Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*

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1 In the guinea-pig colon ascendens, 5-hydroxytryptamine (5-HT) induces contractions, mediated by 5-HT₂, 5-HT₃ and 5-HT₄ receptors, and relaxations, through a 5-HT₁ receptor subtype, that triggers the release of an inhibitory neurotransmitter. Nitric oxide (NO) is one of the main candidates of NANC inhibitory neurotransmission in the gut. The aim of this study was to establish whether NO is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens.

2 Antagonists to block the contractile responses to 5-HT via 5-HT₂, 5-HT₃ and 5-HT₄ receptors were present throughout the experiments and methacholine was administered to precontract the strips. Under these conditions, 5-HT concentration-dependently induced relaxations from 10 nM onwards (EC₅₀ = 258 (172–387) nM). The relaxations were inhibited by metergoline (10 nM) and methiothepine (100 nM) and abolished by tetrodotoxin (TTX, 320 nM). Guanethidine (3.2 µM) did not affect them.

3 N^G-nitro-L-arginine (L-NNA) inhibited the responses to 5-HT (IC₅₀ = 18.7 (13.3–26.3) µM); at the highest 5-HT concentration a maximum inhibition of about 75% was observed with 320 µM L-NNA. This inhibition was reversed with L-arginine. Relaxations to glyceryl trinitrate (GTN) were not inhibited by L-NNA.

4 Haemoglobin (32 µM) inhibited the relaxations to 5-HT and GTN, but not those to isoprenaline (Iso). Methylene blue (10 µM) inhibited the relaxations to 5-HT but did not affect those caused by GTN or Iso.

5 It is concluded that 5-HT induces relaxations that involve NO. We also confirmed that 5-HT induces these relaxations via (a) 5-HT₁ receptor subtype(s), located on neurones.

Keywords: Guinea-pig colon ascendens; longitudinal muscle; nitric oxide; methylene blue; haemoglobin; N^G-nitro-L-arginine; 5-hydroxytryptamine; 5-HT₁-receptors

Introduction

Inhibitory enteric neurones play an important role in gut motility. They are involved in reflex relaxation of the lower oesophageal and the internal anal sphincter, the receptive and adaptive relaxation of the stomach and the descending inhibition during intestinal peristalsis. The exact mechanisms underlying these phenomena remain to be elucidated.

In isolated colon ascendens preparations of the guinea-pig, 5-hydroxytryptamine (5-HT), a neurotransmitter of the enteric nerves, induces both contractions and relaxations (Costa & Furness, 1979). It has previously been shown that the contractions are mediated via 5-HT₂, 5-HT₃ and 5-HT₄ receptors (Briejer *et al.*, 1991; Sato *et al.*, 1991; Elswood *et al.*, 1991), and the relaxations through a 5-HT-receptor different from 5-HT₂, 5-HT₃ or 5-HT₄ receptors (Briejer *et al.*, 1991; Sato *et al.*, 1991) but sensitive to 5-HT₁ receptor antagonists (Kojima, 1991; Elswood & Bunce, 1992). Since TTX abolished these relaxations, they were presumed to be mediated by nerves (Gershon, 1967). In the guinea-pig proximal (Costa & Furness, 1979) and distal colon (Onori *et al.*, 1984), 5-HT-evoked relaxations were not affected by incubation with guanethidine, which suggests that the relaxations are mediated by the intrinsic NANC (non-adrenergic, non-cholinergic) inhibitory neurones. Several compounds have been proposed as a neurotransmitter of these NANC inhibitory neurones: ATP (Burnstock, 1972), vasoactive intestinal

peptide (VIP) (Makhoulf, 1985) and nitric oxide (NO) (Moncada *et al.*, 1991).

Evidence for NO as a transmitter of inhibitory motor neurones in the gastrointestinal tract have been obtained by several authors in various tissues: canine ileocolonic junction (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990), canine terminal ileum (Bogers *et al.*, 1991), guinea-pig ileum (Osthaus & Galligan, 1992), guinea-pig stomach (Desai *et al.*, 1991; Meulemans *et al.*, 1992), rat gastric fundus (Li & Rand, 1990), rat stomach (Lefebvre *et al.*, 1992), opossum internal anal sphincter (Rattan & Chadker, 1992; Tøttrup *et al.*, 1992), human ileum (Maggi *et al.*, 1991), human sigmoid colon and internal anal sphincter (Burleigh, 1992).

Much of this evidence is based on the use of compounds that selectively modify the 'nitrergic' neurotransmission. NO is formed from L-arginine by NO-synthase (Palmer *et al.*, 1988), which has been immunohistochemically demonstrated in the myenteric plexus of the rat (Bredt *et al.*, 1990). NO readily diffuses through the presynaptic membrane into the target cell, where it is thought to activate the soluble guanylate cyclase, thus giving rise to increased guanosine 3':5'-cyclic monophosphate (cyclic GMP)-levels (Arnold *et al.*, 1977; Feelish & Noack, 1987a; Moncada *et al.*, 1991; McCall & Vallance, 1992). This L-arginine-NO-pathway can be inhibited at several levels. Analogues of L-arginine inhibit the synthesis of NO (Moncada *et al.*, 1991; McCall & Vallance, 1992). N^G-nitro-L-arginine has been shown to be a potent inhibitor of the NO-synthesis (Moore *et al.*, 1990; Mülsch & Busse, 1990). Haemoglobin captures NO after it has diffused out of the neurone, which will prevent it from diffusing into the smooth muscle cells (Martin *et al.*, 1985;

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Kelm & Schrader, 1990). The dye and oxidant methylene blue selectivity inhibits the target of NO, the soluble guanylate cyclase (Gruetter *et al.*, 1981; Martin *et al.*, 1985) which is present in the smooth muscle cells.

The aim of this study was to establish whether NO is involved in relaxations of the guinea-pig colon ascendens evoked by 5-HT.

Methods

Tissue preparation

Dunkin-Hartley guinea-pigs of either sex, weighing 400–600 g, were killed by cervical dislocation. The ascending colon was removed, and the luminal contents were washed out with De Jalon solution (mm: KCl 5.6, CaCl₂ 0.5, NaHCO₃ 6.0, NaCl 155, glucose 2.78). The mesentery was carefully removed. Starting at the proximal end, about 1 cm distal from the caecum, the colon was divided into four segments of circa 2.5 cm. These intact segments were individually mounted vertically for isotonic measurement into a tissue bath containing 20 ml De Jalon solution. This solution was kept at 37°C and gassed with 95% O₂:5% CO₂. The strips were subjected to a preload of 2 g and allowed to stabilize for 30 min. Ketanserin (320 nM) and tropisetron (ICS 205–930, 3.2 µM) were continuously present in the De Jalon solution during all experiments to block contractile responses mediated by 5-HT₂, 5-HT₃ and 5-HT₄ receptors. After stabilization, the response of the longitudinal muscle to 320 nM methacholine was measured. After washing and 10 min stabilization, the procedure was repeated in order to stabilize the response of the tissue.

Protocols

For the construction of concentration-response curves, agonists and antagonists were added directly to the tissue bath (added volume ≤ 3% of tissue bath volume). Methacholine (320 nM) was added to the bath in order to precontract the muscle. The mean decrease in length was 5.78 ± 0.24 mm.

5-Hydroxytryptamine Since pilot experiments had revealed that relaxations to 5-HT are subject to tachyphylaxis, non-cumulative concentration-response curves were established, beginning 10 min after the administration of methacholine. The size of the 5-HT-induced relaxation was measured as the maximum relaxation induced during an incubation period of approximately 2 min. Then, the agonists were washed out by replacing the bathing fluid twice. Stabilization of 10 min was allowed. This 20 min dosing cycle was repeated, applying ascending concentrations of 5-HT. The response to repetitive administration of methacholine was perfectly stable throughout the experiment. On each segment, first a concentration-response curve to 5-HT was constructed in the absence of an antagonist (ketanserin and tropisetron present) (= first series). Consecutively, the measurements were repeated in the presence of an antagonist (= second series). Antagonists were added 10 min prior to the methacholine-induced precontraction, and were re-added directly after each washout. The antagonists had no effect on the methacholine-induced contractions, except for methiothepin. When L-arginine was tested against N^G-nitro-L-arginine, they were added together. Four segments were taken per animal; each fourth strip was used as a control, i.e. vehicle control was added during the second series. In these control strips, the concentration-response curve to 5-HT was perfectly reproducible as compared to the curves of the first series. All responses were expressed as a percentage of the relaxation induced by 5-HT 10 µM of the first series for each individual segment. 5-HT (10 µM) induced relaxations of 3.8 ± 0.2 mm (*n* = 32; mean ± s.e.mean).

Glyceryl trinitrate and isoprenaline For the construction of concentration-response curves to glyceryl trinitrate and isoprenaline, the compounds were added in a cumulative fashion, 10 min after precontraction to methacholine (320 nM). After washing and stabilization for 30 min (washing every 10 min), a second concentration-response curve was established in the presence of an antagonist. The responses induced by glyceryl trinitrate or isoprenaline were expressed relative to the response to either glyceryl trinitrate 10 µM or isoprenaline 3.2 µM of the first series for each segment. The relaxations induced by these concentrations were respectively 7.6 ± 0.5 mm (*n* = 18) and 10.5 ± 1.0 mm (*n* = 12).

Chemicals

Methiothepin maleate (Hoffman-La Roche, Switzerland), methacholine HCl (E. Merck, Germany), 5-hydroxytryptamine HCl, tetrodotoxin (Serva, Germany), bovine haemoglobin (Sigma, Belgium), glyceryl trinitrate (Federa, Belgium), metergoline (Gruppo Montedison Farmitalia, Italy), isoprenaline tartrate, methylene blue, N^G-nitro-L-arginine and L-arginine (Janssen Chimica, Belgium) were added to the tissue bath solution in volumes less than 3% of the bath volumes. Ketanserin tartrate (Janssen Pharmaceutica, Belgium) and tropisetron HCl (ICS 205–930; Sandoz, Switzerland) were continuously present in the De Jalon solution. All compounds were dissolved in distilled water, except for 5-hydroxytryptamine (stock containing ascorbic acid 250 nM). This vehicle had no effect on the tone of the strips. Glyceryl trinitrate was further diluted with distilled water from a stock solution of 1% in ethanol. The solution of haemoglobin was always freshly prepared, kept on ice during the experiment and protected from light. The stock solution of TTX was prepared in advance and kept frozen (–25°C) in aliquots of 100 µM. It was kept on ice during the experiment.

Statistical analysis

For graphical representation means ± standard error of the means were calculated. EC₅₀ and IC₅₀ values were determined by linear regression analysis. Differences between mean values were tested by the two-tailed Student's *t* test for unpaired observations. Values of *P* < 0.05 were considered to be statistically different.

Results

5-HT induced concentration-dependent relaxations from 10 nM onwards (Figure 1). The relaxations were fast in onset. In about 1 out of 3 preparations, the relaxations induced by 5-HT in the concentration-range of 320 nM to 3.2 µM were multiphasic, i.e. an initial relaxation was followed by a quick

Figure 1 Tracing of relaxations of the guinea-pig colon ascendens evoked by non-cumulative concentrations of 5-hydroxytryptamine (5-HT). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT₂, 5-HT₃ and 5-HT₄ receptors.

small contraction and consecutively a larger, more sustained relaxation. The concentration-response curve to control 5-HT appeared non-monophasic, starting a second phase at about $1.0 \mu\text{M}$ (Figure 2). The EC_{50} value calculated over the entire concentration-response curve to 5-HT was 258 ($172\text{--}387$) nM. TTX (320 nM) contracted the strips ($18.4 \pm 3.8\%$ ($n = 7$) of the methacholine-induced contractions), and it abolished the relaxations to 5-HT, $10 \mu\text{M}$ ($n = 6$, not shown). The 5-HT_1 receptor antagonist, metergoline (10 nM), inhibited the relaxations to 5-HT (Figure 2). Methiothepin (100 nM) also inhibited the relaxations to 5-HT (by about 45–70% depending on the applied 5-HT concentration) but it inhibited the methacholine-induced contractions as well (by about 25%). Guanethidine ($3.2 \mu\text{M}$) did not affect the concentration-response curve to 5-HT ($n = 4$, results not shown).

NO-antagonists

N^G -nitro-L-arginine concentration-dependently depressed the concentration response curve to 5-HT, the inhibitory effect being statistically significant from $10 \mu\text{M}$ N^G -nitro-L-arginine onwards (Figure 3). The IC_{50} of the inhibitory effects of N^G -nitro-L-arginine against 5-HT-induced relaxations was 18.7 ($13.3\text{--}26.3$) μM (calculated at 5-HT 320 nM). N^G -nitro-L-arginine $320 \mu\text{M}$ abolished relaxations to 5-HT up to 320 nM . However, the relaxations caused by 5-HT $> 320 \text{ nM}$ were not completely blocked: about 25% of the control response remained (Figure 3). The high concentrations of N^G -nitro-L-arginine contracted the preparations slightly ($27.2 \pm 4.5\%$ ($n = 6$) of the methacholine-induced contractions). L-Arginine ($100 \mu\text{M}$) did not affect the concentration-response curve to 5-HT ($n = 3$, results not shown). L-Arginine ($320 \mu\text{M}$) completely reversed the inhibition by N^G -nitro-L-arginine ($10 \mu\text{M}$) of 5-HT (100 nM)-induced relaxations ($n = 6$, results not shown).

Glyceryl trinitrate concentration-dependently induced relaxations from 3.2 nM onwards (Figure 4) that were fast in onset. TTX (320 nM), N^G -nitro-L-arginine ($100 \mu\text{M}$) and L-arginine ($100 \mu\text{M}$) did not inhibit the responses to glyceryl trinitrate (Figure 4).

Figure 2 Concentration-response curves to 5-hydroxytryptamine (5-HT): control (○), or in the presence of metergoline 10 nM (■) or methiothepin 100 nM (▲). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT_2 , 5-HT_3 and 5-HT_4 receptors. Values ($n = 6$) that are significantly different from control values ($P < 0.05$) are marked with an asterisk. The relaxations are expressed as a percentage of the relaxations of 5-HT ($10 \mu\text{M}$).

Figure 3 Concentration-response curves to 5-hydroxytryptamine (5-HT): control (○), or in the presence of N^G -nitro-L-arginine $3.2 \mu\text{M}$ (▲), $10 \mu\text{M}$ (●), $32 \mu\text{M}$ (□), $100 \mu\text{M}$ (■) and $320 \mu\text{M}$ (△). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT_2 , 5-HT_3 and 5-HT_4 receptors. Values (all $n = 6$; control $n = 12$) that are significantly different from control values ($P < 0.05$) are marked with an asterisk. The relaxations are expressed as a percentage of the relaxations of 5-HT ($10 \mu\text{M}$).

Figure 4 Concentration-response curves to glyceryl trinitrate in the absence of an antagonist (○), or in the presence of L-arginine $100 \mu\text{M}$ (▼), N^G -nitro-L-arginine $100 \mu\text{M}$ (■) or tetrodotoxin 320 nM (▽). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT_2 , 5-HT_3 and 5-HT_4 receptors. All values $n = 6$. The relaxations are expressed as a percentage of the relaxations to glyceryl trinitrate ($10 \mu\text{M}$).

Haemoglobin $10 \mu\text{M}$ tended to inhibit the 5-HT-induced relaxations, whereas haemoglobin $32 \mu\text{M}$ significantly inhibited them (Figure 5). Isoprenaline relaxed the preparations from 1 nM onwards. The relaxations were not fast in onset. Haemoglobin $32 \mu\text{M}$ did not affect the concentration-response curves to isoprenaline (Figure 5). In contrast, it depressed the concentration-response curve to glyceryl trinitrate (Figure 5). Haemoglobin slightly contracted the muscle ($4.6 \pm 1.6\%$ of

Figure 5 Concentration-response curves to 5-hydroxytryptamine (5-HT) (a), isoprenaline (b) and glyceryl trinitrate (c): controls (○), or in the presence of haemoglobin 10 μM (■) and 32 μM (▲). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT₂, 5-HT₃ and 5-HT₄ receptors. Values ($n = 6$) that are significantly different from control values ($P < 0.05$) are marked with an asterisk. The relaxations to 5-HT, isoprenaline or glyceryl trinitrate are expressed as a percentage of the relaxations of 5-HT (10 μM), isoprenaline (3.2 μM) or glyceryl trinitrate (10 μM) respectively.

methacholine-induced contractions, $n = 10$). Methylene blue (10 μM) inhibited the responses to 5-HT from 100 nM onwards (Figure 6). The concentration-response curves to isoprenaline or glyceryl trinitrate however were not affected (Figure 6). Methylene blue (10 μM) contracted the muscle slightly ($6.2 \pm 2.1\%$ of the methacholine-induced contractions, $n = 6$). Higher concentrations of methylene blue could not be tested because they markedly inhibited the methacholine-induced contractions.

Figure 6 Concentration-response curves to 5-hydroxytryptamine (5-HT) (a), isoprenaline (b) and glyceryl trinitrate (c): controls (○), or in the presence of methylene blue 10 μM (●). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited via 5-HT₂, 5-HT₃ and 5-HT₄ receptors. Values ($n = 6$) that are significantly different from control values ($P < 0.05$) are marked with an asterisk. The relaxations to 5-HT, isoprenaline or glyceryl trinitrate are expressed as a percentage of the relaxations of 5-HT (10 μM), isoprenaline (3.2 μM) or glyceryl trinitrate (10 μM) respectively.

Discussion

The selective 5-HT₁ receptor antagonists, methiothepin and metergoline, both inhibited the relaxations caused by 5-HT. The selective 5-HT₂ receptor antagonist ketanserin and the 5-HT₃ and 5-HT₄ receptor antagonist, tropisetron, were present throughout our experiments. It therefore seems likely that, according to the criteria for 5-HT receptor classification as proposed by Bradley *et al.* (1986), the relaxations to 5-HT are mediated via 5-HT₁ receptors. Kojima (1991) and Elwood & Bunce (1992) also found a 5-HT₁ receptor to be

involved in 5-HT-induced relaxations in the guinea-pig colon. It was however not established which 5-HT₁ receptor subtype is concerned. In intestinal tissues of the guinea-pig, rat and rabbit, receptors with 5-HT_{1A} (Fozard & Kilbinger, 1985; Matsuyama *et al.*, 1990; Wade *et al.*, 1991; Galligan, 1992), putative 5-HT_{1P} (Mawe *et al.*, 1986; Branchek *et al.*, 1988; Wade *et al.*, 1991), 5-HT_{1C} and 5-HT_{1D} (Baez *et al.*, 1991; Kalkman & Fozard, 1991) characteristics have been demonstrated. Further studies are required to elucidate which 5-HT₁ receptor subtype is involved in the 5-HT-induced relaxations of the guinea-pig colon.

TTX abolished relaxant responses to 5-HT, which confirms that nerves are involved. Since guanethidine did not affect the 5-HT-induced relaxations (Costa & Furness, 1979; present results), it is not likely that noradrenaline is the inhibitory neurotransmitter involved. In the canine terminal ileum and ileocolonic junction, it has been demonstrated that 5-HT-induced relaxations are mediated by NO (Bogers *et al.*, 1991). In our study, evidence was obtained which strongly suggests that NO is involved in 5-HT-induced relaxations in the guinea-pig colon ascendens as well. This evidence consists of three sets of observations.

Firstly, the L-arginine analogue, N^G-nitro-L-arginine, which is known to be a potent inhibitor of NO-synthase (Moore *et al.*, 1990; Mülsch & Busse, 1990), inhibited the relaxations to 5-HT but not those to glyceryl trinitrate. Furthermore, the inhibition could be reversed by L-arginine. These data suggest that NO is involved in the 5-HT-induced relaxations.

Secondly, both the relaxations to glyceryl trinitrate and 5-HT but not those to isoprenaline were inhibited by haemoglobin. Although the exact mechanism of action is still under debate, glyceryl trinitrate is thought to react with thiol groups of co-factors (cysteine) of the soluble guanylate cyclase, which yields the generation of NO (Ignarro *et al.*, 1981; Feelish *et al.*, 1988). NO in turn activates the soluble guanylate cyclase to increase the intracellular cyclic GMP content (Feelish & Noack, 1987a,b). Haemoglobin is a scavenger of NO (Martin *et al.*, 1985; Kelm & Schrader, 1990). It has been shown that haemoglobin does not bind or inactivate glyceryl trinitrate and that it does not affect the reaction between glyceryl trinitrate and the thiols (Martin *et al.*, 1986; Feelish & Noack, 1987b). In our experiments, neither TTX nor N^G-nitro-L-arginine or L-arginine inhibited the relaxations to glyceryl trinitrate, which is in good agreement with the above mechanism of action. Due to its large size, haemoglobin is likely to be confined to the extracellular space. Since NO readily crosses cell membranes, haemoglobin might

avidly bind the extracellular NO which will lower its extracellular concentration, thus increasing the net rate of diffusion of NO out of the cell (Martin *et al.*, 1985; 1986). This might explain the observed inhibition by haemoglobin against the relaxations to glyceryl trinitrate. Other authors have also found haemoglobin to inhibit relaxations to glyceryl trinitrate: Martin *et al.* (1985, 1986; rabbit aorta), Boeckxstaens *et al.* (1990; canine ileocolonic junction), Bogers *et al.* (1991; canine terminal ileum and ileocolonic junction). Isoprenaline, on the other hand, induces relaxations through an increase in intracellular cyclic AMP-content (Martin *et al.*, 1985). Hence, the inhibition by haemoglobin of the relaxations to 5-HT and glyceryl trinitrate, but not isoprenaline, strongly suggests that NO is involved in the action of 5-HT.

Thirdly, the relaxations to 5-HT were inhibited by the oxidant methylene blue, whereas the responses to glyceryl trinitrate and isoprenaline were not. Methylene blue is a selective inhibitor of the target of NO, the soluble guanylate cyclase (Gruetter *et al.*, 1981; Martin *et al.*, 1985). Hence, it would be expected that the responses to glyceryl trinitrate would also be inhibited, as it does so in other tissues (Gruetter *et al.*, 1981; Martin *et al.*, 1985; Desai *et al.*, 1991). We do not have an explanation for this observation. The inhibition of the responses to 5-HT but not to isoprenaline however provides additional proof for the involvement of NO.

Methylene blue, N^G-nitro-L-arginine, haemoglobin and also the neurotoxin TTX all contracted the strips. This might indicate that NO constantly leaks from enteric inhibitory nerves in small quantities.

The concentration-response curve to 5-HT does not seem to be monophasic. Furthermore, N^G-nitro-L-arginine was not able to abolish the responses to 5-HT. Both these findings suggest that, at higher concentrations of 5-HT, inhibitory endogenous substances might be co-released with NO. Whether other candidates for inhibitory NANC-neurotransmission, which implicates VIP and ATP, are involved, needs further investigation.

It is concluded that in the guinea-pig colon ascendens, NO is involved in the relaxation that is induced by 5-HT. Our studies confirm that the relaxations are mediated by (a) 5-HT₁ receptor subtype(s). The involvement of VIP and/or ATP cannot be excluded.

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