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NERVE-INDUCED RELEASE OF NITRIC OXIDE IN GASTROINTESTINAL AND ERECTILE TISSUE

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Nerve-induced release of nitric oxide in gastrointestinal and erectile tissue.
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Bortom all ära och redlighet ligger en dal som kallas Mumindalen. Den befolkas av varelser som lever mer eller mindre på känn och går i ide till vintern.
De vet mycket lite om civilisation…

Dalen där de bodde var mycket vacker. Den var full av lyckliga småkryp och stora gröna träd. Tvärs genom ängarna rann floden, den gjorde en bukt kring det blåa muminhuset och försvann mot andra platser med andra småkryp som undrade varifrån den kom…

Tove Jansson

TOM SIDA

ABSTRACT

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LIST OF PUBLICATIONS

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I OLGART, C., **HALLÉN, K.**, WIKLUND, N.P., IVERSEN, H.H. & GUSTAFSSON, L.E. (1998).
Blockade of nitrergic neuroeffector transmission in guinea-pig colon by a selective inhibitor of soluble guanylyl cyclase. Acta Physiol Scand, 162, 89-95

II **HALLÉN, K.**, OLGART, C., GUSTAFSSON, L.E. & WIKLUND, N.P. (2001).
Modulation of neuronal nitric oxide release by soluble guanylyl cyclase in guinea pig colon. Biochem Biophys Res Commun, 280, 1130-4.

III **HALLÉN, K.**, GUSTAFSSON, L.E. & WIKLUND, N.P.
Nerve-induced release of nitric oxide from the rabbit corpus cavernosum is modulated by cyclic GMP (submitted).

IV **HALLÉN, K.**, WIKLUND, N.P. & GUSTAFSSON, L.E.
PDE 5 inhibitors influence the nerve-induced release of nitric oxide from the rabbit corpus cavernosum (submitted).

CONTENTS

ABBREVIATIONS

ACh	acetylcholine
ANP	atrial natriuretic peptides
ATP	adenosine-5'-triphosphate
cAMP	cyclic adenosine 3'-5' monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
EDRF	endothelium derived relaxing factor
EFS	electrical field stimulation
eNOS	endothelial nitric oxide synthase
FAD	flavine adenine dinucleotide
FMN	flavine mononucleotide
GC	guanylate cyclases
GSNO	S-nitroso-glutathione
GTP	guanosine-5'-triphosphate
HPLC	high performance liquid chromatography
iNOS	inducible nitric oxide synthase
IP_3	inositol 1,4,5-triphosphate receptors
L-NAME	N^{ω} -nitro-L-arginine methyl ester
7-NI	7-nitroindazole
L-NIO	N^{ω} -iminoethyl-L-ornithine
L-NMMA	N^{ω} -monomethyl-L-arginine
L-NOARG	N^{ω}-nitro-L-arginine
mtNOS	mitochondrial nitric oxide synthase
muNOS	muscluar nitric oxide synthase
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NANC	non-adrenergic non-cholinergic
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
NP	natriuretic peptide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PDE	phosphodiesterase
PKA	protein kinases A
PKG	protein kinases G
sGC	soluble guanylate cyclases
SNAP	S-nitroso-acetyl-penicillamine
TTX	tetrodotoxin
VIP	vasoactive intestinal peptide
YC-1	3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole

INTRODUCTION

The autonomic nervous system regulates many responses throughout the organism and contributes to the homoeostasis of various physiological and hence crucial functions such as respiration, circulation, digestion and reproduction. These functions are important for the survival of humans and animals, and are without voluntary input or control. In the present thesis the autonomic nervous system in the gastrointestinal and genital tract has been studied, with special emphasis on nitric oxide (NO) as a messenger molecule. The neuronal release of NO and its feedback mechanism is the main subject for the present study.

Autonomic nervous system

The peripheral autonomic nervous system innervates smooth muscle cells throughout the organism, such as blood vessels, heart, lungs, intestinal and urogenital organs as well as glands. It consists of three parts, the sympathetic, the parasympathetic and the enteric nervous system. The sympathetic and parasympathetic parts have been ascribed opposite effects such as increases or decreases in blood pressure, positive or negative chronotropic effects and increased or decreased motility of the gut. In some organs the sympathetic and parasympathetic nervous system mediates synergistic effects, e.g. in the salivary glands where parasympathetic neurons contributes to the amount of secret, while sympathetic neurons increase the protein content of the saliva. The third part of the autonomic nervous system, the enteric nervous system, is located in the wall of the intestine. It is present in the gastrointestinal tract from the oesophagus to the anal sphincter and functions to coordinate movements and secretion of the different segments of the gastrointestinal tract. It functions by its own, but is under modulation by parasympathetic and sympathetic neurons (Bennett, 1997; Gabella, 1976).

In order for the neurons to communicate, either with other neurons or with smooth muscle cells, molecules, i.e. neurotransmitters, are released upon nerve activation. A century ago, it was believed that the transmission of signals by neurons was of either electrical or chemical character. In a classic experiment it was shown that stimulation of the vagal nerve in frogs inhibited the heart activity and that the inhibition could be transferred to another heart (Löwi, 1921). Therefore it was concluded that a substance (Vagusstoff) was released and that neurotransmission is mediated via release of chemical messenger molecules. The substance has later been identified as acetylcholine (ACh) (Burnstock, 1986; Dale *et al.*, 1929).

The most common neurotransmitter in the parasympathetic part of the autonomic nervous system is ACh whereas noradrenaline (NA) is the most prominent transmitter substance in the sympathetic nervous system. Release of ACh results in an excitatory response of the smooth muscle cells, while NA inhibits the motility in non-sphincter regions of the gut (Furness *et al.*, 1992). However, ACh and NA did not fully explain the nerve-mediated inhibitory responses in the urinary or gastrointestinal tract.

For long time it was believed that noradrenaline and acetylcholine were the only neurotransmitters in the autonomic nervous system. This was questioned in 1960's, by two important and almost simultaneous discoveries. First, it was found that nerve stimulation in the presence of cholinergic and adrenergic blockage resulted in hyper-polarisation of the smooth muscle cells of the intestine (Burnstock *et al.*, 1963; Burnstock *et al.*, 1964). In another study it was demonstrated that vagal stimulation in the presence of the cholinergic blocker atropine elicited relaxations of the cat stomach and the relaxations were unaffected by adrenergic nerve blocking agents (Martinson, 1965). Taken together, this demonstrated the presence of nerves that were neither adrenergic nor cholinergic and the term non-adrenergic non-cholinergic (NANC) neurons was introduced (Burnstock, 1986; Lundberg *et al.*, 1986), and the search for this proposed neurotransmitter started.
Different endogenously formed substances such as adenosine-5'-triphosphate (ATP) and adenosine, (Burnstock, 1972; Burnstock *et al.*, 1970), as well as vasoactive intestinal peptide (VIP) (Fahrenkrug, 1979; Goyal *et al.*, 1980) was suggested to mediate the inhibitory NANC responses. However, none of them fully explained the effects of NANC mediated neurotransmission (Furness *et al.*, 1992). Later, inhibitory NANC nerves have been identified not only in gastrointestinal and urogenital tracts, but also in the respiratory and cardiovascular systems. The discovery of the endothelium derived relaxing factor (EDRF), later shown to be NO, has explained various physiological responses previously described as to be NANC-mediated. However, NO as a mediator of NANC-responses questioned some of the classical criteria's of classical neuro-transmission (Burnstock, 1986).

Neurotransmission

Neurotransmission takes place over synapses or neuroeffector junctions (Burnstock, 1986). Synapses are present in the neuromuscular junctions e.g. in a skeletal muscle cell or at a neural connection within a ganglia. They are characterised by specialisation of both nerve and muscle membranes, and the distance between the nerve and nerve/muscle cell is about 50 nm. The neuroeffector junctions are present in tissues innervated by the autonomic nervous system and are characterised by a thickening in the plasma membrane of the distal part of the nerve endings, so called varicosities. The varicosities indicate the site for release of the neurotransmitter, but there is no corresponding postjunctional specialisation in the membrane of the effector cell. Further, the distance between the nerve and the smooth muscle cell varies from 20 nm in densely innervated tissues to 1 μm in large elastic arteries (Burnstock, 1986). Also, in the autonomic nervous system transmitter release can occur spontaneously at random sites, whereas nerve-induced release occurs at preferred sites of the neurons (Cunnane *et al.*, 1984).
During neurotransmission, messenger molecules are released from the neurons and diffuse to the surrounding cells. Microscopic studies of the nerve terminals revealed the presence of small membrane-enclosed organelles, so called synaptic vesicles (de Robertis, 1958; Robertson, 1956) that later were demonstrated to contain neurotransmitters (von Euler *et al.*, 1956). Upon nerve activation, the concentration of Ca^{2+} is increased locally at the nerve terminal and the process for release of the content of the synaptic vesicles into the synaptic cleft (exocytosis) begins. The intracellular increase of Ca^{2+} triggers the

synaptic vesicles to dock at specialised zones and fuse with the plasma membrane whereby the content of the vesicle is released (Südhof, 1995). For intact exocytosis and release of the neurotransmitters, highly conserved membrane proteins associated with the vesicle (synaptobrevin/VAMP) and the plasma membrane (SNAP-25 and syntaxin) are important (Jahn *et al.*, 1995; Montecucco *et al.*, 1995). These membrane proteins can be cleaved by clostridial neurotoxins (tetanus toxin and botulinum neurotoxins) that result in inhibition of neurotransmitter release. Thus, neurotoxins have been important tools to study the Ca^{2+}-dependent release of neurotransmitters, without affecting propagation of nerve impulses, Ca^{2+} entry, neurotransmitter synthesis, uptake or storage (Jahn *et al.*, 1995; Montecucco *et al.*, 1995).
At present, it is well established that neurotransmitters are substances synthesised in the nerve terminal, stored in vesicles, and released following nerve activation. When the neurotransmitters are released they diffuse across the junctional cleft and occupy receptors in the postjunctional membrane, which leads to changes in the activity of the effector cell (Boron *et al.*, 2003; Burnstock, 1986).
Neurotransmitters are acutely influencing the excitability of innervated cells or tissues and are often referred to as primary transmitters. Initially it was thought that nerves would contain only one neurotransmitter. However, it became evident that neurons could release more than one transmitter and co-transmission of neurotransmitters was suggested (Burnstock, 1976; Hökfelt *et al.*, 1977; Hökfelt *et al.*, 1987). In cases of co-transmission (e.g ACh and substance P), one compound seems to have a dominant role (Furness *et al.*, 1992). There are also substances that contribute to the changes in excitability by enhancing or diminishing the release or action of a primary transmitter. Theses substances are often referred to as neuromodulators (Furness *et al.*, 1992; Hökfelt *et al.*, 1987).

The discovery of nitric oxide

In 1980 Furchgott and co-workers proposed that the endothelium released a factor that mediated smooth muscle relaxation of aortic rings. In preparations of rabbit aortic rings it was found that application of ACh produced a relaxation if the endothelium was intact. In contrast, rings where the endothelium had been removed, responded with a contraction upon application of ACh. Furchgott suggested that ACh released a factor from the endothelial cells that mediated the relaxation and the factor was called endothelium derived relaxing factor (EDRF) (Furchgott *et al.*, 1980).
The process of identification of EDRF began and it was concluded that EDRF was synthesised by endothelial cells and could be transferred from a donor to a detector tissue (Furchgott, 1984; Griffith *et al.*, 1984; Rubanyi *et al.*, 1985). Also, cultured endothelial cells were found to release EDRF (Cocks *et al.*, 1985; Gryglewski *et al.*, 1986a) and EDRF appeared to be a short lived substance with a half-life of a few seconds (Cocks *et al.*, 1985; Griffith *et al.*, 1984). Several substances were found to inhibit the effect of EDRF such as haemoglobin and methylene blue (Martin *et al.*, 1985) and Fe^{2+} (Gryglewski *et al.*, 1986b). Furthermore, it seemed as EDRF was unstable in the presence of increased amounts of oxygen and superoxide (Gryglewski *et al.*, 1986b; Rubanyi *et al.*, 1986), whereas hypoxia stabilised EDRF. It was also found that EDRF, apart from a relaxation, caused increased formation of intracellular cyclic guanosine 3',5'-

monophosphate (cGMP) in the vascular smooth muscle (Bowman *et al.*, 1984; Bowman *et al.*, 1982b; Martin *et al.*, 1985; Rapoport *et al.*, 1983).
At a Mayo clinic conference on Mechanisms in Vasodilatation (1986), Furchgott suggested that EDRF might be nitric oxide (NO) since there were apparent chemical similarities to acidified nitrite (Furchgott, 1990). At the same meeting Ignarro made a similar speculation, the full data were published in 1987 (Ignarro *et al.*, 1987a; Ignarro *et al.*, 1987b). A conclusive experiment to identify EDRF as NO was made by utilising a chemiluminescence technique and determine NO by its reaction with ozone (Palmer *et al.*, 1987). The biological activity of EDRF and NO was compared in terms of relaxation of bioassay tissues and were found to be indistinguishable. Furthermore, the action of EDRF and NO were equal in terms of stability, and the relaxations induced by EDRF and NO were inhibited by haemoglobin and potentiated by superoxide dismutase to a similar degree. Therefore the authors concluded that EDRF and NO were identical (Palmer *et al.*, 1987).

Enzymatic formation of nitric oxide
Once the identity of EDRF had been revealed, the characterisation of the pathway for synthesis began. NO is synthesised from the amino acid L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988) and the reaction forms L-citrulline as a co-product (Palmer *et al.*, 1989). The reaction is enzymatically catalysed and the enzyme was characterised to be a P-450 reductase (Bredt *et al.*, 1991). The formation of NO from L-arginine requires molecular oxygen and NADPH as well as the presence of co-factors such as FAD, FMN, haem and tetrahydrobiopterin (Bredt *et al.*, 1991; Leone *et al.*, 1991; Mayer *et al.*, 1991).
The enzyme responsible for NO synthesis was named NO synthase (NOS). At present, three main isoforms of the enzyme have been identified, out of which two are expressed constitutively and one isoform is inducible. The isoforms of the enzymes were named after the tissue they were initially found in (Alderton *et al.*, 2001).
The first NOS to be identified was neuronal NOS (nNOS, NOS I), which was found to be localised within the peripheral as well as the central nervous system (Bredt *et al.*, 1990; Knowles *et al.*, 1989). Furthermore, endothelial NOS (eNOS, NOS III) was found in porcine vascular endothelial cells (Mayer *et al.*, 1989; Palmer *et al.*, 1989). The activity of both nNOS and eNOS are regulated by intracellular calcium concentrations and the enzymes catalyse the formation of NO influenced by physiological stimuli (Knowles *et al.*, 1989; Mayer *et al.*, 1989). Lack of neurons expressing nNOS results in stenosis of the pyloric sphincter (Huang *et al.*, 1993) and absence of eNOS is accomplished by increased blood pressure and reduced heart rate (Alderton *et al.*, 2001; Moncada *et al.*, 1991; Shesely *et al.*, 1996).
Apart from the constitutive isoforms of NOS, an inducible isoforms was identified (iNOS, NOS II) (Stuehr *et al.*, 1989; Tayeh *et al.*, 1989). By exposing macrophages to interferon-gamma and bacterial lipopolysaccharide, iNOS was induced and found to catalyse the conversion of L-arginine to NO. In contrast to the constitutive NOS, iNOS has a Ca^{2+}-calmodulin complex tightly bound to the protein, hence in calcium independent (Cho *et al.*, 1992; Stuehr *et al.*, 1991). Inducible NOS is immunologically induced by cytokines at the transcriptional level, and is present primarily in macrophages, where it produces NO in high concentrations for long periods of time (Vodovotz *et al.*,

1994; Xie *et al.*, 1992). The inducible NOS is absent in resting cells, but gene expression can be induced upon stimulation in different types of cells including hepatocytes, epithelial cells, as well as in macrophages (Aktan, 2004; Nathan *et al.*, 1994).
The three NOS were identified to be approximately 125-155 kD in size (Pollock *et al.*, 1991; Schmidt *et al.*, 1991; Stuehr *et al.*, 1991). They are structurally related, show 50-60 % homology and requires dimerisation to homodimers for enzymatic activity (Hobbs *et al.*, 1999). The genes that encode the various NOS in humans are located on different chromosomes. nNOS is located on chromosome 12 (Kishimoto *et al.*, 1992; Xu *et al.*, 1993) and eNOS is located on chromosme 7 (Marsden *et al.*, 1993; Xu *et al.*, 1994), whereas iNOS has been found in chromosome 14 and 17 (Chartrain *et al.*, 1994; Xu *et al.*, 1995).
In addition to NOS I-III, evidence for a mitochondrial NOS (mtNOS) in heart, skeletal muscle and kidney has been presented. It was suggested that mtNOS might be involved in regulation of mitochondrial oxidative phosphorylation in mammalian cells (Bates *et al.*, 1995) and it was found that nM concentrations of NO inhibit mitochondrial respiration in a reversible manner. The inhibition occurred at the level of cytochrome oxidase and it was suggested that inhibition of the cytochrome oxidase by NO competes with the activation by oxygen and may occur *in vivo* (Brown *et al.*, 1994). Mitochondrial NOS as well as muscle NOS (muNOS) are alternative splicing products for nNOS (Haynes *et al.*, 2004).
The activity of NOS is regulated by negative feedback (Griscavage *et al.*, 1995). In rat cerebellum it was found that removal of NO by addition of haemoglobin increased the activity of NOS. Further, superoxide dismutase and donors of NO inhibited the activity of nNOS. Therefore it was suggested that NO may function as a negative feedback modulator of its own synthesis (Rogers *et al.*, 1992). The finding that nNOS is subject to modulation by NO was confirmed also for eNOS and iNOS. The NO donor S-nitroso-acetyl-penicillamine (SNAP) or S-nitroso-glutathione (GSNO) markedly inhibited the enzyme activity in activated macrophages (Assreuy *et al.*, 1993). In porcine endothelial cells of the aortic wall it was found that NO and NO-donors inhibited the activity of eNOS and that presence of haemoglobin facilitated the activity of eNOS. Furthermore, pre-treatment of isolated bovine pulmonary arterial rings with an NO-donor diminished the endothelium-dependent relaxation without altering endothelium-independent relaxation to NO itself (Buga *et al.*, 1993).

Nitric oxide as a messenger in the autonomic nervous system
Upon the discovery of NO it was suggested that NO might mediate NANC inhibitory responses in smooth muscle cells (Gillespie *et al.*, 1989; Rand, 1992). In neurons, NO was first found in the central nervous system (Garthwaite *et al.*, 1988; Garthwaite *et al.*, 1989; Knowles *et al.*, 1989), and then identified in the peripheral autonomic nervous system (Bult *et al.*, 1990b; Gillespie *et al.*, 1989; Li *et al.*, 1989), indicating a role for NO not just in the vascular system but also as a neurotransmitter. The first evidence for NO serving as a neurotransmitter was obtained by chemical characterisation of the biological activity of EDRF/NO as well as inhibition of the synthesis by different L-arginine analogues. The presence of nitrergic neurotransmission was further established by identification of NOS in enteric neurons (Bredt *et al.*, 1990; Dalziel *et al.*, 1991; Llewellyn-Smith *et al.*, 1992) and the increased formation of cGMP in the intestinal

smooth muscle cells upon neuronal stimulation (Kanada *et al.*, 1992; Shuttleworth *et al.*, 1993).

Electrical stimulation of enteric neurons in guinea pig ileum and colon elicits release of NO and relaxations of the smooth muscle cells. By utilising HPLC (Wiklund *et al.*, 1993b) or a chemiluminescence technique (Iversen *et al.*, 1994; Wiklund *et al.*, 1993a) the evoked release of NO was quantified in guinea pig ileum and colon, measured as the breakdown product NO_2^-. The formation of NO via the L-arginine/NO-pathway and a neuronal origin was indicated by diminished NO-release in the presence of an NOS-inhibitor and the neurotoxin tetrodotoxin (TTX). Further, the release of NO has been visualised in the myenteric plexus of guinea pig colon, in the hypogastric trunk of rabbits and in cultured cells (Wiklund *et al.*, 1997; Wiklund *et al.*, 1999). These studies confirmed that NO utilise the L-arginine/NO-pathway and identified the nerve-induced release of NO not only at dthe varicosities of the nerve endings, but also along the entire neuron including the axon. The nerve-induced release of NO was not affected by preventing the synaptic vesicles to bind and fuse with the nerve membrane in the nerve terminals, therefore it was suggested that NO is not stored within vesicles (Olgart *et al.*, 2000). Taken together, this indicates that NO is released from the autonomic neurons but is not behaving like the classical transmitters.

Prior to the discovery of NO it was described that extracts from the ox or bovine retractor penis and rat anococcygeus muscle exerted inhibitory responses on various tissues (Ambache *et al.*, 1975; Bowman *et al.*, 1979; Gillespie *et al.*, 1978; Gillespie *et al.*, 1980). It was shown that the isolated material occurred in two different forms, a stable inactive form that upon acidification (pH 2) was converted into the active but instable form. Also, the extracts were thermolabile and the muscular inhibitory responses were unaffected by blockage of catecholamines, ACh and histamine. It was suggested that the inhibitory substance was not ATP, and was also unlikely to be a prostaglandin or bradykinin (Gillespie *et al.*, 1980). Later, it was found that electrical filed stimulation (EFS) of the bovine retractor penis muscle evoked a relaxation that was preceded by a rise in the tissue content of cGMP (Bowman *et al.*, 1984). The inhibitory factor extracted from the bovine retractor penis muscle mimicked the effects of inhibitory nerve stimulation in that both the factor and nerve stimulation evoked a relaxation of the bovine retractor penis (Bowman *et al.*, 1982a; Gillespie *et al.*, 1981) that was associated with a selective rise in cGMP (Bowman *et al.*, 1984). The muscular response and the rise in cGMP were prevented by haemoglobin and methylhydroxylamine (known to block guanylate cyclase (Deguchi *et al.*, 1978)), whereas 8-Br-cGMP produced a relaxation of the muscle, which was not inhibited in the presence of haemoglobin (Bowman *et al.*, 1984). Taken together, the authors suggested that extracts of muscle contained an inhibitory neurotransmitter (Gillespie, 1990). Further, hypoxia impaired the neurally evoked relaxation in different tissues, whereas relaxations induced by VIP, prostaglandin E1, sodium nitroprusside or an inhibitory factor isolated from the bovine retractor penis were unaffected. Therefore, it was suggested that hypoxia impaired neurotransmission prejunctionally at the inhibitory nerve endings (Bowman *et al.*, 1985). In retrospect, the inhibitory factor from the bovine retractor penis muscle most likely is NO_2^- that became "active" upon acidification since nitrite is reduced to NO at low pH (Gillespie, 1990; Martin *et al.*, 1988). Although these studies did not manage to finally characterize the exact nature of the neurotransmitter, they served to significantly advance the field,

boosting interest for NANC neurotransmission and pinpointing cGMP as an important messenger mechanism in this system.

Inhibitors of nitric oxide synthesis

The high level of amino acid conservation and structural similarity in the immediate vicinity of the substrate binding sites of NOS makes it difficult to design substances selective for either NOS. Inhibitors of NOS have utilised different sites of the enzyme in order to diminish NO synthesis (Alderton *et al.*, 2001). The first NOS inhibitors that were used to describe the NO mediated effects acted on the L-arginine site of NOS. L-N^{ω}-substituted analogues compete with L-arginine, hence competitive inhibitors, and inhibit the oxidation of the guanidino group of L-arginine. Prior to the identification of NO, it was found that L-arginine was required for expression of a cytotoxic effector mechanism by activated macrophage and this mechanism was inhibited in the presence of N^{ω}-monomethyl-L-arginine (L-NMMA) (Hibbs *et al.*, 1987). Further it was found that the effect by L-NMMA could be overridden by increasing the concentration of L-arginine in the culture medium, thus NO was proposed to mediate cytotoxicity. After the identity of EDRF as NO had been revealed and L-arginine found to serve as the substrate for NO synthesis, it was shown that L-arginine analogues functions as inhibitors of NOS (Hibbs *et al.*, 1987; Palmer *et al.*, 1988; Sakuma *et al.*, 1988). Other frequently used L-arginine analogues are N^{ω}-nitro-L-arginine (L-NOARG), its methyl ester (L-NAME) and N^{ω}-iminoethyl-L-ornithine (L-NIO). Some differences can be noted between the arginine analogues. The inhibition of nNOS by L-NOARG was found to be irreversible whereas inhibition of iNOS was reversible (Dwyer *et al.*, 1991; Furfine *et al.*, 1993). Also, L-NIO was reported to inhibit iNOS more potently than L-NMMA (McCall *et al.*, 1991). 7-nitroindazole (7-NI) was initially reported to inhibit cerebellar nNOS of mice without increasing mean arterial pressure, suggesting that 7-NI would be selective to nNOS over eNOS (Moore *et al.*, 1993). However, these findings were later questioned since eNOS was inhibited by 7-NI (Prickaerts *et al.*, 1997; Zagvazdin *et al.*, 1996).

Apart from utilising the binding site for L-arginine, NOS-inhibitors can act at NOS binding sites for cofactors, e.g. tetrahydrobiopterin, calmodulin or the binding site for haem (Alderton *et al.*, 2001). Glucocorticosteroids have been shown to inhibit endotoxin-induced production of NO in the lung and liver of the rat, whereas the NO synthesis of constitutive NOS was unaffected (Knowles *et al.*, 1990). Inducible NOS has been suggested to play a major role in tissue damage in inflammatory reactions and might contribute to both tumour progression and defence. Several experimental compounds with *in vitro* selectivity for iNOS have been described, however, it has proven to be difficult to obtain selective inhibition of NOS *in vivo* (Alderton *et al.*, 2001).

Nonenzymatic formation of nitric oxide

NO is unstable in a biological environment, especially in the presence of O_2, where NO rapidly is oxidised to nitrite (NO_2^-) or nitrate (NO_3^-) (Ignarro *et al.*, 1993). However, reduction of NO_2^- and NO_3^- can take place under normal physiologic conditions. Both NO_2^- and NO_3^- are present in excessive amounts in alimentation (Weitzberg *et al.*, 1998) and saliva is rich in NO_3^- (Spiegelhalder *et al.*, 1976). Several bacteria are known to reduce

NO_3^- to NO_2^- (Godden *et al.*, 1991; Prakash *et al.*, 1966), e.g. in the mouth (Bartsch *et al.*, 1988) and in the urine (James *et al.*, 1978). Furthermore, in an acidic environment NO_2^- will be further reduced to NO. This mechanism can be facilitated by the presence of ascorbic acid (Bartsch *et al.*, 1988). The importance of these findings has been under debate and it was questioned whether non-enzymatic formation of NO has any physiological implication. *In vivo* studies revealed that NO formation in the stomach was greatly reduced by inhibiting the proton pump of the gastric mucosa in human subjects. Also, the authors found that the NO_2^- in saliva when swallowed, formed NO the in stomach (Lundberg *et al.*, 1994) and was later confirmed by letting healthy men ingest sodium nitrite and measuring the NO-formation (Lundberg *et al.*, 2004; McKnight *et al.*, 1997). In urine, large amounts of NO were released from infected NO_2^--containing urine after mild acidification. Furthermore, the growth of E. coli was markedly reduced by the addition of NO_2^- to acidified urine (Carlsson *et al.*, 2001; Lundberg *et al.*, 1997). *In vitro* studies of the vascular tissue revealed that NO_2^- evoked vasodilation and this effect was increased when pH was lowered to levels present during ischemia or hypoxia in tissues (Modin *et al.*, 2001). Further, in heart it was reported that NO can also be generated in the ischaemic heart by direct reduction of NO_2^- to NO (Zweier *et al.*, 1995). Thus, formation of NO via acidification of NO_2^- might be of physiological importance.

Soluble guanulate cyclase/cyclic guanosine 3',5'-monophosphate pathway

NO is a small gas, highly lipophilic and therefore does not respect cell membranes. Upon formation, NO will diffuse across the cell membrane and within the cell bind to soluble guanylate cyclase (sGC). In this respect, NO differs from the classical neurotransmitters since it does not bind to a receptor on the membrane of the postjunctional cell.

There are two different forms of guanylate cyclases (GC), one particulate membrane bound and one soluble intracellular form. In common, both forms catalyse the conversion of guanosine-5'-triphosphate (GTP) to cyclic guanosine-3',5'-monophosphate (cGMP). The particulate form of GC is stimulated by natriuretic peptides and consists of a transmembrane domain, a variable extracellular domain binding the natriuretic peptide and an intracellular domain with catalytic capacity (Hamad *et al.*, 2003). Application of atrial natriuretic peptides (ANP) to precontracted bovine tracheal smooth muscle (Ishii *et al.*, 1989) or guinea-pig tracheal smooth muscle (Watanabe *et al.*, 1990) resulted in a relaxation of the tissue along with increasing concentrations of cGMP.

The soluble form of GC (sGC) was first identified in 1969 (Hardman *et al.*, 1969; Ishikawa *et al.*, 1969; Schultz *et al.*, 1969; White *et al.*, 1969). It has been identified in mammalian and non-mammalian species as well as in insects and non-vertebrate animals. The enzyme consists of two different subunits, a large α- and a smaller β-unit, forming a heterodimer (Garbers, 1979) and contains haem (Hamad *et al.*, 2003; Hobbs, 1997). As the particulate GC, sGC can be divided into three different functional domains, a haem-binding domain, a central part and a catalytic domain. Before the discovery of EDRF it was known that NO and other nitro-containing compounds could stimulate GC to form cGMP (Arnold *et al.*, 1977; Katsuki *et al.*, 1977), but it was not until EDRF was identified to be NO that the importance of this pathway became clear. It has not been possible to

obtain animals lacking sGC, indicating that loss of sGC is lethal, which further underlines the importance of sGC (Hobbs, 1997).
NO will bind to the prosthetic haem-group located at the N-terminal of the enzyme and thereby increasing the activity of the enzyme hundred fold (Hobbs, 1997; Ignarro *et al.*, 1982; Ohlstein *et al.*, 1982). A prerequisite for activity of the enzyme is that the haem-moiety is in the ferrous state (Fe^{2+}), ferric (Fe^{3+}) haem results in loss of activity. The catalytic domain of sGC is located at the C-terminal of each subunit, where GTP will bind and the conversion to cGMP takes place. In between the haem-binding and the catalytic domains there is a central domain, thought to be responsible for association of

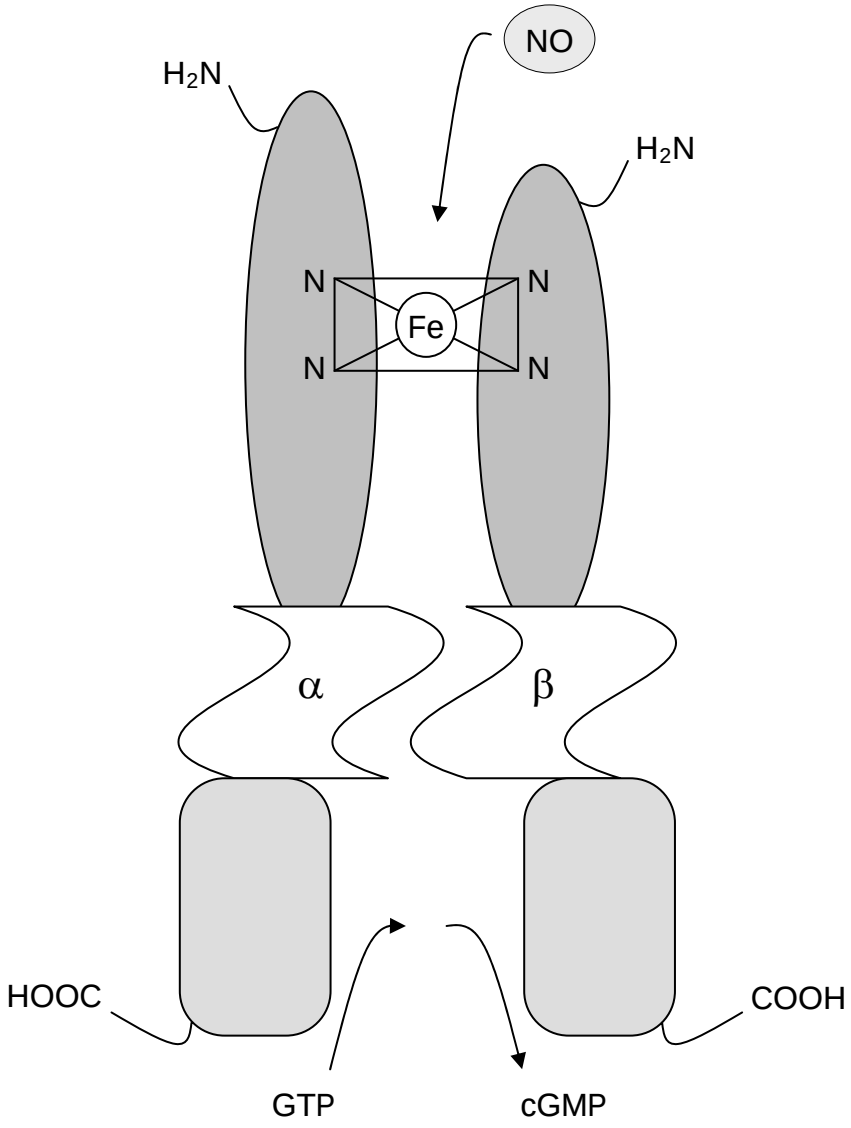


Fig. 1 Schematic presentation of heterodimer soluble guanylate cyclase containing the α- and β-unit. The haem group is attached to the amino-terminal whereas the carboxy-terminal provides the catalytic domain responsible for the formation of cGMP from GTP. The central part of each subunit mediates the dimerisation required for catalytic activity. Modified after Hobbs, 1997.

the subunits to form a heterodimer Although both the α- and β-subunit express catalytic activity co-expression of the subunits is required for catalytic activity (Hobbs, 1997; Russwurm *et al.*, 2002). Also CO can bind to the haem of sGC but this increases the activity of the enzyme only by five to six times (Brune *et al.*, 1990; Stone *et al.*, 1994).
The activity of the sGC can be pharmacologically stimulated or inhibited. In 1994 it was found that the substance 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) showed antiaggregating capacity in platelets harvested from rabbits. Upon application of YC-1 the sGC was stimulated and the content of cGMP was increased, whereas the particulate guanylate cyclase was not affected (Ko *et al.*, 1994). In addition, the phosphodiesterase (PDE) levels were unaffected. Furthermore, the antiplatelet effect of sodium nitroprusside but not of YC-1 was inhibited by haemoglobin, suggesting that YC-1 stimulates sGC independently of the presence of NO (Ko *et al.*, 1994). This finding was confirmed in human platelets (Wu *et al.*, 1995). In endothelial-free aortic rings YC-1 caused a relaxation and induced concentration-dependent activation of sGC along with increased cGMP levels (Mulsch *et al.*, 1997; Wegener *et al.*, 1997). Later studies has revealed that YC-1 sensitise sGC to NO but also to CO (Friebe *et al.*, 1996).
1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was found to inhibit the stimulation of sGC by endogenously formed NO in a reversible fashion (Garthwaite *et al.*, 1995). In incubated slices of cerebellum, ODQ reversibly inhibited the NO-dependent cGMP response, whereas NOS activity was left unaffected. Furthermore, ODQ inhibited the generation of cGMP by NO-donors. These findings were confirmed in human platelets and rat vascular smooth muscle were ODQ inhibited the antiaggregatory and vasodilator actions of endogenously released NO as well as NO-donors (Moro *et al.*, 1996). Also, in rabbit anococcygeus muscle ODQ inhibited relaxations induced by EFS (Cellek *et al.*, 1996). Characterisation of ODQ revealed that sGC is inhibited in a competitive manner by oxidising the haem group of sGC (from ferrous to ferric state) (Schrammel *et al.*, 1996). Thus, over the years ODQ has proven to be an important tool to distinguish between cGMP-dependent and -independent effects of NO signalling.
Stimulation of sGC mediates increased concentrations of cGMP. Intracellularly, there are three receptors for cGMP: cGMP protein kinases (PKG) mediating phosphorylation of proteins, cGMP-regulated ion channels allowing cation influx and cGMP-binding cyclic nucleotide PDE removing cGMP from the cytosol (Lincoln *et al.*, 1993). The rise in intracellular cGMP lowers the intracellular levels of Ca^{2+} and thereby mediates relaxation of smooth muscle cells. As of today, two major mechanisms for reduction of cytosolic Ca^{2+} have been described. First, an increase in Ca^{2+} activates K^{+}-channels, whereby the cellular membrane will be hyperpolarized hence inhibition of the influx of Ca^{2+}. The second mechanism for cGMP mediated decrease in cellular content of Ca^{2+} is mediated via phosphorylation of inositol 1,4,5-triphosphate receptors (IP_3) by PKG (Lincoln *et al.*, 2001; Lohmann *et al.*, 1997).

Phosphodiesterase
Phosphodiesterases (PDE) are terminating the action of the second messengers 3'-5' cyclic adenosine monophosphate (cAMP) and/or cGMP by converting them into the corresponding monophosphate nucleotide AMP or GMP. There are two classes of PDEs, class I present within the cells and class II that are extracellular enzymes or associated with the cell membrane (Francis *et al.*, 2001). In mammalians, all PDEs are of

class I and contain a conserved segment in the C-terminal of the protein. This part of the protein contains the catalytic site of the enzyme with the cGMP binding site as well as the Zn^{2+} binding motif required for the cleavage of the cyclic nucleotide phosphodiester bond. The class I PDEs are divided in 11 families (PDE 1-11), derived from different genes and distributed in various tissues. They show different selectivity for cAMP and cGMP and it appears as if PDE 1, 2, 3, 10 and 11 are nonselective, whereas PDE 4, 7 and 8 are specific for cAMP and PDE 5, 6 and 9 are specific for cGMP. In smooth muscle cells, the most prominent PDE is PDE 5, but also PDE 2, 3 and 4 are present (Francis *et al.*, 2001; Gibson, 2001; Rosen *et al.*, 2003).

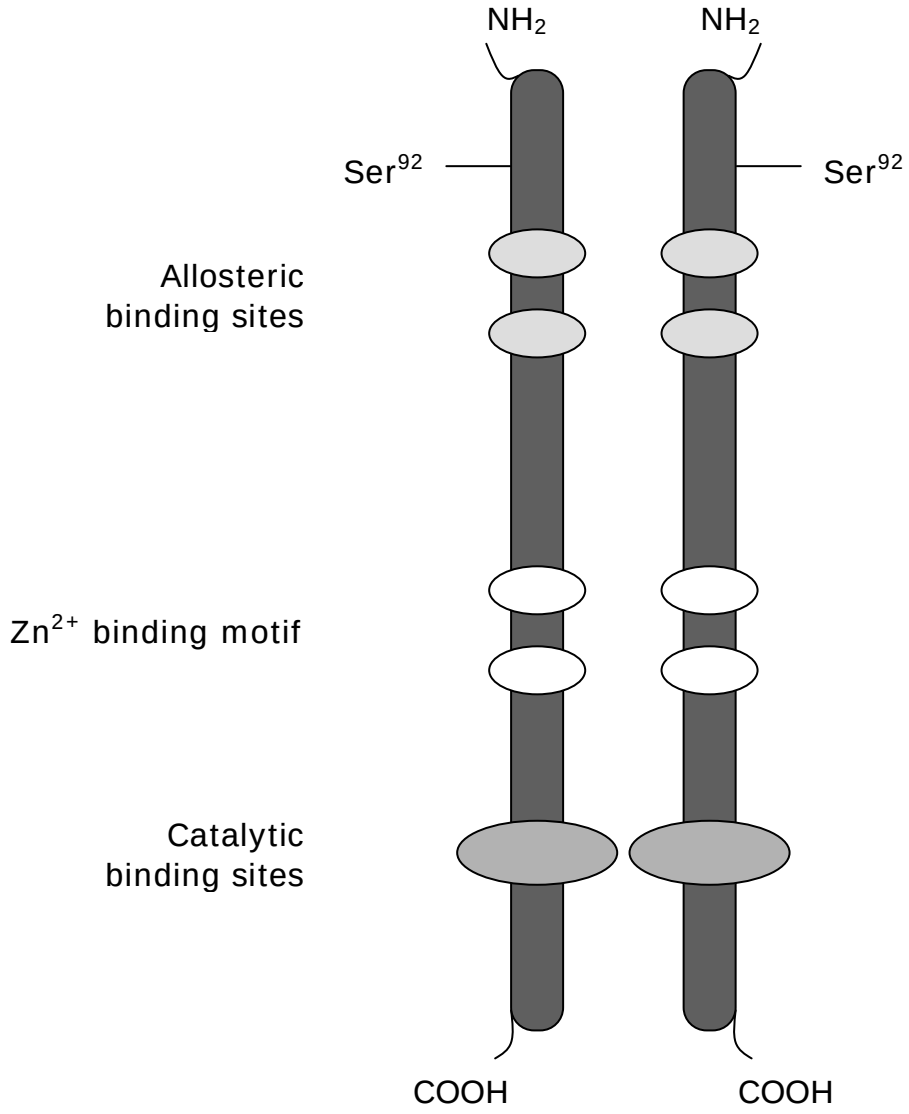


Fig. 2 Schematic presentation of homodimer phosphodiesterase 5. The PDE 5 is a homodimer, and each subunit consists of two allosteric binding sites for cGMP, two binding sites for Zn^{2+} and a catalytic binding site, which promotes the binding of cGMP to the allosteric binding sites. In addition, each subunit contains a Ser^{92}, subject for phosphorylation. Modified after Gibson, 2001.

All known mammalian PDEs are dimeric. The PDE 5 is a homodimer and each subunit consists of the conserved C-terminal and the less conserved N-terminal, containing the regulatory domains. It has been thought that dimerisation is a prerequisite for catalytic activity, however, it was found that a mutant PDE 5 did not require interaction between the catalytic and regulatory domains or interactions between subunits of the dimer (Fink *et al.*, 1999). In PDE 5, the N-terminal regulatory domain contains two allosteric binding sites for cGMP and a Ser^{92}-residue. If cGMP is bound to the allosteric binding sites, Ser^{92} can be phosphorylated by either protein kinase A (PKA) or PKG, thereby increasing the activity of PDE 5 in hydrolysing cGMP to the inactive GMP (Corbin *et al.*, 2000).
Upon the discovery of cAMP in the 1950s (Rall *et al.*, 1958), it was found that xanthine-derivates such as caffeine and theophylline could inhibit the activity of PDE, thereby increasing the cellular content of the second messengers (Butcher *et al.*, 1962). The PDE inhibitor zaprinast was initially developed as an anti-allergy compound (Broughton *et al.*, 1974), but was found to be about 200 times more potent in inhibiting cGMP over cAMP. Further, zaprinast potentiated the effects of nitrovasodilator drugs in the coronary artery (Bergstrand *et al.*, 1977; Bergstrand *et al.*, 1978; Gibson, 2001; Kukovetz *et al.*, 1979), potentiated the NANC-mediated relaxations in the bovine retractor penis muscle (Bowman *et al.*, 1984) and enhanced nerve-stimulated erections in dogs (Trigo-Rocha *et al.*, 1993). At present, it has been found that zaprinast inhibits PDE 1, 5, 6, 9 and 11 (Francis *et al.*, 2001).
The discovery that penile erections are dependent on nitrergic neurons and subject for modulation by zaprinast (Rajfer *et al.*, 1992; Trigo-Rocha *et al.*, 1993) promoted the development of selective PDE 5 inhibitors. At present, selective inhibitors of PDE 5 (sildenafil, tadalafil and vardenafil) are used for treatment of male erectile dysfunction. Sildenafil was initially developed for treatment of cardiovascular conditions, instead it was shown to enhance the erectile response in patients with erectile dysfunction (Boolell *et al.*, 1996). Recently the PDE 5 inhibitors sildenafil, tadalafil and vardenafil were launched as treatment option for erectile dysfunction and in common, they all enhance penile erection by increasing the cellular content of cGMP (Ballard *et al.*, 1998; Eardley *et al.*, 2002; Francis *et al.*, 2003; Jeremy *et al.*, 1997; Klotz *et al.*, 2001).

Importance of nitric oxide in the gastrointestinal tract

The major part of the gastrointestinal tract is organised in two separate muscle layers, consisting of smooth muscle cells and two dense neuronal plexa, one plexus in between the muscle layers (the Auerbach plexus, pl myentericus) and one plexus between the mucosa and the inner muscle layer (the Meissner plexus, pl submucosus). The neuronal plexa consist of three types of neurons, motor-, sensory- and interneurons. The distribution of the neurons in the two plexa differs somewhat, such as that the myenteric plexus to a larger extent consists of motor neurons, while the submucosus contains primarily sensory neurons. These two plexa forms the enteric nervous system and is a part of the autonomic nervous system (Boron *et al.*, 2003).
The physiological effects of NO in the gastrointestinal tract implicate relaxation of the smooth muscle cells of the intestine, such as the lower esophageal sphincter (Oliveira *et al.*, 1992). Adaptive relaxation of the stomach in response to increases in intragastric pressure was mediated via nitrergic neurons (Desai *et al.*, 1991) and inhibition of NOS delayed the gastric emptying and increased contractions of proximal duodenum (Orihata

et al., 1994). Relaxation of the smooth muscle cells in the small and large intestine is mediated by NO (Boeckxstaens *et al.*, 1990; Dalziel *et al.*, 1991; Gustafsson *et al.*, 1990; Hata *et al.*, 1990; Irie *et al.*, 1991). However, there has also been reports that NO mediates contractions in the ileum (Bartho *et al.*, 1992; Olgart *et al.*, 1997). Furthermore, in the anal sphincter, NO mediates the relaxation (Burleigh, 1992; Rattan *et al.*, 1992), and NO appears to be involved in gastrointestinal reflexes (Hata *et al.*, 1990; Izzo *et al.*, 1998). Thus, today it is well accepted that the L-arginine/NO-pathway is of great importance in the gastrointestinal tract.

Importance of nitric oxide in the lower urogenital tract

As in the gastrointestinal canal, NO has been found to mediate NANC-responses in the urogenital tract. In the urinary bladder it was noted that inhibition of the L-arginine/ NO-pathway causes hyperactivity of the urinary bladder (Persson *et al.*, 1992) and nerves within the bladder were found to express NOS (McNeill *et al.*, 1992; Smet *et al.*, 1994). In the bladder neck and urethra relaxations are mediated via NO (Andersson *et al.*, 1992; Thornbury *et al.*, 1992) and it was suggested that NO contributes to intact emptying of the bladder.

Prior to the discovery of NO, it was known that the cavernous tissue was innervated by cholinergic parasympathetic and the adrenergic sympathetic neurons. However, none of these neurons could explain the nerve-induced erection, and therefore erection was designated as NANC-mediated (Burnett, 2002; Cellek, 2000). When the identity of EDRF had been revealed, it was found that in the presence of adrenergic and cholinergic blockade, electrical stimulation of the rabbit corpus cavernosum elicited relaxation of the tissue and an increased formation of NO_2^- and cGMP. This effect was abolished by TTX, and it was suggested that penile erection may be mediated by neuronal release of NO (Ignarro *et al.*, 1990). This hypothesis has later been confirmed and NO was shown to be the major neurotransmitter, responsible for the smooth muscle relaxation of rabbit and human corpus cavernosum (Holmquist *et al.*, 1992). The importance of NO in the erectile tissue has been further established by identification of NOS containing neurons in the corpus cavernosum (Burnett *et al.*, 1992; Burnett *et al.*, 1993; Bush *et al.*, 1992). Furthermore, endothelial NOS is present in the arteries of the corpus cavernosum (Alm *et al.*, 1993; Burnett *et al.*, 1992) and contribute to formation and release of NO.

AIMS OF THE STUDY

The present work was carried through in order to study the nitrergic neurotransmission in peripheral autonomic neurons. The project was designed primarily to study the release of NO, and its regulation by the soluble guanylate cyclase/cyclic guanosine monophosphate pathway. In particular, the following issues were addressed:

- To study if NO released from enteric neurons is mediating its effects solely via the sGC/cGMP-pathway or whether other mechanisms are at hand
- To study if other neurotransmitters apart from NO are utilising sGC/cGMP-pathway in the intestine
- To chemically quantify the nerve-mediated release of NO in the intestine and in the corpus cavernosum
- To investigate if the sGC/cGMP-pathway affects the release of NO from autonomic neurons in enteric and cavernous neurons
- To study the effect of selective PDE 5 inhibitors on the nerve-induced release of NO from cavernous tissue

METHODS

Preparation of colon tissue, guinea-pig (paper I and II)

Male Dunkin-Hartley guinea-pigs (350-500 g) were anaesthetised by carbon dioxide and exsanguinated. Approximately 25 cm of the colon was removed and mounted on a glass pipette. The longitudinal muscle layer with the underlying myenteric plexus was isolated by gentle rubbing with a cotton swab transversally to the direction of the intestine, as previously described (Rang, 1964) and placed in Tyrode's solution (Na^+ 161 mM, K^+ 2.8 mM, Ca^{2+} 1.8 mM, Mg^{2+} 0.5 mM, Cl^- 144 mM, HCO_3^- 24 mM, $H_2PO_3^-$ 0.4 mM, glucose 5.5 mM). The Tyrode's solution was continuously aerated with 6.5% CO_2 in O_2.
In study **II** the superior mesenteric artery was cannulated and perfused with 0.9% saline prior to the isolation of the longitudinal smooth muscle, in order to minimise the content of haemoglobin of the tissue, hence to optimise the conditions for measurements of NO/NO_2^- by chemiluminescence.

Preparation of corpus cavernosum, rabbit (paper III and IV)

Male rabbits of the strain New Zeeland white (2.5-3.2 kg) were anaesthetised by injecting pentobarbituate in the marginal vein of the right ear and exsanguinated. The distal part of the abdominal aorta was cannulated and perfused with 150 ml 0.9% NaCl at 37°C in order to diminish the concentration of haemoglobin in the tissue. The corpora cavernosa were cleaned from muscle and connective tissues and placed in ice-cold modified Kreb's solution (136.9 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.6 mM $MgSO_4$, 11.9 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 11.5 mM glucose). The Kreb's solution was continuously aerated with 6.5% CO_2 in O_2.

Organ bath experiments (paper I, IV)

The isolated longitudinal muscle layer from guinea-pig colon was folded twice, cut in to 10-12 mm long strips (study **I**) and the corpora cavernosa were divided into 10-14 strips, 10-12 mm in length (study **IV**). The preparations were suspended in organ baths containing Tyrode's solution (study **I**) or modified Kreb's solution (study **IV**), at 20°C and after 20 min heated to 37°C. Atropine (10^{-6} M) and histamine (10^{-5} M) (study **I**) or phenylephrine, ($3x10^{-6}$ M), scopolamine (10^{-5} M) and guanethidine (10^{-5} M) (study **IV**) was applied to the organ baths. The muscle preparations were stimulated by transmural EFS (3 Hz, 40 V, 0.2 ms pulse duration, 180 pulses at intervals of 3 min, study **I** and 5 Hz, 40 V, 0.3 ms pulse duration, 25 pulses at intervals of 2 min, study **IV**). The stimulation parameters were chosen to obtain stable and reproducible muscular response over time and in accordance to stimulation parameters utilised in previous work (Cellek *et al.*, 1997; Iversen *et al.*, 1994).
The organ baths were made of Perspex, contained 5.5-6.5 ml and equipped with platinum electrodes 10 mm in length and 10 mm apart. The EFS was applied through a S44 stimulator (Quincy, Mass., USA). In study **I** the mechanical muscular activity was

measured isotonically by Harward smooth muscle transducers (Millis, MA, USA) and recorded by ABB SE 120 (Västerås, Sweden) ordinate recorders. In study **IV** the mechanical muscular activity was monitored isometerically by Grass force-displacement transducer (FT03) and displayed with a Grass model 7P122 or 7DA Polygraph (Grass Instruments, Quincy, MA, USA).

NO Release experiments (paper II - IV)

In study **II**, the isolated longitudinal muscle layer from guinea-pig colon was folded to a 4-5 cm long preparation, and in study **III** and **IV**, the paired corpora cavernosa were divided longitudinally to obtain two similar strips (35 x 6 mm). The tissues were tied between thin nylon monophil threads and mounted in a glass chamber at a load of 10 mN (study **II**) or 6-10 mN (study **III** and **IV**) with continuous superfusion (1 ml/min) of Tyrode's solution (study **II**) or of modified Kreb's solution (study **III** and **IV**), at 1 ml/min.

The superfusing fluid was preheated to 37°C and the NO-substrate L-arginine (10^{-5} M) was added to the superfusion buffer respectively, in order to enhance the synthesis of NO during nerve stimulation. The isolated longitudinal muscle layer from guinea-pig colon was left for 180 min (study **II**) and the rabbit corpus cavernosum 120 min (study **III** and **IV**) in order to equilibrate before pharmacological substances were applied. Transmural electrical stimulation (50 V biphasic, 32 Hz, pulse duration 1 ms, study **II** and 40 V biphasic, 20 Hz, pulse duration 1 ms, study **III** och **IV**) was applied with needle shaped silver electrodes placed approximately 4.5 cm (colon) or 3 cm (corpus cavernosum) apart. The isolated longitudinal muscle layer from guinea-pig colon was stimulated for 1 min every 45 min (study **II**) and of the rabbit corpora cavernosa for 1 min every 30 min (study **III** and **IV**). The EFS was applied with a Grass S88 stimulator and the muscle activity of the tissue was recorded isometrically by Grass force-displacement transducer (FT03) and displayed with a Grass model 7P122 Polygraph (Grass Instruments, Quincy, MA, USA). The pharmacological substances were present for 45 min (study **II**) or 20 min (study **III** and **IV**) before nerve stimulation took place.

Measurements of NO/NO_2^- by chemiluminescence (paper II - IV)

The superfusate of the colonic or cavernous tissue was collected during periods of 1 min prior to and during electrical field stimulation. Aliquots of 1 ml were injected into a reaction glass chamber, containing 100 ml (study **II**) or 150 ml (study **III** and **IV**) of 1% sodium iodide in deoxygenated and concentrated hot (90°C) acetic acid. In the acidic and reducing milieu, NO_2^- is reduced to NO (Walters *et al.*, 1987) and carried further by a stream of N_2 into a reaction chamber. In the reaction chamber NO together with ozone reacts under vacuum to give rise to photons, the amount being counted in a photo-multiplier (Palmer *et al.*, 1987; Walters *et al.*, 1987). The detection limit was 0.5-2 pmol $NaNO_2$ per ml. The system was calibrated by injecting freshly made aliquots of $NaNO_2$ solution and using peak heights for construction of standard curves for calculation of unknown samples (Iversen *et al.*, 1994; Wiklund *et al.*, 1993a). The release of NO was recorded on an ABB SE 120 (Västerås, Sweden) ordinate recorder.

All studies were performed after approval of the local ethics committee for animal experimentation.

Statistical analysis

Experimental data are expressed as mean ± S.E.M. Statistical significance was tested according to Student's *t*-test for paired observations, except where normality failed, where Wilcoxon's test was used. For calculation of statistical difference between repeated measurements One-way ANOVA was used. All statistical calculations were performed with a computer program (SigmaStat, Jandel, San Rafael, CA, USA). Significant differences between treatment groups was accepted for $p<0.05$ and denoted * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). *n* indicates the number of tissues.

RESULTS AND DISCUSSION

Inhibitory NANC transmission of the enteric autonomic neurons

Preparations of the outer longitudinal smooth muscle layer with the underlying myenteric plexus were isolated from the guinea pig colon (study **I**). Electrical field stimulation (EFS) elicited reproducible contractions in these preparations whereas in the presence of atropine and histamine EFS induced relaxations. Also, application of exogenous NO and ATP elicited relaxations in the pre-contracted tissue. The smooth muscle relaxations induced by exogenous application of NO were totally abolished by the sGC inhibitor ODQ (Garthwaite *et al.*, 1995), indicating that the inhibitory effect by NO was mediated via stimulation of sGC. However, ODQ failed to inhibit the muscular relaxations induced by ATP, indicating that ATP utilise another pathway than sGC in agreement with previous studies (Lecci *et al.*, 2002). Previously, it has been shown that the muscular contractions and relaxations induced by EFS were nerve-mediated, as indicated by the abolishment of muscular responses upon application of the neurotoxin TTX (Iversen *et al.*, 1994). In line with earlier studies (Bult *et al.*, 1990a; Li *et al.*, 1989) we found that EFS-induced relaxations were diminished in the presence of the NOS inhibitor L-NOARG, indicating that part of the relaxations were mediated via the L-arginine/NO-pathway. EFS-induced relaxations were also diminished by application of ODQ, indicating that the inhibitory response upon nerve-stimulation was mediated via sGC/cGMP-pathway. Additional application of L-NOARG to preparations treated with ODQ, or additional application of ODQ to preparations treated with L-NOARG did not further alter the EFS induced relaxations. Taken together, this suggests that the inhibitory muscular response to NO is mediated via sGC and that NO does not act as a smooth muscle relaxant via other mechanisms apart from the sGC/cGMP-pathway. Furthermore, no other substance than NO is utilising the sGC/cGMP-pathway during EFS in the guinea pig colon.

In addition, it was noted that the inhibitory responses to EFS was not abolished by either L-NOARG or ODQ. Primarily, it was the latter part of the EFS-induced relaxations that was subject for modulation by L-NOARG and ODQ, while the initial fast relaxation was left unaffected. This indicates that there is an additional inhibitory neurotransmitter released upon nerve-stimulation, earlier in onset than NO. Further, this substance is not formed via the L-arginine/NO-pathway and does not utilise the sGC/cGMP-pathway.

Release of NO from autonomic neurons

Colon

The release of NO was measured in preparations of the longitudinal smooth muscle layer from the guinea pig colon (study **II**). Electrical stimulation of the plexus-containing tissue evoked release of NO measured as NO/NO_2^-. The evoked release of NO was stable over time and has previously been shown to be diminished in the presence of

L-NAME and abolished by TTX (Iversen *et al.*, 1994; Wiklund *et al.*, 1993a). Taken together this indicates that electrical stimulation of the autonomic neurons within the outer smooth muscle layer of the guinea pig colon is releasing NO and that NO is formed via the L-arginine/NO-pathway.

Corpus cavernosum

The nerve-induced release of NO was studied in isolated preparations of rabbit corpus cavernosum (study **III**). Previously it was described that NO was released upon nerve stimulation in preparations of human corpus cavernosum (Leone *et al.*, 1994). We have modified this method by extending the method previously used for studying the release of NO from preparations of the guinea pig colon (Hallén *et al.*, 2001; Iversen *et al.*, 1994). During nerve stimulation, NO was released from the rabbit cavernous tissue, measured as NO/NO_2^-. In the erectile tissue, the release of NO was stable over time. Inhibition of the NO synthase by L-NAME diminished the release of NO, indicating that the substance analysed was formed from the L-arginine/NO-pathway. In the presence of TTX, the evoked release of NO was reduced, thus implying that the formation of NO is originating from the neurons and is dependent of nerve-activity in the erectile tissue. At basal conditions the release of NO was decreased in the presence of L-NAME, indicating that there is a basal formation of NO via the L-arginine/NO-pathway. Further, TTX failed to alter the release of NO under basal conditions suggesting that the neurons do not contribute to the basal formation of NO in the cavernous tissue. Application of scopolamine and guanethidine did not to alter the release of NO from the isolated preparations of corpus cavernosum, either at basal conditions or during nerve stimulation. Thus, the release of NO does not seem to be influenced either by the cholinergic or noradrenergic pathways. This indicates that ACh released during nerve stimulation is not a major contributor to formation of NO via the endothelium.

Modulation of nerve-induced release of NO by cGMP

Colon

Modulation of the NO-release by cGMP and cGMP altering compounds was studied in preparations obtained from the longitudinal smooth muscle layer of the guinea pig colon (study **II**). The cGMP levels were modulated via application of the analogue 8-Br-cGMP (Schultz *et al.*, 1979), the sGC stimulator YC-1 (Ko *et al.*, 1994), the PDE inhibitor zaprinast (Bergstrand *et al.*, 1977) and the sGC inhibitor ODQ (Garthwaite *et al.*, 1995). The basal release of NO was not altered by 8-Br-cGMP, zaprinast or ODQ, indicating that these substances do not influence the release of NO under basal conditions. However, application of YC-1 resulted in increased release of NO suggesting that stimulation of sGC may alter the release of NO at basal conditions.

Upon nerve stimulation, the release of NO was modulated by cGMP and cGMP altering compounds. Application of 8-Br-cGMP, YC-1 and zaprinast all increased the nerve-induced release of NO, whereas ODQ diminished the NO-release. This suggests that cGMP is enhancing the nerve-induced release of NO in the enteric tissue.

The contractile response to nerve stimulation was altered by the cGMP mimicking and modifying compounds. The nerve-induced contractions were decreased in the presence of 8-Br-cGMP and YC-1, whereas ODQ increased the contractile response. Taken together, this indicates that cGMP-elevating compounds increase the formation of NO and is inhibiting the contractile response to EFS in the enteric tissue. Further, blockage

of sGC by ODQ diminished the nerve-induced release of NO and increased the contractile response. The PDE inhibitor zaprinast did not alter the muscular response and the reason for this is at present unclear.

Corpus cavernosum

In isolated preparations of corpus cavernosum the modulation by cGMP on the release of NO was investigated (study **III**). As in the isolated preparations of guinea pig colon, the sGC/cGMP-pathway was modulated by application of 8-Br-cGMP, YC-1, zaprinast or ODQ. The nerve-induced release of NO was increased upon application of 8-Br-cGMP and YC-1. Also, application of zaprinast showed a tendency to increase the nerve-induced release of NO. Further, inhibition of the sGC by application of ODQ diminished the nerve-induced release of NO. Thus, this indicates that modulation of the sGC/cGMP-pathway is altering the nerve-induced release of NO in the cavernous tissue. Together with the results obtained in the enteric tissue, this suggests that the enhancing effect cGMP is exerting on nerve-induced release of NO might be a general mechanism.

The basal release of NO was not altered by 8-Br-cGMP, zaprinast or ODQ indicating that the sGC/cGMP-pathway did not modulate the L-arginine/NO-pathway under basal conditions. Stimulation of sGC by application of YC-1 elicited an increase in the basal release of NO; similar to what was found in guinea pig colon.

Modulation of nerve-induced release of NO by PDE inhibitors

Corpus cavernosum

Isolated preparations of the rabbit corpus cavernosum were studied (study **IV**). In organ bath experiments the muscular response to EFS was monitored. As was found in the guinea pig colon, EFS induced reproducible contractions, whereas in the presence of scopolamine, guanethidine and phenylephrine EFS induced relaxations. Application of the selective phosphodiesterase 5 inhibitors sildenafil, tadalafil and vardenafil elicited a reduction of the muscular tone in a dose dependent fashion. Sildenafil and vardenafil almost totally abolished the muscular tone induced by phenylephrine, whereas there was a remaining muscular tone in the presence of tadalafil. Furthermore, the relaxations induced by EFS were enhanced by sildenafil, tadalafil and vardenafil. It was also noted that the duration of the relaxing phase evoked by EFS was increased in the presence of sildenafil, tadalafil and vardenafil. Taken together, this indicates that the selective PDE 5 inhibitors enhance the nerve-induced inhibitory muscular responses in the rabbit corpus cavernosum.

The release of NO from the erectile tissue was studied and it was found that formation and release of NO was subject to modulation by the selective PDE 5 inhibitors. Sildenafil and tadalafil did not alter the release of NO at basal conditions, whereas application of vardenafil diminished the release of NO in a dose dependent manner. During electrical stimulation of the erectile tissue the nerve-induced release of NO was decreased in a dose dependent fashion. Our results indicate that the evoked release of NO in the rabbit corpus cavernosum is subject to modulation by the selective PDE 5 inhibitors, possibly via negative feedback. This is in contrast to the previous findings with a cGMP-mediated enhancement of nerve-induced release of NO in both enteric and erectile tissue.

GENERAL DISCUSSION

Neuronal release of NO

NO does not act as the classical neurotransmitters in that it is not stored in vesicles, but is released directly at site of synthesis in the nerve-endings (Olgart *et al.*, 2000; Wiklund *et al.*, 1997). Also, when being released as a consequence of nerve activity, NO does not bind to an extracellular receptor postjunctionally. Instead, it is diffusing into the surrounding tissue and across cell membranes.

In order to investigate the nitrergic neurotransmission, several methods have been developed. These methods include: limitation of substrate availability, localisation and measurement of NOS activity, quantification of the by-product L-citrulline, localisation and measurement of sGC activity, quantification and localisation of cGMP and monitoring the muscular responses. However all the above-mentioned methods are indirect methods and do not quantify NO it self. An additional challenge in studying the nitrergic pathway of signal transduction is the rapid oxidation of NO to NO_2^- and the amounts of NO_2^- present in the tissue before nerve stimulation took place.

In the present thesis we have studied the nerve-induced release of NO from guinea pig colon and rabbit corpus cavernosum. Based on previously used methods (Iversen *et al.*, 1994; Wiklund *et al.*, 1993a) the release of NO was quantified by utilising a chemiluminescence technique that selectively determines NO and its primary metabolite by reducing NO_2^- to NO. Therefore, our results are obtained by direct measurement of the true mediator NO, rather than indirect observations such as co-products, secondary metabolites or muscular responses.

We found that the nerve-induced release of NO in the enteric and erectile tissue was decreased by inhibition of NOS, indicating that NO is enzymatically formed. Further, the nerve-induced release of NO was diminished in the presence of the neurotoxin TTX. Taken together, this indicates that the evoked release of NO in enteric and erectile tissue is originating from the autonomic neurons by synthesis catalysed by nNOS. Our results of a neuronal source for formation and release of NO is supported by identification of NOS-containing neurons in the gastrointestinal tract (Bredt *et al.*, 1990; Llewellyn-Smith *et al.*, 1992; Young *et al.*, 1992) and in the corpus cavernosum (Burnett *et al.*, 1992; Keast, 1992).

The nerve-induced release of NO has previously been studied and our results are in line with earlier findings. In the gastrointestinal tract it was found that the evoked release of NO was frequency-dependent, attenuated in the presence of a NOS-inhibitor, Ca^{2+}-dependent and sensitive to TTX (Iversen *et al.*, 1994; Wiklund *et al.*, 1993a; Wiklund *et al.*, 1993b). Together with visualisation of nerve-induced release of NO in guinea pig colon (Wiklund *et al.*, 1997) it can be assumed that the evoked release of NO during nerve stimulation is originating from the autonomic neurons in the enteric tissue.

In the erectile tissue NO can be formed either via stimulation of eNOS by ACh or via nerve activity stimulating nNOS. In rabbit corpus cavernosum we found that the nerve-induced release of NO was not subject to modulation by cholinergic neurons. Thus, ACh-mediated release of NO from the endothelium is not of importance in the erectile

tissue. Further, our results support the hypothesis that autonomic neurons are the primary source for formation and release of NO in the erectile tissue. This is also in line with previous findings that intravenous or intracorporeal injections of atropine did not inhibit nerve-induced erections in humans (Andersson *et al.*, 1995; Brindley, 1986). Also, it was shown that administration of ACh does not induce reliable erection (Andersson *et al.*, 1995; Dorr *et al.*, 1967). Further support for a neuronal source of NO is the observation that lack of nitrergic neurons results in inability to achieve erections (Cellek *et al.*, 1999; Saenz de Tejada *et al.*, 1989).

Nerve-induced release of NO has previously been investigated in the erectile tissue. In corpus cavernosum, EFS evoked release of NO that was enzymatically formed, frequency-dependent and sensitive to TTX (Kuwahara *et al.*, 2003; Leone *et al.*, 1994; Wada *et al.*, 2003). Taken together, the previous and our present findings suggest that the cholinergic pathway does not influence the release of NO. This indicates that the autonomic neurons in the erectile tissue are the major contributor of NO during nerve-activation. Hence, we suggest that the nerve-induced release of NO in the erectile tissue is originating from the autonomic neurons.

We used a chemiluminescence method to analyse NO/NO_2^- that is sensitive in the low nM-range. Other previously used assays are less sensitive (Feelisch *et al.*, 1996), e.g. the Griess-assay that is based on spectrophotometeric determination of NO_2^-, sometimes used after reduction of NO_3^- to NO_2^-. The sensitivity of the Griess-assay can be optimised to low nM-concentrations if combined with a fluorometeric detection. However, the Griess-assay requires incubation of the samples to be analysed, which might accumulate NO_2^- from glassware, plastic material and the air (Feelisch *et al.*, 1996). Also HPLC has previously been used to quantify NO_2^-/NO_3^- (Leone *et al.*, 1994; Wiklund *et al.*, 1993b). The present method to analyse the evoked release of NO offers an advantage in that it quantifies NO and its primary metabolite NO_2^-, rather than NO_2^- and the secondary metabolite NO_3^-.

sGC/cGMP-pathway och PDE

Within the cells NO binds to sGC and will thereby increase the activity of sGC and the conversion of GTP to cGMP is stimulated. This will be followed by relaxation of the smooth muscle cells.

By quantifying the neuronal release of NO from guinea pig colon and rabbit corpus cavernosum we have found that cGMP mimicking or modifying compounds alters the release of NO in a positive manner. The role of the sGC/cGMP-pathway was tested by application of a cGMP-analogue, stimulation or inhibition of sGC, or inhibition of PDE. All four modifications altered the detected levels of NO in the enteric tissue upon nerve stimulation. The same results were obtained in erectile tissue, apart from the partly selective PDE inhibitor (zaprinast) that did not alter the nerve-induced release of NO. Taken together, this suggests that cGMP is modulating the nerve-induced release of NO via a positive feedback and that this proposed positive feedback might be a general mechanism since it is present both in enteric and erectile tissue.

Recently, the selective PDE 5 inhibitors sildenafil, tadalafil and vardenafil were launched to treat male erectile dysfunction. They are clinically widely used and act via increasing the cellular content of cGMP upon stimulation (Corbin *et al.*, 2002; Lincoln, 2004) and thereby facilitating the erectile response. In rabbit cavernous tissue we presently found

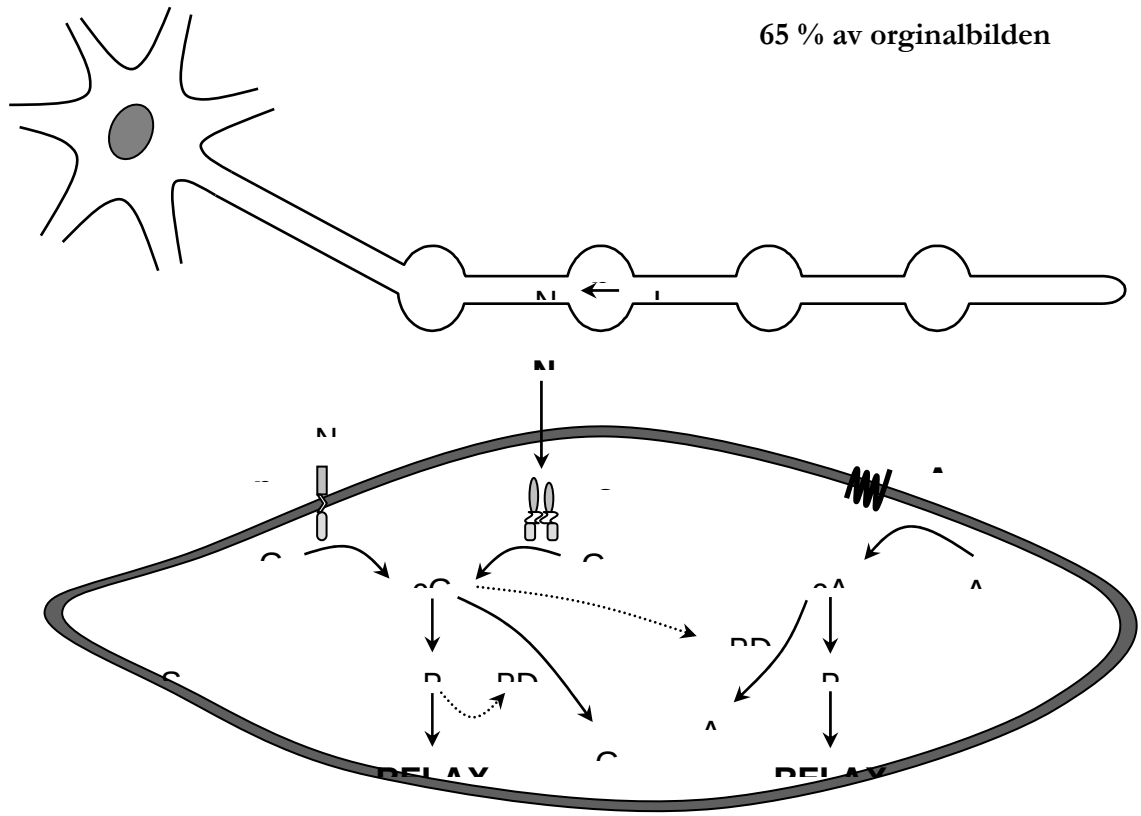


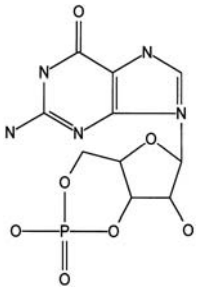
Fig. 3 Schematic presentation of intracellular signal transduction pathways in autonomic innervated tissue. Nitric oxide (NO) diffuses across the cell membrane and stimulates the soluble guanylate cyclase (sGC), whereas natriuretic peptides (NP) activate the particulate guanylate cyclase (pGC) and conversion of GTP to cGMP is catalysed. Cyclic GMP activates cGMP-dependent protein kinases (PKG) and this ultimately leads to relaxation of the smooth muscle cells (SMC) in the tissue. PKG is also catalysing the phosphorylation of phosphodiesterase 5 (PDE 5) and the hydrolysis of cGMP to the inactive GMP is catalysed. Cyclic GMP is also binding to cAMP phosphodiesterase 3 (PDE 3), whereby hydrolysis of cAMP to the inactive AMP is inhibited. The conversion of ATP to cAMP is enhanced during activation of adenylate cyclase (AC) and leads to activation of cAMP-dependent protein kinase (PKA). PKA is mediating various effects via phosphorylation of target substances, including relaxation of smooth muscle cells. Possible cGMP-mediated mechanisms for modulation of the nerve-induced release of NO is via a general enhancing effect (Olgart *et al.*, 1997) or via cGMP-mediated phosphorylation of nNOS (Dinerman *et al.*, 1994). Modified after Lincoln, 2004.

that application of selective PDE 5 inhibitors decreased the muscular tone as previously has been described (Francis *et al.*, 2003). Further, we found that the selective PDE 5 inhibitors altered the nerve-induced release of NO. Application of sildenafil, tadalafil and vardenafil all decreased the nerve-induced release of NO, indicating that PDE 5 is influencing the formation and release of NO during nerve stimulation in the cavernous tissue.

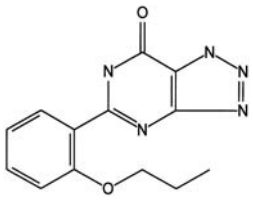
Stimulation of the autonomic neurons in the gastrointestinal and erectile tissue might lead to increases in cGMP in smooth muscle cells, neurons or interneurons. However, it has been suggested that cGMP is not formed in the nitrergic neurons in the intestine (Shuttleworth *et al.*, 1993), and a role (stimulatory or inhibitory) for cGMP directly in the nitrergic neurons seems at first sight difficult to reconcile with the observation by Shuttleworth *et al.* also, with recent studies on PDE 5 localisation in cerebellar neurons (Giordano *et al.*, 2001) where NOS and PDE 5 are localised in different cell types. One possibility could be that upregulation of cGMP in the tissue would lead to release of a factor, possibly from another neuron than the nitrergic, or from endothelium and smooth muscle cells, and this proposed factor may facilitate further release of NO. Another possibility is that cGMP mediates a general enhancing mechanism in neuronal excitability (Olgart *et al.*, 1997) and thereby increases the formation and release of NO. A further possibility is that our understanding of compartmentalisation of cGMP-mediated regulation is inadequate, and that cGMP may exert several roles, stimulatory and inhibitory, in the same neuron. The experiment by Shuttleworth *et al* serves to show that cGMP can be formed in the enteric neurons, a necessary basis to propose a role for cGMP in the regulation of nitrergic transmission. The present data suggest that new methods must be utilised to in more detail investigate localisation of NO-induced cGMP activation in neuronal tissue, in agreement with very specific activation of cGMP-dependent mechanisms in cerebellar Purkinje cells (Shimizu-Albergine *et al.*, 2003).

In agreement with differentiated effects of cGMP in neuronal tissue an effect of selective PDE 5 inhibitors was found in erectile tissue, as compared with the lack of effect of zaprinast. Thus, application of the selective PDE 5 inhibitors caused increased concentration of cGMP in erectile tissue (Corbin *et al.*, 2002) and presently we found that they decreased the nerve-induced release of NO. The reduced release of NO was found upon application of all three PDE 5 inhibitors sildenafil, tadalafil and vardenafil to the cavernous tissue. This is in contrast to what was found using 8-Br-cGMP, YC-1 and zaprinast. A possible explanation for the conflicting results presented in our studies is that PDE 5 inhibitors might exhibit an additional effect in the erectile tissue, resulting from increasing the levels of cGMP. It might be that the PDE 5 inhibitors indirectly are inhibiting the enzymatic formation of NO, likely via accumulation cGMP, which in turn via PKG leads to phosphorylation of nNOS. This is supported by the previous finding that nNOS is subject to phosphorylation by PKA or PKG and hence diminished catalytic activity of nNOS (Dinerman *et al.*, 1994). This is in line with the finding that in the urogenital nNOS and sGC are present in the neurons of the submucosa and detrusor muscle of the urinary bladder in humans and that eNOS and sGC are found in the detrusor muscle (Fathian-Sabet *et al.*, 2001). In another study it was found that incubation of bovine penile arteries with sodium nitroprusside induced cGMP in the smooth muscle cells and in nNOS positive neurons (Vanhatalo *et al.*, 2000).

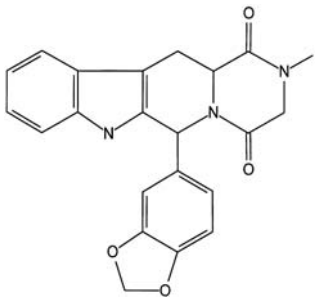
cGMP



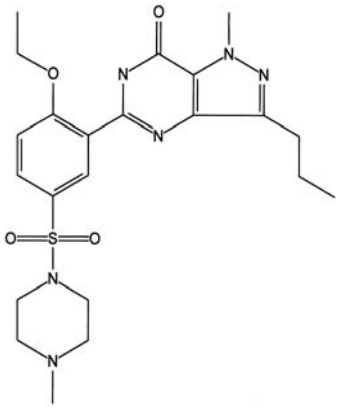
Zaprinast



Tadalafil



Sildenafil



Vardenafil

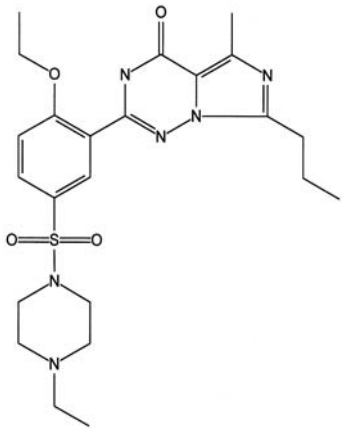


Fig. 4 Chemical structure of cGMP and phosphodiesterase inhibitors. All PDE inhibitors has some resemblance in structure with the purine moiety in cGMP.

The diminished release of NO mediated by the selective PDE 5 inhibitors during nerve stimulation is most likely of significant physiological and pharmacological importance. If the selective PDE 5 inhibitors had increased the release of endogenously formed NO, this would start a positive feedback circle, which might ultimately have induced priapism. However, since the selective PDE 5 inhibitors diminish formation of NO priapism is counteracted through negative feedback by NO on its own formation: a) via cGMP-dependent phosphorylation of NOS, b) via NO-inhibition of NOS. This is in agreement with the induction of priapism upon intake of sildenafil in patients undergoing treatment for angina pectoris with nitrovasodilator substances, where the NO-formation from the NO-donor is not to be expected to be under this feedback control (Burnett, 2003). Recently, Ibrahim *et al.* investigated the regulatory effect of the production of NO in serum and in aortic tissue in rats (Ibrahim *et al.*, 2004). The authors concluded that the PDE 5 inhibitor zaprinast has no regulatory effect on the NO-release in serum and aortic tissue, though the mean arterial pressure was lowered. However, their study quantified the breakdown products NO_2^- and NO_3^- (NOx) in the μM concentration range whereas we measured NO at concentrations a thousand fold lower (nM).

Furthermore, in this study zaprinast was administered to the rats by intraperitoneal injections, hence the concentration of the substance is unknown in the tissue. In contrast, we superfused the tissue at a known concentration of the pharmacological substance and thereby increasing the availability of the substance added. Also, it is likely that the study performed by Ibrahim *et al.* have studied effects mediated via eNOS in the cardiovascular system, whereas we have investigated the modulation of NO released from peripheral autonomic neurons.

Neurotransmission in the enteric tissue

Application of NO to smooth muscle cells stimulates sGC whereby the formation of cGMP is increased and followed by relaxation of the tissue. However, it was not clear if NO also induced cGMP-independent muscular responses. Hence, the introduction of the sGC inhibitor ODQ (Garthwaite *et al.*, 1995) became an important tool to distinguish between cGMP-dependent and –independent mechanisms exerted by NO. The nitrergic neurotransmission was investigated in guinea pig colon. Muscular relaxations induced by nerve stimulation were diminished via inhibition of sGC by ODQ. Additional inhibition of NOS did not alter the inhibitory muscular response further. Thus, the inhibitory nitrergic neurotransmission is exclusively mediated via a cGMP-dependent mechanism. Furthermore, the inhibitory muscular responses were diminished upon inhibition of NOS and application of ODQ did not further alter the muscular response. Thus, no other inhibitory principle is exerting its effects via the sGC/cGMP-pathway.

Our results are in agreement with formation of cGMP upon stimulation of the enteric neurons and exogenous application of NO in the gastrointestinal tract (Kanada *et al.*, 1992; Ward *et al.*, 1992). Also, in line with our results it was shown that inhibitory muscular responses in rabbit anococcygeus muscle was prevented in the presence of ODQ, indicating that NO utilises a cGMP-dependent mechanism (Cellek *et al.*, 1996). Previously there has been reports suggesting that NO might induce muscular responses independent of the sGC/cGMP-pathway in rat duodenum (Martins *et al.*, 1995), colon (Takeuchi *et al.*, 1996) and in mouse caecum (Young *et al.*, 1996). However, our results do not support a cGMP-independent mechanism.

Stimulation of the autonomic neurons released an additional inhibitory component apart from NO. This neurotransmitter was not formed via the L-arginine/NO-pathway and did not utilise the sGC/cGMP-system. The muscular relaxations induced by this neurotransmitter were earlier in onset than relaxations induced by NO. This finding is in agreement with previous studies (Lecci *et al.*, 2002).

CONCLUSIONS

- Nerve stimulation-induced relaxations in guinea pig colon exhibited a rapid and a somewhat slower component. Primarily, the latter part was mediated via stimulation of nitrergic neurons, whereas the initial part was not altered by inhibition of NOS. Thus, the inhibitory NANC transmission in autonomic neurons in the guinea pig colon consists of two inhibitory components, one is NO and the other is clearly different from NO being earlier in onset.

- NO-induced relaxations in the guinea pig colon were mediated via the soluble guanylate cyclase and did not utilise any other pathways for the muscular responses. No other inhibitory principle than NO seems to exert its action through stimulation of the sGC/cGMP-pathway.

- Nerve-induced formation and release of NO/NO_2^- in guinea pig colon was altered by cGMP modulating and mimicking substances in a positive fashion, suggesting an enhancing effect of cGMP on the nitrergic neurotransmission in the enteric tissue.

- Electrical field stimulation of preparations of rabbit corpus cavernosum evoked release of NO. The release of NO was formed enzymatically and was stable over time. Nitrergic neurotransmission in the erectile tissue does not seem to be influenced by the adrenergic and cholinergic pathways. Thus, the release of NO was most likely originating from the autonomic neurons and not via ACh mediated effects on endothelial cells.

- The nerve induced release of NO/NO_2^- in rabbit cavernous tissue was altered by cGMP modulating and mimicking substances in a positive direction, indicating a role for cGMP in regulating neuronal or release of NO. This is in agreement with our finding in the enteric tissue.

- Selective PDE 5 inhibitors diminished the release of NO/NO_2^- during activation of nitrergic neurons in rabbit cavernous tissue. This suggests that selective PDE 5 inhibitors are exhibiting an additional effect apart from inhibiting the hydrolysis of cGMP. It might be that nNOS is under local negative feedback control via cGMP, possibly via phosphorylation nNOS.

There seems to be a dual effect of cGMP in nitrergic neurotransmission. First there might be a general enhancing mechanism on neuronal excitability or on some other aspect of activation of the neurotransmission. Second, the NO formation and release is probably under a direct local inhibitory control via cGMP.

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APPENDIX (PAPER I-IV)

MUMIN

Stormar, ack!
Meningen med dem är antagligen att man ska få en soluppgång efteråt…

Tove Jansson