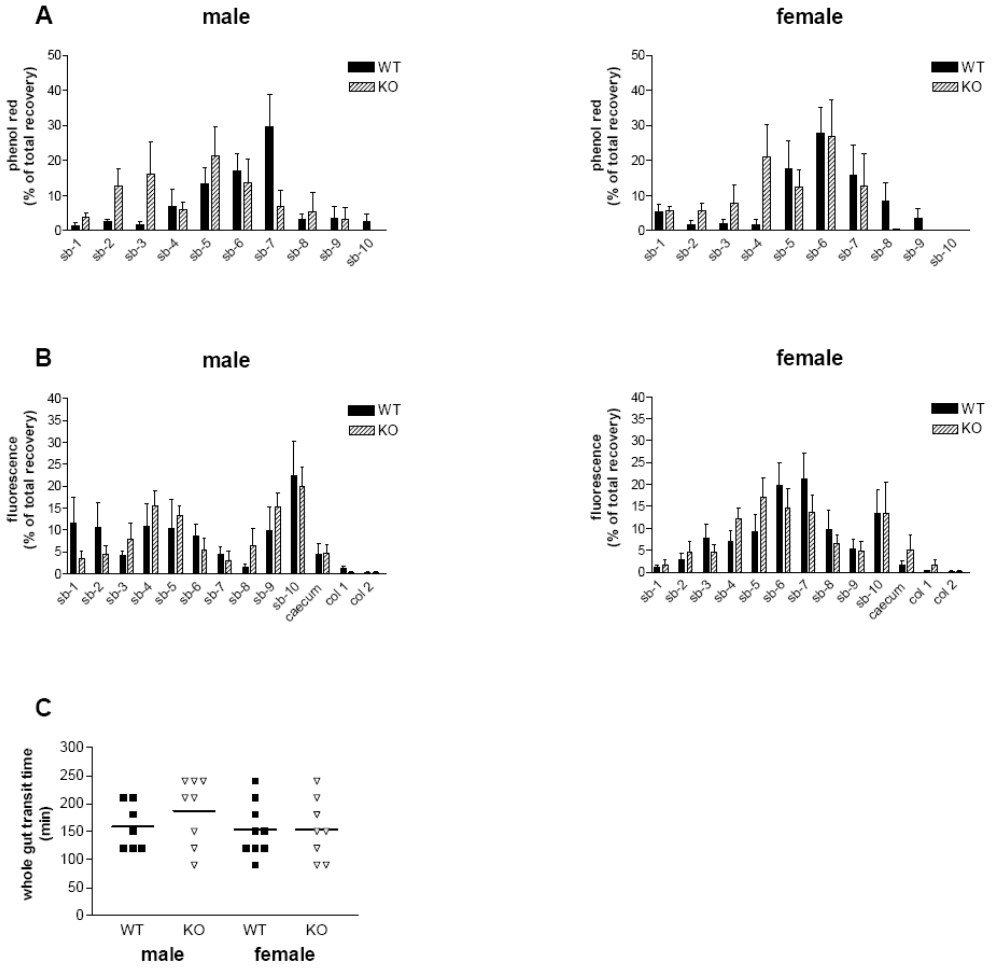



**Figure III.7**

A) Distribution of phenol red in 10 equal small bowel (sb) segments 60 min after gavage of a 250 µl phenol red meal (0.1 % w $v^{-1}$ dissolved in water) in male (left) and female (right) wild-type (WT) and knock-out (KO) mice. Values are means ± S.E.M. of n = 8 animals.

B) Distribution of fluorescein-labelled dextran in 10 equal small bowel (sb) segments, caecum, and 2 equal colon (col) segments 60 min after gavage of 200 µl fluorescein-labelled dextran (70 kDa; 2.5 % w $v^{-1}$ dissolved in water) in male (left) and female (right) wild-type (WT) and knock-out (KO) mice. Values are means ± S.E.M. of n = 7-8 animals.

C) Scatter graph showing the whole gut transit time of a carmine solution (6 % w $v^{-1}$ dissolved in 0.5 % methylcellulose) in male (left) and female (right) wild-type (WT) and knock-out (KO) mice. The mean value is represented by a solid line (n = 7-9 animals).

## *III.5 Discussion*

The aim of our study was to investigate the role of $sGC\alpha_1\beta_1$ in nitrergic regulation of jejunal smooth muscle activity by studying the in vitro and in vivo small intestinal consequences of knocking-out $sGC\alpha_1$.

### III.5.1 Role of sGC in male mice

The observation that L-NAME abolished the EFS-induced responses indicates that NO is the most important inhibitory neurotransmitter in WT jejunal circular muscle strips of male Swiss 129 mice. Additionally, both endogenous NO released upon EFS and exogenous NO induces relaxation through activation of sGC as the sGC inhibitor ODQ clearly reduced these nitrergic relaxations. Furthermore, exogenous NO induced a 10-fold increase in cGMP levels in jejunum strips of male WT mice further supporting the importance of sGC activation in nitrergic relaxation. In contrast, EFS at 4 Hz for 10 s, although inducing an ODQ-sensitive relaxation, did not increase cGMP levels. This corresponds to what we reported previously in the gastric fundus of male Swiss 129 WT mice (Vanneste et al., 2007). Together these data suggest that the subcellular localization, and recuperability by the assay used, of cGMP induced by endogenous NO released from nerve varicosities must differ from that raised by exogenous NO diffusing into the smooth muscle strip (Vanneste et al., 2007). Surprisingly, application of L-NAME induced an increase of the NO-evoked responses; we have no clearcut explanation for this observation. Indeed, increased responses to NO-donors upon removal of endogenous NO have been reported in vascular tissues and were attributed to desensitization of sGC by tonically released NO (Busse et al., 1989; Luscher et al., 1989; Shirasaki and Su, 1985). However, if NO were tonically released, L-NAME can be expected to increase basal tone by abolishing this tonic relaxant drive; this was not the case.

In male sGC$\alpha_1$ KO mice responses evoked by endogenous and exogenous NO were almost absent, and the increase in cGMP levels by exogenous NO was clearly reduced indicating that the major sGC isoform involved in nitrergic relaxation of male jejunum is sGC$\alpha_1\beta_1$. Still, the small NO and EFS-induced responses were sensitive to ODQ, and exogenous NO induced a 2.5-fold increase in cGMP levels in sGC$\alpha_1$ KO mice illustrating that some response is present due to activation of sGC$\alpha_2\beta_1$; ODQ indeed equally inhibits sGC$\alpha_2\beta_1$ as sGC$\alpha_1\beta_1$ (Russwurm et al., 1998).

No compensatory mechanisms in cAMP-induced relaxation nor in contractile mechanisms were observed in sGC$\alpha_1$ KO strips in view of the maintained responses to VIP, carbachol and PGF2$\alpha$. This corresponds to observations in cGKI KO mice (Ny et al., 2000; Bonnevier et al., 2004). As the responses to carbachol are maintained in sGC$\alpha_1$ KO strips, this suggests that the decreased EFS-induced cholinergic contractions observed in male sGC$\alpha_1$ KO strips are not related to an alternation at the level of the muscarinic receptors located on the smooth muscle cells or at their transduction mechanism. The $L_o$ of the KO strips was increased. This might correlate with the partial disappearance of the nitrergic inhibitory pathway so that the strips require more load to relax and to reach their optimal length-tension relationship. The weight of WT and KO strips was the same showing that an adaptive

increase in muscle mass in response to the decrease in nitrergic inhibitory influence is unlikely. Additionally, in gastric fundus strips of KO mice, we did not observe an increase in $L_o$ (Vanneste et al., 2007).

### III.5.2 Role of sGC in female mice

The results in female WT mice were very similar as in male WT mice except that in female mice L-NAME did not significantly increase the NO-induced response. We have no explanation for these differences. Most strikingly however, jejunum strips of female $sGC\alpha_1$ KO mice still produced a clear-cut relaxation upon EFS and application of exogenous NO. The clearly remaining responses to EFS (2-8 Hz) and to exogenous NO were sensitive to ODQ, suggesting that $sGC\alpha_2\beta_1$ plays a more important role in nitrergic relaxation of jejunum strips of female $sGC\alpha_1$ KO mice than in male $sGC\alpha_1$ KO mice. Exogenous NO indeed still induced a significant 2.5-fold increase in cGMP levels in female KO tissue, which was unexpectedly not more pronounced than the 2.5-fold increase in cGMP levels in male KO tissues by exogenous NO in view of the better maintained relaxation to exogenous NO in female KO tissues. As mentioned above, the maintained NO-induced relaxation in female $sGC\alpha_1$ KO tissues results from the activation of $sGC\alpha_2\beta_1$. It is possible that the subcellular localization of cGMP raised by the membrane-associated $sGC\alpha_2\beta_1$ (Russwurm et al., 2001) differs from that raised by the cytosolic $sGC\alpha_1\beta_1$ and that this has consequences on the recuperability of cGMP by the assay used.

Buys et al. (2008) also observed a gender-specific alteration in $sGC\alpha_1$ KO mice when studying the cardiovascular consequences of the $sGC\alpha_1$ KO mice as male KO mice developed testosteron-dependent hypertension while female mice did not. As we found no up-regulation of $sGC\alpha_2$ and $sGC\beta_1$ mRNA in jejunum tissue of male as well as female $sGC\alpha_1$ KO mice, a gender-differential compensatory mechanism via an increase in the $sGC\alpha_2\beta_1$ isoform in the female mice seems not present. Whatever mechanism contributes to the less pronounced decrease in nitrergic relaxation in female $sGC\alpha_1$ KO mice compared to male $sGC\alpha_1$ KO mice, it does not occur in the whole GI tract as we previously reported that the influence of knocking-out $sGC\alpha_1$ at the level of the gastric fundus and the colon is the same in male and female KO mice (Vanneste et al., 2007; Dhaese et al., 2008).

### III.5.3 The $sGC\alpha_1$ KO model

Buys et al. (2008) describe the generation of the $sGC\alpha_1$ KO mice by targeted deletion of the sixth exon which is essential for the enzymatic activity. As a result, $sGC\alpha_1$ KO mice express an enzymatically inactive mutant $sGC\alpha_1\beta_1$ protein. Mice heterozygous for this deletion express the active as well as the mutant inactive $sGC\alpha_1\beta_1$ protein. NO-induced increases in sGC enzyme activity in aortic, lung and left ventricular tissue were intermediate in

heterozygous mice to those measured in WT and sGC$\alpha_1$ KO mice, suggesting a gene dose effect and indicating that the mutant sGC$\alpha_1\beta_1$ protein does not function as a dominant negative (Buys et al., 2008). Still, no differences were observed in systolic blood pressure between male WT and heterozygous mice.

In the jejunum, EFS-induced relaxations were reduced in male heterozygous mice compared to male WT mice, whereas NO-induced relaxations were not altered in male heterozygous mice. In the light of the observations made by Buys et al. (2008), we do not believe that the reduced EFS-evoked relaxations in the heterozygous mice are due to the non-specific effects of the mutant sGC$\alpha_1\beta_1$ protein. This observation could possibly be related to the fact that endogenous NO is released from nerve varicosities and thus enters the smooth muscle cell more locally whereas exogenous NO is diffusing from the surrounding extracellular solution and therefore enters the smooth muscle over the whole surface. As sGC$\alpha_1\beta_1$ is a cytosolic enzyme, it is possible that endogenous NO is thus not as effective as exogenous NO in reaching and thus activating the more limited amount of active sGC$\alpha_1\beta_1$ in heterozygous mice, resulting in a decreased response to EFS but a maintained response to exogenous NO.

### III.5.4 Implication on intestinal transit

In agreement with observations in cGKI KO mice (Ny et al., 2000), no morphological abnormalities at the level of the small bowel were observed in sGC$\alpha_1$ KO mice although the small bowel length was slightly increased in male KO compared to male WT mice; this increase was not observed in female mice.

Inhibition of nitrergic relaxation at the level of the small intestine is expected to delay intestinal transit as manifest from studies with NOS-inhibitors in different species illustrating the essential role of NO in intestinal peristalsis (Chiba et al., 2002; Fraser et al., 2005; Karmeli et al., 1997). Also cGKI KO mice showed a delay in intestinal transit (Pfeifer et al., 1998). In male and female sGC$\alpha_1$ KO mice, we found that the small intestinal transit of a phenol red solution tended to be delayed. However, the intestinal transit of a fluorescein-labelled dextran solution and the whole gut transit time of a carmine solution were not delayed in female nor in male sGC$\alpha_1$ KO mice. Taken together, these results suggest that knocking out sGC$\alpha_1$ has no important in vivo effects on intestinal motility. This seems plausible for the female sGC$\alpha_1$ KO mice, as also in vitro NO-induced relaxations were only moderately influenced, indicating that in female mice sGC$\alpha_2\beta_1$ is able to sustain the NO-induced motility effects. However, in male sGC$\alpha_1$ KO mice, an important decrease in in vitro NO-induced relaxations was observed. Still, this is not accompanied by a decrease in in vivo intestinal motility. This might be explained by the concomitant reduction of cholinergic nerve activity in male sGC$\alpha_1$ KO mice as demonstrated by the decrease in EFS-induced cholinergic

contractions. Indeed, the propagation of peristaltic activity is the result of an interplay between ascending excitatory and descending inhibitory enteric pathways (Waterman et al., 1994). The effects of decreased inhibitory (nitrergic) as well as decreased excitatory (cholinergic) responses in $sGC\alpha_1$ KO mice might neutralize each other, leaving the balance between these two players of peristaltic activity unaltered.

In conclusion, in vitro, $sGC\alpha_1\beta_1$ is the most important mediator in nitrergic relaxation of jejunum but nitrergic relaxation can also occur via activation of $sGC\alpha_2\beta_1$. This latter mechanism is more pronounced in female than in male $sGC\alpha_1$ KO mice. In vivo, no important implications of knocking out $sGC\alpha_1$ on intestinal motility were observed in male and female KO mice.

## *III.6 References*

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# Chapter IV

# INVOLVEMENT OF SOLUBLE GUANYLATE CYCLASE $\alpha_1$ AND $\alpha_2$, AND $SK_{Ca}$ CHANNELS IN NANC RELAXATION OF MOUSE DISTAL COLON

**Ingeborg Dhaese [1], Gwen Vanneste [1], Patrick Sips [2,3], Emmanuel Buys [2,3,4], Peter Brouckaert [2,3], Romain A. Lefebvre [1]**

[1] Heymans Institute of Pharmacology, Ghent University, Ghent, Belgium

[2] Department of Molecular Biomedical Research, VIB, Ghent, Belgium

[3] Department of Molecular Biology, Ghent University, Ghent, Belgium

[4] Anesthesia Center for Critical Care Research, Department of Anesthesia & Critical Care, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

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# Chapter IV Involvement of soluble guanylate cyclase $\alpha_1$ and $\alpha_2$, and $SK_{Ca}$ channels in NANC relaxation of mouse distal colon

## *IV.1 Abstract*

In distal colon, both nitric oxide (NO) and ATP are involved in non-adrenergic non-cholinergic (NANC) inhibitory neurotransmission. The role of the soluble guanylate cyclase (sGC) isoforms $\alpha_1\beta_1$ and $\alpha_2\beta_1$, and of the small conductance $Ca^{2+}$-dependent $K^+$ channels ($SK_{Ca}$ channels) in the relaxation of distal colon by exogenous NO and by NANC nerve stimulation was investigated, comparing wild type (WT) and sGC$\alpha_1$ knockout (KO) mice. In WT strips, the relaxation induced by electrical field stimulation (EFS) at 1 Hz but not at 2-8 Hz was significantly reduced by the NO-synthase inhibitor L-NAME or the sGC inhibitor ODQ. In sGC$\alpha_1$ KO strips, the EFS-induced relaxation at 1 Hz was significantly reduced and no longer influenced by L-NAME or ODQ. The $SK_{Ca}$ channel blocker apamin alone had no inhibitory effect on EFS-induced relaxation, but combined with ODQ or L-NAME, apamin inhibited the relaxation induced by EFS at 2-8 Hz in WT strips and at 8 Hz in sGC$\alpha_1$ KO strips. Relaxation by exogenous NO was significantly attenuated in sGC$\alpha_1$ KO strips, but could still be reduced further by ODQ. Basal cGMP levels were lower in sGC$\alpha_1$ KO strips but NO still significantly increased cGMP levels versus basal.

In conclusion, in the absence of sGC$\alpha_1\beta_1$, exogenous NO is able to partially act through sGC$\alpha_2\beta_1$. NO, acting via sGC$\alpha_1\beta_1$, is the principal neurotransmitter in EFS-evoked responses at 1 Hz. At higher stimulation frequencies, NO, acting at sGC$\alpha_1\beta_1$ and/or sGC$\alpha_2\beta_1$, functions together with another transmitter, probably ATP acting via $SK_{Ca}$ channels, with some degree of redundancy.

## *IV.2 Introduction*

Nitric oxide (NO), synthesized from L-arginine by neuronal NO synthase (nNOS, NOS-1), is considered to play a major role as non-adrenergic, non-cholinergic (NANC) neurotransmitter in the gastrointestinal (GI) tract. In colon however, NO is not the sole neurotransmitter as also ATP was reported to play a role in inhibitory neurotransmission of mouse (Serio et al., 2003a), rat (Pluja et al., 1999), hamster (El Mahmoudy et al., 2006) and human colon (Benko et al., 2007; Boeckxstaens et al., 1993; Keef et al., 1993). ATP is suggested to induce localized $Ca^{2+}$ release via a mechanism involving P2Y receptors, phospholipase C (PLC) and inositol 1,4,5-trisphosphate ($IP_3$) receptors. The resulting increase in $Ca^{2+}$ near the plasma membrane ($Ca^{2+}$ puffs) leads to activation of small conductance $Ca^{2+}$-dependent $K^+$ channels ($SK_{Ca}$ channels) and consequently hyperpolarization (Bayguinov et al., 2000).

The predominant target of the GI NANC neurotransmitter NO in smooth muscle cells is soluble guanylate cyclase (sGC). Activation of sGC results in increased intracellular cGMP levels, which consequently activates cGMP-dependent protein kinase (PKG, cGK), leading to relaxation by lowering the intracellular $Ca^{2+}$ concentration and by desensitization of the contractile apparatus to $Ca^{2+}$ (Lucas et al., 2000; Toda and Herman, 2005). sGC is a heterodimeric hemoprotein that is composed of a larger α and a smaller β subunit (Harteneck et al., 1991), both necessary for catalytic activity (Harteneck et al., 1990). For each subunit, two isoforms ($\alpha_1/\alpha_2$ and $\beta_1/\beta_2$) have been identified (Harteneck and al., 1991). The $sGC\alpha_1\beta_1$ and the $sGC\alpha_2\beta_1$ heterodimers seem to be the only physiologically active isoforms (Mergia et al., 2003; Russwurm et al., 1998). Both isoforms are functionally indistinguishable: no differences in kinetic properties and sensitivity towards NO between the 2 isoforms were found (Russwurm et al., 1998). In all other tissues than brain, including ileum and colon, the $sGC\alpha_1\beta_1$ heterodimer is the predominating isoform (Mergia and al., 2003). In brain, the $sGC\alpha_2\beta_1$ isoform has been reported to be associated with PSD-95. As a consequence of this interaction, the $sGC\alpha_2\beta_1$ isoform is recruited to the membrane fraction. This suggests that, at least in brain, the $sGC\alpha_2\beta_1$ isoform works as a sensor for the NO formed by the PSD-95-associated neuronal NO synthase (Russwurm et al., 2001).

Endogenous NO also has been shown to induce sGC-independent relaxation via the activation of $SK_{Ca}$ channels in mouse (Serio et al., 2003b) and rat duodenum (Martins et al., 1995) and in rat colon (Van Crombruggen and Lefebvre, 2004).

Recently, $sGC\alpha_1$ knockout (KO) mice were developed, lacking exon 6 of the $sGC\alpha_1$ gene, coding for an essential part in the catalytical domain (Buys et al., 2008). We previously reported that $sGC\alpha_1\beta_1$ plays an important role in nitrergic relaxation of gastric smooth muscle cells, but some nitrergic relaxation can occur in $sGC\alpha_1$ KO mice via $sGC\alpha_2\beta_1$ activation. As a result, knocking out $sGC\alpha_1$ only has a moderate in-vivo consequence on gastric emptying

(Vanneste et al., 2007). In the present study, the role of the two physiologically active sGC isoforms and the $SK_{Ca}$ channels in relaxation of distal colon by exogenous NO and by endogenous NANC neurotransmitters was investigated using circular muscle strips of distal colon isolated from $sGC\alpha_1$ KO mice.

## *IV.3 Materials and Methods*

### IV.3.1 Animals

$sGC\alpha_1$ KO mice were generated as described by Buys et al. (2008). Briefly, $sGC\alpha_1$ KO mice were generated by targeting exon 6 of the $sGC\alpha_1$ gene which codes for an essential part of the catalytical domain. Chimeras were generated by aggregating R1 embryonic stem cells carrying the mutant $sGC\alpha_1$ allele with Swiss morula-stage embryos.

$sGC\alpha_1$ KO Swiss/129 mice and wildtype (WT) littermates of both sexes (male: n = 48-50, 8-37 weeks, 20-50 g; female: n = 28-29, 8-34 weeks, 22-43 g) had free access to water and commercially available chow (ssniff Spezialdiäten GmbH). All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

### IV.3.2 Muscle tension experiments

#### IV.3.2.1 Tissue preparation and tension recording

Animals were sacrificed by cervical dislocation. The distal colon was isolated as an approximately four centimetre segment, taken above the pelvic brim.

This segment was then opened along the mesenteric border and pinned mucosa side up in Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The mucosa was removed by sharp dissection under a microscope and 2 full-thickness muscle strips (3 × 5 mm) were cut along the circular axis. After a silk thread (USP 4/0) was attached to both ends of the muscle strips, strips were mounted in 5 ml organ baths between 2 platinum electrodes (7 mm apart). The organ baths contained aerated (5% $CO_2$ in $O_2$) Krebs solution maintained at 37°C.

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

#### IV.3.2.2 Protocol

After an equilibration period of 30 min with refreshing of the Krebs solution every 10 min at a load of 0.25 g, the length-tension relationship was determined. To determine the optimal load ($L_o$; the load at which maximal response to the contractile agent occurred), muscle strips were stretched by load increments of 0.25 g and at each load level exposed to 1 µM carbachol. Strips were then allowed to equilibrate for 1 h at $L_o$ with flushing every 10 min in Krebs solution.

Cumulative contractile responses to carbachol (1 nM – 30 µM) and prostaglandin F2$\alpha$ ($PGF_{2\alpha}$) (1 nM – 3 µM) were obtained in separate strips in Krebs solution without atropine and guanethidine; concentrations were increased at 2 min intervals. All other experiments were performed after switching to Krebs solution containing 1 µM atropine and 4 µM guanethidine to block cholinergic and noradrenergic responses respectively (NANC conditions).

Strips were then pre-contracted with 3 µM $PGF_{2\alpha}$. 15 Min after its administration, when the contractile response was surely stabilized, relaxations were induced at 5 min interval by application of electrical field stimulation (EFS; 40V, 0.1 ms, 1-8 Hz for 30 s) via the platinum plate electrodes by means of a Hugo Sachs Stimulator I type 215/I, followed by the application of exogenous NO (1 µM-100 µM) and vasoactive intestinal polypeptide (VIP, 100 nM); these relaxant stimuli were always applied in this order. Strips were then washed and equilibrated at $L_o$ for 30 min with refreshing of the Krebs solution every 10 min; the NOS inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 300 µM) or the sGC inhibitor 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 µM) was then incubated for 30 min. Strips were re-contracted with 3 µM $PGF_{2\alpha}$ and the responses to EFS, NO and VIP were studied again in the presence of L-NAME or ODQ. The concentrations of ODQ (10 µM) and L-NAME (300 µM) have been shown to fully inhibit purified sGC (Garthwaite et al., 1995) and NO-synthases (Heinzel et al., 1992). The responses to EFS and NO were also studied, in male mice only, in the presence of ODQ and of ODQ plus the $SK_{Ca}$ channel blocker apamin (500 nM) (administered 30 min before a 3$^{rd}$ cycle of $PGF_{2\alpha}$-EFS-NO). The influence of apamin and apamin plus ODQ, apamin and apamin plus L-NAME, and L-NAME and L-NAME plus ODQ was tested in the same way on EFS- and NO-induced relaxation. The reproducibility of the responses to EFS, NO and VIP were evaluated by running time-control vehicle treated strips in parallel. The concentration of apamin used has been shown to fully inhibit $SK_1$, $SK_2$ and $SK_3$ channels expressed in Xenopus oocytes (Grunnet et al., 2001). These 3 SK channel types are indeed expressed in murine colon (Ro et al., 2001).

In a separate series, the influence of ODQ was also studied versus the relaxing effect of 8-bromoguanosine 3',5' cyclic monophosphate (8-Br-cGMP; 100 µM).

At the end of each experiment, the wet weight of the muscle tissue between the two silk thread knots was measured (mg wet weight; see data analysis).

#### IV.3.2.3 Data analysis

To measure the contractile responses to carbachol and $PGF_{2\alpha}$, the area under the curve (AUC) above baseline was determined. The mean phasic activity induced by $PGF_{2\alpha}$ was determined as the difference between the mean peak level and the mean trough level of the phasic component of the response to $PGF_{2\alpha}$. To measure inhibitory responses, the AUC for a given response was determined and subtracted from the AUC of a corresponding period just before applying the relaxing drug or stimulus, yielding the area above the curve for the inhibitory response. Rebound contractions occurring after EFS-induced inhibitory responses were measured as amplitude above the mean peak level of phasic activity during the 2 min before EFS.

The duration of the inhibitory responses was determined as 30 s for EFS (i.e. the length of the stimulus train applied), as the response immediately disappeared upon ending the 30 s stimulation train. VIP and 8-Br-cGMP induced a sustained response and the duration was fixed at 5 min for VIP and 10 min for 8-Br-cGMP. NO abolished phasic activity with a concentration-dependent duration, after which phasic activity progressively reoccurred. The duration of the inhibitory responses to NO was therefore determined as the time necessary for phasic activity to regain 50 % of the interval between the mean peak level of phasic activity during the 2 min before administration of NO and the minimum tone level during the NO response. This calculation was performed during the $1^{st}$ cycle of $PGF_{2\alpha}$-EFS-NO and the determined duration was further used for the NO-induced responses in the $2^{nd}$ and $3^{rd}$ cycle of $PGF_{2\alpha}$-EFS-NO. The responses are expressed as (g.s)/mg wet weight, except for the mean phasic activity induced by $PGF_{2\alpha}$ and the rebound contractions (g/mg wet weight).

### IV.3.3 cGMP analysis

From each mouse, 3 circular muscle strips of distal colon were prepared analogous to the strips prepared for the muscle tension experiments. The muscle strips were weighed to obtain the tissue wet weight and were mounted as described above, under the mean optimal load determined in the tension experiments (0.5 g). $PGF_{2\alpha}$ pre-contracted strips were snap frozen in liquid nitrogen without inducing relaxation (basal), or at maximal relaxation by NO (10 µM) or upon EFS (40 V, 0.1 ms, 1 Hz for 30 s) via platinum plate electrodes by means of a Grass S88 Stimulator (Grass, W. Warwick, RI, USA).

Snap frozen tissues were stored at – 80 °C until further processing. They were then pulverized by a Mikro-dismembrator U (B-Braun Biotech International, Germany) and dissolved in cold 6 % trichloroacetic acid to give a 10 % (w/v) homogenate. The homogenate

was centrifuged at 2000 x g for 15 min at 4 °C; the supernatant was recovered and washed four times with five volumes of water saturated diethyl ether. The aqueous extract was then dried under a stream of nitrogen at 60 °C and dissolved in a 10 or 50 times volume of assay buffer depending on the cGMP content detected in preliminary experiments. cGMP concentrations were determined using an enzyme immunoassay kit (EIA Biotrak System; Amersham Biosciences; UK) after acetylation of the samples and according to the manufacturer's instructions. The optimal density (OD) was measured with a 96-well plate reader (Biotrak II, Amersham Biosciences) at 450 nm. Tissue cGMP concentration was expressed as pmol per g tissue wet weight.

### IV.3.4 Western blot

After removal of the mucosa, colonic muscle tissues were homogenized in denaturing cell extraction buffer (Invitrogen), containing protease inhibiting cocktail (Roche), subjected to NuPAGE electrophoresis on Novex 4-12% Bis-Tris gels (Invitrogen) and subsequently transferred to nitrocellulose membranes (Amersham Bioscience). Blots were incubated with the following antibodies: rabbit polyclonal antibody specific for $sGC\alpha_1$ (dilution 1:2500), $sGC\alpha_2$ (dilution 1:200), $sGC\beta_1$ (dilution 1:500) and β-tubulin (dilution 1:500) (Abcam). After incubation with anti-rabbit IgG HRP-linked antibody (Cell signalling Technology), blots were developed by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and quantification of the bands was done by densitometry (ImageJ).

### IV.3.5 Statistics

All results are expressed as means ± S.E.M. n refers to tissues obtained from different animals unless indicated otherwise. Comparison between $sGC\alpha_1$ KO and WT tissues was done with an unpaired Student's *t*-test. Comparison within tissues of either WT or $sGC\alpha_1$ KO was done by a paired Student's *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. $P < 0.05$ was considered to be statistically significant (GRAPHPAD, San Diego, CA, USA).

### IV.3.6 Drugs used

The following drugs were used: apamin (obtained from Alomone Labs), atropine sulphate, 8-bromoguanosine 3',5' cyclic monophosphate sodium salt, guanethidine sulphate, L-NAME, prostaglandin F2α tris salt, VIP (all obtained from Sigma-Aldrich), carbachol (from Fluka AG), ODQ (from Tocris Cookson). All drugs were dissolved in de-ionized water except ODQ, which was dissolved in 100 % ethanol. Saturated NO solution was prepared from gas (Air Liquide, Belgium) as described by Kelm and Schrader (1990).

## *IV.4 Results*

The series of experiments where the influence of L-NAME or ODQ was tested on relaxation by EFS, NO and VIP, were performed in mice of both sexes. However, no systematic differences between sexes were found in distal colon strips. Therefore, results are presented for male mice and further experiments investigating the effect of apamin alone or in combination with ODQ or L-NAME were carried out in male mice only. No gross morphological GI differences between WT and $sGC\alpha_1$ KO animals were observed.

### IV.4.1 Western blot

The $sGC\alpha_1$ subunit was not detected in $sGC\alpha_1$ KO distal colon tissues, whereas this subunit was clearly present in the WT tissues (Fig. IV.1A). The relative expression versus β-tubulin of the $sGC\alpha_2$ subunit was not significantly up-regulated in the $sGC\alpha_1$ KO compared to the WT as shown in Fig. IV.1B (0.35 ± 0.02 in the $sGC\alpha_1$ KO tissues vs. 0.32 ± 0.04 in the WT tissues, n = 5). The $sGC\beta_1$ subunit was however significantly lower in the $sGC\alpha_1$ KO versus the WT (0.44 ± 0.02 in the $sGC\alpha_1$ KO tissues vs. 0.64 ± 0.06 in the WT tissues, n = 5; Fig. IV.1B).

### IV.4.2 General observations of the distal colon muscle strips

In the muscle tension experiments, the length of the strips, measured after equilibration and determination of optimal load, and the tissue wet weight, measured immediately after the experiment, were similar in WT and $sGC\alpha_1$ KO mice. The length and weight of the strips was respectively 5.4 ± 0.2 mm and 0.69 ± 0.06 mg in WT (n = 30 strips from 20 animals) and 5.0 ± 0.2 mm and 0.89 ± 0.11 mg in $sGC\alpha_1$ KO tissues (n = 28 strips from 16 animals).

WT strips as well as $sGC\alpha_1$ KO strips showed no or only moderate phasic activity during their equilibration at optimal load. The total basal contractile activity, measured during 5 min before the first administration of $PGF_{2\alpha}$, was similar in $sGC\alpha_1$ KO strips and WT strips (204.34 ± 25.54 g.s/mg wet weight in $sGC\alpha_1$ KO vs. 254.52 ± 55.20 g.s/mg wet weight in WT, n = 28-30 strips from 16-20 animals).

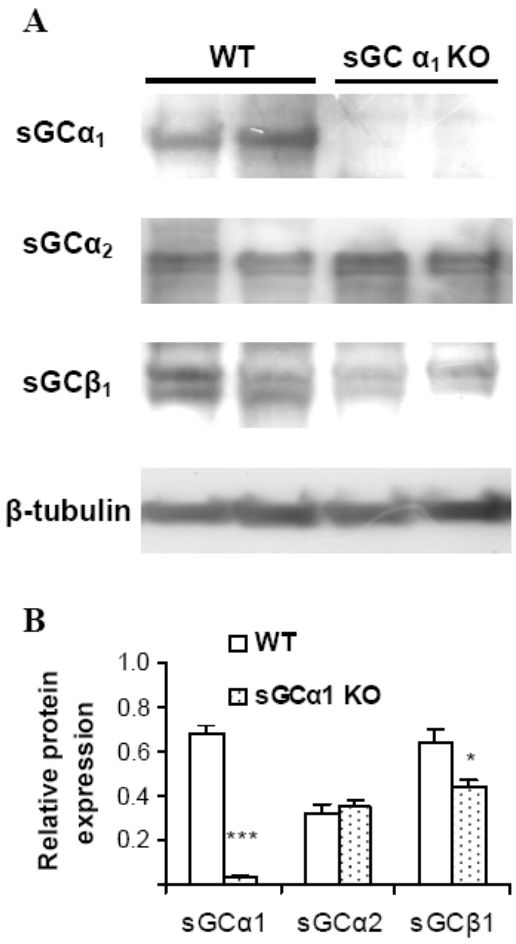


**Figure IV.1**

Western blot analysis of $sGC\alpha_1$, $sGC\alpha_2$, $sGC\beta_1$ and β-tubulin in colonic muscle tissues from two WT and two $sGC\alpha_1$ KO mice (A) and the relative protein expression levels of $sGC\alpha_1$, $sGC\alpha_2$, $sGC\beta_1$ versus β-tubulin in colonic muscle tissues from WT (□) and $sGC\alpha_1$ KO (⊡) mice. Means ± S.E.M. of n = 5 are shown (B). * $P < 0.05$, *** $P < 0.001$: $sGC\alpha_1$ KO versus WT (unpaired Student's *t*-test).

Contractile responses to cumulatively added concentrations of carbachol and $PGF_{2\alpha}$ tended to be lower for the examined concentration range in $sGC\alpha_1$ KO strips but did not significantly differ from these observed in WT strips (Fig. IV.2). A concentration of 3 μM PGF2α was used both in WT and $sGC\alpha_1$ KO strips to establish pre-contraction in further experiments studying inhibitory responses.

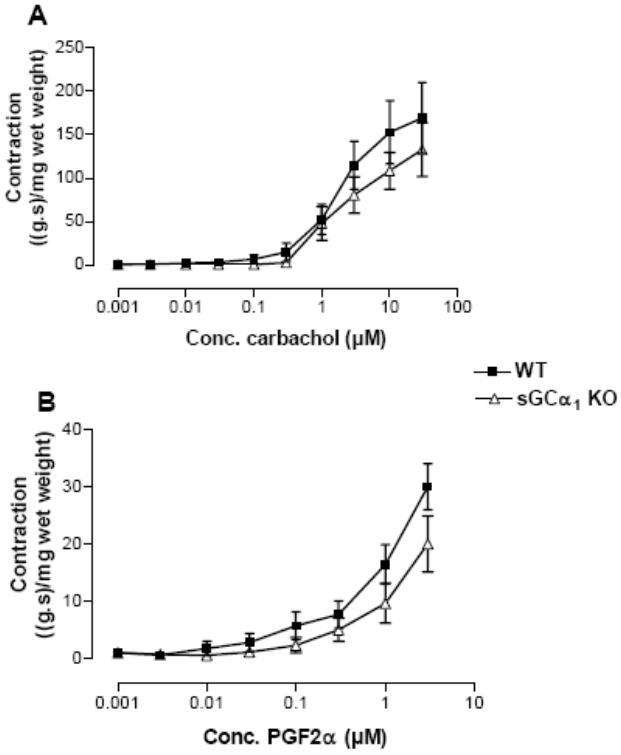


**Figure IV.2**

Concentration-response curves of A) carbachol (1 nM – 30 µM) and B) $PGF_{2\alpha}$ (1 nM – 3 µM) in WT (■) and $sGC\alpha_1$ KO (Δ) strips. Means ± S.E.M. of n = 8-9 are shown.

## IV.4.3 Inhibitory responses

### IV.4.3.1 Inhibitory responses to EFS and NO

In WT and $sGC\alpha_1$ KO strips, the addition of 3 µM $PGF_{2\alpha}$ induced an initial fast increase in tone, which then declined to a lower level with superimposed phasic activity. The mean phasic activity induced by 3 µM $PGF_{2\alpha}$, as measured during 5 min before the first EFS at 1 Hz, was not significantly different in $sGC\alpha_1$ KO strips compared to WT strips in these series of experiments (0.42 ± 0.07 g/mg wet weight in $sGC\alpha_1$ KO strips vs. 0.37 ± 0.06 g/mg wet weight in WT strips, n = 28-30 strips from 16-20 animals). However, the total contractile activity induced by 3 µM $PGF_{2\alpha}$, measured during the same period, was clearly lower in $sGC\alpha_1$ KO strips (44.21 ± 8.25 g.s/mg wet weight in $sGC\alpha_1$ KO strips vs. 61.95 ± 6.25 g.s/mg wet weight in WT strips, n = 28-30 strips from 16-20 animals). This means that there is less $PGF_{2\alpha}$-induced motor activity to suppress in the $sGC\alpha_1$ KO mice, which might per se lead to smaller absolute responses to EFS and NO. To compare the AUC of the relaxing responses in the $sGC\alpha_1$ KO strips to that in WT strips, the AUC in $sGC\alpha_1$ KO strips was multiplied with a factor "$PGF_{2\alpha}$ response in WT / $PGF_{2\alpha}$ response in $sGC\alpha_1$ KO".

In WT strips, the 30 s trains of EFS induced a decrease in tone and suppression of phasic activity. Upon ending stimulation, a fast, high amplitude rebound contraction occurred and phasic activity returned. The inhibitory response during the 30 s stimulation train was very similar at the four frequencies (Fig. IV.3A).

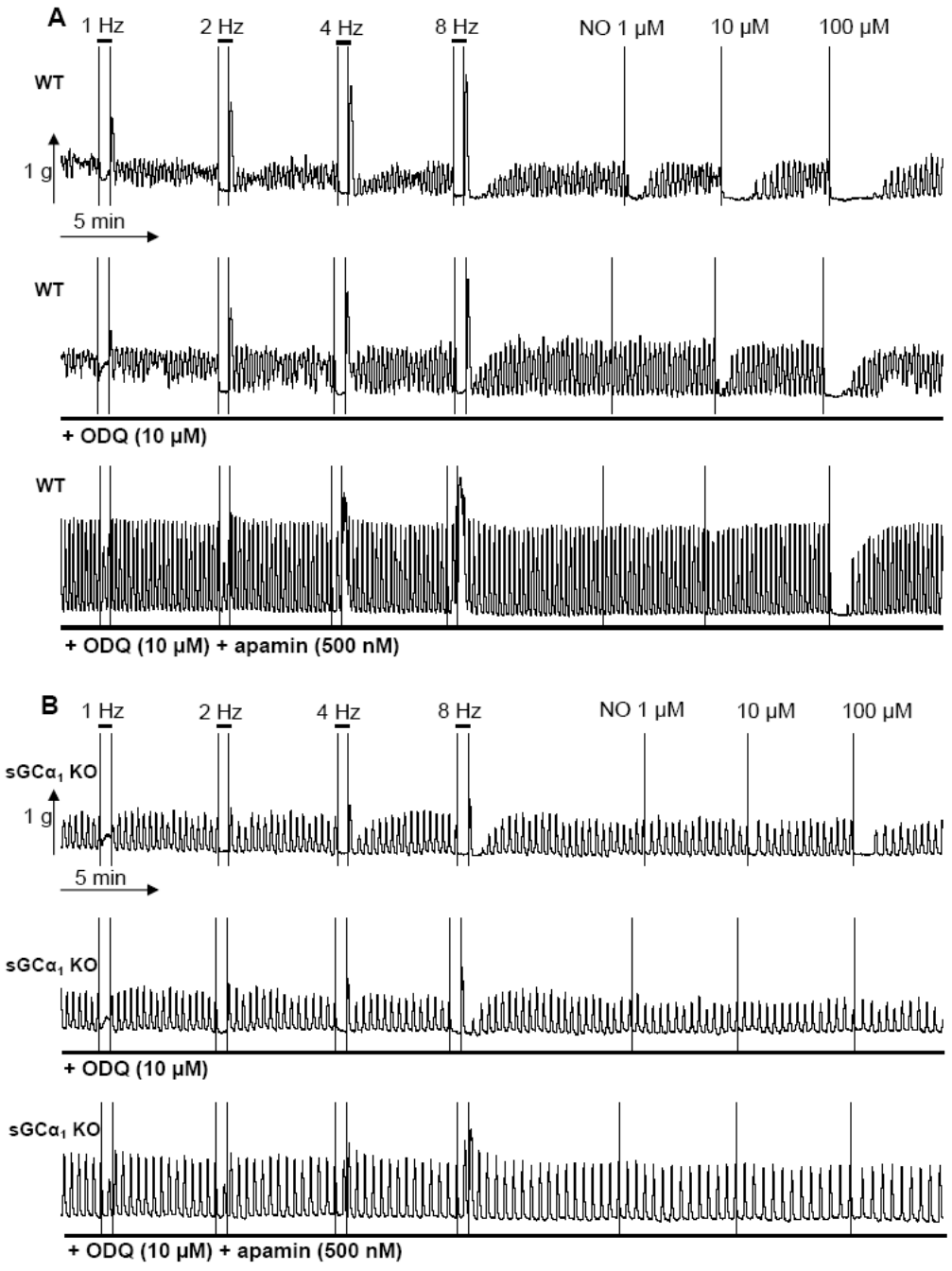


**Figure IV.3**

Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 1-8 Hz, 30 s trains) and exogenously applied NO (1-100 µM) in precontracted (3 µM $PGF_{2\alpha}$) circular muscle strips of distal colon from a WT mouse (A) and a $sGC\alpha_1$ KO mouse (B) in the absence (upper trace) or the presence of ODQ (10 µM, middle trace), or of ODQ (10 µM) plus apamin (500 nM, lower trace).

In sGC$\alpha_1$ KO strips, the relaxant response induced by EFS at 1 Hz was clearly decreased compared to WT strips: after a short initial decrease, tone already recovered during stimulation; the rebound contraction was suppressed (from 0.45 ± 0.06 g/mg wet weight in WT strips to 0.11 ± 0.07 g/mg wet weight in sGC$\alpha_1$ KO strips, n = 28 strips from 16-18 animals, P < 0.001). For EFS at 2 to 8 Hz, the relaxant response was not changed (Figs. IV.3B and IV.4A), nor were the rebound contractions (0.61 ± 0.09, 0.80 ± 0.14 and 0.98 ± 0.21 g/mg wet weight in WT strips; 0.42 ± 0.08, 0.74 ± 0.15 and 0.94 ± 0.20 g/mg wet weight in sGC$\alpha_1$ KO strips for 2, 4 and 8 Hz respectively, n = 28 strips from 16-18 animals).

In WT strips, application of exogenous NO (1-100 µM) decreased tone and suppressed pre-imposed phasic activity with a concentration-dependent duration. In sGC$\alpha_1$ KO strips, the NO-induced responses were significantly reduced compared to WT strips at all concentrations tested (Figs. IV.3B and IV.4B).

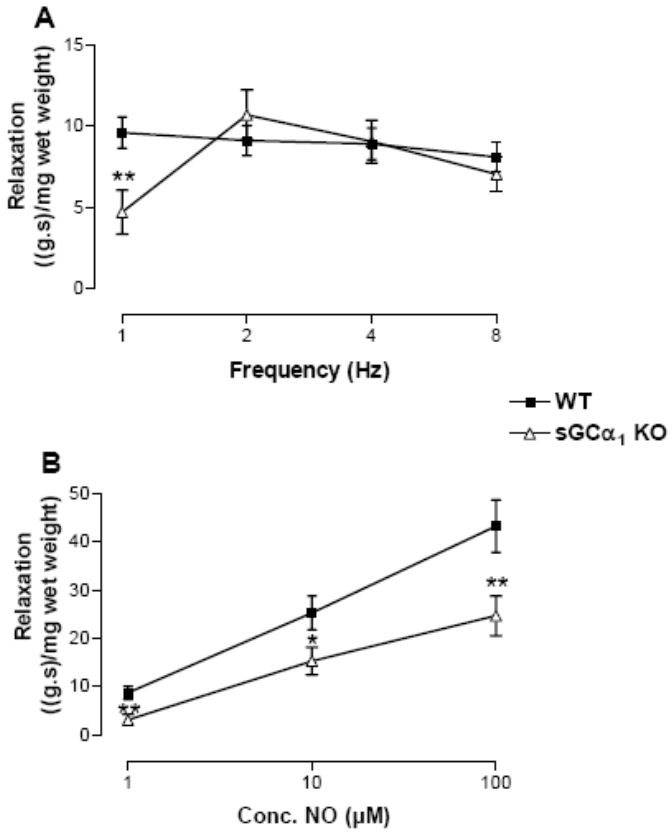


**Figure IV.4**

Frequency-response curves of EFS (40 V; 0.1 ms; 1-8 Hz, 30 s trains) (panel A) and concentration-response curves of NO (1 µM – 100 µM) (panel B) in WT (■) and sGC$\alpha_1$ KO (Δ) strips. Means ± S.E.M. of n = 28-30 strips from 14-15 animals are shown. * P < 0.05, ** P < 0.01: sGC$\alpha_1$ KO versus WT (unpaired Student's *t*-test).

### IV.4.3.2 Influence of ODQ and L-NAME

The inhibitory responses to EFS and exogenous NO were examined following pre-incubation with ODQ (10 µM) and L-NAME (300 µM) (Fig. IV.5).

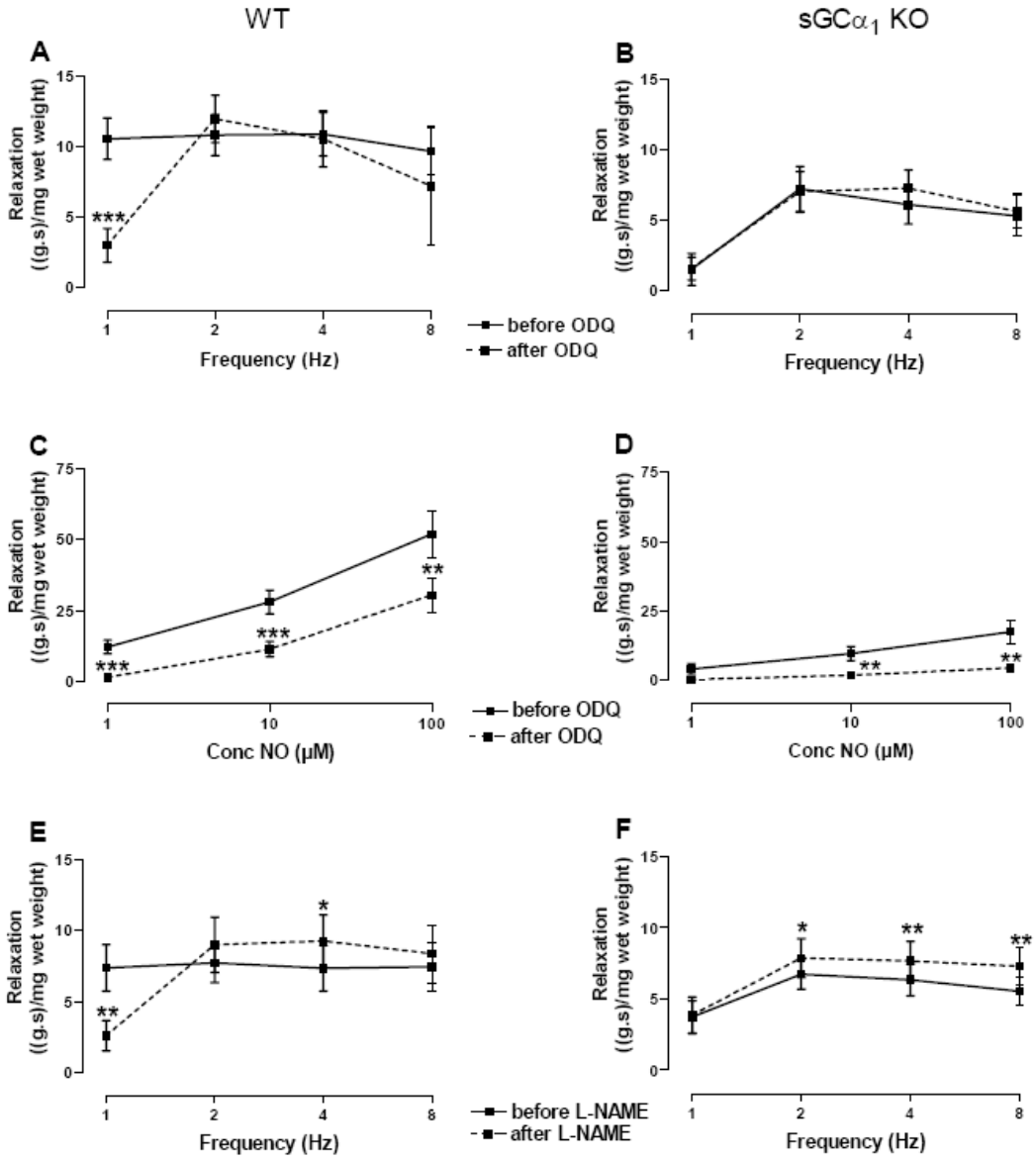


**Figure IV.5**

Panel A, B, E and F: frequency-response curves of EFS (40 V; 0.1 ms; 1-8 Hz, 30 s trains) before and after incubation with ODQ (10 µM; A and B) and before and after incubation with L-NAME (300 µM; E and F) in WT (A and E) and sGC$\alpha_1$ KO strips (B and F). Panel C and D: concentration-response curves of NO (1 µM – 100 µM) before and after incubation with ODQ (10 µM) in WT (C) and sGC$\alpha_1$ KO strips (D). Means ± S.E.M. of n = 11-15 are shown. * P < 0.05, ** P < 0.01, *** P < 0.001: after incubation versus before (paired Student's *t*-test).

ODQ and L-NAME had no systematic influence on basal contractility, nor on the $PGF_{2\alpha}$-induced response, either when total contractile activity (AUC) or mean phasic activity was assessed. The response to EFS at 1 Hz was significantly reduced by ODQ in WT strips, but

was not influenced by ODQ in sGC$\alpha_1$ KO strips (Figs. IV.3A and B and IV.5A and B). In WT strips, ODQ abolished the relaxing response to 1 µM exogenous NO and significantly reduced the relaxing responses to 10 and 100 µM exogenous NO (Figs. IV.3A and IV.5C). In sGC$\alpha_1$ KO strips, the remaining NO-induced relaxations were virtually abolished by ODQ (Figs. IV.3B and IV.5D).

L-NAME only reduced the response to EFS at 1 Hz in WT strips but not in sGC$\alpha_1$ KO strips (Fig. IV.5E and IV.F). In both WT and sGC$\alpha_1$ KO strips, relaxation induced by EFS at 2-8 Hz was slightly increased in the presence of L-NAME. L-NAME did not influence the relaxations to exogenous NO in WT nor in sGC$\alpha_1$ KO strips (results not shown). Adding ODQ to L-NAME had no additional inhibitory effect on the EFS-induced responses than had L-NAME per se (results not shown).

#### IV.4.3.3 Influence of apamin and apamin plus ODQ or L-NAME

In 8 out of 12 WT strips (Fig. IV.6A), but not in sGC$\alpha_1$ KO strips (Fig. IV.6B), administration of apamin (500 nM) induced phasic activity. However, the $PGF_{2\alpha}$-induced response was clearly influenced by apamin in WT as well as in sGC$\alpha_1$ KO strips (Fig. IV.6). When comparing the total contractile activity in response to $PGF_{2\alpha}$ in the presence versus in the absence of apamin, no significant differences were detected. However, the mean phasic activity in response to $PGF_{2\alpha}$, as measured during 5 min before the first EFS at 1 Hz, was significantly increased in the presence of apamin in both WT and sGC$\alpha_1$ KO strips (WT strips: 0.76 ± 0.16 g/mg wet weight vs. 0.28 ± 0.06 g/mg wet weight before apamin, n = 12, $P < 0.01$; sGC$\alpha_1$ KO strips: 0.80 ± 0.23 g/mg wet weight vs. 0.24 ± 0.07 g/mg wet weight before apamin, n = 11, $P < 0.01$). The effect of apamin on basal and $PGF_{2\alpha}$-induced contractile activity was not altered when adding it concomitantly with ODQ or L-NAME.

In a first series, the influence of ODQ (10 µM) on the EFS- and NO-induced responses was studied again, first alone, and then when apamin (500 nM) was added. ODQ alone, again only influenced relaxation by EFS at 1 Hz in WT strips; when adding apamin, the responses to EFS at 2, 4 and 8 Hz were also depressed (Fig. IV.7A). At 8 Hz, EFS induced, after an initial suppression of phasic activity, a clear-cut contraction above $PGF_{2\alpha}$-induced motor activity during the stimulation period (Fig. IV.3A). In sGC$\alpha_1$ KO strips, ODQ alone had no influence but ODQ plus apamin influenced the EFS-induced responses in that EFS no longer fully suppressed phasic activity and at 8 Hz, EFS even induced more pronounced phasic contraction (Fig. IV.3B). Only the reduction by ODQ plus apamin of the relaxation by EFS at 8 Hz reached significance (Fig. IV.7B). The combination of ODQ and apamin had no systematic additional inhibitory effect compared to ODQ alone on the responses to exogenous NO in both WT and sGC$\alpha_1$ KO strips (Fig. IV.3).

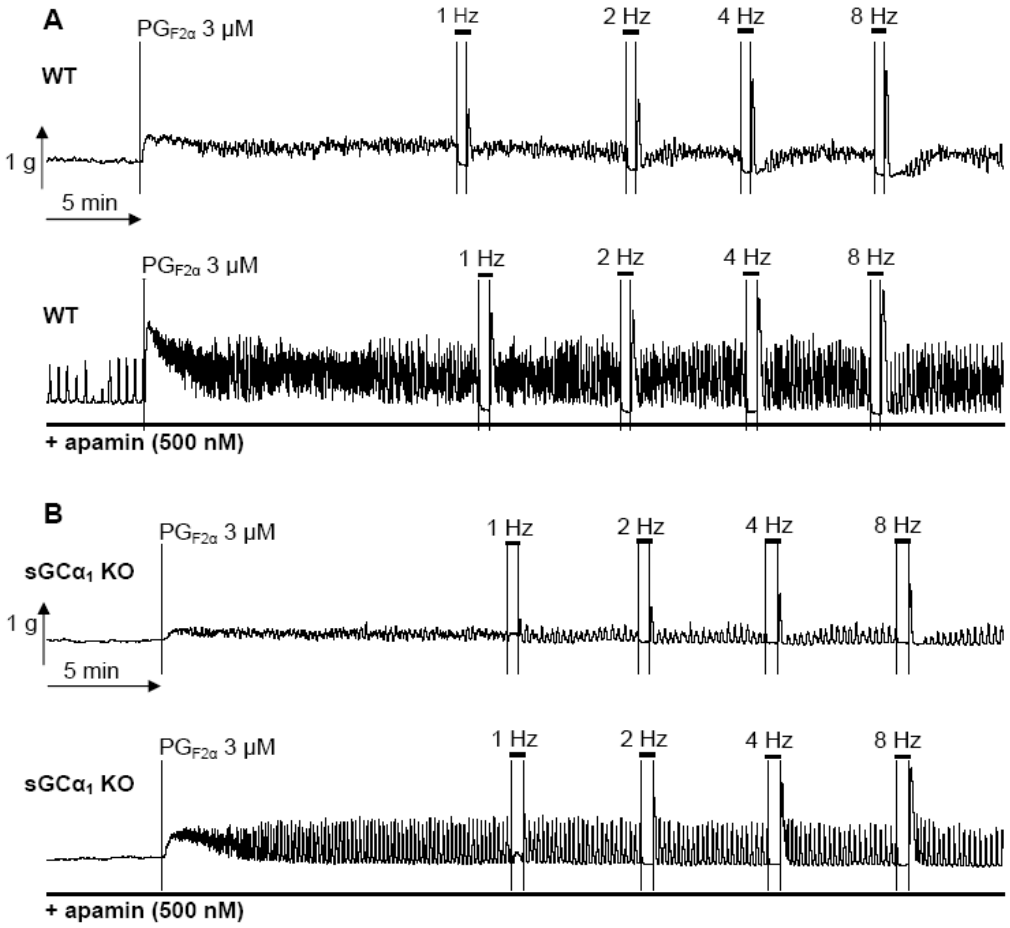


**Figure IV.6**

Representative traces showing basal contractile activity before adding $PGF_{2\alpha}$ and the inhibitory responses to EFS (40 V; 0.1 ms; 1-8 Hz, 30 s trains) after pre-contraction with $PGF_{2\alpha}$ (3 µM) in the absence (upper trace) and the presence of apamin (500 nM; bottom trace) in a circular muscle strip of distal colon from a WT mouse (A) and a $sGC\alpha_1$ KO mouse (B).

Apamin alone had no significant inhibitory influence on EFS-induced responses, neither in WT strips (Figs. IV.6A and IV.7C and E) nor in $sGC\alpha_1$ KO strips (Figs. IV.6B and IV.7D and F). However, when strips were treated concomitantly with apamin plus ODQ or plus L-NAME (300 µM), the EFS-induced responses were influenced in a similar way as described above in the presence of ODQ plus apamin: in WT strips, the relaxing response by EFS at 1, 2, 4 and 8 Hz was decreased (Fig. IV.7C and E), while in $sGC\alpha_1$ KO strips only the response at 8 Hz was clearly decreased although this did not reach significance (Fig. IV.7D and F). The responses to exogenous NO were not influenced by apamin alone nor apamin plus L-NAME in WT and $sGC\alpha_1$ KO strips; adding ODQ to apamin had the same inhibitory effect on the NO-induced responses as ODQ alone (results not shown).

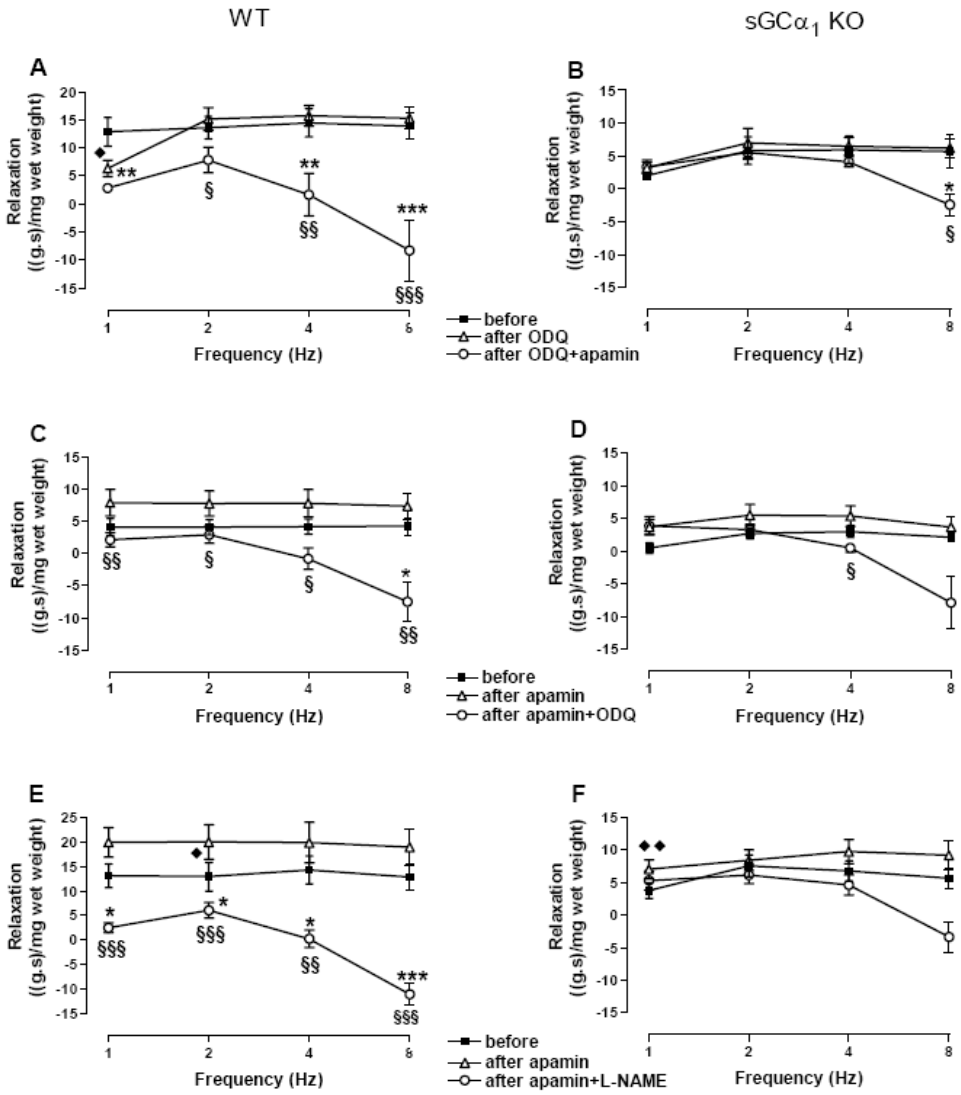


**Figure IV.7**

Frequency-response curves of EFS (40 V; 0.1 ms; 1-8 Hz, 30 s trains) in WT (A, C and E) and sGCα$_1$ KO strips (B, D and F) before and after incubation of ODQ and ODQ plus apamin (A and B), before and after incubation of apamin and apamin plus ODQ (C and D) and before and after incubation of apamin and apamin plus L-NAME (E and F). Negative values in the y-axis indicate that a contractile response instead of a relaxation was obtained. Means ± S.E.M. of n = 5-6 are shown. ♦ P < 0.05, ♦♦ P < 0.01: after incubation of ODQ or apamin versus before; * P < 0.05, ** P < 0.01, *** P < 0.001: after incubation of ODQ plus apamin, apamin plus ODQ or apamin plus L-NAME versus before; § P < 0.05, §§ P < 0.01, §§§ P < 0.001: after incubation of ODQ plus apamin, apamin plus ODQ or apamin plus L-NAME versus after incubation of ODQ or apamin (repeated measures ANOVA followed by Bonferroni multiple comparison *t*-test).

## IV.4.4 Inhibitory responses to VIP and 8-Br-cGMP

Complementary to the EFS and NO-induced relaxations (see IV.4.3.1), the relaxations to VIP and 8-Br-cGMP in the sGCα$_1$ KO strips were also multiplied with a factor "PGF$_{2\alpha}$ response in WT / PGF$_{2\alpha}$ response in sGCα$_1$ KO" in order to avoid an influence of the PGF$_{2\alpha}$-induced

motor activity per se in the interpretation of these inhibitory responses when comparing WT and sGCα$_1$ KO strips.

In both WT and sGCα$_1$ KO strips, exogenously applied VIP (100 nM) resulted in a persistent suppression of phasic activity. The response to VIP (100 nM), measured for 5 min, was not significantly different in sGCα$_1$ KO strips when compared to WT strips (120.00 ± 16.77 g.s/mg wet weight in sGCα$_1$ KO strips vs. 95.97 ± 10.13 g.s/mg wet weight in WT strips, n = 28-30 strips from 16-19 animals). The response to VIP was not influenced by ODQ nor by L-NAME in either WT strips or sGCα$_1$ KO strips (results not shown).

Also the sustained relaxing response to 8-Br-cGMP, measured for 10 min, was similar in WT strips (142.04 ± 33.93 g.s/mg wet weight, n = 13 strips from 7 animals) and in sGCα$_1$ KO strips (160.29 ± 29.90 g.s/mg wet weight, n = 12 strips from 7 animals). The response to 8-Br-cGMP was not reduced by ODQ in either WT or sGCα$_1$ KO strips (results not shown).

## IV.4.5 cGMP measurements

In sGCα$_1$ KO strips, contracted with $PGF_{2\alpha}$, basal cGMP levels (2.14 ± 0.40 pmol/g tissue, n = 6) were much lower as compared to WT strips (8.61 ± 0.88 pmol/g tissue, n = 6). In both WT and sGCα$_1$ KO strips, EFS at 1 Hz did not significantly change cGMP levels compared to basal levels. On the other hand, 10 µM NO caused a significant 4 to 5-fold increase in cGMP levels versus basal in WT as well as sGCα$_1$ KO strips (Fig. IV.8).

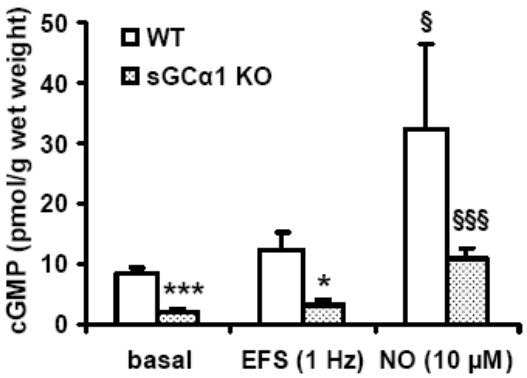


**Figure IV.8**

cGMP levels measured in basal conditions and at maximal relaxation by EFS (40 V, 0.1 ms, 1 Hz, 30 s) or NO (10 µM) in WT (□) and sGCα$_1$ KO (⊡) strips. Means ± S.E.M. of n = 6 are shown. * P < 0.05, *** P < 0.001: sGCα$_1$ KO versus WT (unpaired Student's *t*-test). §: P<0.05; §§§: P<0.001: NO versus basal (unpaired Student's *t*-test).

## *IV.5 Discussion*

In the present study, we investigated the impact of $sGC\alpha_1$-deficiency on distal colon motility. We used recently developed $sGC\alpha_1$ KO mice lacking exon 6 of the $sGC\alpha_1$ gene, coding for an essential part in the catalytical domain (Buys et al., 2008). This $sGC\alpha_1$ knockout model provides a valuable tool to get a better insight in the relative importance of the two sGC isoforms in inhibitory NANC neurotransmission and in the mechanism of action of the neurotransmitters involved. There are indeed many reports that NO is not the sole neurotransmitter responsible for NANC relaxation in colonic tissue, but that also ATP is involved (Benko et al., 2007; Boeckxstaens et al., 1993; El Mahmoudy et al., 2006; Keef et al., 1993; Pluja et al., 1999; Serio et al., 2003a; Van Crombruggen and Lefebvre, 2004).

We started the experiments in both male and female mice, as it was found that male but not female $sGC\alpha_1$ KO mice develop hypertension (Buys et al., 2008). However, no systematic differences between sexes were found in distal colon strips. The experiments were therefore continued using male mice only.

### IV.5.1 Inhibitory responses to 8-Br-cGMP and VIP

The finding that both WT and $sGC\alpha_1$ KO distal colon strips responded in a similar way to the cell-permeable cGMP analog 8-Br-cGMP excludes that relaxant mechanisms downstream of sGC are affected by knocking out $sGC\alpha_1$. This corresponds to observations in the gastric fundus (Vanneste et al., 2007) and the aorta and femoral artery (Nimmegeers et al., 2007) of the $sGC\alpha_1$ KO mice. In contrast, in the $sGC\alpha_1$ KO mice developed by Mergia et al. (2006), increased sensitivity to a cGMP analog in vascular tissue was reported; in these mice exon 5 of the $sGC\alpha_1$ gene is targeted. Because Mergia et al. started their numbering from the first coding exon while Buys et al. (2008) included the first 5' non-coding exon in their numbering, exon 4 of Mergia et al. indeed corresponds to exon 5 in the exon count of Buys et al.. The difference in response to 8-Br-cGMP suggests that different compensatory regulations might exist between the 2 knockout models or between the strains of mice used, although other results were similar (see below).

Knocking out $sGC\alpha_1$ did not induce changes in cAMP-mediated relaxation. This was demonstrated by the fact that relaxation by the vasoactive intestinal polypeptide (VIP), acting via activation of adenylate cyclase (Simon and Kather, 1978; Laburthe et al., 2002), was well maintained in the $sGC\alpha_1$ KO mice. A contribution of muscular NO to the relaxing effect of VIP as proposed by Grider (1993) in circular muscle strips of rat colon, was not found as L-NAME did not influence the response to VIP.

### IV.5.2 Inhibitory responses to exogenous NO

To assess the role of $sGC\alpha_2\beta_1$ in nitrergic relaxation, we studied the influence of ODQ on the relaxation by exogenous NO. In $sGC\alpha_1$ KO strips, relaxations induced by exogenous NO were significantly decreased, but not abolished, when compared to WT strips, and were still sensitive to ODQ. This suggests that the remaining relaxation by exogenous NO in the $sGC\alpha_1$ KO strips is due to the activation of sGC, which can only be $sGC\alpha_2\beta_1$. This is corroborated by the fact that exogenous NO still induced an increase in cGMP levels in $sGC\alpha_1$ KO strips. Similarly, a contribution of $sGC\alpha_2\beta_1$ in NO-induced relaxation was previously observed in the aorta, the femoral artery (Nimmegeers et al., 2007) and the gastric fundus (Vanneste et al., 2007) of the $sGC\alpha_1$ KO mice, and in the aorta of the $sGC\alpha_1$ KO mice developed by Mergia et al. (2006). Similar to what was reported in gastric fundus (Vanneste et al., 2007) and aorta (Mergia et al., 2006) at mRNA level, no increase in the $sGC\alpha_2$ gene function was detected in distal colon as the $sGC\alpha_2$ protein level was not changed. Similar to what has been described in lung tissue of our $sGC\alpha_1$ KO mice (Vermeersch et al., 2007), we found decreased $sGC\beta_1$ protein levels, which is probably due to the absence of the $sGC\alpha_1$ subunit. The contribution of $sGC\alpha_2$ to GI nitrergic relaxation is furthermore demonstrated by the fact that no gross GI abnormalities in the $sGC\alpha_1$ KO mice were observed, whereas recently developed mice deficient for the $\beta_1$ subunit of sGC, resulting in a complete loss of enzyme activity, suffered from fatal GI obstruction (Friebe et al., 2007).

Interestingly, in WT strips, the relaxation to 10 µM and 100 µM NO is not abolished by ODQ. First, this finding could point in the direction of a sGC-independent mechanism of NO, responsible for the remaining, ODQ-insensitive, relaxation. NO has for example been reported to induce relaxation via cGMP-independent $SK_{Ca}$ channel activation in GI smooth muscle (Martins and al., 1995; Serio and al., 2003b). However, the $SK_{Ca}$ channel blocker apamin did not influence the responses to NO, excluding the possibility that NO-induced relaxation in the murine distal colon is mediated via a direct –cGMP-independent- activation of $SK_{Ca}$ channels. The addition of apamin to ODQ did not add to the inhibitory effect of ODQ versus the relaxation induced by exogenous NO, which is in contrast to results obtained in rat distal colon circular muscle (Van Crombruggen and Lefebvre, 2004); in this species, the responses to higher concentrations of exogenous NO were only blocked by a combination of apamin and ODQ, indicating that the response to exogenous NO is mediated by both sGC and $SK_{Ca}$ channels. Alternatively, the most plausible explanation for the remaining NO-induced relaxation in the presence of ODQ in WT strips in our study is the assumption that, due to the competitive nature of the sGC inhibitor ODQ (Fedele et al., 1996; Schrammel et al., 1996), 10 µM ODQ might not be sufficient to fully antagonize the sGC activation by

higher concentrations of NO. We indeed reported before in rat gastric fundus that 10 μM ODQ inhibited the relaxation by NO more extensively than 1 μM ODQ did (Lefebvre,1998). In $sGC\alpha_1$ KO strips, where the degree of sGC activation to overcome by ODQ might be smaller in view of the inactivity of $sGC\alpha_1\beta_1$ and the lower cGMP levels when exposed to NO, 10 μM ODQ is sufficient to abolish the responses to exogenous NO.

### IV.5.3 Inhibitory responses to EFS

To study the role of sGC as a messenger and of NO as a neurotransmitter in the NANC relaxation in mouse distal colon, we investigated the EFS-evoked responses in WT and $sGC\alpha_1$ KO mice, in the absence and presence of L-NAME and ODQ. In WT strips, the response to EFS at 1 Hz, but not at 2, 4 and 8 Hz, was significantly reduced by L-NAME and ODQ, indicating that only at this frequency NO is the main neurotransmitter liberated in the distal colon, and that the relaxing effect of endogenous NO is mediated via sGC. Accordingly, only the response to EFS at 1 Hz was reduced in $sGC\alpha_1$ KO strips when compared to WT strips. The small remaining response to 1 Hz in $sGC\alpha_1$ KO strips was no longer influenced by L-NAME or ODQ, indicating that the nitrergic response to EFS at 1 Hz in WT strips must be mediated via $sGC\alpha_1\beta_1$. The cGMP measurements of WT strips snap frozen during the 30 s EFS-train at 1 Hz did not show a significant increase in cGMP levels versus basal levels. This finding might be related to very localised cGMP rises induced by the release of endogenous NO along the varicosities during EFS, which could not be detected by the cGMP analysis. It should be noted that the cGMP measurements were performed in the absence of the phosphodiesterase-5 (PDE-5) inhibitor zaprinast, which was required to measure a cGMP increase for the EFS-evoked response in gastric fundus strips of WT mice (Vanneste et al., 2007).

The EFS-evoked responses at 2 to 8 Hz were not reduced in $sGC\alpha_1$ KO strips compared to WT strips, nor were they sensitive to inhibition by L-NAME or ODQ. They were also not influenced by apamin, excluding a dominant role of an apamin-sensitive factor, such as ATP, as single neurotransmitter in the EFS-evoked responses in mouse distal colon. However, the addition of ODQ to apamin, or apamin to ODQ clearly reduced the EFS-induced responses at 2, 4 and 8 Hz in WT strips, and at 8 Hz in $sGC\alpha_1$ KO strips; similar results were obtained with the combination of apamin plus L-NAME. These results are indicative of a redundant action of NO, acting at sGC, and another NANC neurotransmitter, acting at $SK_{Ca}$ channels when NANC neurons in mouse distal colon are stimulated by EFS at 2 to 8 Hz. This implies that both neurotransmitters can act together such that the loss of any one is compensated by the other. The fact that this interactive system seems to be maintained in $sGC\alpha_1$ KO strips points to the ability of endogenous NO, released by EFS at 2 to 8 Hz, to act via $sGC\alpha_2$ in the absence of $sGC\alpha_1$.

A similar redundant inhibitory mechanism was suggested for the guinea-pig isolated taenia coli by Selemidis et al. (1997). They proposed that NO acts as a “backup” or redundant NANC inhibitory neurotransmitter to the dominant apamin-sensitive, nerve-derived hyperpolarizing factor (NDHF). This hypothesis was based on the observation that L-NOARG and ODQ had no effect on the EFS-evoked relaxation, but that the apamin-induced reduction in the EFS-evoked relaxations was increased when L-NOARG or ODQ was added to apamin. In our experiments only the combinations of apamin with ODQ or L-NAME – and not apamin alone- inhibited the EFS-evoked responses at 2 to 8 Hz. These results suggest that, in mouse distal colon, both NO as well as the apamin-sensitive neurotransmitter are redundant to each other in the EFS-evoked responses at higher frequencies. Also for the circular muscle of human colon (Boeckxstaens et al., 1993) it was suggested that NO and another apamin-sensitive neurotransmitter, probably ATP, are involved in the inhibitory neurotransmission, albeit not in a redundant manner. In rat distal colon (Van Crombruggen and Lefebvre, 2004), both the nitrergic and the purinergic pathway needed to be blocked to inhibit maintained EFS-induced relaxations. Indeed, only the combination of L-NAME plus Reactive Blue 2 (RB2) or L-NAME plus apamin was able to almost abolish the EFS-induced responses, whereas RB2 or L-NAME alone did not show any effect.

### IV.5.4 Basal and $PGF_{2\alpha}$-induced contractile activity

In colonic tissue of other species such as rat, rabbit and human, NOS inhibition increased the amplitude or frequency of the spontaneous contractions (Ciccocioppo et al., 1994; Keef et al., 1993; Mulè et al., 1999). Spontaneous contractions in GI smooth muscle preparations are related to spontaneous depolarizations, produced by interstitial cells of Cajal (ICC). In cultured ICCs, NO donors inhibited the frequency of pacemaker currents via cGMP generation (Koh et al., 2000). However, neither L-NAME nor ODQ had a systematic effect on basal contractile activity in distal colon strips of WT and $sGC\alpha_1$ KO mice in this study, arguing against a tonic inhibitory effect of NO via sGC activation. Administration of apamin induced phasic activity in 67 % of the WT strips, suggesting that $SK_{Ca}$ channels might be involved in a tonic suppression of contractile activity in mouse colon. $SK_{Ca}$ channels are expressed in ICCs (Fujita et al., 2001). The effect of apamin on basal contractile activity corresponds to observations by Powel and Bywater (2001) in an intact mouse colon preparation, where apamin induced non-migrating contractions in distal colon. The tonic suppression of contractile activity via $SK_{Ca}$ channels might also counteract $PGF_{2\alpha}$-induced phasic activity, as apamin also increased $PGF_{2\alpha}$-induced mean phasic activity in WT strips. It is not clear why the effect of apamin on basal contractile activity was not present in $sGC\alpha_1$ KO strips, while the effect on $PGF_{2\alpha}$-induced phasic activity was maintained.

### IV.5.5 Conclusion

In conclusion, our data indicate that in mouse distal colon exogenous NO is able to act at least partially through $sGC\alpha_2\beta_1$ in the absence of $sGC\alpha_1\beta_1$ activity. Endogenous NO, acting through $sGC\alpha_1\beta_1$, is the principal neurotransmitter released by EFS at 1 Hz. At higher stimulation frequencies, a redundant action of NO, acting at sGC, and another NANC neurotransmitter, acting at the $SK_{Ca}$ channels, is proposed. This interactive system is maintained in $sGC\alpha_1$ KO strips highlighting the ability of endogenous NO, released in response to EFS at 2 to 8 Hz, to act via $sGC\alpha_2\beta_1$ in the absence of $sGC\alpha_1\beta_1$ activity.

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# Chapter V

# ROLE OF SOLUBLE GUANYLATE CYCLASE IN GASTROINTESTINAL MOTILITY

## Gastrointestinal phenotyping of NO resistant sGCbeta1his105phe knock in mice

# Chapter V Role of soluble guanylate cyclase in gastrointestinal motility: Gastrointestinal phenotyping of NO resistant sGCbeta1his105phe knock in mice

## *V.1 Abstract*

In the gastrointestinal (GI) tract, the importance of nitric oxide (NO) in non-adrenergic non-cholinergic (NANC) relaxation is well established. The aim of this study was to investigate the role of nitrergic activation of sGC in regulation of gastrointestinal motility, comparing wild-type (WT) and sGC$\beta_1$his105phe knock in (KI) mice, which lack heme-bound, NO-sensitive sGC. As a consequence, diethylenetriamine NONOate (DETA-NO) failed to increase sGC activity in GI tissue of KI mice and exogenous NO did not induce relaxation in KI fundic, jejunal and colonic strips. The KI mice showed an enlarged stomach with hypertrophy of the circular muscle of the fundus and delayed gastric emptying. In addition, delayed intestinal transit and increased whole gut transit time were observed in the KI mice. The nitrergic relaxant responses to electrical field stimulation (EFS) at 1-8 Hz were abolished in the KI fundic and jejunal strips; in the KI colonic strips, only the response at 1 Hz was abolished.

In conclusion, the GI consequences of lacking NO-sensitive sGC were most pronounced at the level of the stomach establishing the pivotal role of the activation of sGC by NO in normal gastric functioning. In addition, delayed transit was observed in the absence of NO-sensitive sGC, indicating that nitrergic activation of sGC also plays a role in the lower GI tract. In the distal colon, other neurotransmitters besides NO take part in the relaxations to higher stimulation frequencies.

## V.2 Introduction

In the GI tract, nitric oxide (NO) is synthesized from L-arginine by neuronal NO synthase (nNOS, NOS-1) in non-adrenergic non-cholinergic (NANC) neurons, from where it diffuses to the smooth muscle cells. The importance of NO in NANC relaxation and hence GI motility is well established. Experiments with NOS inhibitors resulted in delayed small intestinal transit in rats (Karmeli et al., 1997), dogs (Chiba et al., 2002) and humans (Fraser et al., 2005) and delayed colonic transit in rats (Mizuta et al., 1999) and dogs (Chiba et al., 2002). nNOS knockout (KO) mice, which are chronically deprived of nNOS generated NO, showed an enlarged stomach and delayed gastric emptying of solids and liquids (Huang et al., 1993; Mashimo et al., 2000). The principal intracellular target of NO is the heme-protein soluble guanylate cyclase (sGC). Once activated by NO, sGC leads to the production of the second messenger cyclic guanosine 3'-5'-monophosphate (cGMP) and subsequently relaxation (Toda and Herman, 2005). However, relaxant effects of NO not involving the sGC-cGMP pathway but related to activation of small conductance $Ca^{2+}$-dependent $K^+$ channels have been described in mouse (Serio et al., 2003) and rat duodenum (Martins et al., 1995) and in rat colon (Van Crombruggen and Lefebvre, 2004), corresponding with direct activation of $K^+$ channels by NO observed in colonic smooth muscle cells by Koh et al. (1995) and Lang and Watson (1998). Additionally, sGC can be activated by other stimuli than NO such as carbon monoxide (CO; Schmidt et al., 1992). CO represents another putative NANC neurotransmitter, albeit the evidence for this is much weaker compared to NO.

sGC is composed of an α subunit and a β subunit (Harteneck et al., 1991). The physiologically active isoforms are $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ with $sGC\alpha_1\beta_1$ being the predominant isoform in the GI tract (Mergia et al., 2003). However, we showed before that $sGC\alpha_2\beta_1$ can compensate at least partially for the absence of $sGC\alpha_1\beta_1$ such that knocking out $sGC\alpha_1$ does not induce important implications on in vivo GI motility (Vanneste et al., 2007; Dhaese et al., 2009). Knocking out exon 10 of the $\beta_1$ subunit, eliminating activation of both sGC isoforms by NO but also basal activity, led to a phenotype with severely delayed gut transit, grossly enlarged and dilated caecum, growth retardation and premature death (Friebe et al., 2007). Essential for the activation of both sGC isoforms by NO is the prosthetic heme group. The histidine 105 residu of the $\beta_1$ subunit is a crucial amino acid for the binding of the heme group to the sGC protein (Schmidt et al., 2004). Recently, $sGC\beta_1$his105phe knock in (KI) mice in which the histidine 105 amino acid was replaced by phenylalanine were developed by Thoonen et al. (2009); the resulting heme-deficient sGC isoforms retain their basal activity but can no longer be activated by NO (Wedel et al., 1994). The $sGC\beta_1$his105phe KI mice show a reduced live span, growth retardation and cardiovascular implications such as elevated blood pressure and reduced heart rate (Thoonen et al., 2009).

In the present study, the importance of nitrergic activation of sGC in regulation of GI motility was investigated using sGC$\beta_1$his105phe KI mice.

## *V.3 Methods*

### V.3.1 Ethical approval

All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

### V.3.2 Animals

Homozygous sGC$\beta_1$his105phe knock in (KI) mice and wild type (WT) controls were derived from a heterozygous breeding (genetic background: mixed 129/SvJ-C57BL/6J; Thoonen et al., 2009). WT and KI mice of both sexes (male: n = 47 (WT) and 48 (KI), 7-13 weeks; female: n = 36 (WT) and 36 (KI), 7-16 weeks) had free access to water and commercially available chow. However, when investigating transit using the phenol red or fluorescein-labelled dextran method (see below), food was withheld for 16 hours overnight with free access to water.

### V.3.3 Muscle tension experiments

#### V.3.3.1 Tissue preparation

Animals were killed by cervical dislocation and the GI tract was removed and put in aerated Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The stomach was emptied from its contents and weighed. Then the gastric fundus was separated from the rest of the stomach and two full wall thickness fundus strips (2 x 11 mm) were prepared by cutting in the direction of the circular muscle layer. A ± 5 cm long fragment of small bowel, starting approximately 10 cm distal to the pylorus, and a ± 4 cm long segment of distal colon, taken above the pelvic brim, were isolated and opened along the mesenteric border. The fragments were pinned mucosa side up in Krebs solution. The mucosa was removed by sharp dissection under a microscope and two full-thickness muscle strips (4 × 5 mm) were cut along the circular axis.

#### V.3.3.2 Isometric tension recording

After a cotton thread (fundus) or a silk thread (USP 4/0; jejunum and colon) was attached to both ends of the strips, strips were mounted in 5 (jejunum and colon) or 15 ml (fundus) organ baths between 2 platinum plate electrodes (6 or 7 mm apart). The organ baths contained aerated (5% $CO_2$ in $O_2$) Krebs solution, maintained at 37°C. Changes in isometric tension

were measured using MLT 050/D force transducers (ADInstruments) and recorded on a Graphtec linearcorder F WR3701 (Graphtec, Yokohama, Japan; fundus)) or on a PowerLab/8sp data recording system (ADInstruments) with Chart software (jejunum and colon).

After an equilibration period of 30 min with flushing every 10 min at a load of 0.75 g (fundus), 0.25 g (colon) or 0.125 g (jejunum), the length-tension relationship was determined. Muscle tissues were stretched by load increments of 0.25 g (fundus and colon) or 0.125 g (jejunum) and at each load level exposed to 0.1 (fundus and jejunum) or 1 (colon) μM carbachol to determine the optimal load ($L_o$; the load at which maximal response to the contractile agent occurred). Tissues were allowed to equilibrate for 60 min at $L_o$ with flushing every 15 min in Krebs solution.

#### V.3.3.3 Protocols

All experiments were performed after switching to Krebs solution containing 1 μM atropine and 4 μM guanethidine to block cholinergic and noradrenergic responses respectively (NANC conditions), except for the cumulative contractile responses to carbachol and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which were obtained in Krebs solution without atropine and guanethidine. All relaxant stimuli were examined after pre-contraction of the strips with 300 nM (fundus and jejunum) or 3 μM (colon) $PGF_{2\alpha}$. In a first series, 10 to 15 min after adding $PGF_{2\alpha}$ and when the contractile response was surely stabilized, relaxations were induced by application of EFS (40V, 0.1 ms, 1-2-4-8 Hz for 10 s [jejunum], 30 s [colon] or 60 s [fundus] at 5 min interval) via the platinum plate electrodes by means of a Grass S88 Stimulator (Grass, W. Warwick, RI, USA; fundus) or a Hugo Sachs Stimulator I type 215/I (jejunum and colon). This was followed by the application of exogenous NO (1-10-100 μM with an interval of at least 5 min during which the effect of a given concentration of NO had disappeared) and finally vasoactive intestinal polypeptide (VIP, 100 nM), which was left in contact with the tissue for 5 min. Strips were washed for 30 min, and were subsequently incubated with the sGC inhibitor 1H[1,2,4,]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ; 10 μM) for 30 min. $PGF_{2\alpha}$ was then applied again and the responses to EFS, NO and VIP were studied again in the presence of ODQ. In a second series, cumulative contractile responses to carbachol (1 nM – 30 μM) or $PGF_{2\alpha}$ (1 nM – 30 μM) were obtained. In jejunal and colonic strips, the contact time for each concentration of carbachol and $PGF_{2\alpha}$ was fixed at 2 min; in fundic strips, a higher concentration of carbachol and $PGF_{2\alpha}$ was given when the former concentration reached its maximal contractile effect. Then, the influence of ODQ (10 μM) versus 10 μM (fundus) or 100 μM (jejunum and colon) 8-bromoguanosine 3',5' cyclic monophosphate (8-Br-cGMP) was studied. 8-Br-cGMP was left in contact with the tissue for

10 min. In a third series, the influence of the NOS inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 300 µM) was tested against the relaxation evoked by EFS. In colonic tissues, the $PGF_{2\alpha}$-EFS cycle was repeated a third time in order to test subsequently the combination of L-NAME (300 µM) plus the small conductance $Ca^{2+}$-dependent $K^+$ channel blocker apamin (500 nM). In all series, the reproducibility of the relaxant responses was evaluated by running time-control vehicle treated strips in parallel. At the end of each experiment, the tissue wet weight was determined (mg wet weight, see data analysis).

#### V.3.3.4 Data analysis

As jejunal and colonic strips showed phasic activity, the area under the curve (AUC) above baseline was determined to measure the contractile responses to carbachol and $PGF_{2\alpha}$. $EC_{50}$ values of the concentration-response curves were calculated by linear interpolation. To measure relaxant responses in jejunal and colonic strips, the AUC for a given response was determined and subtracted from the AUC of a corresponding period just before applying the relaxing drug or stimulus, yielding the area above the curve for the relaxant response. The duration of the relaxant responses was determined as 10 s (jejunum) or 30 s (colon) for EFS (i.e. the length of the stimulus train applied). VIP and 8-Br-cGMP induced a sustained response and the duration was fixed at 5 min for VIP and 10 min for 8-Br-cGMP. NO abolished phasic activity for a concentration-dependent period, after which phasic activity progressively reoccurred. The duration of the relaxant responses to NO was therefore determined as the time necessary for phasic activity to regain 50 % of the interval between the mean peak level of phasic activity during the 2 min before administration of NO and the minimum tone level during the NO response. This calculation was performed during the $1^{st}$ cycle of $PGF_{2\alpha}$-EFS-NO-VIP and the determined duration was further used for the NO-induced responses in the $2^{nd}$ cycle of $PGF_{2\alpha}$-EFS-NO-VIP. The responses are expressed as (g.s)/mg wet weight.

Fundic strips showed tonic responses and the amplitude of the response to EFS was measured at the end of the 60 s stimulation train; the responses to carbachol, $PGF_{2\alpha}$, NO and 8-br-cGMP were measured at their maximal effect. The amplitude of contractile and relaxant responses was expressed in g/g wet weight. The relaxant responses were then expressed as % of the contraction evoked by $PGF_{2\alpha}$. $EC_{50}$ values of the concentration-response curves were calculated by linear interpolation.

### V.3.4 Gastric emptying

As modified from de Rosalmeida et al. (2003), mice were, after food was withheld overnight, administered 250 µl of a phenol red meal (0.1 % w $v^{-1}$ dissolved in water) by gavage with a

feeding needle. 15 min later, mice were killed by cervical dislocation and the stomach and small bowel were clamped at both sides. Both organs were cut into small fragments and placed into 20 ml of 0.1 N NaOH in a 50 ml Falcon tube. This mixture was homogenised for approximately 30 s and allowed to stand for 20 min at room temperature. 10 ml of supernatant was placed into a 15 ml Falcon tube and centrifuged for 10 min at 2800 rpm. Proteins in 5 ml supernatant were precipitated with 0.5 ml of 20 % (w/v) trichloroacetic acid and the solution was centrifuged for 20 min at 2800 rpm. 0.5 ml of supernatant was added to 0.667 ml of 0.5 N NaOH and the absorbance of 300 µl of this mixture was spectrophotometrically determined at 540 nm in a Biotrak II plate reader (Amersham Biosciences). Gastric emptying was calculated as the amount of phenol red that left the stomach as % of the total amount of phenol red recovered and the phenol red recovery was determined as the amount of phenol red recovered, expressed as % of the amount of phenol red administered.

## V.3.5 Transit

### V.3.5.1 Intestinal transit (fluorescein-labelled dextran method)

Mice were, after food was withheld overnight, administered 200 µl of non-absorbable fluorescein-labelled dextran (FD70; 70 kDa, 2.5 % w $v^{-1}$ dissolved in water) by gavage with a feeding needle. Ninety minutes later, mice were killed by cervical dislocation. For a full description of the technical details of this method, we refer to De Backer et al. (2008). Briefly, the entire GI tract was excised and the mesenterium was removed. The GI tract was then pinned down in a custom-made Petri dish filled with Krebs solution. Immediately after, FD70 was visualized using the Syngene Geneflash system (Syngene, Cambridge, UK). Two full-field images- one in normal illumination mode and another in fluorescent mode- were taken and matched for analysis. The fluorescent intensity throughout the intestinal tract was calculated and data were expressed as the percentage of fluorescence intensity per segment (sb, small bowel segments 1-10; caecum; col, colon segments 1-2). The geometric centre was calculated as ($\Sigma$ (% FD70 per segment x segment number))/100.

### V.3.5.2 Whole gut transit time (carmine method)

As adapted from Friebe et al. (2007), mice were administered 200 µl carmine (6 % w $v^{-1}$ dissolved in 0.5 % methylcellulose) by gavage with a feeding needle. Mice were then returned to individual cages, without food deprivation. The time taken for excretion of the first red coloured faeces was determined at 30 min intervals.

### V.3.6 Small intestinal contractility

Immediately after the evaluation of intestinal transit by fluorescence imaging (see above), the spontaneous contractile activity in the jejunum was recorded. For a full description of the technical details of this method, we refer to De Backer et al. (2008). Briefly, a 6 cm long segment of jejunum was recorded for 30 s and the video files were imported in ImageJ. After the contrast threshold value was set, the images were converted to black-and-white and the mean diameter of the jejunal segment was measured in the first frame. Next, all 750 frames were sequentially analysed using Amplitude Profiler software- written as ImageJ plugin. The change in intestinal diameter within this 30 s-period for every pixel (768 pixels) along this 6 cm long jejunal segment was calculated as % contraction amplitude by the following equation: [(maximal diameter – minimal diameter)/maximal diameter] * 100. Finally, the mean value of these 768 amplitude values was calculated. The oscillatory changes were also represented in a three-dimensional (3-D) plot using Spatiotemporal Motility Mapping software - written as ImageJ plugin with a GnuPlot backend – allowing to see contractility in function of time.

### V.3.7 Histology

Specimens of fundic, jejunal and colonic tissues were taken from 1 WT and 1 KI mouse of both sexes. The tissues were fixed in 4% neutral buffered formalin (NBF), dehydrated through a graded series of ethanol and embedded in paraffin wax. Serial transverse sections of 5 µm thickness were cut at 500 µm intervals, using a rotary microtome (SLEE CUT 4060) and stained with hematoxylin and eosin for morphological observation.

### V.3.8 sGC enzyme activity

sGC enzyme activity was measured as described (Buys et al., 2008). Fundus, jejunum and distal colon were harvested. The fundus was immediately snap-frozen in liquid nitrogen; the jejunum and distal colon were snap-frozen after removal of the mucosa. The tissues were stored at -80°C until further processing. They were then thawn and homogenized in buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris).HCl (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol (DTT), and 2 mM phenylmethylsulfonyl fluoride. Extracts were centrifuged at 20,000 *g* for 20 min at 4°C. Supernatants (containing 40 µg protein) were incubated for 10 min at 37°C in a reaction mixture containing 50 mM Tris.HCl (pH 7.5), 4 mM $MgCl_2$, 0.5 mM 1-methyl-3-isobutylxanthine, 7.5 mM creatine phosphate, 0.2 mg $ml^{-1}$ creatine phosphokinase, 1 mM L-NAME, and 1 mM GTP with or without 1 mM diethylenetriamine NONOate (DETA-NO). The reaction was terminated by the addition of 0.9 ml of 0.05 M HCl. cGMP in the reaction mixture was measured using a commercial radioimmunoassay

(Biomedical Technologies, Stoughton, MA). sGC enzyme activity is expressed as pmol of cGMP produced per min per milligram of protein in GI extract supernatant.

### V.3.9 Drugs used

The following drugs were used: apamin (obtained from Alomone Labs), atropine sulphate, 8-Br-cGMP sodium salt, carmine, guanethidine sulphate, L-NAME, phenol red, $PGF_{2\alpha}$ tris salt, VIP (all obtained from Sigma-Aldrich), DETA-NO (from Alexis Biochemicals), carbachol (from Fluka AG), fluorescein-labelled dextran (70 kDa, FD70; Invitrogen), ODQ (from Tocris Cookson). All drugs were dissolved in de-ionized water except for the following: ODQ, which was dissolved in 100 % ethanol and DETA-NO, which was dissolved in sGC enzyme activity buffer (see above). Saturated NO solution was prepared from gas (Air Liquide, Belgium) as described by Kelm and Schrader (1990).

### V.3.10 Statistics

All results are expressed as means ± S.E.M. n refers to tissues obtained from different animals unless otherwise indicated. Comparison between KI and WT tissues was done with an unpaired Student's *t*-test. Comparison within tissues of either WT or KI was done by a paired Student's *t*-test. When more than 2 sets of results within the same tissue had to be compared, repeated measures ANOVA followed by a Bonferroni corrected *t*-test was applied. A P-value less than 0.05 was considered to be statistically significant (GRAPHPAD, San Diego, CA, USA).

## *V.4 Results*

All in vivo experiments and all in vitro experiments were performed in mice of both sexes, except for the measurement of sGC activity which was investigated in male mice only. As no systematic differences between the sexes were observed, results are presented for male mice.

### V.4.1 General observations

The body weight of the KI mice was significantly smaller than that of the WT mice (Table V.1). However, in contrast, the stomach of the KI mice was significantly enlarged; in some KI mice the stomach occupied most of the abdominal cavity. The mean empty stomach weight of the KI mice was significantly larger than this of the WT mice (Table V.1).

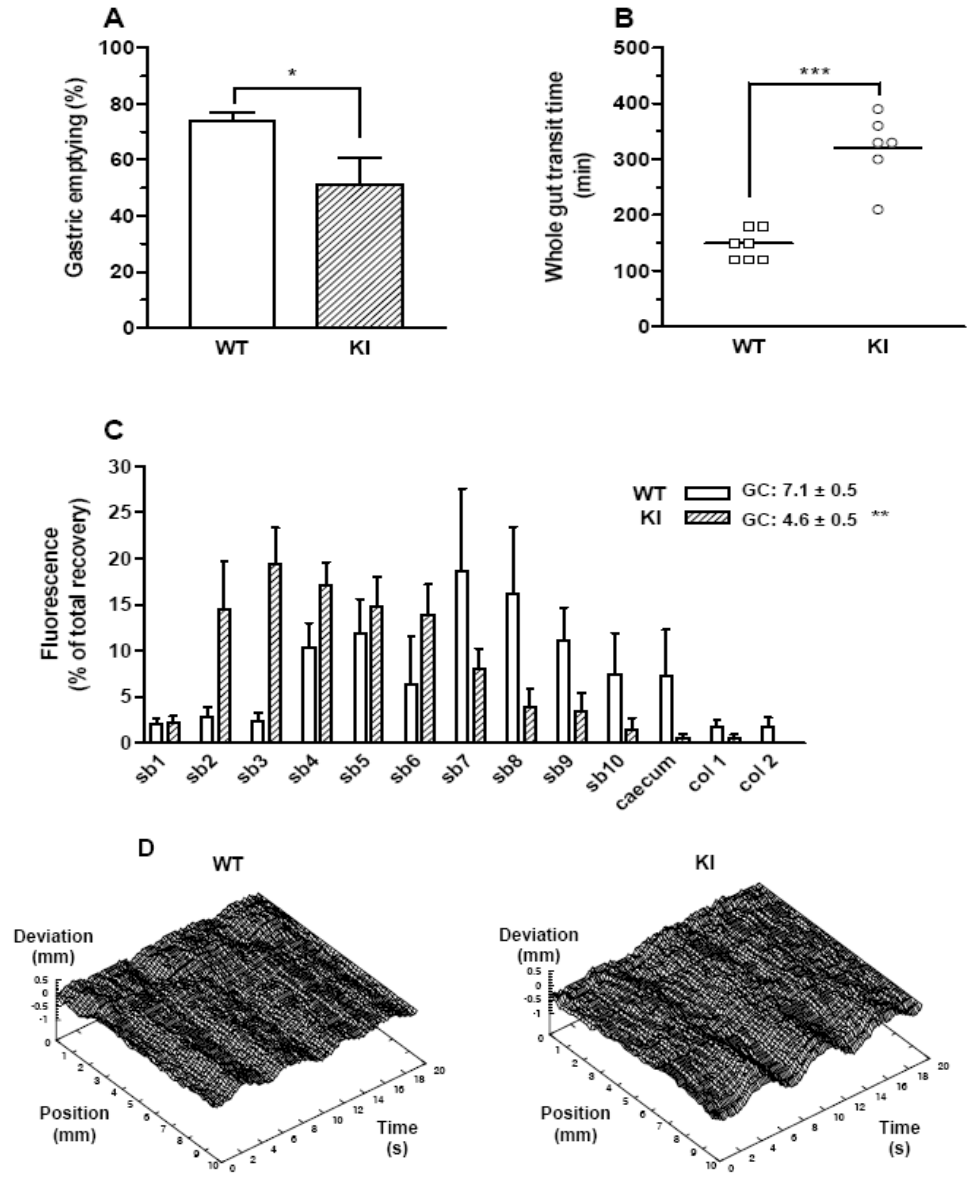
**Table V.1:** Body weight, stomach weight and weight of the gastro-intestinal preparations.

| | WT | KI |
|---|---|---|
| Body weight (g) | 30 ± 1 (n = 23) | 22 ± 1 (n = 25) *** |
| Stomach weight (mg) | 209 ± 10 (n = 23) | 379 ± 28 (n = 25) *** |
| Fundus strips (mg) | 5.83 ± 0.27 (n = 46) | 15.73 ± 1.55 (n = 50) *** |
| Jejunum strips (mg) | 0.54 ± 0.05 (n = 47) | 0.45 ± 0.05 (n = 45) |
| Colon strips (mg) | 0.53 ± 0.03 (n = 41) | 0.44 ± 0.03 (n = 41) * |

For the weight of the strips (2 strips per animal), values are means ± SEM of n = 41-50 out of 23-25 animals, i.e. all male animals used in the muscle tension experiments. * $P < 0.05$, *** $P < 0.001$: unpaired Student's *t* test (KI vs. WT).

### V.4.2 Gastric emptying, small intestinal transit and whole gut transit time

The phenol red recovery was 74 ± 5 % in WT mice (n = 10) and 77 ± 4 % in KI mice (n = 8). Fifteen min after gavage, gastric emptying of a phenol red solution was significantly delayed in KI mice compared to WT mice (Fig. V.1A). At ninety min after gavage, we observed a delayed intestinal transit of a fluorescein-labelled dextran solution in KI versus WT mice as manifested from the significant decrease in geometric center (Fig. V.1C). Small intestinal contractility at that time point was however not different between WT and KI mice (% contraction amplitude in KI: 23 ± 3 % versus WT: 22 ± 3 %, n = 5-6), which is illustrated in Fig. V.1D. The whole gut transit time, determined as the time taken for excretion of the first red-coloured faeces after gavage with a carmine solution, was between 120 and 180 min in WT mice. In KI mice, the whole gut transit time was more variable and the mean value was significantly increased (KI: 320 ± 25 min versus WT: 146 ± 10 min, n = 6-7; $P < 0.001$; Fig. V.1B).




## Figure V.1

A) Gastric emptying 15 min after gavage of 250 µl of a phenol red meal (0.1 % w $v^{-1}$ dissolved in water) in WT and KI mice. Values are means ± S.E.M. of n = 8-10 animals.

B) Scatter graph showing the whole gut transit time of a carmine solution (6 % w $v^{-1}$ dissolved in 0.5 % methylcellulose) in WT and KI mice. The mean value is represented by a solid line (n = 6-7 animals).

C) Distribution of fluorescein-labelled dextran in 10 equal small bowel (sb) segments, caecum, and 2 equal colon (col) segments 90 min after gavage of 200 µl fluorescein-labelled dextran (70 kDa; 2.5 % w $v^{-1}$ dissolved in water) and geometric center (GC) in WT and KI mice. Values are means ± S.E.M. of n = 5-6 animals.

* P < 0.05; ** P < 0.01; *** P < 0.001: unpaired Student's *t* test (KI versus WT).

D) Representative contractility traces showing spontaneous oscillatory contractions in a 1 cm jejunal segment (*X-axis*) as deviations in mm (*Y-axis*) for a period of 20 s (*Z-axis*); the intestinal diameter measured at t = 20 s was used as reference value. The 1 cm jejunal segment used for this illustration is part of the 6 cm long segment of jejunum that was recorded for 30 s in order to calculate the small intestinal contractility.

### V.4.3 Histology

As demonstrated in Fig. V.2, the fundus of the KI mouse showed a marked thickening of the smooth muscle layer compared to that of the WT mouse. No histological differences between WT and KI mouse at the level of the jejunum and the colon were observed.

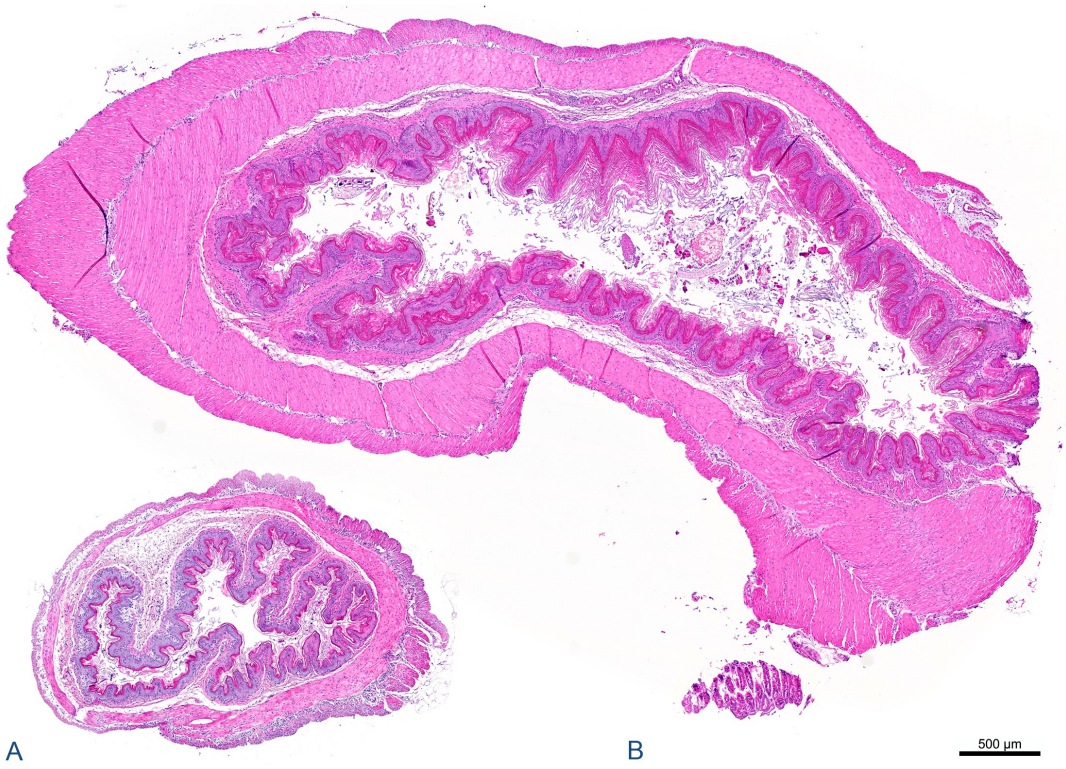



**Figure V.2**

Comparison of fundic histologic transverse sections from a WT mouse (A) and a KI mouse (B). The transverse sections of 5µm thickness were stained with Hematoxylin and Eosin for morphological observation.

### V.4.4 Muscle tension experiments

#### V.4.4.1 Tissue weight

The fundic strips of the KI mice weighed significantly more than these prepared from the WT mice (Table V.1). On the other hand, the colonic strips of the KI mice were slightly lighter than these of the WT mice (Table V.1). All obtained functional data were corrected for these differences in weight between WT and KI strips by taking into account the wet weight of the strips (see V.3.3.4).

#### V.4.4.2 Contractile responses to carbachol and PGF2α

The cumulative concentration-response curves of carbachol and $PGF_{2\alpha}$ (1 nM – 30 µM) did not significantly differ in fundic, jejunal or colonic strips from KI mice compared to these from

WT mice. For example, in colonic strips, the $EC_{50}$ (nM) and $E_{max}$ ([g.s]/mg wet weight) for carbachol were 433 ± 72 and 170 ± 35 in WT (n = 6) and 709 ± 144 and 211 ± 77 in KI (n = 6). For $PGF_{2\alpha}$, the $EC_{50}$ (μM) and $E_{max}$ ([g.s]/mg wet weight) in this tissue were 7.68 ± 1.04 and 53 ± 13 in WT (n = 6) and 9.14 ± 1.86 and 44 ± 9 in KI (n = 6). $PGF_{2\alpha}$ at a concentration of 300 nM was chosen to pre-contract fundic and jejunal strips in order to investigate the relaxant responses to EFS, NO, VIP and 8-br-cGMP (see below); in colonic strips 3 μM $PGF_{2\alpha}$ was used. In fundic strips, the $PGF_{2\alpha}$-induced response consisted solely of an increase in tone. Instead, in jejunal and colonic strips, albeit $PGF_{2\alpha}$ induced an initial fast increase in tone, this then declined to a lower level, or in the case of the jejunal strips even to baseline, with superimposed phasic activity (Fig. V.3). Despite the similar concentration-response curve of $PGF_{2\alpha}$ in the WT and KI colonic strips, the response to 3 μM $PGF_{2\alpha}$ was decreased in the KI versus WT colonic strips in the first series where ODQ was tested against the responses to EFS, NO and VIP (see below).

#### V.4.4.3 Relaxant responses to EFS

In fundic strips of WT mice, EFS (1-8 Hz) induced frequency-dependent relaxations, consisting of a progressive decline in tone which recovered upon ending stimulation (see Fig. V.3A for the response at 8 Hz and Fig. V.4A for mean responses). In WT jejunal and colonic strips, the magnitude of the EFS-induced relaxations was rather similar at the different stimulation frequencies (Fig. V.3B and C and Fig. V.4B and C). In WT jejunal strips, EFS only induced suppression of phasic activity; in WT colonic strips, EFS induced suppression of phasic activity as well as a decrease in tone. Upon ending stimulation, a rebound contraction was observed at 8 Hz in WT jejunal strips and at all tested frequencies in WT colonic strips (Fig. V.3B and C). ODQ (10 μM; Fig. V.4A and B) and L-NAME (300 μM, data not shown) (nearly) abolished the EFS-induced responses at all frequencies in WT fundic and jejunal strips. In WT colonic strips, only the response to 1 Hz was reduced by ODQ (Fig. V.4C) or L-NAME, albeit the latter did not reach significance (Fig. V.5); the combination of L-NAME plus apamin was able to decrease the response at all frequencies in these strips but did not abolish them (Fig. V.5).

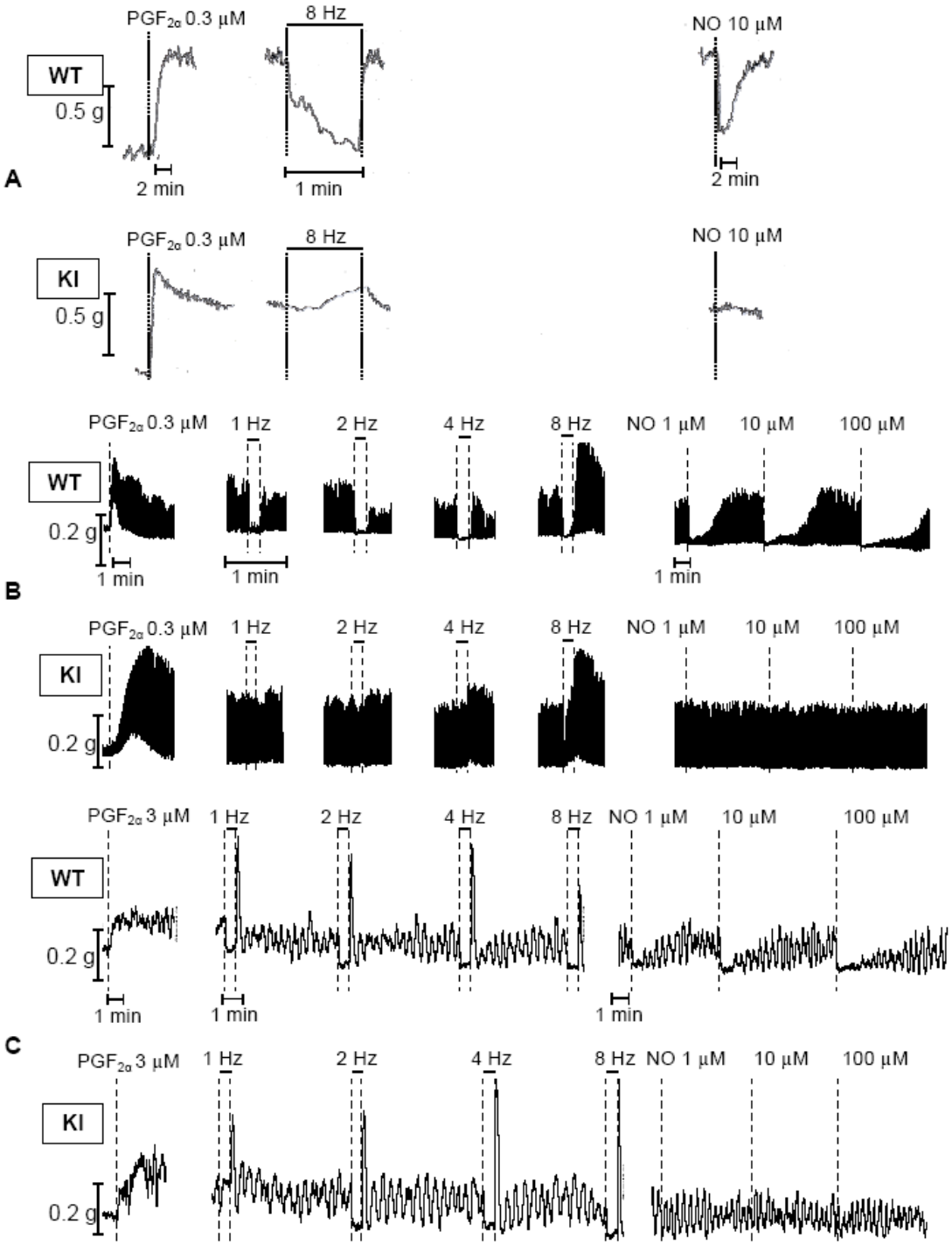
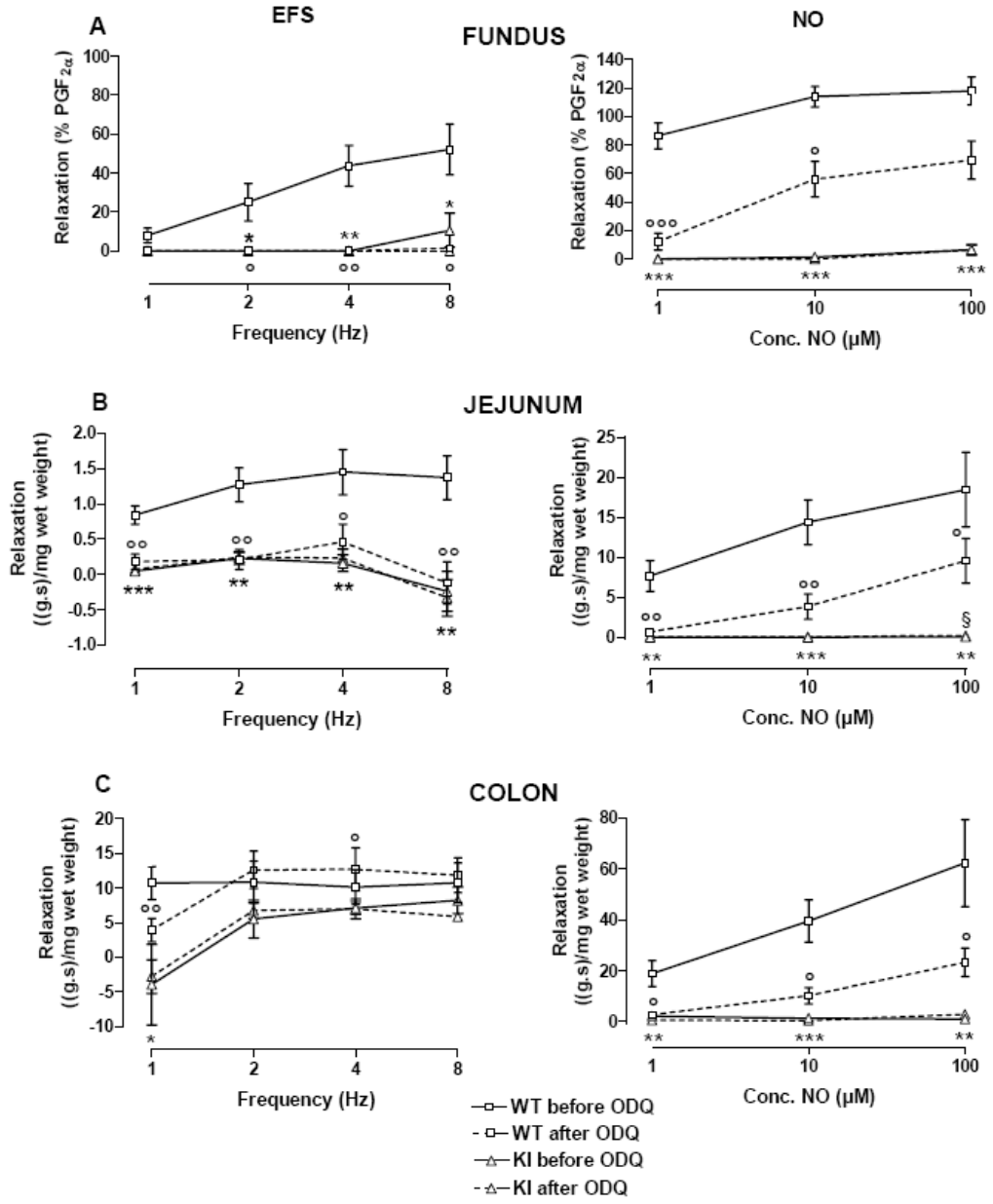



**Figure V.3**

Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 1-8 Hz) and exogenously applied NO (1-100 μM) in $PGF_{2\alpha}$-pre-contracted circular muscle strips of gastric fundus (A), jejunum (B) or distal colon (C) from a WT mouse (upper trace) and a KI mouse (lower trace).




## Figure V.4

Frequency-response curves of EFS (40 V; 0.1 ms; 1-8 Hz) (*left*) and concentration-response curves of NO (1 μM – 100 μM) (*right*) in WT (□) and KI (Δ) strips of fundus (A), jejunum (B) and colon (C) before and after incubation with ODQ (10 μM). Negative values in the y-axis of panel B (*left*, EFS in jejunum) indicate that a contractile response instead of a relaxation was obtained. The responses to EFS and NO in the KI colonic strips (panel C) were multiplied with a factor "$PGF_{2\alpha}$ response in WT colonic strips / $PGF_{2\alpha}$ response in KI colonic strips" in order to correct for the significantly smaller $PGF_{2\alpha}$ (3 μM)-induced pre-contraction in the KI versus the WT colonic strips in these series (KI: 32.00 ± 4.94 (g.s)/mg wet weight versus WT: 89.92 ± 17.94 (g.s)/mg wet weight, n = 6, $P < 0.05$). Means ± SEM of n = 6-8 are shown. * $P < 0.05$, ** $P < 0.01$: KI before incubation versus WT before incubation (unpaired Student's *t*-test); ○ $P < 0.05$, ○○ $P < 0.01$, ○○○ $P < 0.001$: WT after incubation with ODQ versus before (paired Student's *t*-test). § $P < 0.05$: KI after incubation with ODQ versus before (paired Student's *t*-test).

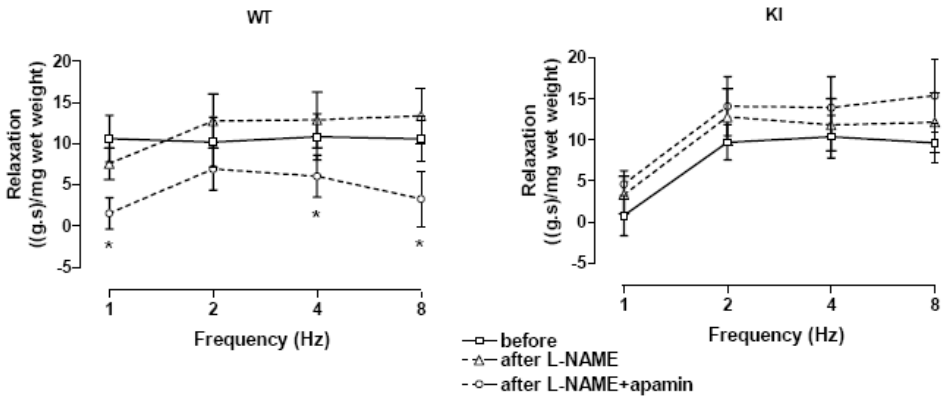



**Figure V.5**

Frequency-response curves of EFS (40 V; 0.1 ms; 1-8 Hz) in WT (*left*) and KI (*right*) colonic strips before (□) and after incubation with L-NAME (300 µM; ∆ ) and L-NAME (300 µM) plus apamin (500 nM; ○). Means ± SEM of n = 6-7 are shown. * P < 0.05: after incubation with L-NAME plus apamin versus after incubation with L-NAME (repeated measures ANOVA followed by a Bonferroni corrected *t*-test).

In fundic strips of KI mice, the relaxant responses to EFS at 1-4 Hz were totally abolished; at 8 Hz 2 out of 6 strips showed a small relaxant response, 2 strips showed no response and 2 strips contracted (see Fig. V.3A for an example of the contractile response at 8 Hz). To obtain the mean results, the 2 contractile responses to EFS were interpreted as zero relaxation, resulting in a small relaxant response at 8 Hz in the KI fundic strips (Fig. V.4A). ODQ (see Fig. V.4A) and L-NAME (see Fig. V.6) did not significantly influence the relaxant responses to 8 Hz that occurred in some of the KI fundic strips. In KI jejunal strips, the responses to EFS at 1-4 Hz were virtually abolished. At 8 Hz, the phasic activity was initially suppressed, however near the end of the stimulation train an increase in phasic activity was systematically observed, which further progressed when the stimulation train was ended (Fig. V.3B). In KI colonic strips, EFS at 1 Hz induced a short initial decrease in tone, which already recovered during stimulation. At higher frequencies a pronounced relaxant response was present (Fig. V.3C). Neither ODQ (see Fig. V.4C), nor L-NAME, or L-NAME plus apamin (see Fig. V.5) influenced the remaining EFS-induced responses at 2 to 8 Hz in KI colonic strips.

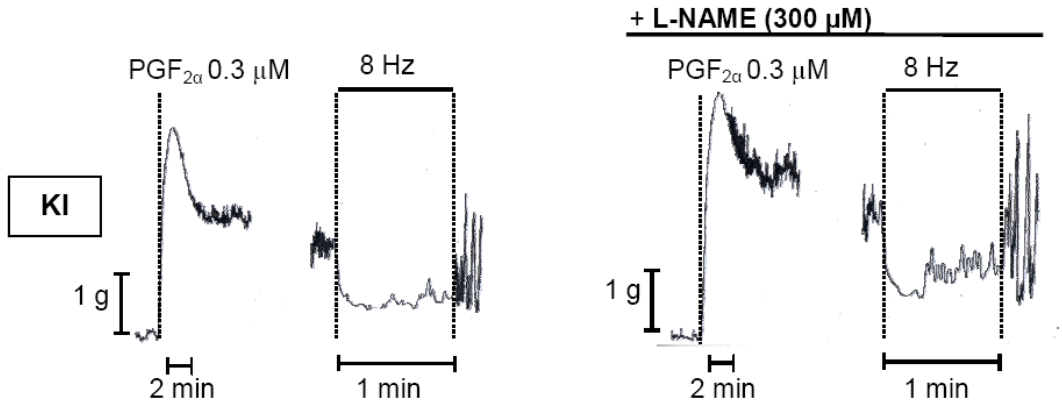



**Figure V.6**

Representative trace showing the inhibitory response to EFS (40 V; 0.1 ms; 8 Hz) in a $PGF_{2\alpha}$-pre-contracted circular gastric fundus muscle strip from a KI mouse before and after incubation with the NOS inhibitor L-NAME (300 µM).

### V.4.4.4 Relaxant responses to NO

NO induced concentration-dependent relaxations in the fundic, jejunal and colonic strips from WT mice (Fig. V.4). In WT fundic strips, the response to NO consisted of a quick and transient decline in tone (Fig. V.3A). In WT jejunal and colonic strips, NO suppressed pre-imposed phasic activity with a concentration-dependent duration; in colonic strips also a decrease in tone was sometimes observed (Fig. V.3B and C). ODQ reduced the relaxant responses to NO in the WT strips, with a nearly complete abolition at 1 µM (Fig. V.4). The responses to NO were totally abolished in the fundic, jejunal and colonic strips from KI mice (Fig.V. 3 and Fig. V.4).

### V.4.4.5 Inhibitory responses to 8-Br-cGMP and VIP

8-Br-cGMP (10 or 100 µM; see V.3.3.3) and VIP (100 nM) induced a sustained response, consisting of a decrease in tone in fundic strips, a suppression of phasic activity in jejunal strips, and suppression of phasic activity sometimes accompanied by a decrease in tone in colonic strips. Mean responses to 8-Br-cGMP and VIP were not different between WT and KI strips (Fig. V.7). ODQ did not significantly decrease the responses to 8-Br-cGMP and VIP in either WT strips or KI strips from fundus, jejunum or colon (results not shown).

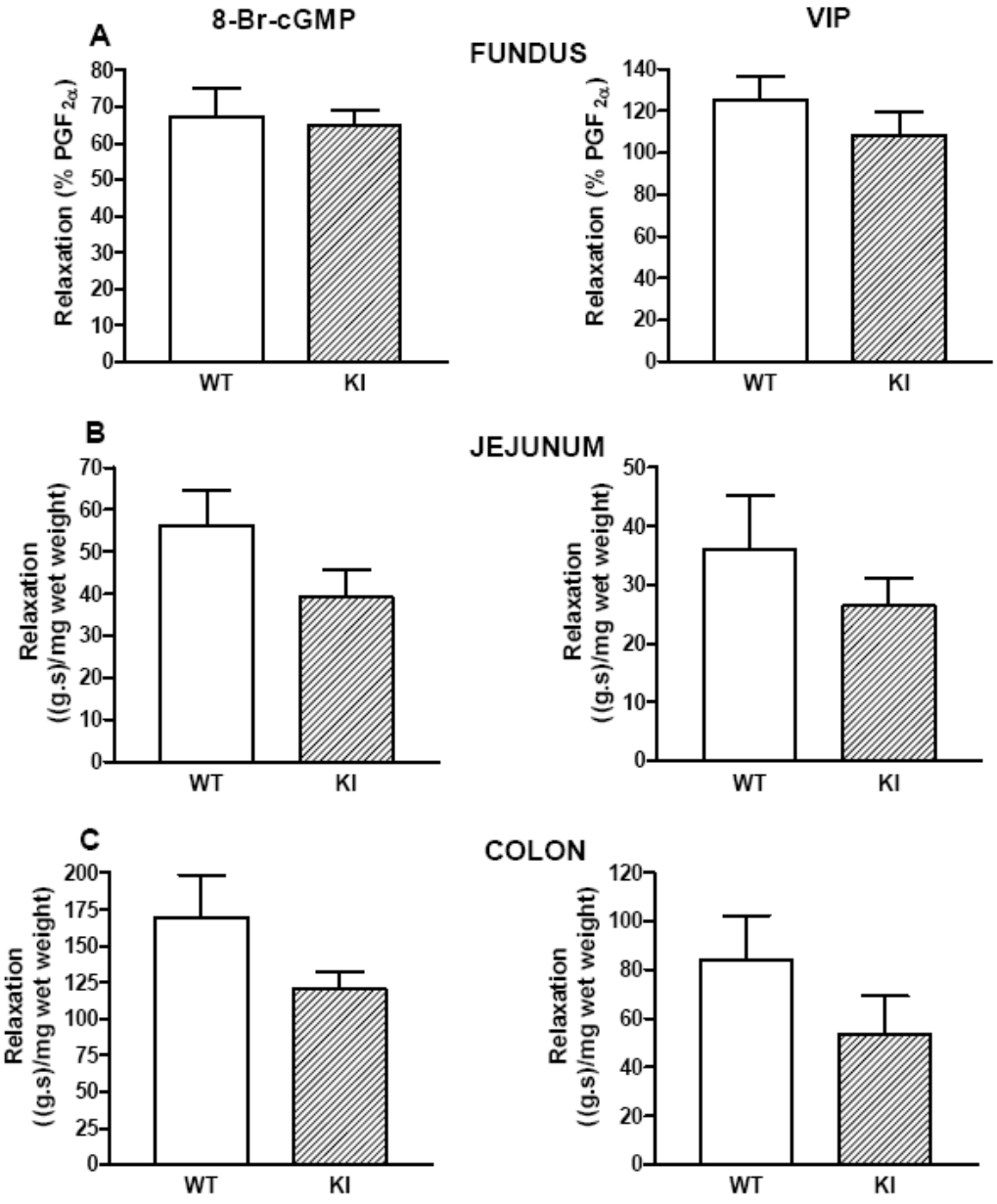



**Figure V.7**

Relaxant responses to 10 µM (fundus, A) or 100 µM (jejunum, B and colon, C) 8-Br-cGMP (*left*) and to 100 nM VIP (*right*) in WT and KI strips. Complementary to the EFS- and NO-induced relaxations (see legend Fig. V.4C), the responses to VIP in the KI colonic strips were multiplied with the correction factor "$PGF_{2\alpha}$ response in WT colonic strips / $PGF_{2\alpha}$ response in KI colonic strips". Means ± SEM of n = 9-12 out of 5-6 animals are shown.

## V.4.5 sGC enzyme activity

Baseline sGC activity was slightly increased in KI tissues compared to WT tissues; this reached only significance in the colonic tissues (Fig. V.8). DETA-NO significantly increased sGC activity in WT tissues, but not in KI tissues. The level of sGC activity in the presence of DETA-NO was as a result significantly lower in the KI versus the WT tissues.

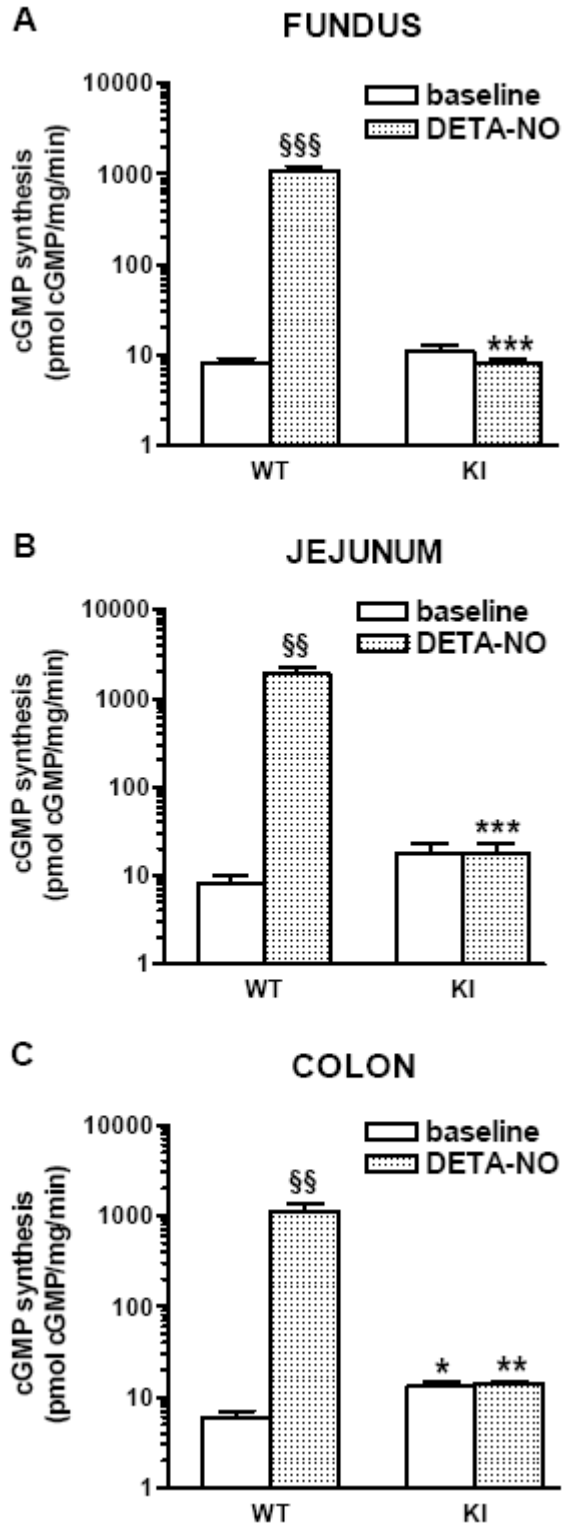



**Figure V.8**

Un-stimulated (baseline) and DETA-NO-stimulated sGC enzyme activity (expressed as picomoles cGMP produced per mg protein per minute) in fundic (A), jejunal (B) and colonic (C) extracts of WT and KI mice. Means ± SEM of n = 5-6. ** P < 0.01, *** P < 0.001: KI versus WT (unpaired Student's *t*-test); §§ P < 0.01, §§§ P < 0.001: DETA-NO versus baseline (paired Student's *t*-test).

## *V.5 Discussion*

The aim of this study was to investigate the importance of the nitrergic activation of sGC in regulation of GI motility by use of sGC$\beta_1$his105phe knock-in (KI) mice, possessing heme-deficient sGC. Heme-deficient sGC isoforms retain their basal activity but can not be activated by NO (Wedel et al., 1994). Indeed exogenous NO was not able to induce in vitro relaxation in the fundic, jejunal and colonic smooth muscle strips of KI mice. Correspondingly, the NO-donor DETA-NO failed to increase the sGC activity in the GI tissues of KI mice.

In vivo, the observed GI phenotype of the KI mice included delayed gastric emptying, delayed intestinal transit and increased whole gut transit time. The maintained responses to respectively the cell-permeable cGMP analogue 8-Br-cGMP and VIP, that acts through adenylate cyclase coupled VIP receptors, in the KI fundic, jejunal and colonic strips, indicate that there are no compensatory mechanisms in the relaxant pathway downstream of sGC nor in the cAMP-induced relaxation in the KI mice. Also contractile mechanisms are not influenced as contractions to carbachol and $PGF_{2\alpha}$ did not consistently differ between KI and WT mice. The severely disturbed gastric emptying and intestinal transit in KI mice illustrate the importance of NO-sensitive sGC in GI motility and probably contribute to the reduced lifespan of KI mice (median survival of 29-36 weeks; Thoonen et al., 2009). The latter values contrast with the much shorter lifespan of sGC $\beta_1$ KO mice, where less than 10 % survived for 1 month due to intestinal dysmotility (Friebe et al., 2007), suggesting that basal sGC activity, maintained in the KI mice, is important for longer survival.

NO is the principal relaxant neurotransmitter in the mouse gastric fundus, as EFS-induced relaxations are totally abolished by L-NAME in WT fundic strips. The fact that ODQ has a similar effect as L-NAME in the WT fundic strips indicates that these NO-induced relaxations are mediated through activation of sGC. However, a relaxant response to EFS occurred in response to EFS at 8 Hz in some KI fundic strips, being insensitive to L-NAME. The latter suggests the release of another neurotransmitter than NO, at higher stimulation frequencies in the gastric fundus of some KI mice. We could speculate this neurotransmitter to be VIP, as VIP was shown to be released at higher stimulation frequencies in rat gastric fundus strips (D'Amato et al., 1992). In compensation for the loss of nitrergic relaxation, VIP release might start at lower frequencies in some KI mice. Fundic nitrergic relaxation is essential for gastric accommodation (Desai et al., 1991) and its deficiency in KI mice might be expected to speed up liquid gastric emptying as fundic storage of the liquids is impaired. Instead, we observed delayed liquid gastric emptying in the KI mice, which is in accordance with reports of delayed gastric emptying in nNOS KO mice (Mashimo et al., 2000) and cGMP-dependent protein kinase (cGKI) KO mice (Pfeifer et al., 1998). Whereas nNOS is involved in the synthesis of

NO and thus is situated upstream of sGC, cGKI is an important downstream effector of sGC. Moreover, the KI mice, similar to the nNOS (Huang et al., 1993; Mashimo et al., 2000) and cGKI (Pfeifer et al., 1998) KO mice, showed a marked enlargement of the stomach with hypertrophy of the circular muscle of the fundus. Mashimo et al. (2000) suggested this gastric smooth muscle thickening to be secondary to functional pyloric obstruction and to represent work hypertrophy. NOS inhibition is indeed reported to increase the pyloric tone (Anvari et al., 1998) and impairment of pyloric relaxation will counteract the accelerating effect of the deficient fundic relaxation on gastric emptying (Anvari et al., 1998; Mashimo et al., 2000), as such explaining the observed delay in gastric emptying in the KI mice in this study.

The delay in small intestinal transit might at least in part result from the disturbances in gastric emptying, retarding the liquid solution to enter the small intestine in KI mice and creating a lag compared to the WT mice that cannot be compensated anymore. The abolition of the EFS-induced relaxations in the WT jejunal strips by L-NAME points to NO as the principal neurotransmitter in mouse jejunum. In the KI jejunal strips, due to the lack of NO-sensitive sGC, nitrergic relaxation is abolished. It seems therefore inevitable that in the KI mice an imbalance between the inhibitory (nitrergic) and excitatory (cholinergic) input during peristalsis develops, which is expected to contribute to the delay in intestinal transit observed in these mice. Similarly, interrupting the pathway downstream of NO-sGC in the cGKI KO mice indeed resulted in disturbed intestinal motility as spastic contractions of long intestinal segments followed by scarce and slow relaxations occurred (Pfeifer et al., 1998). Still, in the KI mice, the in vitro small intestinal contractility was not different from that in the WT mice, which seems in contrast to the observed delay in intestinal transit in the KI mice. The in vitro small intestinal contractility was assessed by measuring the change in intestinal diameter along jejunal segments within a 30 s-period. Although the results thus indicate that the in vitro spontaneous oscillatory contractions are preserved in the jejunum of the KI mice, the coordinated interplay between the ascending contractions and descending relaxations, essential for the propagation of the peristaltic contraction (Waterman et al., 1994), is most probably disturbed in KI mice and contributes to the delay in small intestinal transit.

The delays in gastric emptying and in intestinal transit certainly contribute to the increase in whole gut transit time in KI mice. In addition to the delays in the upper GI transit, also retardation of the colonic transit might take part in the increase in whole gut transit time in the KI mice. NOS inhibition was previously indeed found to inhibit the colonic propulsion of artificial pellets in guinea pig colonic segments (Foxx-Orenstein & Grider, 1996) and to delay colonic transit in rats (Mizuta et al., 1999). Still, in the KI mice, the extent of delay in colonic transit per se will probably be limited, considering the fact that in mouse distal colon, NO - acting via sGC- is only the principal neurotransmitter at a stimulation frequency of 1 Hz.

Indeed, in WT colonic strips, only the response to EFS at 1 Hz was reduced by L-NAME or ODQ. Accordingly, in KI colonic strips, only the relaxant response to EFS at 1 Hz was abolished, whereas the responses to EFS at 2 to 8 Hz were not influenced. We previously suggested that a redundant action of NO, acting at sGC, and another neurotransmitter, acting at the small conductance $Ca^{2+}$-dependent $K^+$ channels, is responsible for the relaxant responses to EFS at 2 to 8 Hz in mouse distal colon. This proposal was based on the observation that in mouse colonic strips, the relaxant responses at these higher stimulation frequencies were nearly abolished by the combined addition of L-NAME plus apamin, or ODQ plus apamin (Dhaese et al., 2008). However, in the KI mice, L-NAME plus apamin failed to influence the relaxant responses by EFS at 2 to 8 Hz. This indicates the presence of another, possibly third, neurotransmitter, responsible for the relaxations at 2 to 8 Hz, and could represent a compensatory mechanism in the KI mice. In the WT colonic strips in the actual study, L-NAME plus apamin could only reduce -and not abolish- the relaxations to EFS at 2 to 8 Hz; thus also in the WT mice used in this study, a third neurotransmitter is likely to be present. The presence of this unidentified neurotransmitter could be related to the genetic background of the mice, as the latter is different between this study (mixed 129/SvJ-C57BL/6J) and the referred study (mixed Swiss-129; Dhaese et al., 2008), where L-NAME plus apamin was able to nearly abolish the responses to EFS at 2 to 8 Hz in the WT colonic strips. The presence of a third neurotransmitter, besides NO and ATP, was also reported in rat distal colon by Van Crombruggen & Lefebvre (2004).

In conclusion, the GI consequences of lacking NO-sensitive sGC were most pronounced at the level of the stomach; the observed enlargement of the stomach with hypertrophy of the circular muscle of the fundus and the delayed gastric emptying establish the pivotal role of the activation of sGC by NO in normal gastric functioning. In addition, the absence of NO-sensitive sGC induced delayed intestinal transit and increased whole gut transit time. In mouse distal colon, other neurotransmitters besides NO play a role in the in vitro relaxant responses to higher stimulation frequencies.

## *V.6 References*

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# Chapter VI

# MYOSIN LIGHT CHAIN PHOSPHATASE ACTIVATION IS INVOLVED IN THE HYDROGEN SULFIDE-INDUCED RELAXATION IN MOUSE GASTRIC FUNDUS

**Ingeborg Dhaese, Romain A. Lefebvre**

**Based on**

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# Chapter VI Myosin light chain phosphatase activation is involved in the hydrogen sulfide-induced relaxation in mouse gastric fundus

## *VI.1 Abstract*

The relaxant effect of hydrogen sulfide ($H_2S$) in the vascular tree is well established but its influence and mechanism of action in gastrointestinal (GI) smooth muscle was hardly investigated. The influence of $H_2S$ on contractility in mouse gastric fundus was therefore examined. Sodium hydrogen sulfide (NaHS; $H_2S$ donor) was administered to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-contracted circular muscle strips of mouse gastric fundus, before and after incubation with interfering drugs. NaHS caused a concentration-dependent relaxation of the pre-contracted mouse gastric fundus strips. The $K^+$ channels blockers glibenclamide, apamin, charybdotoxin, 4-aminopyridin and barium chloride had no influence on the NaHS-induced relaxation. The relaxation by NaHS was also not influenced by L-NAME, ODQ and SQ 22536, inhibitors of the cGMP and cAMP pathway, by nerve blockers capsazepine, ω-conotoxin and tetrodotoxin or by several channel and receptor blockers (ouabain, nifedipine, 2-aminoethyl diphenylborinate, ryanodine and thapsigargin). The myosin light chain phosphatase (MLCP) inhibitor calyculin-A reduced the NaHS-induced relaxation, but the Rho-kinase inhibitor Y-27632 had no influence.

We show that NaHS is able to relax $PGF_{2\alpha}$-contracted mouse gastric fundus strips. The results suggest that in the mouse gastric fundus, $H_2S$ causes relaxation at least partially via activation of MLCP.

## *VI.2 Introduction*

Hydrogen sulfide ($H_2S$), a colourless gas with the smell of rotten eggs, has traditionally been considered to be toxic leading to brain intoxication and the inhibition of the respiratory system (Beauchamp et al., 1984). However, it was found that $H_2S$ can also be produced endogenously in mammalian tissues from L-cysteine by two enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Stipanuk and Beck, 1982). It was suggested that $H_2S$ may operate as an endogenous neurotransmitter based on its endogenous production and its biological effects at physiological concentrations. $H_2S$ is as such a possible third gaseous transmitter, besides nitric oxide (NO) and carbon monoxide (CO), and has been reported to play a role in hippocampal long-term potentiation (Abe and Kimura, 1996) and to exert antinociceptive (Distrutti et al., 2006), exocrine prosecretory (Schicho et al., 2006) and smooth muscle relaxing (Cheng et al., 2004, Hosoki et al., 1997, Kubo et al., 2007a, Teague et al., 2002, Wang et al., 2008, Zhao et al., 2001) effects.

Smooth muscle relaxation by $H_2S$ has been shown in different parts of the vascular tree. In this system, the ATP-dependent $K^+$ channel ($K_{ATP}$ channel) blocker glibenclamide generally inhibits at least partially the vasorelaxation induced by $H_2S$, indicating that $H_2S$ is an activator of $K_{ATP}$ channels, leading to hyperpolarization and subsequently relaxation (Cheng et al., 2004, Tang et al., 2005, Webb et al., 2008; Zhao et al., 2001). Both $H_2S$-synthetizing enzymes have been shown in human and guinea-pig myenteric neurons (Schicho et al., 2006). Recently, CSE but not CBS was shown to be present in mouse colon myenteric neurons, and the intact colonic muscle layer containing the myenteric plexus generated detectable levels of $H_2S$ (Linden et al., 2008). Still, there are only a few reports concerning the influence of $H_2S$ on GI smooth muscle. $H_2S$ relaxed concentration-dependently acetylcholine-induced contraction in guinea-pig ileum (Hosoki et al., 1997). This was confirmed by Teague et al. (2002); these authors also showed that $H_2S$ inhibited spontaneous contractions of rabbit ileum and electrically induced contractions of the guinea-pig ileum. The latter response was unaffected by glibenclamide, indicating that the mechanism by which $H_2S$ induces inhibition of GI smooth muscle differs from that in the cardiovascular system.

The gastric fundus contains a smooth muscle layer with tonic activity, essential in gastric receptive relaxation. Although a principal role for NO in nonadrenergic noncholinergic relaxation at this level was established, evidence for neuronal release of a relaxant agent that is not NO, vasoactive intestinal peptide (VIP), ATP or CO has been reported (Curro et al., 2004). The aim of this study was to investigate the influence of $H_2S$ on contractility in mouse gastric fundus, and to explore its mechanism of action.

The data obtained in this study indicate that in mouse gastric fundus, $H_2S$ induces relaxation at least partially via activation of myosin light chain phosphatase; this seems not related to an inhibition of the RhoA/Rho-kinase pathway.

## *VI.3 Methods*

### VI.3.1 Animals

Male Swiss (SPF Orl) mice (7-12 weeks, 31-43 g, n = 102) were purchased from Janvier, Le Genest St-Isle, France and had free access to water and commercially available chow. All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health sciences at Ghent university.

### VI.3.2 Tissue preparation

Mice were sacrificed by cervical dislocation. The gastric fundus was isolated and put in Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1).

Two full-thickness fundus strips (2 x 15 mm) were prepared from the gastric fundus by cutting in the direction of the circular muscle layer. After a cotton thread was attached to both ends of the fundus strips, strips were mounted in 5 or 10 ml organ baths, containing aerated (5% $CO_2$ in $O_2$) Krebs solution maintained at 37°C.

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

All experiments were performed at optimal load. Therefore, after an equilibration period of 30 min at a load of 0.75 g with refreshing of the Krebs solution every 10 min, tissues were exposed to 100 nM carbachol. Muscle strips were then stretched by load increments of 0.25 g and at each load level exposed to 100 nM carbachol, to determine the optimal load (the load at which maximal response to the contractile agent occurred). Once this was determined, strips were allowed to further equilibrate for 45 min at their optimal load with flushing every 10 min in Krebs solution.

### VI.3.3 Protocols

Preliminary experiments had shown that, at basal tone, NaHS, the experimental source of $H_2S$, had no contractile effect but induced small relaxations. After equilibration and determination of optimal load, strips were therefore contracted with 300 nM $PGF_{2\alpha}$. When the contractile response to $PGF_{2\alpha}$ was stabilized, NaHS was cumulatively added. Strips were then washed with Krebs solution every 10 min for 1 hour. Inhibitors (4-aminopyridine (4-AP),

2-aminoethyl diphenylborinate (2-APB), apamin, barium chloride, calyculin-A, capsazepine, charybdotoxin, ω-conotoxin, glibenclamide, $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME), nifedipine, 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), ouabain, ryanodine, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536), thapsigargin, tetrodotoxin or (R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide (Y-27632)) were then added and allowed to incubate for 30 min. The strips were re-contracted with 300 nM $PGF_{2\alpha}$, and the responses to NaHS were studied again. The reproducibility of the responses to NaHS was evaluated by running time-control solvent treated strips in parallel. All these inhibitors were used in concentrations shown to inhibit their target in the literature. When the inhibitors caused per se a contraction of the strip during the incubation period, the $PGF_{2\alpha}$ concentration used to induce a re-contraction of the strip was adjusted by starting at a lower concentration of 10 nM $PGF_{2\alpha}$ and titrating higher concentrations of $PGF_{2\alpha}$ until a similar degree of contraction as before application of the inhibitor was obtained. This $PGF_{2\alpha}$ titration was applied for the following inhibitors: 4-AP, calyculin-A, thapsigargin and ryanodine. Two inhibitors, nifedipine and Y-27632, decreased basal tone during incubation and had an inhibiting effect on the $PGF_{2\alpha}$-induced re-contraction. Here higher concentrations of $PGF_{2\alpha}$ than 300 nM were titrated until a similar absolute tone level was reached during the re-contraction compared to the first pre-contraction.

### VI.3.4 Data analysis

The amplitude of the inhibitory responses to NaHS was measured and expressed as a percentage of the induced contraction. The relaxant response at the highest concentration of NaHS (3 mM), that could practically be administered, was considered as $E_{max}$. The $EC_{50}$ value of the concentration-response curve for NaHS was calculated by linear interpolation.

### VI.3.5 Statistics

All results are expressed as means ± S.E.M. and *n* refers to the number of strips obtained from different animals. The concentration-response curves for NaHS obtained in parallel strips in the presence of inhibitors or solvent (aqua, ethanol or DMSO) were compared by an unpaired Student's *t*-test (2 groups) or an one-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparison *t*-test (more than 2 groups). $P < 0.05$ was considered to be statistically significant (GRAPHPAD, San Diego, CA, USA).

### VI.3.6 Drugs used

The following drugs were used: apamin, charybdotoxin, ω-conotoxin GVIA, ryanodine, tetrodotoxin (obtained from Alomone Labs), Y-27632 dihydrochloride monohydrate (from

Calbiochem), 4-AP, 2-APB, capsazepine, glibenclamide, L-NAME, nifedipine, ouabain octahydrate, prostaglandin F2$\alpha$ tris salt, NaHS hydrate, SQ 22536, thapsigargin (from Sigma-Aldrich), barium chloride (from Merck), carbachol (from Fluka AG), calyculin-A, ODQ (from Tocris Cookson). Drugs were dissolved in de-ionized water except 2-APB, capsazepine, glibenclamide, nifedipine, ODQ, ryanodine, thapsigargin which were dissolved in ethanol and calyculin-A which was dissolved in dimethyl sulfoxide (DMSO). Solutions of NaHS were prepared fresh on the morning of each experiment and kept on ice.

## *VI.4 Results*

### VI.4.1 Basic observations

In $PGF_{2\alpha}$-contracted gastric fundus strips, cumulatively added NaHS induced concentration-dependent relaxations. Concentrations higher than 30 µM NaHS were needed to induce relaxation (Fig. VI.1). In the majority of the strips, the NaHS-induced relaxations progressed to a stabile sustained degree. However, in 31/145 (21 %) of the strips tested, at one or more concentrations of NaHS, the tone partially recovered after reaching a maximal degree of relaxation; the latter was used for calculations. In all strips treated with aqua, ethanol or DMSO (i.e. control strips, n = 54), this yielded an overall mean $E_{max}$ and $EC_{50}$ of respectively 132 ± 3 % and 514 ± 36 µM for the NaHS-induced relaxation.

The cumulative concentration-response curves were reproducible within the same strip, except for 300 µM NaHS. In the majority of the control strips, independently whether they were treated with aqua, ethanol (Fig. VI.1) or DSMO before constructing the second concentration-response curve to NaHS, NaHS at a concentration of 300 µM induced a larger relaxation during the second compared to the first concentration-response curve. As the first concentration-response curves of NaHS in parallel strips were always comparable, the influence of inhibitors on the relaxations by NaHS was thus assessed by comparing the concentration-response curves in the presence of the inhibitors with those in the presence of their solvent in the parallel tissues (see VI.3.5).

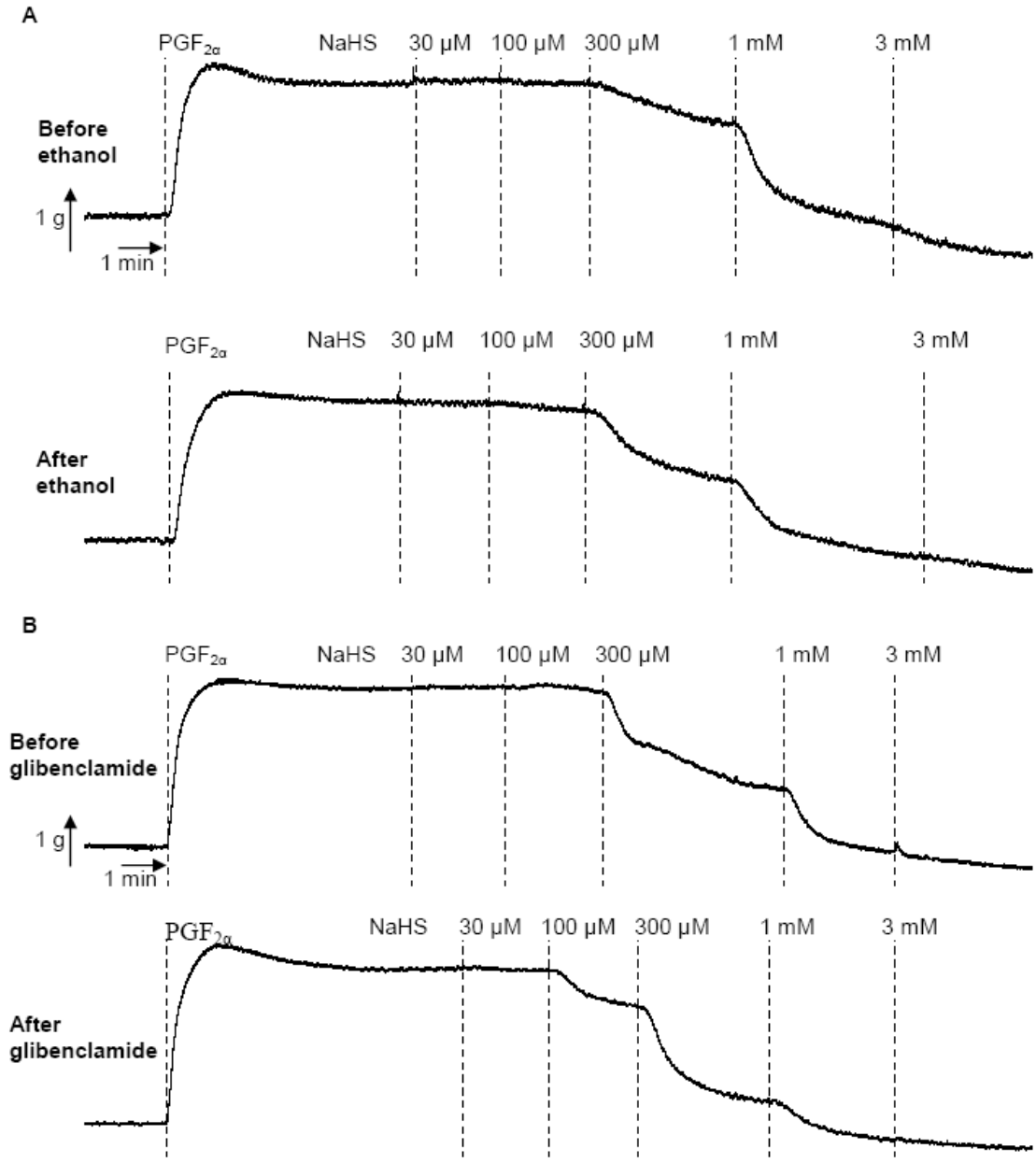


**Figure VI.1**

Representative traces showing concentration-response curves of NaHS (30 µM - 3 mM) in pre-contracted (300 nM $PGF_{2\alpha}$) gastric fundus strips before and after incubation with ethanol (A) or 10 µM glibenclamide (B).

## VI.4.2 Influence of glibenclamide, ODQ plus SQ 22536, L-NAME, tetrodotoxin and ω-conotoxin

The influence of the $K_{ATP}$ channel blocker glibenclamide on the NaHS-induced relaxation was first examined; the NaHS-induced relaxation was unaltered by incubation of the strips with glibenclamide (10 μM) (Figs. VI.1B and VI.2A). The combination of ODQ (10 μM) and SQ 22536 (100 μM), inhibitors of respectively soluble guanylate cyclase (sGC) and adenylate cyclase (AC), did also not influence the NaHS-induced relaxation (Fig. VI.2B). The NO synthase inhibitor L-NAME (300 μM), the $Na^+$ channel blocker tetrodotoxin (3 μM) and the N-type voltage dependent $Ca^{2+}$ channel blocker ω-conotoxin (3 μM) did not have any effect on the NaHS-induced relaxation (Fig. VI.2C and D).

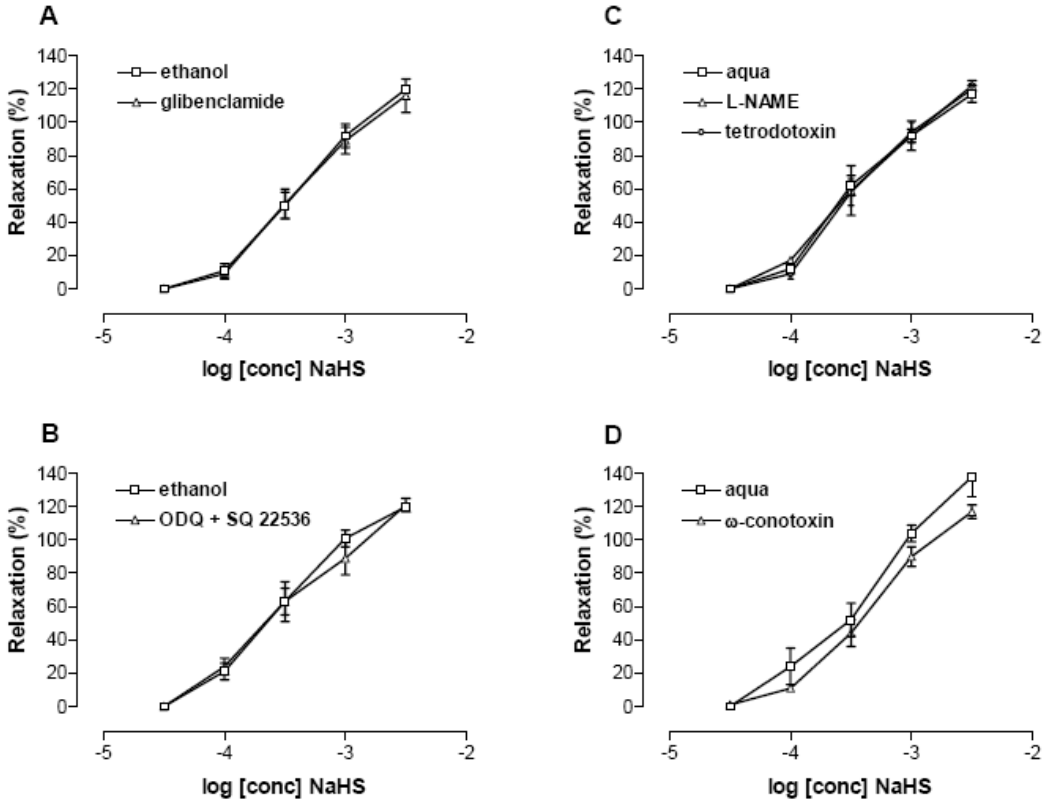


**Figure VI.2**

Concentration-response curves of NaHS (30 μM - 3 mM) in the presence of A) ethanol (□) or glibenclamide (10 μM ; ∆), B) ethanol (□) or ODQ (10 μM) plus SQ 22536 (100 μM ; ∆), C) aqua (□) or L-NAME (300 μM, ∆) or tetrodotoxin (3 μM , ○), D) aqua (□) or ω-conotoxin (3 μM, ∆). Means ± S.E.M. of n = 8 (except for 3 mM: n = 4) are shown in A, means ± S.E.M. of n = 4-5 are shown in B, C and D.

## VI.4.3 Influence of channel and receptor blockers

A second set of inhibitors was tested, targeting ion channels/receptors located in the plasma membrane of smooth muscle cells or neurons or the membrane of the sarcoplasmatic reticulum of smooth muscle cells, possibly involved in relaxation. The full list of the tested inhibitors is shown in Table VI.1. 4-AP, ryanodine and thapsigargin caused per se a contraction and elevated the basal tone by respectively 93 ± 5 % (n = 4), 45 ± 3 % (n = 6) and 68 ± 12 % (n = 6) expressed as a percentage of the first $PGF_{2\alpha}$-induced contraction. Nifedipine (30 nM) on the other hand decreased the basal tone by 14 ± 4 % (n = 6) compared to the first $PGF_{2\alpha}$-induced contraction. None of the inhibitors listed in Table VI.1 had a significant influence on the $E_{max}$ and $EC_{50}$ of the NaHS-induced relaxation.

**Table VI.1: $E_{max}$ (%) and $EC_{50}$ (µM) for the relaxant effect of NaHS in the presence of inhibitors of the listed targets, or their solvent (control)**

| inhibitor | target | n | Emax (%) | $EC_{50}$ (µM) |
|---|---|---|---|---|
| * Control (ethanol) | | 4 | 120 ± 1 | 429 ± 147 |
| ODQ (10 µM) + Apamin (500 nM) | sGC + small conductance $Ca^{2+}$ dependent $K^+$ channel | 4 | 117 ± 7 | 301 ± 57 |
| * Control (aqua) | | 5 | 117 ± 5 | 364 ± 73 |
| Apamin (500 nM) + Charybdotoxin (100 nM) | small conductance + intermediate and large conductance $Ca^{2+}$ dependent $K^+$ channel | 4 | 131 ± 8 | 354 ± 77 |
| * Control (aqua) | | 3 | 117 ± 1 | 369 ± 64 |
| Ouabain (100 µM) | $Na^+/K^+$ ATPase | 4 | 123 ± 9 | 476 ± 150 |
| 4-AP (5 mM) | voltage dependent $K^+$ channel | 4 | 122 ± 6 | 360 ± 84 |
| Barium chloride (30 µM) | inward rectifier $K^+$ channel | 4 | 131 ± 3 | 445 ± 63 |
| * Control (ethanol) | | 4 | 120 ± 1 | 334 ± 56 |
| 2-APB (50 µM) | $IP_3$ receptor | 4 | 110 ± 3 | 442 ± 179 |
| Capsazepine (3 µM) | transient receptor potential vanilloid type 1 (TRPV1) | 4 | 118 ± 3 | 407 ± 116 |
| * Control (ethanol) | | 6 | 123 ± 6 | 411 ± 85 |
| Nifedipine (30 nM) | L-type voltage dependent $Ca^{2+}$ channel | 6 | 113 ± 4 | 563 ± 67 |
| * Control (ethanol) | | 6 | 141 ± 5 | 590 ± 119 |
| Ryanodine (10 µM) | ryanodine receptor | 6 | 133 ± 7 | 428 ± 61 |
| * Control (ethanol) | | 5 | 129 ± 6 | 820 ± 96 |
| Thapsigargin (1 µM) | sarcoplasmatic reticulum $Ca^{2+}$ ATPase | 6 | 161 ± 20 | 633 ± 37 |

## VI.4.4 Influence of calyculin-A and Y-27632

Calyculin-A is an inhibitor of myosin light chain phosphatase (MLCP) and Y-27632 an inhibitor of Rho-kinase. Both targets are involved in the desensitization of the smooth muscle contractile apparatus to the intracellular $Ca^{2+}$-concentration.

Calyculin-A (1 µM) elevated the basal tone by 79 ± 10 % (n = 8) compared to the first $PGF_{2\alpha}$-induced contraction (Fig. VI.3A). $PGF_{2\alpha}$ administered on top of the calyculin-A-induced contraction only induced a minor additional contraction. Still, the active contraction level obtained in the presence of calyculin-A plus $PGF_{2\alpha}$ attained 86 ± 9 % (n = 8) of that by the first administration of $PGF_{2\alpha}$.

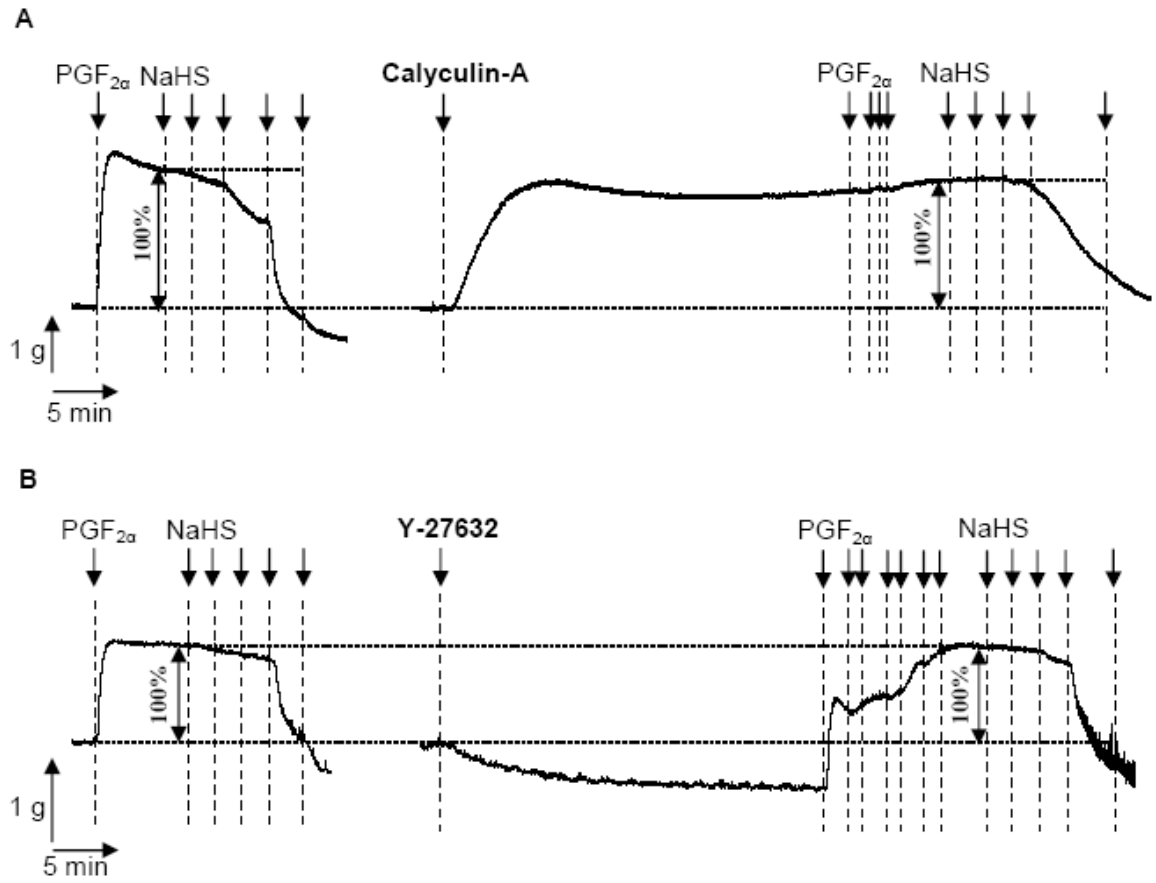


**Figure VI.3**

Representative traces showing concentration-response curves of NaHS (30 µM, 100 µM, 300 µM, 1 mM, 3mM administered at the arrows) in $PGF_{2\alpha}$-pre-contracted gastric fundus strips before and after incubation with 1 µM calyculin-A (A) or 10 µM Y-27632 (B). For the first pre-contraction 300 nM $PGF_{2\alpha}$ was administered. For the second pre-contraction, $PGF_{2\alpha}$ was administered in concentrations of 10, 30, 100 and 300 nM after incubation with calyculin-A (A) or of 0.3, 0.5, 0.7, 1, 3, 5 and 7 µM after incubation with Y-27632 (B), aiming for a similar absolute tone level (passive plus active) after versus before incubation with calyculin-A or Y-27632. The reference contraction level used to express the NaHS-induced relaxations is indicated as 100 %.

The relaxation induced by NaHS was reduced in the strips incubated with calyculin-A (Figs. VI.3A and VI.4A). This was reflected in a significantly lower maximal effect of NaHS in the presence of calyculin-A versus solvent DMSO ($E_{max}$ = 98 ± 6 % versus 123 ± 5 %, n = 8, P < 0.01). Calyculin-A also shifted the concentration-response curve of NaHS to the right and significantly raised the $EC_{50}$ for NaHS (987 ± 129 µM versus 509 ± 132 µM, n = 8, P < 0.05).

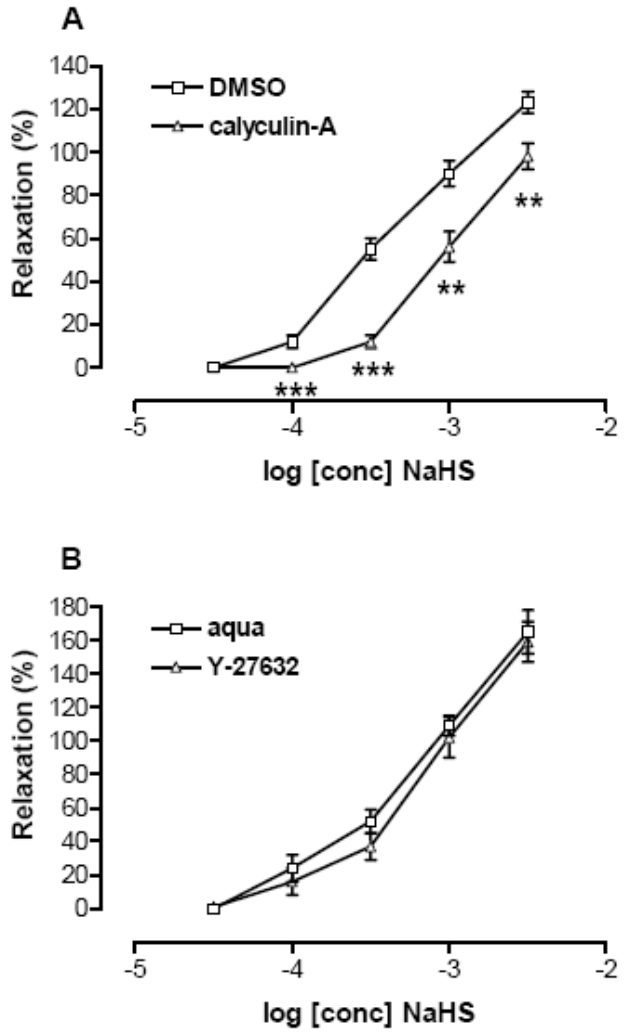


**Figure VI.4**

Concentration-response curves of NaHS (30 µM - 3 mM) in the presence of A) DMSO (□) or calyculin-A (1 µM , Δ), B) aqua (□) or Y-27632 (10 µM, Δ). Means ± S.E.M. of n = 8 are shown. ** P < 0.01, *** P < 0.001: in the presence of calyculin-A versus in the presence of DMSO (unpaired Student's *t*-test).

Incubation of the strips with Y-27632 (10 µM) decreased the basal tone by 64 ± 14 % (n = 8) compared to the first $PGF_{2\alpha}$-induced contraction (Fig. VI.3B). By adding higher concentrations of $PGF_{2\alpha}$ than 300 nM, a similar degree of absolute tone level (passive plus active) as with the first administration of $PGF_{2\alpha}$ could be achieved (Fig. VI.3B). As reference

value to express the NaHS-induced relaxations, the second pre-contraction was measured as the difference between the $PGF_{2\alpha}$-induced absolute tone level and the tone level present before the administration of Y-27632 (Fig. VI.3B). Using active $PGF_{2\alpha}$-induced contraction as reference would indeed mean that NaHS-induced relaxations are expressed as percentage of a much higher value than for the first administration cycle. In the presence of Y-27632, the maximal relaxing effect of NaHS as well as the $EC_{50}$ for NaHS were not significantly influenced versus in the presence of the solvent aqua ($E_{max}$ = 159 ± 12 % versus 165 ± 13 %; $EC_{50}$ = 805 ± 127 µM versus 630 ± 85 µM, n = 8) (Fig. VI.4B).

## *VI.5 Discussion*

In this study, we investigate the relaxing effect of $H_2S$ on the tonic smooth muscle of the mouse gastric fundus. Using NaHS makes it possible to define the concentrations of $H_2S$ in solution more accurately and reproducibly than bubbling $H_2S$ gas (Hosoki et al., 1997).

In pre-contracted mouse gastric fundus strips, NaHS induced concentration-dependent relaxations with a threshold of 100 µM and an $EC_{50}$ of 514 ± 36 µM. These values are higher than previously reported for NaHS in GI tissues. For inhibition of spontaneous contractions in rabbit ileum: $EC_{50}$, 76 µM, threshold, 10 µM; inhibition of electrically evoked contractions in rat and guinea-pig ileum: $EC_{50}$, 65 µM and 63 µM, threshold, 10 µM (Teague et al., 2002). For relaxation of acetylcholine pre-contracted guinea-pig ileum: $EC_{50}$, 180 µM, lowest tested concentration 100 µM (Hosoki et al.,1997). Also vascular tissues seem more sensitive to NaHS: rat aorta, $EC_{50}$, 136 µM, threshold, 60 µM (Zhao and Wang, 2002); rat mesenteric artery, $EC_{50}$, 104 µM, threshold, 0.1 µM (Cheng et al., 2004). But the concentration-response curve is similar to that reported in mouse carbachol-contracted bronchial rings (Kubo et al., 2007a, $EC_{50}$ not reported) so that species differences might be important.

In our study, the $K_{ATP}$ channel blocker glibenclamide had no influence on the relaxing effect of NaHS, arguing against a role of $K_{ATP}$ channels in the NaHS-induced relaxation in mouse gastric fundus. This is in contrast to cardiovascular tissues, where $K_{ATP}$ channels are reported to be involved in the NaHS-induced relaxation, such as in rat aorta (Zhao et al., 2001), rat mesenteric artery (Cheng et al., 2004) and human internal mammary artery (Webb et al., 2008). An exception however is the mouse aorta, where glibenclamide failed to affect the relaxing effect of NaHS (Kubo et al., 2007b). Our result is also in correspondence with the non-effect of glibenclamide on the NaHS-induced bronchodilation in mice (Kubo et al., 2007a) and on the NaHS-induced inhibition of the electrically induced contractions in the guinea-pig ileum (Teague et al., 2002).

Zhao and Wang (2002) reported the release of endothelium-derived NO by $H_2S$ in endothelium-intact rat aortic tissues. The possible contribution of NO, released from nitrergic nerves, in the relaxation by NaHS of mouse gastric fundus was excluded as the NO-

synthase inhibitor L-NAME had no influence. This is in correspondence with Teague et al. (2002), who reported that the inhibitory effect of NaHS on electrically induced contractions of the guinea-pig ileum was unaffected by L-NAME, and is corroborated by our observation that inhibiting the principal target of NO in mouse gastric fundus (Vanneste et al., 2007), i.e. soluble guanylate cyclase, with ODQ did not influence the NaHS-induced relaxation. Activation of another non-nitrergic relaxant neuron type is also excluded as the $Na^+$ channel blocker tetrodotoxin, inhibiting action potential propagation, and the N-type voltage dependent $Ca^{2+}$ channel blocker ω-conotoxin, interfering with exocytosis at the nerve ending, had no influence. In human and guinea-pig colon, the prosecretory effect of NaHS involves the activation of the transient receptor potential vanilloid type 1 (TRPV1) on the extrinsic primary afferent nerve terminals, which in turn activates the enteric neurons (Schicho et al., 2006). In the rat urinary bladder, NaHS stimulates the primary afferent nerve terminals to induce contraction (Patacchini et al., 2004). But the TRPV1 blocker capsazepine had no influence on NaHS-induced relaxation in mouse gastric fundus.

ODQ was tested together with the adenylate cyclase inhibitor SQ 22536, as cAMP generation is also a classic relaxant transduction pathway in smooth muscle e.g. for VIP (Hagen et al., 2006), a peptide involved in sustained non-adrenergic non-cholinergic relaxation of gastric fundus (De Beurme and Lefebvre, 1987). As ODQ plus SQ 22536 was ineffective also the cAMP transduction pathway can be excluded for the relaxant effect of NaHS. ODQ was also tested in combination with the small conductance $Ca^{2+}$ dependent $K^+$ channel ($SK_{Ca}$ channel) blocker apamin, as this combination was required to fully inhibit the relaxation by exogenous NO in rat colon (Van Crombruggen and Lefebvre, 2004), but this had no influence versus NaHS. In mouse gastric fundus, the relaxation by exogenous CO is significantly reduced by apamin plus the intermediate ($IK_{Ca}$) and large ($BK_{Ca}$) conductance $Ca^{2+}$ dependent $K^+$ channel blocker charybdotoxin, while either agent alone had no influence (De Backer et al., 2007). However, the relaxant effect of NaHS in mouse gastric fundus was not influenced by apamin plus charybdotoxin, excluding an interactive contribution of a $SK_{Ca}$ channel and a $IK_{Ca}$ or $BK_{Ca}$ channel. The influence of barium chloride and 4-AP, blockers of respectively the voltage dependent and the inward rectifier $K^+$ channel, was therefore tested. 4-AP caused per se a contraction of the gastric fundus strips, which was also observed in mouse trachea rings by Li et al. (1998). An inhibiting effect of 4-AP and barium on the acetylcholine-induced relaxation in canine basilar artery was previously shown by Elliott et al. (1991), indicating that the voltage dependent and the inward rectifier $K^+$ channel can play a role in smooth muscle relaxation. However, 4-AP as well as barium chloride did not affect the NaHS-induced relaxation. Taken together, this indicates that the above mentioned $K^+$ channels, which were reported to play a role in neurotransmitter-induced relaxation, are not involved in the NaHS-induced relaxation in mouse gastric fundus.

In rat aortic tissues, the $H_2S$-induced vasorelaxation was reduced by the L-type voltage dependent $Ca^{2+}$ channel blocker nifedipine and was as such shown to be dependent on interference with extracellular calcium entry (Zhao and Wang, 2002). In mouse gastric fundus strips, nifedipine per se decreased the basal tone, as was previously observed in dog cerebral arteries by Asano et al. (1996), and reduced the $PGF_{2\alpha}$-induced contraction, as was previously reported in rat gastric fundus by Van Geldre and Lefebvre (2004). Consequently, higher concentrations of $PGF_{2\alpha}$ were needed to induce contraction in the presence of nifedipine but in these conditions, nifedipine had no effect on the NaHS-induced relaxation in mouse gastric fundus. As stimulation of $Na^+/K^+$ ATPase was reported to be involved in the NO-induced relaxation in mouse gastric fundus strips and rabbit aorta by respectively Yaktubay et al. (1998) and Gupta et al. (1994), we investigated whether $Na^+/K^+$ ATPase could also be involved in the NaHS-induced relaxation. However, the $Na^+/K^+$ ATPase inhibitor ouabain had no influence on the relaxation induced by NaHS. The sarcoplasmatic reticulum $Ca^{2+}$ ATPase (SERCA) inhibitor thapsigargin was reported to decrease the sodium nitroprusside (SNP)-induced inhibitory effects in mouse ileum (Zizzo et al., 2005), whereas ryanodine and 2-APB, inhibitors of respectively the ryanodine receptor and the $IP_3$ receptor, decreased the SNP-induced relaxation of mouse gastric fundus (Kim et al., 2006). Thapsigargin and ryanodine caused per se a contraction of the mouse gastric fundus strips, as previously observed in respectively mouse anococcygeus (Wallace et al., 1999) and dog cerebral arteries (Asano et al., 1996). Thapsigargin nor ryanodine or 2-APB influenced the NaHS-induced relaxation, arguing against the involvement of calcium mobilization/sequestration at the level of the sarcoplasmatic reticulum in the NaHS-induced relaxation.

Relaxation in smooth muscle cells can only be induced through a decrease in the intracellular calcium concentration, and/or through a desensitization of the contractile proteins to the intracellular calcium concentration. Calcium desensitization is accomplished by the activation of myosin light chain phosphatase (MLCP), leading to a higher degree of dephosphorylation of myosin light chain (MLC) and subsequently relaxation. The phosphatase inhibitor calyculin-A (1 µM) was reported to induce contraction of canine antral smooth muscle together with MLC phosphorylation but without increasing the intracellular calcium concentration (Ozaki et al., 1991). Similarly, calyculin-A (1 µM) per se caused contraction of mouse gastric fundus strips, illustrating that MLCP is tonically active in this tissue. Although Ozaki et al. (1991) mention in their discussion that calyculin-A is much more effective versus phosphatase type 1 than versus phosphatase type 2, Ishihara et al. (1989a) reported similar $IC_{50}$ values of calyculin-A versus type 1 and type 2A phosphatase (0.5-2 nM). Phosphatase type 1c is part of the physiologically active MLCP in smooth muscle cells (Somlyo and Somlyo, 2003; Ito et al., 2004) so that the effects of calyculin-A in smooth

muscle can probably be ascribed to interference with phosphatase type 1. In intact smooth muscle preparations, higher concentrations than the low nM range are required to induce effects. In guinea pig taenia caeci e.g., calyculin-A induced contraction with an $EC_{50}$ of 210 nM and an $E_{max}$ at 3 µM (Ishihara et al., 1989b). In the mouse anococcygeus, calyculin-A (1 µM) nearly abolished relaxations induced by endogenous NO, liberated by electrical field stimulation; this implies that calcium desensitization by activation of MLCP is the major cellular mechanism underlying nitrergic relaxation in this tissue (Gibson et al., 2003). Similarly, calyculin-A (1 µM) reduced the NaHS-induced relaxations in mouse gastric fundus, suggesting that activation of MLCP is also involved in the relaxant effect of NaHS.

An important pathway controlling smooth muscle contraction is the RhoA/Rho-kinase pathway, acting by inhibition of MLCP through phosphorylation of the MYPT1 unit and thus promoting calcium sensitization and contraction (Somlyo and Somlyo, 2003). Inhibition of the RhoA/Rho-kinase pathway will thus lead to relaxation. The Rho-kinase inhibitor Y-27632 lowered the basal tone of the mouse gastric strips, as was also observed in the rat internal anal sphincter by Patel and Rattan (2006), illustrating that the RhoA/Rho-kinase pathway is tonically active in the mouse gastric fundus under basal conditions. Büyükafşar and Levent (2003) have shown before that Y-27632 concentration-dependently relaxed carbachol-contracted mouse gastric fundus illustrating that also muscarinic receptor activation in this tissue activates RhoA/Rho-kinase. In blood vessels, it was shown that the vasorelaxant effect of NO can be mediated through inhibition of the RhoA/Rho-kinase pathway, and thus subsequent increased activity of MLCP (Sauzeau et al., 2000). To investigate whether the activation of MLCP by NaHS in the mouse gastric fundus, as revealed in the experiments with calyculin-A, might also be related to suppression of the RhoA/Rho-kinase pathway, we inhibited the latter with Y-27632; but in this condition, the relaxant effect of NaHS was maintained excluding this possibility. Our results are similar to those reported for the relaxant effect of the β-receptor agonist isoprenaline in guinea-pig tracheal smooth muscle, that was abolished by calyculin-A but not influenced by Y-27632; also in this study it was concluded that isoprenaline acts through activation of MLCP, without affecting the RhoA/Rho-kinase pathway (Oguma et al., 2006).

In conclusion, we show that NaHS (donor of $H_2S$) is able to relax $PGF_{2\alpha}$-contracted mouse gastric fundus strips. The mechanism of action of the NaHS-induced relaxation in mouse gastric fundus is different from that in the cardiovascular system as the relaxation induced by NaHS does not involve activation of $K_{ATP}$ channels. The results obtained in this study suggest that in the mouse gastric fundus, $H_2S$ causes relaxation at least partially via activation of myosin light chain phosphatase; this seems not related to an inhibition of the RhoA/Rho-kinase pathway.

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# Chapter VII

# MECHANISMS OF ACTION OF HYDROGEN SULFIDE IN RELAXATION OF MOUSE DISTAL COLONIC SMOOTH MUSCLE

**Based on**

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# Chapter VII Mechanisms of action of hydrogen sulfide in relaxation of mouse distal colonic smooth muscle

## *VII.1 Abstract*

Hydrogen sulfide ($H_2S$) has been suggested as a gaseous neuromodulator in mammals. The aim of this study was to examine the influence of $H_2S$ on contractility in mouse distal colon. The effect of sodium hydrogen sulfide (NaHS; $H_2S$ donor) on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-contracted circular muscle strips of mouse distal colon was investigated. In addition, tension and cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in the mouse distal colon strips were measured simultaneously in the presence of NaHS. NaHS caused concentration-dependent relaxation of the pre-contracted mouse distal colon strips. The NaHS-induced relaxation was not influenced by the $K^+$ channels blockers glibenclamide, apamin, charybdotoxin, barium chloride and 4-aminopyridine. The relaxation by NaHS was also not influenced by the nitric oxide inhibitor L-NAME, by the soluble guanylate cyclase respectively adenylate cyclase inhibitors ODQ and SQ 22536, by the nerve blockers capsazepine, ω-conotoxin and tetrodotoxin or by several channel and receptor blockers (ouabain, nifedipine, 2-aminoethyl diphenylborinate, ryanodine and thapsigargin). The initiation of the NaHS-induced relaxation was accompanied by an increase in $[Ca^{2+}]_{cyt}$, but once the relaxation was maximal and sustained, no change in $[Ca^{2+}]_{cyt}$ was measured. This calcium desensitization is not related to the best known calcium desensitizing mechanism as the myosin light chain phosphatase (MLCP) inhibitor calyculin-A and the Rho-kinase inhibitor Y-27632 had no influence.

We conclude that NaHS caused concentration-dependent relaxations in mouse distal colon not involving the major known $K^+$ channels and without a change in $[Ca^{2+}]_{cyt}$. This calcium desensitization is not related to inhibition of Rho-kinase or activation of MLCP.

## *VII.2 Introduction*

Hydrogen sulfide ($H_2S$) has been suggested as a third gaseous signal molecule and neuromodulator in mammals, besides nitric oxide (NO) and carbon monoxide (CO). $H_2S$ can be produced endogenously in mammalian tissues from L-cysteine by two enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Stipanuk and Beck, 1982). Besides the detection of CBS mRNA and/or CSE mRNA and $H_2S$ generation in the brain (Abe and Kimura, 1996; Linden et al., 2008) and in cardiovascular tissues (Zhao et al., 2001), CBS and CSE mRNA as well as $H_2S$ generation were reported in guinea pig ileum (Hosoki et al., 1997). Both CBS and CSE have also been shown in human and guinea-pig colon myenteric neurons (Schicho et al., 2006).

In the cardiovascular system, $H_2S$ induces vasorelaxation at least partially due to activation of ATP-dependent $K^+$ channels ($K_{ATP}$ channels) (Cheng et al., 2004; Tang et al., 2005; Webb et al., 2008; Zhao et al., 2001). In the gastrointestinal (GI) tract, $H_2S$ inhibits acetylcholine-induced (Hosoki et al., 1997) and electrically induced contractions of the guinea-pig ileum; the latter effect was unaffected by the $K_{ATP}$ channel inhibitor glibenclamide (Teague et al., 2002). Similarly, we recently reported that the $H_2S$-induced relaxation of mouse gastric fundus is not sensitive to glibenclamide but appears at least partially related to the activation of myosin light chain phosphatase and calcium desensitization (Dhaese and Lefebvre, 2009). In mouse colon, CSE but not CBS was shown to be present in myenteric neurons, and the intact colonic muscle layer containing the myenteric plexus generated detectable levels of $H_2S$ (Linden et al., 2008). It is thus important to know the effect and mechanism of action of $H_2S$ on the distal colon circular muscle layer, as $H_2S$ might play a physiological role in smooth muscle regulation; a role for $H_2S$ as oxygen sensor, as suggested in vascular smooth muscle by Olson et al. (2006), can not be excluded in the distal colon. Additionally, the colonic muscle layer might be reached by luminally produced $H_2S$ in pathological conditions. Indeed, ulcerative colitis is associated with an increased fecal $H_2S$ production (Levine et al., 1998).

In the present experiments, we therefore investigated the influence and mechanism of action of $H_2S$ on pre-contracted mouse distal colon circular muscle strips. Recently, while finishing our study, Gallego et al. (2008) reported that $H_2S$ inhibited spontaneous motor complexes in perfused 3 cm segments of mouse colon, an effect reduced by the small conductance $Ca^{2+}$-dependent $K^+$ channel ($SK_{Ca}$ channel) blocker apamin. In the present study however, we show that in mouse distal colon strips apamin was not effective versus $H_2S$, nor were inhibitors of other major known $K^+$ channels and ion channels; the sustained $H_2S$-induced relaxation in mouse distal colon strips was not accompanied by a change in $[Ca^{2+}]_{cyt}$.

## *VII.3 Material and Methods*

### VII.3.1 Animals

Male Swiss (SPF Orl) mice (7-12 weeks, 26-46 g, n = 91) were purchased from Janvier, Le Genest St-Isle, France and had free access to water and commercially available chow. All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health sciences at Ghent university.

### VII.3.2 Tissue preparation

Animals were sacrificed by cervical dislocation. The distal colon was taken above the pelvic brim as an approximately four cm segment and was put in Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The segment of distal colon was opened along the mesenteric border and pinned mucosa side up. The mucosa was removed by sharp dissection under a microscope and four full-thickness muscle strips (3 × 5 mm) were cut along the circular axis. After a silk thread (USP 4/0) was attached to both ends of the muscle strips, strips were mounted along the circular axis in 5 ml organ baths. The organ baths contained aerated (5% $CO_2$ in $O_2$) Krebs solution maintained at 37°C. Changes in isometric tension were measured using MLT 050/D force transducers (ADInstruments) and recorded on a PowerLab/8sp data recording system (ADInstruments) with Chart software.

### VII.3.3 Measurement of contractile tension

NaHS, the experimental source of $H_2S$ was used. Preliminary experiments showed that cumulative administration of NaHS yielded less pronounced relaxant responses than isolated administration with a threshold concentration varying between strips; this cumulative concentration-response curve was not reproducible nor was the response to a single dose of NaHS within the same strip. After an equilibration period of 1 hour at a preliminary determined optimal pre-load of 0.4 g with refreshing of the Krebs solution every 10 min, strips were pre-contracted with 3 µM $PGF_{2\alpha}$. 15 Min after its administration, each of the four strips was given a single concentration of NaHS (0.1, 0.3, 1 or 3 mM) and the effect was followed for 10 min. From the isolated concentration-response curve of NaHS, a concentration of 1 mM NaHS was chosen to investigate the mechanism of action of NaHS in mouse distal colon.

In these experiments, inhibitors were added after 1 hour of equilibration at 0.4 g. The drugs were allowed to incubate for 30 min before inducing pre-contraction with 3 µM $PGF_{2\alpha}$ followed by the administration of 1 mM NaHS, again examining the effect for 10 min. Control

strips that received the solvent of these drugs (aqua, ethanol or DMSO), were evaluated in parallel. When studying the influence of calyculin-A versus NaHS, an additional series was performed using 80 mM KCl as the contractile agent instead of $PGF_{2\alpha}$. When we examined the influence of glibenclamide, we used carbachol instead of $PGF_{2\alpha}$ to induce pre-contraction as preliminary experiments showed that in 3 out of 4 strips the $PGF_{2\alpha}$-induced contraction was abolished after incubation with glibenclamide; this was previously observed in rat arteries (Delaey and Van de Voorde, 1997). In parallel, carbachol-precontracted control strips receiving ethanol (solvent of glibenclamide) were evaluated.

### VII.3.4 Simultaneous measurement of contractile tension and cytosolic calcium concentration

After removal of the mucosa, thin sheets of full-thickness muscle of distal colon (5 x 5 mm) were cut along the circular axis. The tissue was clipped at both ends with titanium clips and incubated for 4 h at 37°C in physiological salt solution (PSS; Salomone et al., 1995; composition in mM: NaCl 122, KCl 5.9, $MgCl_2$ 1.25, $CaCl_2$ 1.25, $NaHCO_3$ 15 and glucose 10.1), containing 5 µM of the fluorescent calcium indicator fura-2 acetoxymethyl ester (fura-2 AM) and 0.05% cremophor EL. The fura-2 loaded tissues were then transferred to fresh PSS, washed for 5 min to remove uncleaved fura-2 AM, and mounted in a 3 ml cuvette filled with PSS kept at 37°C and aerated with 5% $CO_2$ in $O_2$. The cuvette was part of a spectrofluorimeter (Quantamaster QM-2000-4, Photon Technology International (PTI)). Changes in isometric tension were measured using an isometric force transducer (Grass FT03). The flat rectangular area of the tissue between the clips was positioned in the cuvette with the luminal side (i.e. circular muscle layer) towards the excitation light beam. Changes in cytosolic calcium concentration were measured simultaneously with the contractile tension by alternatively illuminating the circular muscle side with two excitation wavelengths (340 and 380 nm). The emitted fluorescence light from the circular muscle surface was collected by a photomultiplier through an emission monochromator set at 510 nm. The fluorescence signals and the contractile tension were recorded using the data acquisition hardware (PTI) and the data recording software FeliX$^{TM}$ (PTI).

After being mounted in the cuvette, the muscle sheet was equilibrated for 30 min at a pre-load of 0.4 g, meanwhile being perfused with PSS at a flow rate of 8 ml/min. Subsequently, the perfusion was stopped and 3 µM $PGF_{2\alpha}$ was added and left in contact with the tissues for 16 min. 6 Min after the addition of $PGF_{2\alpha}$, nitric oxide (NO; 10 µM) or NaHS (1 mM) was administered in 1/3 of the tissues respectively.

At the end of each experiment, the fura-2 $Ca^{2+}$ signal was calibrated. The maximal ratio ($R_{max}$) was measured in calcium saturating medium by adding ionomycin (20 µM) in high KCl

solution (100 mM), while the minimal ratio ($R_{min}$) was obtained in calcium-free medium in the presence of EGTA (8 mM). The autofluorescence was measured at 340 and at 380 nm by quenching the fura-2 signal with $MnCl_2$ (5 mM) and was subtracted from all values.

### VII.3.5 Data analysis and statistics

To evaluate contractile activity , the area under the curve (AUC; g.s; measured from the zero baseline) was calculated. Measurements were done for 10 min just before adding the inhibitor or solvent, from 21 to 30 min after adding the inhibitor or solvent and from 6 to 15 min after adding the contractile agent. To measure the inhibitory responses to NaHS, the AUC (again from the zero baseline) from 1 to 10 min after adding NaHS was calculated and subtracted from the AUC of the pre-contraction, yielding the area above the curve or the relaxing effect. This relaxing effect was then expressed as % of the AUC of the pre-contraction. The AUCs were measured from the zero baseline and thus integrated passive tone (= 0.4 g) and $PGF_{2\alpha}$-induced contractile activity. This was done because the troughs of $PGF_{2\alpha}$-induced phasic activity sometimes reached levels below the imposed passive tone of 0.4 g (see for example bottom trace of Fig. VII.1A), making it impossible to use active $PGF_{2\alpha}$-induced contractility as a reference for the inhibitory responses to NaHS.

The cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) was calculated according to the equation of Grynkiewicz et al. (1985). $PGF_{2\alpha}$ induced an increase in $[Ca^{2+}]_{cyt}$ versus basal. The mean $[Ca^{2+}]_{cyt}$ was determined for the time interval 1-6 min after adding $PGF_{2\alpha}$ and used as a reference. The mean $[Ca^{2+}]_{cyt}$ in the time intervals 0-1 min, 4-5 min and 9-10 min after adding NaHS or NO, or in corresponding time intervals in the control tissues was calculated and the difference versus the $PGF_{2\alpha}$-induced reference was determined; these values were expressed as % of the $PGF_{2\alpha}$-induced increase in $[Ca^{2+}]_{cyt}$.

All results are expressed as means ± S.E.M. and *n* refers to the number of strips obtained from different animals unless indicated otherwise. In the four groups of parallel strips receiving 0.1, 0.3, 1 or 3 mM NaHS, the AUC for the level of pre-contraction after adding $PGF_{2\alpha}$ was compared by a one-way ANOVA followed by Bonferroni multiple comparison *t*-test. In the solvent- or inhibitor-treated strips, the AUC for the spontaneous activity before and after adding the solvent or inhibitor and for the level of pre-contraction after adding the contractile agent were compared by a repeated measures ANOVA followed by Bonferroni multiple comparison *t*-test. The level of pre-contraction and the inhibitory responses to NaHS obtained in parallel strips in the presence of inhibitors versus in the presence of solvent (aqua, ethanol or DMSO) were compared by an unpaired Student's *t*-test (2 groups) or an one-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparison *t*-test (more than 2 groups). The changes in mean $[Ca^{2+}]_{cyt}$ at the different time intervals after

adding NaHS or NO or at the corresponding time intervals in the control strips were compared by an one-way ANOVA followed by a Bonferroni multiple comparison *t*-test. $P < 0.05$ was considered to be statistically significant (GRAPHPAD, San Diego, CA, USA).

### VII.3.6 Drugs used

Apamin, charybdotoxin, ω-conotoxin GVIA, ryanodine, tetrodotoxin (obtained from Alomone Labs), fura-2 AM, (R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide (Y-27632) dihydrochloride monohydrate (from Calbiochem), 4-aminopyridine (4-AP), 2-aminoethyl diphenylborinate (2-APB), capsazepine, cremophor EL, EGTA, glibenclamide, ionomycin calcium salt, $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME), manganese chloride tetrahydrate, nifedipine, ouabain octahydrate, prostaglandin F2$\alpha$ tris salt, sodium hydrosulfide hydrate, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536), thapsigargin (from Sigma-Aldrich), barium chloride, potassium chloride (from Merck), carbachol (from Fluka AG), calyculin-A, 1H[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (from Tocris Cookson). All drugs were dissolved in de-ionized water except 2-APB, capsazepine, glibenclamide, ionomycin calcium salt, nifedipine, ODQ, ryanodine and thapsigargin which were dissolved in ethanol and calyculin-A which was dissolved in DMSO. Solutions of NaHS were prepared fresh on the morning of each experiment and kept on ice. Saturated NO solution was prepared from gas (Air Liquide, Belgium) as described by Kelm and Schrader (1990).

## *VII.4 Results*

### VII.4.1 Effect of NaHS on contractility

At the imposed passive tone, only in 2 out of 4 mouse distal colon strips tested a response, which consisted of a decrease in tone, was observed after NaHS (1 mM) administration. Therefore, the responses to NaHS were investigated in the presence of $PGF_{2\alpha}$. $PGF_{2\alpha}$ induced (an increase in) phasic activity or an increase of tone plus superimposed phasic activity (Fig. VII.1A). In the four groups of parallel strips receiving 0.1, 0.3, 1 or 3 mM NaHS, the AUC for contractile activity increased from 234 ± 6, 230 ± 6, 239 ± 15 and 243 ± 3 g.s before administration of $PGF_{2\alpha}$ to 300 ± 24, 310 ± 39, 383 ± 72 and 332 ± 13 g.s (n = 6 or 7) after its administration; the AUC after $PGF_{2\alpha}$ did not significantly differ between the four groups.

In the $PGF_{2\alpha}$-pre-contracted mouse distal colon circular muscle strips, NaHS induced concentration-dependent relaxations (Fig. VII.1). At higher concentrations, phasic activity was often completely suppressed in the course of the 10 min incubation time, so that responses were measured as the decrease in overall mechanical activity (AUC before NaHS minus that after NaHS). The concentration-response curve of NaHS yielded a mean $E_{max}$ and $EC_{50}$ of respectively 51 ± 6 % and 444 ± 71 µM (n = 7) for the NaHS-induced relaxation.

A concentration of 1 mM NaHS was chosen to investigate the mechanism of action of NaHS in the mouse distal colon strips, as this concentration induced the largest relaxation; at a higher concentration, the NaHS-induced relaxation started to decline (Fig. VII.1B).

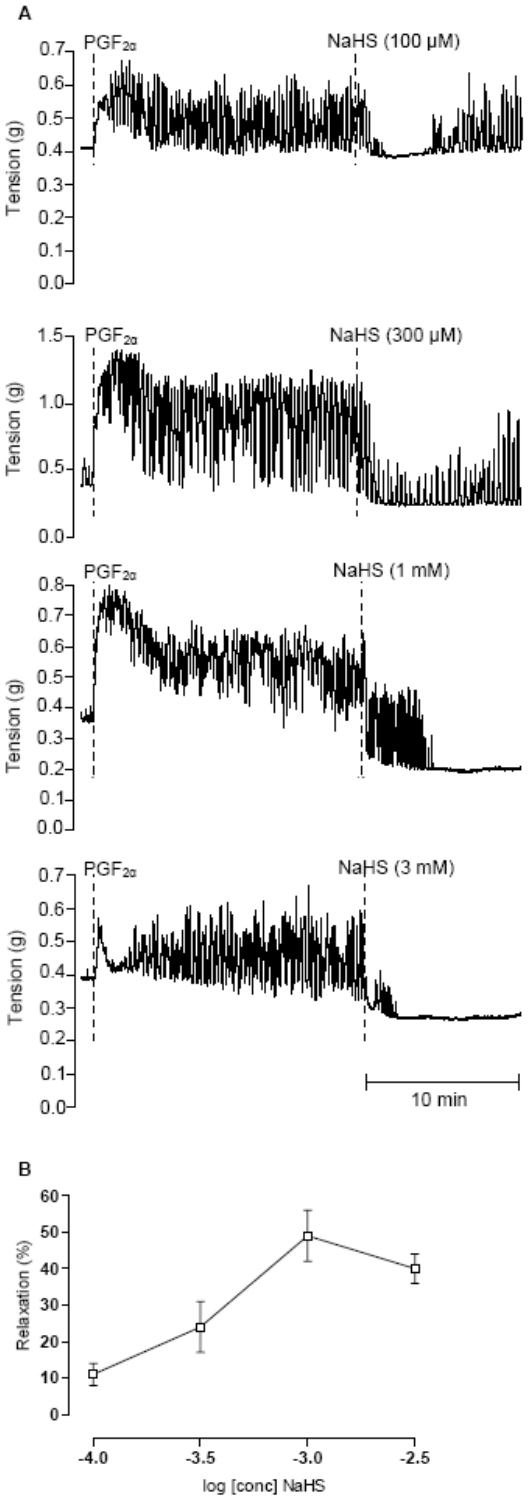



**Figure VII.1**

(A) Representative traces showing the relaxation induced by respectively 100 µM, 300 µM, 1 mM and 3 mM NaHS in pre-contracted (3 µM $PGF_{2\alpha}$) mouse distal colon circular muscle strips. (B) Concentration-response curve of NaHS (100 µM – 3 mM) in pre-contracted (3 µM $PGF_{2\alpha}$) mouse distal colon circular muscle strips. Means ± S.E.M. of n = 7 are shown.

## VII.4.2 Influence of channel, receptor, nerve and enzyme blockers on NaHS-induced relaxation

In order to try to elucidate the mechanism of action of NaHS in the mouse distal colon strips, the influence of a large series of inhibitors was tested. The full list of all tested inhibitors is shown in Tables VII.1 and VII.2. These inhibitors were used in concentrations shown to inhibit their target in the literature.

**Table VII.1: Contractile activity of mouse distal colon strips after pre-contraction with $PGF_{2\alpha}$ or carbachol (in the set with glibenclamide) and after application of NaHS**

| | After pre-contraction | After NaHS | |
|---|---|---|---|
| | AUC | AUC | R |
| Control ethanol | 515 ± 107 | 163 ± 19 | 64 ± 6 |
| Glibenclamide (10 µM) | 433 ± 109 | 161 ± 19 | 54 ± 7 |
| Control aqua | 358 ± 38 | 150 ± 22 | 55 ± 8 |
| Apamin (500 nM) | 327 ± 58 | 159 ± 20 | 48 ± 6 |
| Charybdotoxin (100 nM) | 325 ± 33 | 166 ± 16 | 47 ± 6 |
| Control aqua | 409 ± 49 | 167 ± 19 | 57 ± 7 |
| Apamin (500 nM)+Charybdotoxin (100 nM) | 541 ± 153 | 164 ± 23 | 66 ± 5 |
| Control aqua | 325 ± 33 | 126 ± 32 | 58 ± 15 |
| 4-AP (5 mM) | 551 ± 112 | 173 ± 37 | 68 ± 6 |
| Control aqua | 357 ± 37 | 159 ± 3 | 55 ± 4 |
| Bariumchloride (30 µM) | 327 ± 47 | 194 ± 12 | 39 ± 4 |
| Control ethanol | 242 ± 79 | 135 ± 27 | 39 ± 9 |
| Nifedipine (3 nM) | 250 ± 40 | 165 ± 47 | 41 ± 13 |
| Control aqua | 367 ± 53 | 161 ± 32 | 51 ± 13 |
| Ouabain (100 µM) | 267 ± 18 | 172 ± 22 | 38 ± 9 |
| Control ethanol | 381 ± 41 | 160 ± 15 | 56 ± 5 |
| Ryanodine (10 µM) | 310 ± 80 | 165 ± 22 | 50 ± 7 |
| Control ethanol | 432 ± 100 | 167 ± 23 | 54 ± 11 |
| 2-APB (50 µM) | 246 ± 36 | 116 ± 14 | 48 ± 12 |
| Control ethanol | 274 ± 28 | 130 ± 12 | 52 ± 1 |
| Thapsigargin (1 µM) | 276 ± 16 | 141 ± 22 | 48 ± 9 |

Contractile activity was analysed from 6 to 15 min after adding the contractile agent and from 1 to 10 min after adding NaHS. AUC represents the area under the curve (g.s); R represents the NaHS-induced relaxation (AUC after precontraction-AUC after NaHS/AUC after precontraction x 100; %). Means ± S.E.M. of n = 4-6 are shown, except for the control strips for 4-AP: n = 3 and the control strips for Bariumchloride: n = 2.

**Table VII.2: Contractile activity of mouse distal colon strips after pre-contraction with $PGF_{2\alpha}$ or KCl (in one of the 2 sets with calyculin-A) and after application of NaHS**

| | After pre-contraction | After NaHS | |
|---|---|---|---|
| | AUC | AUC | R |
| Control aqua | 319 ± 35 | 169 ± 18 | 47 ± 3 |
| Tetrodotoxin (3 µM) | 412 ± 82 | 201 ± 16 | 48 ± 6 |
| Control aqua | 272 ± 32 | 171 ± 14 | 38 ± 8 |
| ω-Conotoxin (3 µM) | 320 ± 11 | 124 ± 22 | 58 ± 7 |
| Control ethanol | 287 ± 18 | 166 ± 23 | 42 ± 7 |
| Capsazepine (3 µM) | 233 ± 21 | 158 ± 18 | 30 ± 10 |
| Control ethanol | 356 ± 43 | 160 ± 26 | 51 ± 11 |
| L-NAME (300 µM) | 420 ± 82 | 169 ± 30 | 47 ± 13 |
| ODQ (10 µM) | 364 ± 49 | 148 ± 13 | 56 ± 9 |
| Control ethanol | 383 ± 64 | 184 ± 48 | 53 ± 5 |
| ODQ (10 µM) + Apamin (500 nM) | 349 ± 30 | 126 ± 25 | 62 ±10 |
| Control aqua | 333 ± 39 | 157 ± 16 | 51 ± 10 |
| SQ 22536 (100 µM) | 363 ± 30 | 149 ± 11 | 58 ± 5 |
| Control ethanol | 330 ± 28 | 156 ± 12 | 51 ± 7 |
| SQ 22536 (100 µM) + ODQ (10 µM) | 355 ± 69 | 138 ± 27 | 53 ± 15 |
| Control DMSO (†) | 442 ± 137 | 160 ± 32 | 55 ± 11 |
| Calyculin-A (1 µM; †) | 1013 ± 223 | 566 ± 125 | 44 ± 1 |
| Control DMSO (††) | 524 ± 34 | 275 ± 9 | 46 ± 5 |
| Calyculin-A (1 µM; ††) | 989 ± 129 ** | 591 ± 102 | 42 ± 4 |
| Control aqua | 513 ± 126 | 215 ± 16 | 48 ± 11 |
| Y-27632 (10 µM) | 311 ± 31 | 212 ± 23 | 32 ± 6 |

Contractile activity was analysed from 6 to 15 min after adding the contractile agent and from 1 to 10 min after adding NaHS. AUC represents the area under the curve (g.s); R represents the NaHS-induced relaxation (AUC after precontraction-AUC after NaHS/AUC after precontraction x 100; %). Means ± S.E.M. of n = 4-6 are shown, except for the control strips for SQ 22536: n = 3 and the control strips for ODQ + Apamin: n = 2. (†): series of calyculin-A in $PGF_{2\alpha}$-precontracted strips; (††): series of calyculin-A in KCl-precontracted strips. ** $P < 0.01$: in the presence of inhibitor versus in the presence of solvent (unpaired Student's *t*-test).

Only two inhibitors influenced the spontaneous activity of the mouse distal colon strips. Calyculin-A induced a significant increase of the AUC from 210 ± 18 g.s during the 10 min before its incubation to 1143 ± 198 g.s ($n = 4$, $P < 0.001$) during the 21$^{st}$ to 30$^{th}$ min after its administration, due to a fully tonic response (Fig. VII.2). Y-27632 induced a moderate reduction in AUC (208 ± 15 g.s after incubation with Y-27632 versus 261 ± 15 g.s before incubation with Y-27632, $n = 4$, $P < 0.001$).

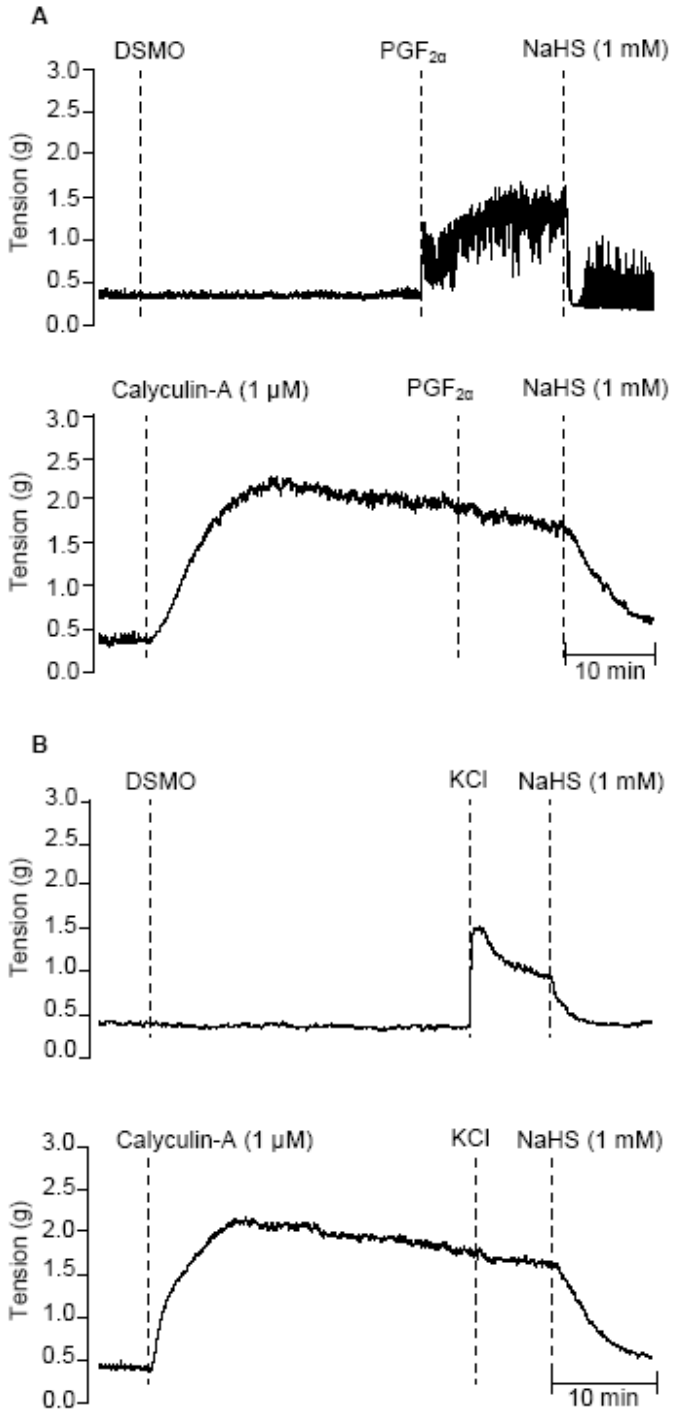



**Figure VII.2**

Representative traces showing the relaxation induced by 1 mM NaHS in mouse distal colon circular muscle strips pre-contracted with 3 μM $PGF_{2\alpha}$ (A) or 80 mM KCl (B), in the presence of DMSO (upper trace) or 1 μM calyculin-A (bottom trace).

In the presence of most inhibitors the response to $PGF_{2\alpha}$ was comparable to that in the controls. However, in the strips treated with glibenclamide, preliminary experiments showed that in 3 out of 4 strips the $PGF_{2\alpha}$-induced contraction was abolished, even when higher concentrations than 3 μM $PGF_{2\alpha}$ were applied; we therefore used carbachol instead of

$PGF_{2\alpha}$ in the presence of glibenclamide. Carbachol induced an elevation of tone with superimposed phasic activity (Fig. VII.3). Preliminary experiments had also learned that nifedipine and Y-27632 reduced the response to $PGF_{2\alpha}$, therefore concentrations higher than 3 µM were applied to induce a comparable level of pre-contraction as in the control strips (Tables VII.1 and VII.2). In the calyculin-A-treated strips, where calyculin-A had induced a merely tonic contraction, $PGF_{2\alpha}$ was not able to further raise the contraction level nor to induce phasic activity, this in contrast to the DMSO-treated control strips where $PGF_{2\alpha}$ raised tonic and phasic activity (Fig. VII.2A); the AUC in the calyculin-A-treated strips was clearly higher than the AUC induced by $PGF_{2\alpha}$ in the DMSO-treated control strips although this did not reach significance (Table VII.2). An additional series was therefore performed in which KCl, in the highest effective concentration of 80 mM as determined from preliminary experiments, was used to pre-contract the DMSO-treated control strips, as KCl also primarily induces a tonic response (Fig. VII.2B) and in order to try matching the AUC in the calyculin-A-treated and the control strips. The AUC of the contractile response to KCl in the control strips was even significantly lower than the AUC of the contraction in the calyculin-A-treated strips, where addition of KCl on top of calyculin-A was not able to further enhance contractile activity (Fig. VII.2B; Table VII.2).

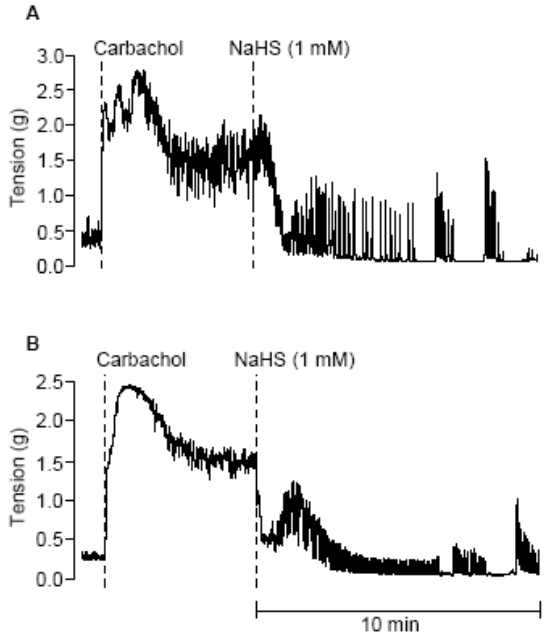



**Figure VII.3**

Representative traces showing the relaxation induced by 1 mM NaHS in pre-contracted (3 µM carbachol) mouse distal colon circular muscle strips, in the presence of ethanol (A) or 10 µM glibenclamide (B).

None of the inhibitors listed in Tables VII.1 and VII.2 had a significant influence on the NaHS-induced relaxation. In calyculin-A-treated strips, the response to NaHS was not different from that in control strips, either when these were contracted by $PGF_{2\alpha}$ or by KCl.

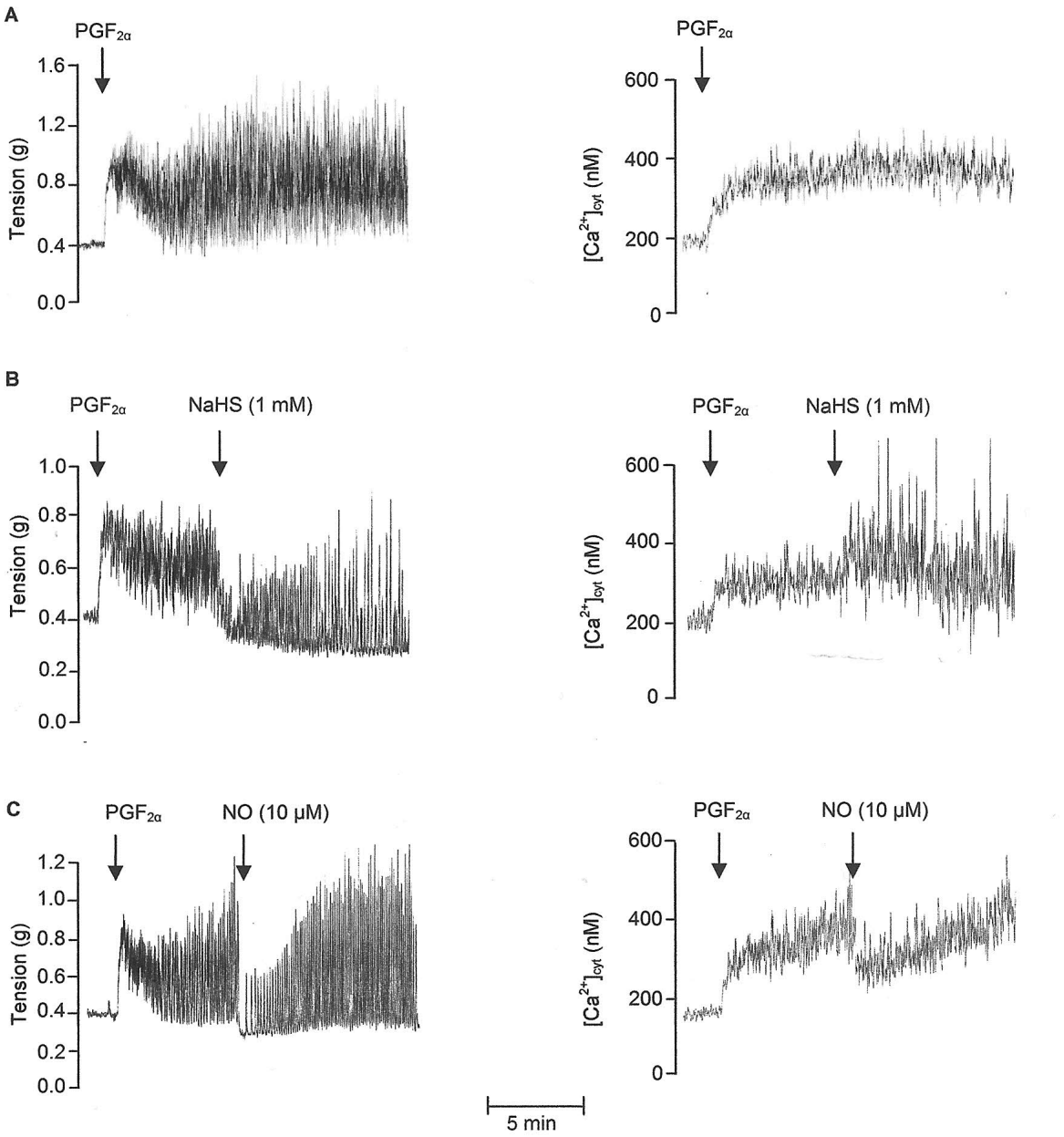
### VII.4.3 Effect of NaHS on cytosolic calcium concentration

We simultaneously measured tension and cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in the mouse distal colon strips. The mean resting $[Ca^{2+}]_{cyt}$ was 181 ± 10 nM (n = 22). Administration of $PGF_{2\alpha}$ induced an increase in tension/phasic activity, that stabilized within 5 min after its administration; $PGF_{2\alpha}$ induced an elevation of $[Ca^{2+}]_{cyt}$ to 331 ± 19 µM (n = 22) as measured for the 2$^{nd}$ to 6$^{th}$ min after its administration.

In control strips, the contractile response to $PGF_{2\alpha}$ remained stable from 6 to 16 min after its administration; in this period the $[Ca^{2+}]_{cyt}$ at 0-1 min, and to a lesser extent at 4-5 and 9-10 min, was slightly increased (Figs. VII.4A and VII.5A).

When NaHS was administered, relaxation was initiated which progressively evolved to a sustained level (Fig. VII.4B). The $[Ca^{2+}]_{cyt}$ however significantly increased immediately after adding NaHS (increase of 38 ± 9 % versus 11 ± 3 % in control strips, n = 7, $P < 0.01$) (Figs. VII.4B and VII.5B). At 4-5 and 9-10 min after administration of NaHS, no differences in $[Ca^{2+}]_{cyt}$ between NaHS-treated and control strips were observed.

The quick relaxation induced by NO was clearly accompanied by an immediate significant ($P < 0.001$) reduction in $[Ca^{2+}]_{cyt}$ of 39 ± 3 % (n = 7-8). The NO-induced relaxation and decrease in $[Ca^{2+}]_{cyt}$ were transient (Figs. VII.4C and VII.5C).




## Figure VII.4

Representative traces showing the tension and the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in control (A), NaHS (1 mM)-treated (B) and NO (10 µM)-treated (C) pre-contracted (3 µM $PGF_{2\alpha}$) mouse distal colon circular muscle strips.

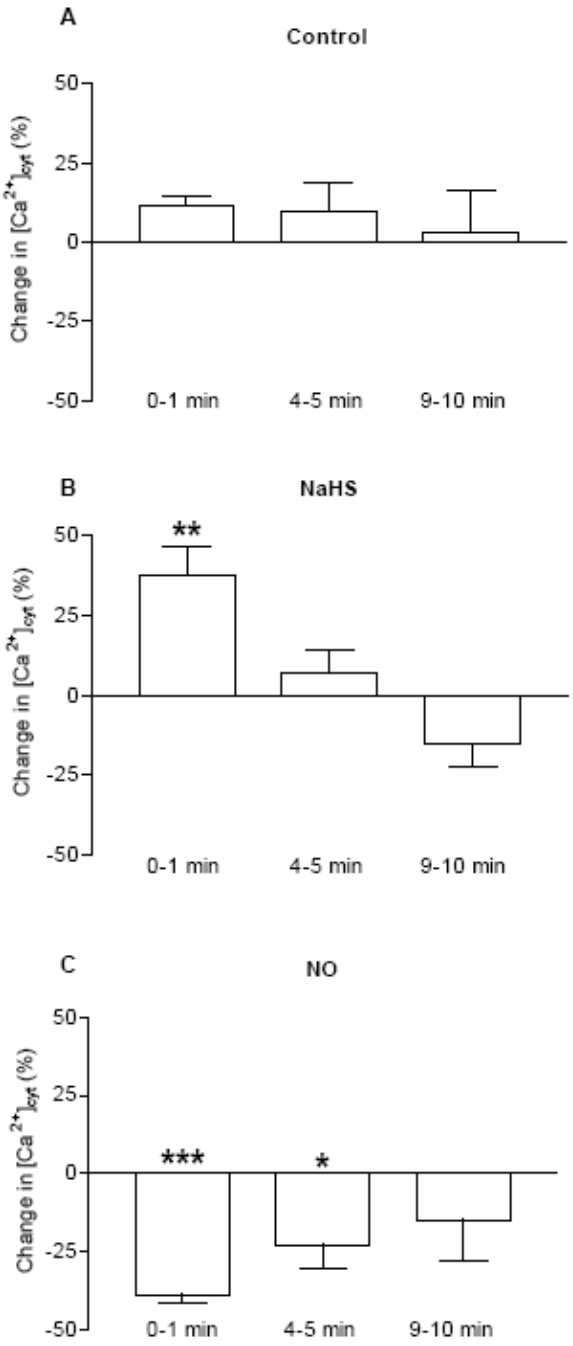



**Figure VII.5**

The change in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in the time intervals 0-1 min, 4-5 min and 9-10 min after administration of NaHS (1 mM) (B) or NO (10 µM) (C) or in corresponding time intervals (control; A) in pre-contracted (3 µM $PGF_{2\alpha}$) mouse distal colon circular muscle strips. Values are represented as % of $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_{cyt}$. The positive and negative values in the y-axis represent respectively increases and decreases in $[Ca^{2+}]_{cyt}$. Means ± S.E.M. of n = 7-8 are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: NaHS- or NO-treated versus control (one-way ANOVA followed by Bonferroni multiple comparison *t*-test).

## *VII.5 Discussion*

The aim of this study was to examine the influence and mechanism of action of $H_2S$ on contractility in mouse distal colon. In pre-contracted mouse distal colon circular muscle strips, $H_2S$-donor NaHS induced concentration-dependent relaxations with an $EC_{50}$ of 444 ± 71 µM. This value is somewhat higher than the previously reported $EC_{50}$ values for NaHS, ranging from 31 to 261 µM, in GI tissues of other species (Gallego et al., 2008; Hosoki et al., 1997; Teague et al., 2002). Also rat vascular tissues seem more sensitive to NaHS with $EC_{50}$ values between 104 and 136 µM (Cheng et al., 2004; Zhao and Wang, 2002). However, in pre-contracted mouse gastric fundus strips, we found that NaHS induced relaxations with an $EC_{50}$ of 514 ± 36 µM (Dhaese and Lefebvre, 2009). In mouse bronchial rings, an $EC_{50}$ above 300 µM was observed for NaHS (Kubo et al., 2007a). These observations suggest that a species difference could exist with mouse smooth muscle being less sensitive to NaHS. Still, an $EC_{50}$ of 121 and 150 µM was reported for the NaHS-induced effects in isolated segments of mouse colon and jejunum (Gallego et al., 2008).

The non-effect of tetrodotoxin and L-NAME on spontaneous contractile activity corresponds with previous data reported in mouse colon strips (Porcher et al., 2004; Dhaese et al., 2008) and illustrates that there is no prevailing tonic neurogenic nitrergic inhibition of mouse distal colon under the conditions used. The inhibitor of myosin light chain phosphatase (MLCP) calyculin-A induced tonic contraction illustrating that MLCP is tonically active in this tissue, possibly under the inhibitory control of Rho-kinase as the Rho-kinase inhibitor Y-27632 reduced spontaneous contractile activity.

NaHS seems to act at the muscular level in mouse distal colon strips since the $Na^+$ channel blocker tetrodotoxin and the N-type voltage dependent $Ca^{2+}$ channel blocker ω-conotoxin did not influence NaHS-induced relaxation. Additionally, the transient receptor potential vanilloid type 1 (TRPV1) blocker capsazepine, tested since TRPV1 receptors were reported to be involved in the prosecretory effect of NaHS in human and guinea-pig colon (Schicho et al., 2006) and in the contractile effect of NaHS in the rat urinary bladder (Patacchini et al., 2004), did not influence NaHS-induced relaxation in the mouse distal colon strips.

$K_{ATP}$ channels are reported to play a role in the NaHS-induced relaxation in cardiovascular tissues (Cheng et al., 2004; Webb et al., 2008; Zhao et al., 2001), and recently, also in human and rat colonic strips (Gallego et al., 2008). Glibenclamide, a blocker of the $K_{ATP}$ channels, however did not inhibit the NaHS-induced relaxation in mouse distal colon strips, corresponding with the non-effect of glibenclamide on the NaHS-induced inhibition in mouse aorta (Kubo et al., 2007b), mouse bronchial rings (Kubo et al., 2007a), mouse gastric fundus (Dhaese and Lefebvre, 2009) and guinea pig ileum (Teague et al., 2002). The $SK_{Ca}$ channel blocker apamin (3 µM) significantly reduced the NaHS-induced effects in rat and human

colonic strips and in mouse distal colon full segments (Gallego et al., 2008). In our study, 500 nM apamin did not influence the NaHS-induced relaxation. $IC_{50}$ values as low as 83 pM to 28 nM were reported for apamin to block the three $SK_{Ca}$ channel subtypes (SK1, SK2 and SK3; Ro et al., 2001; Strøbæk et al., 2000) and Van Crombruggen and Lefebvre (2004) showed that 500 nM apamin reduced the relaxation by ATP in rat distal colon to the same extent as 30 nM apamin. 500 nM apamin is thus sufficient to investigate the possible role of $SK_{Ca}$ channels. The difference in effect of apamin between our study and that of Gallego et al. (2008) might be related to the difference in tissues used: circular muscle strips in the actual study versus full segments. In the latter preparation, NaHS inhibited spontaneous motor complexes that were abolished by tetrodotoxin; precisely this effect of NaHS on the neuronally driven motor complexes was inhibited by apamin; in the presence of tetrodotoxin, NaHS reduced basal tone but the influence of apamin on this effect is not reported (Gallego et al., 2008). Thus probably in mouse distal colon, NaHS has a direct muscular effect not involving $SK_{Ca}$ channels, as seen in smooth muscle strips in our study, and a neurogenic effect involving $SK_{Ca}$ channels, as seen in full segments in the study of Gallego et al. (2008). Recently, SK2 channels were indeed detected in enteric neurons and were found to negatively regulate the acetylcholine release (Nakajima et al., 2007). Still, our results differ from these in human and rat colon circular muscle strips where Gallego et al. (2008) showed that the NaHS-induced inhibition of spontaneous activity is sensitive to apamin in the presence of tetrodotoxin. Also the intermediate ($IK_{Ca}$) and large ($BK_{Ca}$) conductance $Ca^{2+}$ dependent $K^+$ channel blocker charybdotoxin did not influence the NaHS-induced relaxation in mouse distal colon strips, neither alone, nor in combination with apamin; the latter combination was required to reduce significantly the relaxation by exogenous CO in mouse gastric fundus (De Backer and Lefebvre, 2007). Finally, the influence of barium chloride and 4-AP, blockers of respectively the inward rectifier and the voltage dependent $K^+$ channel, was tested as both $K^+$ channels can play a role in smooth muscle relaxation (Horinouchi et al., 2003; Orie et al., 2006). However, 4-AP and barium chloride did not affect the NaHS-induced relaxation.

Zhao and Wang (2002) reported the release of endothelium-derived NO by $H_2S$ in rat aortic tissues. Contribution of endogenous NO and its downstream target soluble guanylate cyclase (sGC) in the relaxation by NaHS of mouse distal colon strips was excluded as the NO synthase inhibitor L-NAME nor the sGC inhibitor ODQ had an influence. As in rat colon the combination of ODQ plus apamin was required to fully inhibit the relaxation by exogenous NO (Van Crombruggen and Lefebvre, 2004), ODQ plus apamin was tested, but this had no influence versus NaHS. The non-effect of the adenylate cyclase (AC) inhibitor SQ 22536 indicates that also the cAMP pathway is not involved in the relaxant effect of NaHS.

In rat aortic tissues, the $H_2S$-induced vasorelaxation was reduced by the L-type voltage dependent $Ca^{2+}$ channel blocker nifedipine (Zhao and Wang, 2002). In our study, nifedipine was used at a concentration (3 nM) able to reduce the $PGF_{2\alpha}$ (3 µM)-induced contractile response by 50 %. Higher concentrations of $PGF_{2\alpha}$ brought about a comparable degree of pre-contraction as in the control strip. Nifedipine showed no effect on the NaHS-induced relaxation in mouse distal colon. As the $Na^+/K^+$ ATPase, the ryanodine receptor, the $IP_3$ receptor and the sarcoplasmatic reticulum $Ca^{2+}$ ATPase (SERCA) can be involved in relaxation of mouse GI tissues (Kim et al., 2006; Yaktubay et al., 1999; Zizzo et al., 2005), we tested respectively ouabain, ryanodine, 2-APB and thapsigargin against the NaHS-induced relaxation. However, none of these inhibitors had an influence on the relaxant effect of NaHS.

Relaxation in smooth muscle cells results from a decrease in the intracellular calcium concentration, and/or a desensitization of the contractile proteins to the intracellular calcium concentration. We therefore performed experiments simultaneously measuring tension and $[Ca^{2+}]_{cyt}$ in the mouse distal colon strips. Exogenous NO induced a relaxation accompanied by a decrease in $[Ca^{2+}]_{cyt}$. This was previously reported for the sodium nitroprusside-induced relaxation in guinea pig taenia coli (Kwon et al., 2000) and the NO-induced relaxation in rat distal colon (Colpaert et al., 2005). The relaxation starting immediately after administration of NaHS was however accompanied by a significant increase in $[Ca^{2+}]_{cyt}$. We have no clear-cut explanation for this rise in $[Ca^{2+}]_{cyt}$. In the literature, only localized increases in $[Ca^{2+}]_{cyt}$ caused by calcium release from the sarcoplasmatic reticulum via ryanodine receptors ($Ca^{2+}$ sparks) or $IP_3$ receptors ($Ca^{2+}$ puffs), leading to the activation of $BK_{Ca}$ or $SK_{Ca}$ channels (Bayguinov et al., 2000; Jaggar et al., 2000), have been associated with relaxation. These localized increases in $[Ca^{2+}]_{cyt}$ can however not account for the rise in global $[Ca^{2+}]_{cyt}$ that we measured. Moreover, as already discussed, inhibiting the ryanodine receptor or the $IP_3$ receptor did not affect the NaHS-induced relaxation, nor did inhibition of the $BK_{Ca}$ and $SK_{Ca}$ channels. At later time intervals, $[Ca^{2+}]_{cyt}$ was not different from that in control strips, despite the fact that the maximal relaxing effect of NaHS was reached and sustained. Smooth muscle relaxation without a change in $[Ca^{2+}]_{cyt}$ is mediated through calcium desensitization. One of the most important calcium desensitizing mechanisms is inhibition of the RhoA/Rho-kinase pathway leading to the activation of myosin light chain phosphatase (MLCP; Somlyo and Somlyo, 2003) but this pathway is excluded as the Rho-kinase inhibitor Y-27632 did not influence NaHS-induced relaxation. Also other pathways leading to MLCP activation can be excluded as the inhibitor of MLCP calyculin-A did not affect NaHS-induced relaxation, which is in contrast to our previous data obtained in the mouse gastric fundus (Dhaese and Lefebvre, 2009). A calcium desensitization mechanism, independent of MLCP, is the

inhibitory phosphorylation of the myosin light chain kinase (MLCK) by activation of $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII; Tansey et al., 1994). However, this requires an increase in $[Ca^{2+}]_{cyt}$. There was an initial short lasting increase in $[Ca^{2+}]_{cyt}$ by NaHS in our experiments, but in the desensitization model through CaMKII , this increase is expected to first activate MLCK and thus to induce contraction which was not the case (Tansey et al., 1994). Some kinases, such as integrin-linked kinase, p21-activated protein kinase and zip kinase that have been shown to be present in smooth muscle cells and were obtained by recombinant technology, have been shown to directly phosphorylate the myosin light chain (MLC) in an $Ca^{2+}$-independent manner in an in vitro assay and to induce smooth muscle contraction when their catalytic domains were introduced as recombinant proteins into permeabilized smooth muscle tissues (Hirano et al., 2004). Inhibition of these kinases would, independently of MLCP, lead to a lower amount of phosphorylated MLC and relaxation. As it still remains to be clarified whether this(these) kinase(s) play(s) a physiological role in the regulation of smooth muscle contractility (Hirano et al., 2004), we can only speculate that NaHS might induce relaxation by inhibition of one of these kinases.
In conclusion, our study demonstrates that in mouse distal colon strips $H_2S$ induced concentration-dependent relaxations, not involving the major known $K^+$ channels and not accompanied by a decrease in $[Ca^{2+}]_{cyt}$. The latter indicates that the $H_2S$ -induced relaxations are mediated via calcium desensitization. This calcium desensitizing mechanism seems not related to inhibition of Rho-kinase or activation of MLCP.

## *VII.6 References*

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# Chapter VIII

# GENERAL DISCUSSION AND CONCLUSION

# Chapter VIII General Discussion and Conclusion

## *VIII.1 The relative importance of the sGC isoforms $\alpha_1\beta_1$ and $\alpha_2\beta_1$ in the nitrergic relaxation of gastrointestinal smooth muscle*

During the past two decades, several studies established a role for the neurotransmitter NO in relaxation of gastrointestinal smooth muscle and hence motility of the gastrointestinal (GI) tract. The principal target of NO is the heme-containing enzyme sGC (Lucas et al., 2000). Two physiological active isoforms of sGC have been described: the predominantly expressed heterodimer $sGC\alpha_1\beta_1$ and the less abundantly expressed $sGC\alpha_2\beta_1$. In mouse ileum and colon, the presence of $\alpha_1$, $\alpha_2$ and $\beta_1$ subunit mRNA was reported (Mergia et al., 2003), albeit the amount of $\alpha_2$ mRNA was clearly lower than this of $\alpha_1$ mRNA, as is the case in most tissues. Still, the presence of $sGC\alpha_1\beta_1$ as well as $sGC\alpha_2\beta_1$ in GI tissues triggers the question on the relative importance of each isoform in the NO-induced relaxation in these tissues. Research on this subject has however been hampered by the fact that there are no isoform-specific inhibitors. We tried to address this topic using genetically engineered mice, focussing our research on the NO-mediated relaxation in the jejunum and the distal colon of the $sGC\alpha_1$ knockout (KO) mice in which a mutant $sGC\alpha_1\beta_1$ isoform is no longer functionally active.

***Exogenous NO*.** We found that the relaxant response to exogenous NO was reduced in the jejunal and distal colonic strips isolated from $sGC\alpha_1$ KO mice, pointing to a role of $sGC\alpha_1\beta_1$ in exogenous NO-induced relaxation in these tissues. The (small) remaining responses in the $sGC\alpha_1$ KO mice were still sensitive to the sGC inhibitor ODQ, indicating that also $sGC\alpha_2\beta_1$ contributes to the relaxing effect of NO. The $sGC\alpha_1$ KO mice did not show an increase in $\alpha_2$ mRNA in the jejunum nor an elevated expression of the $\alpha_2$ subunit protein in the distal colon, indicating that the participation of $sGC\alpha_2\beta_1$ in the exogenous NO-induced relaxation is not a compensation mechanism by upregulation of the $sGC\alpha_2\beta_1$ isoform due to $sGC\alpha_1$ deficiency. At the level of the distal colon, the contribution of $sGC\alpha_2\beta_1$ in the relaxant response to exogenous NO did not differ between male and female mice. In the jejunum however, the participation of $sGC\alpha_2\beta_1$ in the exogenous NO-induced relaxation differed depending on the gender. The contribution of $sGC\alpha_2\beta_1$ in the relaxation by exogenous NO was most pronounced in the jejunum of female mice, as the relaxant responses to exogenous NO were only slightly reduced in the jejunal strips from female $sGC\alpha_1$ KO mice. In contrast, the role of $sGC\alpha_2\beta_1$ in the exogenous NO-induced responses was least pronounced in the jejunum of

male mice, as a near abolition of these responses was observed in the jejunal strips from male sGC$\alpha_1$ KO mice. These observations contrast with previous results of our group in pyloric tissue, where the opposite was found i.e. a more pronounced reduction of relaxation by exogenous NO in female than in male sGC$\alpha_1$ KO mice (Vanneste et al., 2007). The more pronounced consequences of knocking out sGC$\alpha_1$ on the exogenous NO-induced relaxation at the level of the jejunum in the male versus the female mice are however in line with the finding that male sGC$\alpha_1$ KO mice developed hypertension whereas female sGC$\alpha_1$ KO mice maintained normal blood pressure (Buys et al., 2008). But these in vivo data were not accompanied by gender differences in exogenous NO-induced relaxation of aortic rings in vitro, as the latter was similarly attenuated in both male and female sGC$\alpha_1$ KO mice (Nimmegeers et al., 2007), leading to the conclusion that the observed gender dimorphism in blood pressure in the sGC$\alpha_1$ KO mice is not per se related to gender-specific impairments in NO-induced relaxation. Further findings in the study by Buys et al. (2008) suggested a role of androgens in the hypertension associated with sGC$\alpha_1$ deficiency. Taken our results and the results previously reported by Vanneste et al. (2007) together, we can state that exogenous NO is able to induce relaxation via the activation of sGC$\alpha_1\beta_1$ as well as sGC$\alpha_2\beta_1$, but that the relative contribution of sGC$\alpha_2\beta_1$ to the response to exogenous NO can somewhat differ depending on GI tissue and even gender.

***Endogenous NO (in comparison to exogenous NO).*** Whereas exogenous NO diffuses from the surrounding extracellular solution and enters the smooth muscle cells over the whole surface, endogenous i.e. nNOS-derived NO is released from the nerve varicosities and thus enters the smooth muscle cell more locally. The different mechanism of entering could have implications towards the relative contribution of each sGC isoform in mediating relaxation by exogenous respectively endogenous NO. We assessed the responses to endogenous NO by electrical field stimulation (EFS) of the GI tissues in NANC conditions. Within the experimental conditions used, this yielded purely nitrergic responses at all frequencies tested in the jejunum, corresponding to what our group reported before for the gastric fundus (Vanneste et al., 2007) but in the distal colon only the response at 1 Hz was a purely nitrergic one. In the gastric fundus of the sGC$\alpha_1$ KO mice, the responses to endogenous NO, released during 10 s trains of EFS, were reduced to a much greater extent than the relaxations by exogenous NO (Vanneste et al., 2007). These results point to a more dominant role of sGC$\alpha_1\beta_1$ in the relaxant response to endogenous versus exogenous NO in the gastric fundus. Similar findings were observed in the distal colon, where sGC$\alpha_1\beta_1$ as well as sGC$\alpha_2\beta_1$ are involved in the responses to exogenous NO, but the response to endogenous NO released by EFS at 1 Hz was mediated solely through activation of sGC$\alpha_1\beta_1$, as it was abolished in the sGC$\alpha_1$ KO mice. In the jejunum however, the relative

contribution of $sGC\alpha_2\beta_1$ versus $sGC\alpha_1\beta_1$ in response to endogenous NO was similar as this in response to exogenous NO, with $sGC\alpha_2\beta_1$ being only minimally involved in the NO-induced relaxation in the jejunum of male mice but contributing in a much more pronounced way in female mice.

At the level of the gastric fundus and the distal colon, endogenous NO seems to act mainly via $sGC\alpha_1\beta_1$. Although both $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ are regarded to be soluble, it was shown in rat brain that, whereas the $sGC\alpha_1\beta_1$ isoform was found in the cytosol, the $\alpha_2\beta_1$ isoform is actually membrane-associated via interaction with the PDZ-containing post-synaptic density protein-95 (PSD-95; Russwurm et al., 2001). Also in epithelial cells in human colon, $sGC\alpha_2\beta_1$ was found to be localized to the apical plasma membrane (Bellingham & Evans, 2007). It is possible that the spatially more confined and lower concentrations of endogenous NO are not as effective as the more diffused and higher concentrations of exogenous NO in reaching the membrane-located $sGC\alpha_2\beta_1$, which is present in lower amounts in the GI smooth muscle cells than the cytosolic $sGC\alpha_1\beta_1$.

## *VIII.2 sGC-dependency of the nitrergic component of GI smooth muscle relaxation*

***Exogenous NO.*** The $sGC\alpha_1$ KO model showed us that in the absence of $sGC\alpha_1\beta_1$, $sGC\alpha_2\beta_1$ seems able to maintain some degree of nitrergic relaxation in the investigated GI tissues. Conclusive evidence for this supporting role of $sGC\alpha_2\beta_1$ came from our observations in the heme-deficient $sGC\beta_1$ knock in (KI) mice. Due to the impairment of the heme-dependent and thus nitrergic activation of $sGC\alpha_1\beta_1$ as well as $sGC\alpha_2\beta_1$, the relaxant responses to exogenous NO were abolished in the gastric fundic, jejunal and distal colonic strips isolated from the $sGC\beta_1$ KI mice. This illustrates that it is indeed $sGC\alpha_2\beta_1$ that is responsible for the remaining nitrergic responses in the $sGC\alpha_1$ KO mice. Moreover, this indicates that exogenous NO, within the experimental conditions used, completely relies on sGC to induce relaxation; our results do consequently not comply with possible sGC-independent actions, such as the direct activation of $K^+$ channels (Lang & Watson, 1998; Serio et al., 2003b) by NO in the investigated GI tissues. In accordance with this conclusion, one would expect that in the GI tissues isolated from the WT mice, responses to exogenous NO should be abolished by ODQ, an inhibitor of both sGC isoforms by oxidation of their prosthetic heme group. Our observations that ODQ only reduced –not abolished- the relaxation induced by higher concentrations of exogenous NO in the gastric fundic, jejunal and distal colonic strips from the WT mice can however be explained by the NO-competitive nature of ODQ (Schrammel et al., 1996). The latter should indeed be taken into account when interpreting

results obtained with ODQ, illustrating the additional value of our mouse transgenic approach for differentiating and evaluating sGC-dependent versus sGC-independent effects of NO.

***Endogenous NO in gastric fundus.*** Besides exogenous NO, also endogenous NO seems to act solely via sGC in the investigated GI tissues. Indeed, in the gastric fundic strips, the EFS-induced relaxations, which were found to be L-NAME-sensitive in the WT mice, were virtually abolished in the sGC$\beta_1$ KI mice, indicating that NO mediates its relaxation via sGC, and corroborating that NO is the main neurotransmitter in mouse gastric fundus. An exception was the remaining relaxant response to EFS at 8 Hz in some gastric fundic strips of the sGC$\beta_1$ KI mice, notwithstanding this response was found to be abolished by L-NAME in the WT mice. This pointed to a possible sGC-independent action of endogenous NO in the gastric fundus; however further experiments showed that this remaining response in the sGC$\beta_1$ KI mice was unsensitive to L-NAME. The latter thus suggests the emerge of a compensatory mechanism, involving another neurotransmitter than NO, at higher stimulation frequencies in the sGC$\beta_1$ KI mice. We speculate this neurotransmitter to be VIP, as VIP is reported to be released at higher stimulation frequencies in rat gastric fundus strips (D'Amato et al., 1992). In compensation for the loss of nitrergic relaxation in the gastric fundus, VIP release might start at lower frequencies in some sGC$\beta_1$ KI mice.

***Endogenous NO in jejunum.*** NO –acting via sGC- seems also the main neurotransmitter in the mouse jejunum, as the L-NAME-sensitive relaxations observed at the four applied stimulation frequencies in the jejunal strips from the WT mice were abolished in the sGC$\beta_1$ KI mice. This is in contrast to the report of De Man et al. (2003) in which a role for ATP (De Man et al., 2003) in mouse jejunum was suggested. In this study, EFS was applied at the same four frequencies and for 10 s as in our study, but the applied pulse duration and voltage of the stimulations (respectively 0.1 ms and 40 V in our study) are not mentioned and could thus differ from our study. Our results obtained in mouse jejunum however correspond to what our group reported in rat jejunum under similar - but not identical - stimulation parameters (40 V; 0.1 ms; 1-8 Hz; but 20 s trains; Vanneste et al., 2004).

***Endogenous NO in distal colon.*** In mouse distal colon, the response to EFS at 1 Hz, being purely nitrergic, was abolished in distal colonic strips of the sGC$\alpha_1$ KO and sGC$\beta_1$ KI mice, whereas the responses to EFS at 2 to 8 Hz were not influenced. For the relaxant responses to EFS at 2 to 8 Hz, a redundant action of NO, acting at sGC, and another neurotransmitter, acting at small conductance $Ca^{2+}$-dependent $K^+$ channels, is suggested. This proposal is based on the observation that the combined addition of L-NAME plus apamin or ODQ plus apamin was able to nearly abolish the relaxant responses at these higher stimulation frequencies in distal colonic strips of the WT mice, studied in comparison with the sGC$\alpha_1$ KO mice. This second neurotransmitter, besides NO, is probably ATP, as ATP indeed leads to

activation of small conductance $Ca^{2+}$-dependent $K^+$ channels (Bayguinov et al., 2000) and has previously been suggested to be involved in the NANC inhibitory neurotransmission in the mouse (Serio et al., 2003a), rat (Pluja et al., 1999; Van Crombruggen & Lefebvre, 2004), hamster (El Mahmoudy et al., 2006) and human (Benko et al., 2007) colon. In the WT mice from the $sGC\beta_1$ KI study, L-NAME plus apamin could only reduce -and not abolish- the relaxations to EFS at 2 to 8 Hz, suggesting the presence of another, possibly third, neurotransmitter in these mice. The latter could be related to the genetic background of the mice, as this was different between the study of the $sGC\alpha_1$ KO mice (mixed Swiss/129) and the study of the $sGC\beta_1$ KI mice (mixed 129/SvJ-C57BL/6J). Consistent with this, the involvement of a third neurotransmitter was also suggested in the $sGC\beta_1$ KI mice, as L-NAME plus apamin failed to influence the relaxant responses by EFS at 2 to 8 Hz in the distal colonic strips of these mice. Previously, the presence of a third neurotransmitter, besides NO and ATP, was suggested in rat distal colon by our group (Van Crombruggen & Lefebvre, 2004) and in hamster distal colon by El-Mahmoudy et al. (2006).

## *VIII.3 Role of the nitrergic activation of sGC in the regulation of GI motility*

In the $sGC\alpha_1$ KO mice, no morphological GI abnormalities or important GI malfunctions were observed. However, the total impairment of the heme-dependent and thus nitrergic activation of $sGC\alpha_1\beta_1$ as well as $sGC\alpha_2\beta_1$ induced delayed gastric emptying, delayed intestinal transit and increased whole gut transit time in the $sGC\beta_1$ KI mice.

***Gastric emptying.*** Disturbances in liquid gastric emptying were indeed expected in the $sGC\beta_1$ KI mice as fundic nitrergic relaxation, essential for gastric accommodation (Desai et al., 1991), was abolished. The impaired fundic storage of the liquids should be expected to enhance liquid gastric emptying, but we observed delayed liquid gastric emptying in the $sGC\beta_1$ mice. This is in accordance with reports of delayed gastric emptying in nNOS KO mice (Mashimo *et al*., 2000) and cGMP-dependent protein kinase I (cGKI) KO mice (Pfeifer et al., 1998). The $sGC\beta_1$ KI mice also showed, similar to the nNOS (Huang et al., 1993; Mashimo et al., 2000) and cGKI (Pfeifer et al., 1998) KO mice, a marked enlargement of the stomach with hypertrophy of the circular muscle of the fundus. Mashimo et al. (2000) suggested that this gastric smooth muscle thickening is secondary to functional pyloric obstruction and represents work hypertrophy. NOS inhibition is indeed reported to increase the pyloric tone (Anvari et al., 1998) and impairment of pyloric relaxation will counteract the accelerating effect of the deficient fundic relaxation on gastric emptying (Mashimo et al., 2000; Anvari et al., 1998), as such explaining the observed delay in gastric emptying in the $sGC\beta_1$ KI mice. With the study of the $sGC\beta_1$ KI mice, we provide the link between the nNOS

KO mice and the cGKI KO mice. The severe morphological changes at the level of the stomach and the delayed gastric liquid emptying observed in these three transgenic mouse models illustrate the pivotal role of the NO-sGC-cGMP-cGKI pathway in normal gastric functioning.

***Small intestinal transit.*** In addition to and most probably in part resulting from the disturbances in gastric emptying, sGC$\beta_1$ KI mice showed delayed small intestinal transit. Previously, studies in different species with NOS-inhibitors showed delayed small intestinal transit (Chiba et al., 2002; Fraser et al., 2005; Karmeli et al., 1997). In the cGKI KO mice, spastic contractions of long intestinal segments followed by scarce and slow relaxations were observed as a result of the impairment of the pathway downstream of NO (Pfeifer et al., 1998). Considering the impaired response to neurally released NO in the jejunal strips from the sGC$\beta_1$ KI mice, it seems inevitable that also in the sGC$\beta_1$ KI mice an imbalance between the excitatory (cholinergic) and inhibitory (nitrergic) input during peristalsis develops, interrupting the coordinated interplay between the ascending contractions and descending relaxations, which is essential for the propagation of the peristaltic contraction (Waterman et al., 1994).

***Colonic transit.*** Concomitant to the delays in the upper GI transit, also retardation of the colonic transit might take place in the sGC$\beta_1$ KI mice, as an increase in whole gut transit time was observed. NOS inhibition was previously indeed found to inhibit the colonic propulsion of artificial pellets in guinea pig colonic segments (Foxx-Orenstein & Grider, 1996) and to delay colonic transit in rats (Mizuta et al., 1999). Still, in sGC$\beta_1$ KI mice, the extent of delay in colonic transit per se will probably be limited, considering the fact that in mouse distal colon, NO - acting via sGC- is only the principal neurotransmitter at a stimulation frequency of 1 Hz. The severely disturbed gastric emptying and intestinal transit in sGC$\beta_1$ KI mice probably contribute to the reduced lifespan of sGC$\beta_1$ KI mice (median survival of 29-36 weeks; Thoonen et al., 2009). However, the latter values contrast with the much shorter lifespan of sGC $\beta_1$ KO mice, generated by knocking out exon 10 of the $\beta_1$ subunit, eliminating activation of both sGC isoforms by NO but also basal activity (Friebe et al., 2007). Less than 10 % of the sGC $\beta_1$ KO mice survived for 1 month due to intestinal dysmotility (Friebe et al., 2007), suggesting that basal sGC activity, maintained in the sGC $\beta_1$ KI mice, is important for longer survival.

**In conclusion**, our findings indicate that NO is the principal relaxant neurotransmitter in the mouse gastric fundus and the mouse jejunum. In the mouse distal colon, NO is the sole relaxant neurotransmitter released by EFS at 1 Hz, however at higher stimulation frequencies, other neurotransmitters contribute to the smooth muscle relaxation. Our results

further show that the relaxant effect of endogenous and exogenous NO in the investigated GI tissues completely relies on the activation of sGC isoforms $sGC\alpha_1\beta_1$ and $\alpha_2\beta_1$. Interestingly, at the level of the gastric fundus and the distal colon, endogenous NO seems less efficient than exogenous NO in activating $sGC\alpha_2\beta_1$. Still, in the absence of $sGC\alpha_1\beta_1$, $sGC\alpha_2\beta_1$ is able to maintain normal GI functioning. Impairment of the NO-induced activation of $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ induces GI malfunctions, which are most pronounced at the level of the stomach, indicating the important role of the NO-sGC pathway in GI motility.

## *VIII.4 Influence of $H_2S$ on GI contractility*

After NO and CO, a third gaseous molecule, $H_2S$, was recently shown to be generated naturally in mammalian tissues and to exert a number of physiological effects such as smooth muscle relaxation. As $H_2S$-induced smooth muscle relaxation was initially observed in the vascular system, the mechanism of action of $H_2S$ is already most extensively studied in this system. Interestingly, the sGC-cGMP pathway seems not to be involved in the $H_2S$-induced vasorelaxation (Zhao & Wang, 2002), indicating that the mechanism of action of $H_2S$ thus differs from that of NO and CO, as the latter both induce relaxation via the activation of sGC. Instead, most of the studies report the involvement of the ATP-dependent $K^+$ channel ($K_{ATP}$ channel) in the $H_2S$-induced vasorelaxation (Cheng et al., 2004; Webb et al., 2008; Zhao et al., 2001). In a number of GI preparations, $H_2S$ was shown to inhibit spontaneous or induced contractions (Hosoki et al., 1997; Teague et al., 2002; Gallego et al., 2008). In the GI tract however, the possible role of the $K_{ATP}$ channel in the relaxant effect of NaHS remains more controversial with results reported in favour (Distrutti et al., 2006) as well as against (Teague et al.; 2002) its involvement. As the mechanism of action by which $H_2S$ brings about relaxation is thus far from being understood in the GI tract, our aim was to investigate the effect of $H_2S$ on the contractility of the mouse gastric fundus and distal colon and to explore its mechanism of action in these two tissues. We used sodium hydrogen sulfide (NaHS) as experimental source of $H_2S$.

In pre-contracted mouse gastric fundic and distal colonic strips, NaHS was able to induce concentration-dependent relaxations with an $EC_{50}$ of respectively 514 µM and 444 µM. These values are higher than those previously reported for the inhibiting effect of NaHS in GI tissues of other species, ranging from 31 to 261 µM (Gallego et al., 2008; Hosoki et al.,1997; Teague et al., 2002), indicating that a species difference could exist with mouse smooth muscle being less sensitive to NaHS. However, Gallego et al. (2008) recently reported an $EC_{50}$ of 121 µM for the $H_2S$-induced inhibition of spontaneous motor complexes in isolated segments of mouse colon. The difference in tissues used, circular muscle strips in our study versus full segments in the study by Gallego et al. (2008), cannot serve as an explanation for the observed difference in $EC_{50}$ between both studies, as on the contrary, the use of muscle

strips is expected to optimize the penetration of the drugs under study and thus potentially allow a higher effect of lower drug concentrations. Still, we have to take notice of the fact that Gallego et al. (2008) investigate the NaHS-induced inhibition of spontaneous motor complexes whereas we measure the NaHS-induced inhibition of $PGF_{2\alpha}$-induced pre-contraction; these different motility patterns might explain the difference in $EC_{50}$ between both studies.

In agreement with the observations in rat aortic tissues (Zhao & Wang, 2002), the sGC inhibitor ODQ failed to reduce the NaHS-mediated relaxation in the mouse gastric fundic and distal colonic strips, indicating that also in these two GI tissues $H_2S$ does not induce relaxation via the sGC-cGMP pathway. Furthermore, this indicates that NO, released from nitrergic nerves, does probably not contribute in the relaxation by NaHS, which was confirmed by the non-effect of the NOS-inhibitor L-NAME versus the NaHS-mediated relaxation in the mouse gastric fundic and distal colonic strips. Previously, the contribution of NO in the relaxation by NaHS was also excluded in the guinea pig ileum and the mouse colon and jejunum by respectively Teague et al. (2002) and Gallego et al. (2008). In addition, we excluded the involvement of non-nitrergic relaxant neuron types as the $Na^+$ channel blocker tetrodotoxin, inhibiting action potential propagation, and the N-type voltage dependent $Ca^{2+}$ channel blocker ω-conotoxin, interfering with exocytosis at the nerve ending, did not affect the NaHS-mediated relaxation in the mouse gastric fundic and distal colonic strips. This indicates that NaHS seems to act directly at the muscular level in the mouse gastric fundic and distal colonic strips. In contrast to the observations in cardiovascular tissues, which indicate a role of the $K_{ATP}$ channel in the NaHS-induced relaxation, we could not find any evidence supporting a role of the $K_{ATP}$ channel - nor another major $K^+$ channel - in the NaHS-induced relaxation in the mouse gastric fundic and distal colonic strips. This is also in contrast to the results recently reported by Gallego et al. (2008) in mouse distal colonic segments, where the inhibition of neuronally driven spontaneous motor complexes by NaHS was inhibited by the small conductance $Ca^{2+}$-dependent $K^+$ channel ($SK_{Ca}$ channel) blocker apamin. Probably, in mouse distal colon, NaHS has a direct muscular effect not involving $SK_{Ca}$ channels, as seen in smooth muscle strips in our study, and a neuronal effect involving $SK_{Ca}$ channels, as seen in full segments in the study of Gallego et al. (2008).

Besides the major $K^+$ channels, also the L-type voltage dependent $Ca^{2+}$ channel does not seem to play a role in the NaHS-induced relaxation in the mouse gastric fundic and distal colonic strips, arguing against an interference of NaHS with the extracellular calcium entry to induce relaxation. Relaxation can also be achieved by increasing the extrusion of intracellular calcium through the activation of $Na^+/K^+$ ATPase, which leads to hyperpolarization and increased calcium extrusion via the $Na^+/Ca^{2+}$ exchanger. However, inhibiting the $Na^+/K^+$ ATPase did not affect the NaHS-induced relaxation, excluding that NaHS induces relaxation

via this mechanism. As inhibitors of the sarcoplasmatic reticulum $Ca^{2+}$ ATPase (SERCA), the ryanodine receptor and the $IP_3$ receptor did not influence the NaHS-induced relaxation, also the involvement of calcium mobilization/sequestration at the level of the sarcoplasmatic reticulum in the NaHS-induced relaxation in the mouse gastric fundic and distal colonic strips is unlikely. Taken together, we could thus not find any evidence that would point to NaHS being able to induce relaxation via a decrease in the intracellular calcium concentration ($[Ca^{2+}]_{cyt}$).

Relaxation in smooth muscle cells can also take place without an underlying decrease in $[Ca^{2+}]_{cyt}$; this mechanism of action is called calcium desensitization as relaxation of the contractile apparatus then occurs in the presence of unaltered $[Ca^{2+}]_{cyt}$ (Somlyo & Somlyo, 2003). Calcium desensitization is most frequently achieved by the activation of myosin light chain phosphatase (MLCP), leading to a higher degree of dephosphorylation of myosin light chain (MLC) and subsequently relaxation. The MLCP inhibitor calyculin-A reduced the NaHS-induced relaxations in the mouse gastric fundic strips, suggesting that the activation of MLCP is indeed involved in the relaxant effect of NaHS in mouse gastric fundus. The most important mechanism leading to an increased activity of MLCP is relieving MLCP from the inhibitory actions of the Rho-A/Rho-kinase pathway (Somlyo & Somlyo, 2003). But as the Rho-kinase inhibitor Y-27632 did not influence NaHS-induced relaxation, the activation of MLCP by NaHS in the mouse gastric fundus is not related to suppression of the RhoA/Rho-kinase pathway. Similar to the mouse gastric fundus, also in the mouse distal colon, the NaHS-induced relaxation is mediated via a calcium desensitizing mechanism, as simultaneous measurement of tension and $[Ca^{2+}]_{cyt}$ in the mouse distal colonic strips showed that the maximal relaxing effect of NaHS was reached without a concomitant decrease in $[Ca^{2+}]_{cyt}$. However, MLCP activation seems not involved as calyculin-A did not affect the NaHS-induced relaxation.

Altogether, our results suggest that the effectors involved in the relaxation by NaHS differ between the mouse gastric fundus and distal colon. This implies that the mechanism of action of NaHS to induce smooth muscle relaxation not only varies between the cardiovascular system and the GI tract (involvement or not of the $K_{ATP}$ channel), but also within the GI tract (involvement or not of MLCP activation). The fact that the mechanism of action of a substance can vary between organs – even parts of the same body system - was previously illustrated by Cogolludo et al. (2001). They showed that NO-donor sodium nitroprusside (SNP) relaxes the piglet pulmonary and mesenteric arteries through different effectors, as only in the mesenteric arteries the activation of $Na^+/K^+$ ATPase plays a role in the SNP-induced decrease in $[Ca^{2+}]_{cyt}$.

**In conclusion**, our study demonstrates that in pre-contracted mouse gastric fundic and distal colonic strips, NaHS (donor of $H_2S$) induces concentration-dependent relaxations. The relaxing effect of NaHS in the mouse gastric fundus and distal colon is not mediated by the activation of the $K_{ATP}$ channel, the latter being an important mechanism of action of the NaHS-induced relaxation in the cardiovascular system. We propose that a calcium desensitizing mechanism is involved in the NaHS-induced relaxation in the mouse gastric fundus and distal colon. However, the underlying mechanism can differ between organs as this calcium desensitization is mediated via the activation of MLCP in the mouse gastric fundus but not in the mouse distal colon.

## *VIII.5 Future perspectives*

While a role for the NO-sGC pathway in GI motility under physiological conditions is established, it is still unclear as to which role this pathway plays in inflammatory bowel diseases and in postoperative ileus. Conflicting results have been reported, demonstrating that NO may promote, attenuate or have little effect on gut inflammation and injury (Grisham et al., 2002). Moreover, the possible contribution of sGC in these pro- or anti-inflammatory effects of NO still needs to be elucidated. Our group previously tried to address this issue by studying the effect of the sGC inhibitor ODQ in an experimental rat model for inflammatory bowel diseases (Van Crombruggen et al., 2008) and postoperative ileus (Vanneste et al., 2008). In rats with colitis induced by exposure to dextran sulphate sodium (DSS) in the drinking water for 6 days, concomitant intraperitoneal treatment with ODQ moderately reduced the colonic and plasmatic levels of cGMP, but this did not prevent the colonic inflammation and motility changes induced by DSS (Van Crombruggen et al., 2008). Subcutaneous injection of ODQ 30 min before and 12 h after laparotomy and small intestinal manipulation, was not able to reduce the plasmatic and small intestinal levels of cGMP measured at 24 h after intestinal manipulation so that the pro-/anti-inflammatory role of sGC in this model could not be assessed (Vanneste et al., 2008). Ideal sGC inhibitors for in vivo use are currently not available. This problem can be circumvented by using the sGC $\beta_1$ KI mice to investigate the pro-/anti-inflammatory effect of sGC in inflammatory models of postoperative ileus and inflammatory bowel disease.

In the second part of this thesis, we showed that the $H_2S$-donor NaHS is able to induce relaxation in the mouse gastric fundus and distal colon and that calcium desensitization is involved. In the mouse gastric fundus, NaHS induces calcium desensitization via activation of MLCP, but the most important mechanism leading to an increased activity of MLCP, i.e. relieving MLCP from the inhibitory actions of the Rho-A/Rho-kinase pathway, is not involved. Besides the Rho-A/Rho-kinase pathway, another frequently-cited mechanism for inhibition of MLCP is via the peptide CPI-17, whose phosphorylation enhances its potency for inhibiting

MLCP. The kinase responsible for the phosphorylation of CPI-17 is primarily protein kinase C (PKC) (Ito et al., 2004; Somlyo & Somlyo, 2003). It might thus be useful to investigate whether inhibition of PKC, thereby relieving MLCP from the inhibitory action of CPI-17, is involved in the NaHS-induced relaxation in mouse gastric fundus.

Also the role – if any - of $H_2S$ in the inhibitory neurotransmission in the mouse GI tissues should be clarified. Recently, cystathionine γ-lyase (CSE), one of the two $H_2S$-synthetizing enzymes, was shown to be present in mouse colon myenteric neurons, and the intact colonic muscle layer containing the myenteric plexus generated detectable levels of $H_2S$ (Linden et al., 2008). The observation that mouse colon thus generates and releases $H_2S$ together with our finding that mouse colon responds to exogenously applied NaHS, suggest that $H_2S$ could function as an endogenous gaseous neurotransmitter ("gasotransmitter") in mouse colon, however further research is warranted. It might thus be useful to investigate the influence of CSE inhibition on the EFS-induced responses in the mouse distal colon. Although the membrane permeability of CSE-inhibitors D,L-propargylglycine (PAG) and β -cyano-L-alanine (β-CA) has been unclear, it would appear that at least PAG is well absorbed and readily crosses biological membranes (Teague et al., 2002; Zhao et al., 2003), making PAG suitable for use in in vitro intact tissue studies.

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# Chapter IX

## SUMMARY

# Chapter IX Summary

Neuronal control mechanisms, mediated via the extrinsic nervous system and the enteric nervous system (ENS), play an important role in the regulation of gastrointestinal (GI) motility. As part of the ENS, the myenteric plexus, which lies between the longitudinal and circular smooth muscle layers, controls the contraction and relaxation of GI smooth muscle through the release of respectively contractile and inhibitory neurotransmitters. Besides contractile cholinergic and inhibitory adrenergic neurotransmitters, also other, so-called non-cholinergic non-adrenergic (NANC) neurotransmitters are released. An important inhibitory NANC neurotransmitter is nitric oxide (NO), generated by neuronal NO synthase (nNOS). NO induces relaxation of GI smooth muscle through the activation of its principal intracellular target, soluble guanylate cyclase (sGC). sGC has 2 physiologically active isoforms, i.e. the predominantly expressed $sGC\alpha_1\beta_1$ and the less abundantly expressed $sGC\alpha_2\beta_1$. However, also sGC-independent actions of NO have been shown, such as nitration and S-nitrosylation of proteins. Reversely, sGC can also be activated by other stimuli than NO, such as carbon monoxide (CO). In addition, depending on the region of the GI tract, besides NO, also other inhibitory NANC neurotransmitters such as adenosine triphosphate (ATP), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP) and CO are reported to be involved in the relaxation of GI smooth muscle. In the first part of this work, the relative importance of the sGC isoforms $\alpha_1\beta_1$ and $\alpha_2\beta_1$ in GI nitrergic relaxation, the dependency of GI nitrergic relaxation upon sGC and the importance of sGC for GI motility were investigated using genetically modified mice.

Previously, our group studied the relative importance of the two isoforms of sGC in the NO-mediated relaxation at the level of the stomach. As no isoform-specific inhibitors are available, this topic was addressed using $sGC\alpha_1$ knockout (KO) mice in which a mutant $sGC\alpha_1\beta_1$ isoform is no longer functionally active. We have now investigated the consequences of knocking out the $\alpha_1$-subunit of sGC in the jejunum (**Chapter III**) and the distal colon (**Chapter IV**). In the jejunum exogenous and endogenous NO are able to induce relaxation via the activation of $sGC\alpha_1\beta_1$ as well as $sGC\alpha_2\beta_1$. However, the relative contribution of $sGC\alpha_2\beta_1$ versus $sGC\alpha_1\beta_1$ to the response to exogenous and endogenous NO depends on gender, with $sGC\alpha_2\beta_1$ being only minimally involved in the NO-induced relaxation in the jejunum of male mice but contributing in a much more pronounced way in female mice. Still, in the jejunum of both sexes, the less abundantly expressed $sGC\alpha_2\beta_1$ seems able to maintain some degree of nitrergic relaxation in the absence of $sGC\alpha_1\beta_1$, so

that no important in vivo implications on intestinal motility were observed in the sGC$\alpha_1$ KO mice. Also in the distal colon, we found that exogenous NO is able to induce relaxation via the activation of sGC$\alpha_1\beta_1$ as well as sGC$\alpha_2\beta_1$. The relative contribution of sGC$\alpha_2\beta_1$ versus sGC$\alpha_1\beta_1$ in the NO-induced response did not differ between sexes. Opposed to exogenous NO, endogenous NO released by electrical field stimulation (EFS) of the intrinsic neurones at the lowest frequency applied - 1Hz – acts solely via sGC$\alpha_1\beta_1$. At higher stimulation frequencies (2 to 8 Hz), endogenous NO seems able to also act via sGC$\alpha_2\beta_1$. An important finding is that at these higher stimulation frequencies, NO functions together with another neurotransmitter, probably ATP acting via small conductance $Ca^{2+}$-dependent $K^+$ channels ($SK_{Ca}$ channels), with some degree of redundancy. This implies that both neurotransmitters can interact such that the loss of one is compensated by the other.

In a further step, we investigated to what extent 1) nitrergic relaxation of stomach, jejunum and colon is sGC-dependent and 2) in vivo GI motility is influenced when heme-dependent sGC activation is lacking (**Chapter V**). As reliable specific sGC inhibitors with in vivo usefulness are lacking, we approached these questions using sGC$\beta_1$his105phe knock in (KI) mice, where neither sGC$\alpha_1\beta_1$ nor sGC$\alpha_2\beta_1$ can be activated by heme-dependent sGC activators such as NO. Within our experimental conditions, both exogenous and endogenous NO seem to act solely via sGC in the investigated gastrointestinal tissues i.e. gastric fundus, jejunum and distal colon. Our results do consequently not comply with possible sGC-independent actions of NO. The total impairment of the heme-dependent and thus nitrergic activation of sGC$\alpha_1\beta_1$ as well as sGC$\alpha_2\beta_1$ induced delayed gastric emptying, delayed intestinal transit and increased whole gut transit time in the sGC$\beta_1$ KI mice. As our results show that NO - acting via sGC - is the main neurotransmitter in the gastric fundus and the jejunum, disturbances in gastric emptying and intestinal transit were expected in the sGC$\beta_1$ KI mice, in which nitrergic relaxation is abolished due to the lack of NO-sensitive sGC, corresponding to observations obtained in animals treated with NO-synthase inhibitors. The sGC$\beta_1$ KI mice showed also a marked enlargement of the stomach with hypertrophy of the circular muscle of the fundus, which is suggested to be secondary to deficient pyloric relaxation. As our results confirm that in the distal colon, NO - acting via sGC - is only the principal neurotransmitter at a stimulation frequency of 1 Hz, the extent of delay in colonic transit will probably be limited. In addition to NO and probably ATP, released and interacting in a redundant way in response to EFS at 2 to 8 Hz, as observed in the sGC$\alpha_1$ KO mice and their wild type (WT) controls, evidence for the release of yet another neurotransmitter in response to EFS at these higher stimulation frequencies was obtained in the distal colon of the sGC$\beta_1$ KI mice and their WT controls. The presence of an additional neurotransmitter in the latter mice could be related to their genetic background, i.e. a mixed 129/SvJ-C57BL/6J

background; this is different from the mixed Swiss/129 background of the sGC$\alpha_1$ KO mice and their WT controls.

Recently, after NO and CO, a third gaseous molecule, $H_2S$, came to the notice as it was also shown to be generated endogenously and to induce smooth muscle relaxation. As $H_2S$-induced smooth muscle relaxation was initially observed in the vascular system, the mechanism of action of $H_2S$ is already most extensively studied in this system. Interestingly, in contrast to NO and CO, $H_2S$ does not relax vascular smooth muscle through activation of sGC but mainly by activation of ATP-dependent $K^+$ channels ($K_{ATP}$ channels). As up to date only a few reports are available concerning the influence of $H_2S$ on GI smooth muscle, the second part of this work focused on the effect of $H_2S$ on the contractility of the mouse gastric fundus (**Chapter VI**) and distal colon (**Chapter VII**) and its mechanism of action in these two tissues.

In the mouse gastric fundus and distal colon, NaHS, an $H_2S$-donor, is able to induce concentration-dependent relaxations that are not linked to activation of the sGC-cGMP pathway. In contrast to the cardiovascular tissues however, the $K_{ATP}$ channel - nor any other major $K^+$ channel – plays a role in the NaHS-induced relaxation in the mouse gastric fundus and the distal colon. By extension, we could not find any evidence that would point to NaHS being able to induce relaxation via a decrease in the intracellular calcium concentration ($[Ca^{2+}]_{cyt}$). However, relaxation in smooth muscle cells can also take place without an underlying decrease in $[Ca^{2+}]_{cyt}$; this mechanism of action is called calcium desensitization as relaxation of the contractile apparatus then occurs in the presence of unaltered $[Ca^{2+}]_{cyt}$. Calcium desensitization is most frequently achieved by the activation of myosin light chain phosphatase (MLCP), leading to a higher degree of dephosphorylation of myosin light chain (MLC) and subsequently relaxation. The MLCP inhibitor calyculin-A reduced the NaHS-induced relaxations in the mouse gastric fundic strips, suggesting that the activation of MLCP is indeed involved in the relaxant effect of NaHS in mouse gastric fundus. The most important mechanism leading to an increased activity of MLCP is relieving MLCP from the inhibitory actions of the Rho-A/Rho-kinase pathway. However, the Rho-kinase inhibitor Y-27632 did not influence NaHS-induced relaxation; the activation of MLCP by NaHS in the mouse gastric fundus is thus not related to suppression of the Rho-A/Rho-kinase pathway. Similar to the mouse gastric fundus, also in the mouse distal colon, the NaHS-induced relaxation is mediated via a calcium desensitizing mechanism, as simultaneous measurement of tension and $[Ca^{2+}]_{cyt}$ in the mouse distal colonic strips showed that the maximal relaxing effect of NaHS was reached without a concomitant decrease in $[Ca^{2+}]_{cyt}$. However, MLCP activation seems not involved as calyculin-A did not affect the NaHS-induced relaxation in mouse distal colon.

**In conclusion**, our findings indicate that NO is the principal relaxant neurotransmitter in the mouse gastric fundus and the mouse jejunum. In the mouse distal colon, NO is the sole relaxant neurotransmitter released by EFS at 1 Hz, however at higher stimulation frequencies, other neurotransmitters contribute to the smooth muscle relaxation. Our results further show that the relaxant effect of endogenous and exogenous NO in the investigated GI tissues completely relies on the activation of sGC isoforms $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$. In the absence of $sGC\alpha_1\beta_1$, $sGC\alpha_2\beta_1$ is able to maintain normal GI functioning. Impairment of the NO-induced activation of $sGC\alpha_1\beta_1$ and $sGC\alpha_2\beta_1$ induces GI malfunctions, indicating the important role of the NO-sGC pathway in GI motility.

In the mouse gastric fundus and distal colon, the $H_2S$-donor NaHS is able to induce concentration-dependent relaxations via a calcium desensitizing mechanism. In the mouse gastric fundus, this NaHS-induced calcium desensitization involves activation of MLCP; the most important mechanism leading to an increased activity of MLCP, i.e. suppression of the Rho-A/Rho-kinase pathway, is however not involved. In the mouse distal colon, the NaHS-induced calcium desensitization is not related to activation of MLCP.

# Chapter X

# SAMENVATTING

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Neuronale controle-mechanismen, tot stand gebracht via het extrinsieke zenuwstelsel en het intrinsieke of enterische zenuwstelsel, spelen een belangrijke rol in de regulatie van de gastro-intestinale motiliteit. De myenterische plexus, die een onderdeel is van het enterische zenuwstelsel en gelegen is tussen de longitudinale en circulaire gladde spierlaag, controleert de contractie en relaxatie van gastro-intestinale gladde spieren via de vrijstellling van respectievelijk contractiele en relaxerende neurotransmitters. Naast contractiele cholinerge en relaxerende adrenerge neurotransmitters worden ook andere, zogeheten niet-adrenerge niet-cholinerge (NANC) neurotransmitters vrijgesteld. Een belangrijke relaxerende NANC neurotransmitter is stikstofmonoxide (NO), geproduceerd door het neuronale NO synthase (nNOS). NO veroorzaakt relaxatie van gastro-intestinale gladde spiercellen door middel van activering van oplosbaar guanylaat cyclase (sGC), zijn belangrijkste intracellulaire doelwit. sGC bestaat in twee fysiologisch actieve isovormen namelijk $sGC\alpha_1\beta_1$ en $sGC\alpha_2\beta_1$, waarvan $sGC\alpha_1\beta_1$ de meest voorkomende isovorm is in de gastro-intestinale tractus. Niettemin werden ook sGC-onafhankelijke effecten van NO aangetoond, zoals nitratie en S-nitrosylatie van eiwitten. Daarentegen kan sGC ook geactiveerd worden door andere neurotransmitters dan NO, zoals koolstofmonoxide (CO). Bovendien blijken, afhankelijk van de onderzochte diersoort en gastro-intestinale regio, ook andere NANC neurotransmitters, naast NO, betrokken te zijn in de relaxatie van gastro-intestinale gladde spiercellen, zoals adenosine-trifosfaat (ATP), vaso-actief intestinaal peptide (VIP), hypofysair adenylaat cyclase activerend peptide (PACAP) en CO. In het eerste deel van dit proefschrift onderzochten we, gebruikmakend van genetisch gemanipuleerde muizen, het relatieve belang van de sGC isovormen $\alpha_1\beta_1$ and $\alpha_2\beta_1$ in de gastro-intestinale nitrerge relaxatie, de afhankelijkheid van de gastro-intestinale nitrerge relaxatie ten opzichte van sGC en het belang van sGC in de gastro-intestinale motiliteit.

In een vorige studie onderzocht onze groep het relatief belang van de twee sGC isovormen in de nitrerge relaxatie ter hoogte van de maag. Aangezien geen isovorm-specifieke remmers beschikbaar zijn, werd dit onderwerp onderzocht door middel van $sGC\alpha_1$ knock-out (KO) muizen, waarbij de mutante $sGC\alpha_1\beta_1$ isoform niet langer functioneel actief is. In deze studie werden de gevolgen van het elimineren van de $\alpha_1$-subeenheid van het sGC enzym bestudeerd ter hoogte van het jejunum (**Hoofdstuk III**) en het distaal colon (**Hoofdstuk IV**). In het jejunum kunnen zowel exogeen NO als endogeen NO relaxatie veroorzaken door

activering van $sGC\alpha_1\beta_1$ en $sGC\alpha_2\beta_1$. De relatieve bijdrage van $sGC\alpha_1\beta_1$ versus $sGC\alpha_2\beta_1$ in het antwoord op exogeen NO en endogeen NO is echter geslachtsafhankelijk, waarbij $sGC\alpha_2\beta_1$ slechts in beperkte mate betrokken is in de nitrerge relaxatie in het jejunum van mannelijke muizen maar een meer uitgesproken bijdrage levert in de vrouwelijke muizen. Niettemin is, in de afwezigheid van $sGC\alpha_1\beta_1$, het minder voorkomende $sGC\alpha_2\beta_1$ in staat om een zekere mate van nitrerge relaxatie te behouden in het jejunum van zowel mannelijke als vrouwelijke muizen. Er werden dan ook geen belangrijke in vivo implicaties betreffende de intestinale relaxatie geobserveerd in de $sGC\alpha_1$ KO muizen. Ook in het distaal colon vonden we dat exogeen NO relaxaties kan veroorzaken via activering van $sGC\alpha_1\beta_1$ en $sGC\alpha_2\beta_1$. De relatieve bijdrage van $sGC\alpha_2\beta_1$ versus $sGC\alpha_1\beta_1$ in de NO-geïnduceerde relaxatie was niet verschillend in beide geslachten. In tegenstelling tot exogeen NO, werkt endogeen NO, dat vrijgesteld wordt na elektrische veldstimulatie (EVS) van de intrinsieke neuronen aan de laagst toegepaste frequentie, namelijk 1 Hz, enkel via $sGC\alpha_1\beta_1$. Endogeen NO dat vrijgesteld wordt bij hogere stimulatie-frequenties (2 tot 8 Hz) blijkt wel in staat te zijn om ook via $sGC\alpha_2\beta_1$ te functioneren. Een belangrijke bevinding is dat bij deze hogere stimulatie-frequenties, NO op een redundante wijze samenwerkt met een andere neurotransmitter. Deze tweede neurotransmitter is waarschijnlijk ATP, dat relaxatie veroorzaakt via de activering van de $Ca^{2+}$-afhankelijke $K^+$ kanalen met lage begeleiding ($SK_{Ca}$ kanalen). Beide neurotransmitters werken op zodanige wijze samen dat de afwezigheid van de ene neurotransmitter gecompenseerd wordt door de aanwezigheid van de andere neurotransmitter.

In een volgende stap gingen we na in hoeverre 1) de nitrerge relaxatie in de maag, het jejunum en het distaal colon sGC-afhankelijk is, en 2) de in vivo motiliteit beïnvloed wordt wanneer haem-afhankelijke sGC-activatie afwezig is (**Hoofdstuk V**). Aangezien betrouwbare en specifieke remmers van sGC met in vivo bruikbaarheid niet beschikbaar zijn, hebben we deze vragen benaderd aan de hand van $sGC\beta_1$his105phe knock-in (KI) muizen, waarbij $sGC\alpha_1\beta_1$ noch $sGC\alpha_2\beta_1$ geactiveerd kunnen worden door haem-afhankelijke sGC activatoren zoals NO. Binnen onze experimentele condities blijken zowel exogeen NO als endogeen NO enkel via sGC te functioneren in de onderzochte gastro-intestinale weefsels, namelijk de maagfundus, het jejunum en het distaal colon. Onze resultaten zijn bijgevolg niet verenigbaar met mogelijke sGC-onafhankelijke acties van NO. De totale afwezigheid van de haem-afhankelijke en dus ook nitrerge activatie van zowel $sGC\alpha_1\beta_1$ als $sGC\alpha_2\beta_1$ gaf aanleiding tot vertraagde maaglediging, vertraging in de darmtransit en een verhoging van de totale transit-tijd in de $sGC\beta_1$ KI muizen. Aangezien onze resultaten aantoonden dat NO – werkzaam via sGC – de belangrijkste neurotransmitter is in de maagfundus en het jejunum, werden deze stoornissen in maaglediging en darmtransit, vergelijkbaar met deze geobserveerd in dieren behandeld met remmers van NO-synthase, verwacht in de $sGC\beta_1$ KI muizen, waarin de

nitrerge relaxatie uitgeschakeld is wegens het afwezig zijn van NO-activeerbaar sGC. De $sGC\beta_1$ KI muizen vertoonden ook een sterk vergrootte maag en een hypertrofe circulaire spierlaag ter hoogte van de fundus, die te wijten zou zijn aan de gebrekkige relaxatie van de pylorus. Onze resultaten bevestigen dat in het distaal colon NO – werkzaam via sGC- enkel een essentiële rol speelt bij een stimulatie-frequentie van 1 Hz. Hieruit kunnen we afleiden dat de transit in het colon wellicht slechts in beperkte mate vertraagd is in de $sGC\beta_1$ KI muizen. Bovenop NO en ATP, die vrijgesteld worden in antwoord op EVS aan 2 tot 8 Hz en op een redundante wijze samenwerken in de $sGC\alpha_1$ KO muizen en de overeenkomstige wild-type (WT) controle muizen, werden aanwijzigingen voor de vrijstelling van een bijkomende neurotransmitter in antwoord op EVS aan deze hogere stimulatie-frequenties bekomen in het distaal colon van de $sGC\beta_1$ KI muizen en hun overeenkomstige WT controle muizen. De aanwezigheid van een bijkomende neurotransmitter in deze laatst vermelde muizen zou gerelateerd kunnen zijn aan hun genetische achtergrond, namelijk een gemixte 129/SvJ-C57BL/6J achtergrond; dit is verschillend van de gemixte Swiss/129 achtergrond van de $sGC\alpha_1$ KO muizen en hun overeenkomstige WT controle muizen.

Na NO en CO komt nu ook een derde gasvormige molecule, namelijk $H_2S$, in de belangstelling nadat aangetoond werd dat ook $H_2S$ endogeen aangemaakt wordt en in staat is relaxatie van gladde spiercellen te induceren. Het werkingsmechanisme van $H_2S$ is reeds het meest bestudeerd in het vasculaire stelsel, waar de $H_2S$-geïnduceerde relaxatie initieel werd vastgesteld. In tegenstelling tot NO en CO relaxeert $H_2S$ de vasculaire gladde spiercellen niet via de activatie van sGC maar voornamelijk via de activatie van de ATP-afhankelijke $K^+$ kanalen ($K_{ATP}$ kanalen). Aangezien enkel een beperkt aantal studies betreffende de invloed van $H_2S$ op gastro-intestinale gladde spiercellen beschikbaar zijn, werd in het tweede deel van dit proefschrift de invloed van $H_2S$ op de contractiliteit van de maagfundus (**Hoofdstuk VI**) en het distaal colon (**Hoofdstuk VII**) nagegaan en werd het werkingsmechanisme van het $H_2S$ in deze twee weefsels onderzocht.
In de maagfundus en het distaal colon van de muis was NaHS, een $H_2S$-donor, in staat concentratie-afhankelijke relaxaties te induceren die niet door de activatie van de sGC-cGMP transductieweg bewerkstelligd werden. In tegenstelling tot de cardiovasculaire weefsels speelde noch het $K_{ATP}$ kanaal noch één van de andere belangrijke $K^+$ kanalen een rol in de NaHS-geïnduceerde relaxatie in de maagfundus en het distaal colon. Bij uitbreiding vonden we geen enkel bewijs dat zou kunnen suggereren dat NaHS relaxatie veroorzaakt via een daling in de intracellulaire calciumconcentratie ($[Ca^{2+}]_{cyt}$). Relaxatie in gladde spiercellen kan echter ook bewerkstelligd zonder een daling in $[Ca^{2+}]_{cyt}$; dit werkingsmechanisme noemt men calcium desensitisatie aangezien relaxatie van het contractiele apparaat gebeurt zonder verandering in $[Ca^{2+}]_{cyt}$. Calcium desensitisatie wordt meestal tot stand gebracht door de

activatie van het fosfatase van de lichte keten van myosine (MLCP), hetgeen leidt tot een hogere graad van defosforylatie van de lichte keten van myosine (MLC) met relaxatie tot gevolg. Calyculin-A, een remmer van het MLCP, verminderde de NaHS-geïnduceerde relaxatie in de maagfundus, hetgeen aangeeft dat activatie van het MLCP inderdaad een rol speelt in het relaxerend effect van NaHS in de maagfundus van de muis. Het belangrijkste mechanisme om een verhoogde activiteit van het MLCP te bewerkstelligen is de opheffing van de remmende effecten van de Rho-A/Rho-kinase transductieweg. De Rho-kinase inhibitor Y-27632 beïnvloedde echter de NaHS-geïnduceerde relaxatie niet; de activatie van het MLCP door NaHS in de maagfundus van de muis is dus niet gerelateerd met onderdrukking van de Rho-A/Rho-kinase transductieweg. Gelijkaardig aan de maagfundus, vonden we dat ook in het distaal colon de NaHS-geïnduceerde relaxatie tot stand komt via een calcium desensitizerend mechanisme. Inderdaad, door middel van simultane meting van tensie en $[Ca^{2+}]_{cyt}$ in het distaal colon toonden we aan dat NaHS een maximale relaxatie veroorzaakt zonder een daling in $[Ca^{2+}]_{cyt}$ te bewerkstelligen. Echter, activatie van het MLCP blijkt niet betrokken te zijn in de relaxatie door NaHS in het distaal colon van de muis aangezien calyculin-A geen invloed had.

**Samenvattend** tonen de resultaten in dit proefschrift aan dat NO de belangrijkste relaxerende neurotransmitter is in de maagfundus en het jejunum van de muis. In het distaal colon van de muis is NO de enige relaxerende neurotransmitter die vrijgesteld wordt door elektrische stimulatie aan een frequentie van 1 Hz, bij hogere stimulatie-frequenties zijn ook andere neurotransmitters betrokken in de relaxatie van de gladde spiercellen. Verder toonden we aan dat het relaxerend effect van endogeen NO en exogeen NO in de drie onderzochte gastro-intestinale weefsels volledig afhangt van de activatie van de sGC isovormen $sGC\alpha_1\beta_1$ en $sGC\alpha_2\beta_1$. In de afwezigheid van $sGC\alpha_1\beta_1$ is $sGC\alpha_2\beta_1$ in staat om het normale gastro-intestinale functioneren te vrijwaren. De totale afwezigheid van de nitrerge activatie van zowel $sGC\alpha_1\beta_1$ als $sGC\alpha_2\beta_1$ veroorzaakt gastro-intestinale stoornissen; dit wijst op de belangrijke rol van de NO-sGC transductieweg in de gastro-intestinale motiliteit.
In de maagfundus en het distaal colon van de muis is $H_2S$-donor NaHS in staat om concentratie-afhankelijke relaxaties te veroorzaken door middel van een calcium desensitizerend mechanisme; in de maagfundus van de muis induceert NaHS calcium desensitisatie door middel van activatie van het MLCP; het belangrijkste mechanisme dat tot een verhoogde activiteit van het MLCP leidt, namelijk de onderdrukking van de Rho-A/Rho-kinase transductieweg, is echter niet betrokken. In het distaal colon van de muis is de calcium desensitisatie die door NaHS geïnduceerd wordt niet bewerkstelligd door middel van activatie van het MLCP.

# DANKWOORD

# Dankwoord

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