Is Nitric Oxide Involved in 5-HT_3 Receptor-Mediated Neurogenic Relaxation of Guinea Pig Proximal Colon?

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ABSTRACT—The relaxations mediated by the activation of 5-HT receptors in the guinea pig proximal colon were investigated. Longitudinal strips were cut from the colon segment and placed into the bath. In the presence of atropine (0.2 μM), the relaxations were evoked by adding increasing concentrations of 5-HT (1–100 μM). Noncumulative concentration-response curves were established in the absence and presence of either 5-HT or nitric oxide synthase (NOS) antagonists. Selective 5-HT_3 antagonists tropisetron (10 and 100 nM) and ondansetron (1 μM) inhibited the relaxations and shifted the concentration-response curves to the right. Similar effects were observed in the presence of the NOS inhibitor N^G-nitro-L-arginine (3.2, 10, 32 μM) and partly reversed with L-arginine (100, 320 μM). N^G-nitro-D-arginine, serving as a negative control, was ineffective. The relaxations were further inhibited in the presence of the soluble guanylate cyclase blocker methylene blue (10 μM) or NO scavenger hemoglobin (32 μM). These results suggest that the 5-HT_3 receptor plays a role in neurogenic relaxations of guinea pig proximal colon, which are at least partly mediated via release of NO from nerve endings.

Keywords: Proximal colon (guinea pig), Longitudinal muscle, Relaxation, 5-HT_3 receptor, Nitric oxide

5-Hydroxytryptamine (5-HT) present in the mammalian digestive tract (1) exerts multiple types of action including direct contraction or relaxation of smooth muscle and stimulation of the intramural nerve plexus with resulting neurotransmitter release (2–4). In guinea pig proximal colon, the 5-HT evoked response is usually biphasic. It starts with a quick and transient relaxation (5) that is followed by a contraction that tends to fade rapidly within few repeated applications (6).

The relaxation of the guinea pig colon is neurogenic because it is abolished in the presence of tetrodotoxin (5, 7–9). Since 5-HT exerts its effects on multiple receptor types (10), it is not quite clear at present which subtype of the 5-HT receptor is responsible for this phenomenon. Results of some studies suggest the involvement of the 5-HT_1 (7, 11, 12) or 5-HT_2 receptor (13) and other results including the observations of our laboratory support a possible involvement of the 5-HT_3 receptor (8, 9, 14). Studies favoring the role of 5-HT_1/5-HT_2 receptors in the relaxation concluded that its final mediator is nitric oxide (NO) (11, 15) and possibly adenosine 5-triphosphate (ATP) (16). However, studies favoring the role of 5-HT_3 receptors did not investigate the nature of a final mediator yet. The aim of this study was therefore to establish whether NO might be involved also in the relaxation mediated via 5-HT_3 receptors.

MATERIALS AND METHODS

Preparations and solutions

Male guinea pigs (200–400 g) were stunned by a blow on the head and exsanguinated. The most proximal portion of the colon, 4–5 cm from the caecum, was dissected from the surrounding fascia, and strips of approximately 3.0 × 0.3 cm were cut for each experiment (7). The preparations were set up in a 10-ml organ bath filled with Krebs solution (37℃) of the following composition: 120 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 15.4 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 11.5 mM glucose and gassed with 95% O_2, 5% CO_2. Atropine (0.2 μM) was present in the solution throughout all the experiments to block the cholinergic component of contraction; thus the initial relaxation was enhanced (5). The preparations were suspended vertically under a 10-mN tension and allowed to equilibrate for at least 40 min; during this period, the solution was exchanged every 10 min. Changes in the mechanical activity of the preparation were registered isotonically.

Experimental protocol

Tissues were initially given two priming doses of 5-HT (10 μM) before construction of sequential 5-HT concentration-response curves (CRCs). Non-cumulative CRCs were established by adding incremental concentrations of 5-HT (spaced at 0.5 log units) at time interval range of 5–25 min with respect to the concentration used. These intervals were chosen after preliminary experiments to avoid any possibility of desensitization. 5-HT was added to the bath and was present there until the peak of relaxation was achieved and relaxation changed to contraction (usually not more than 1 min); 5-HT was then washed out. Continuous perfusion by an antagonist was started at least 10 min prior to the initial exposure to 5-HT to allow time for equilibration. When L-arginine (L-ARG) was tested against N^G-nitro-L-arginine (L-NOARG), they were added together.

The strips were cut alongside each other from the same colon segment. In most experiments, the following arrangement was adopted: Four preparations were used and 5-HT-CRCs were obtained in the absence of an antagonist (first series). The concentration evoking the maximum relaxation was either 30 or 100 μM. Consecutively, 5-HT-CRCs were repeated in the continuous presence of differing concentrations of an antagonist in three preparations, while the fourth strip served as a control (5-HT-CRC again in the absence of any antagonist) (second series). The control curves of the first and second series were identical. All responses of the second series were expressed as a percentage of the relaxation induced by the most effective 5-HT concentration of the first series for each individual strip. 5-HT (30 or 100 μM) induced maximal relaxations of 3.198 ± 0.088 mN (n=226).

Chemicals

The agents used were: N^G-nitro-D-arginine (D-NOARG), L-NOARG (Calbiochem, La Jolla, CA, USA); L-ARG hydrochloride, bovine hemoglobin, methylene blue trihydrate (Sigma, Prague, Czech Republic); atropine sulphate (Spofa, Prague, Czech Republic); 5-HT creatininsulphate (Fluka AG, Buchs, Switzerland); ondansetron hydrochloride dihydrate (Glaxo, Greenford, UK); and tropisetron hydrochloride (Sandoz, Basle, Switzerland).

Statistics

The results were each expressed as a mean±S.E.M., with the number of experiments in parentheses (n). EC_{50}

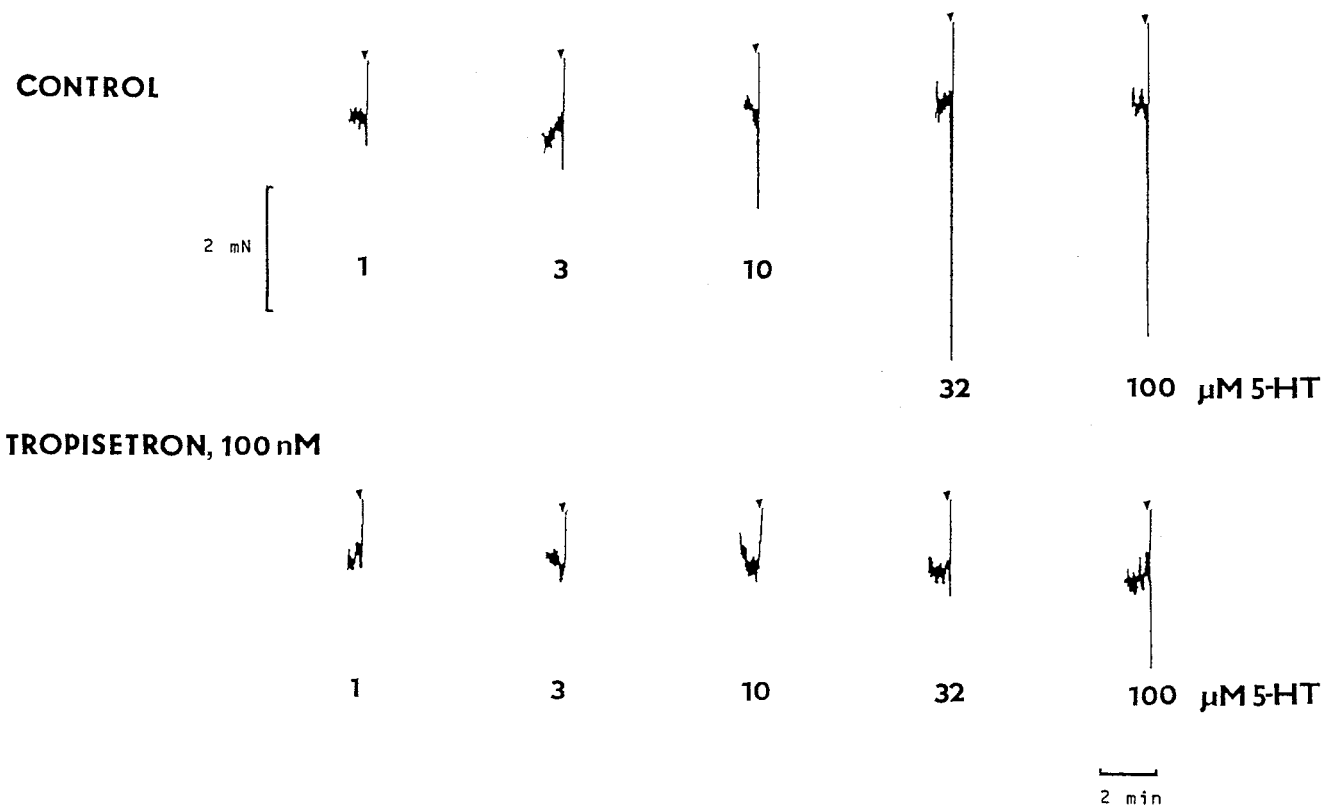

Fig. 1. Recordings of concentration-dependent relaxations evoked by 5-HT in the longitudinal muscle of the guinea pig proximal colon in the absence (upper panel) or presence (lower panel) of 5-HT_3 antagonist tropisetron (100 nM). The addition of 5-HT is indicated by arrows.

values were determined by linear regression analysis. The significance of differences was assessed with two-tailed Student's *t*-test for unpaired data, and a probability level of 0.05 or less was considered to be statistically significant.

RESULTS

The effect of 5-HT in the presence of 5-HT antagonists

Application of 5-HT to the preparation evoked a fast and transient relaxation (Fig. 1) followed immediately by a contraction. Control CRCs of the first and second series were obtained. Their EC_{50}s calculated over the interval 3.2–32 μM 5-HT were 18.2 μM (11.6–21.7 μM, n=6) and 15.8 μM (11.6–17.8 μM, n=6), respectively. There was no significant difference between these two curves at any concentration, suggesting that the effect of 5-HT did not change with time.

5-HT-CRCs were then obtained in the absence and presence of the 5-HT$_3$ antagonists tropisetron (10, 100 nM) or ondansetron (1 μM). The presence of the antagonists caused a shift of the curve to the right; the relaxation evoked by 10 μM 5-HT was abolished (Fig. 2).

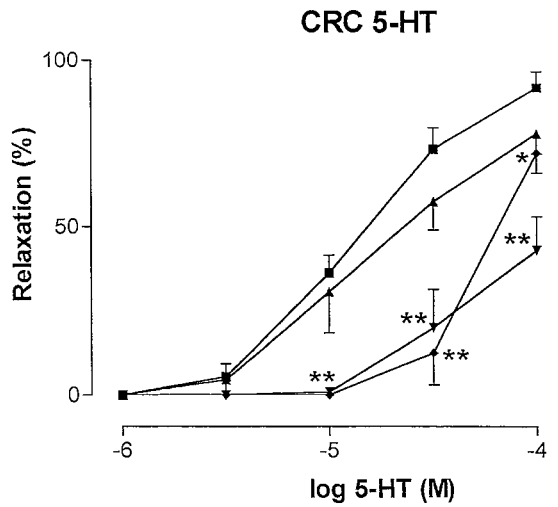

Fig. 2. Concentration-response curves (second series) for 5-HT under control conditions (■) or in the presence of the 5-HT$_3$ antagonist tropisetron at 10 nM (▲) or 100 nM (▼) or ondansetron (1 μM) (◆). Values (all n=6) significantly different from the control values are marked with an asterisk (*P≤0.05, **P≤0.01). The relaxations are expressed as a percentage of the maximal relaxation evoked by 5-HT (30 or 100 μM) in the first series. Atropine (0.2 μM) was present in all experiments.

The effect of 5-HT in the presence of L-NOARG

5-HT-CRCs were obtained in the absence and presence of the NO synthase (NOS) antagonist L-NOARG (3.2, 10, 32 μM). While 3.2 μM L-NOARG was without a major effect, both 10 and 32 μM L-NOARG significantly inhibited 5-HT-evoked relaxations (Fig. 3a). In contrast, the enantiomer D-NOARG (32 μM), serving as a negative control, was ineffective (Fig. 3b). The effect of 10 μM L-NOARG was completely reversed by L-ARG, whereas the reversal was only partial at 32 μM L-NOARG (Figs. 4 and 5). The presence of L-ARG (32, 100, 320 μM) on its own caused only a slight and inconsistent increase of the 5-HT-induced relaxation, which was maximal at 100 μM L-

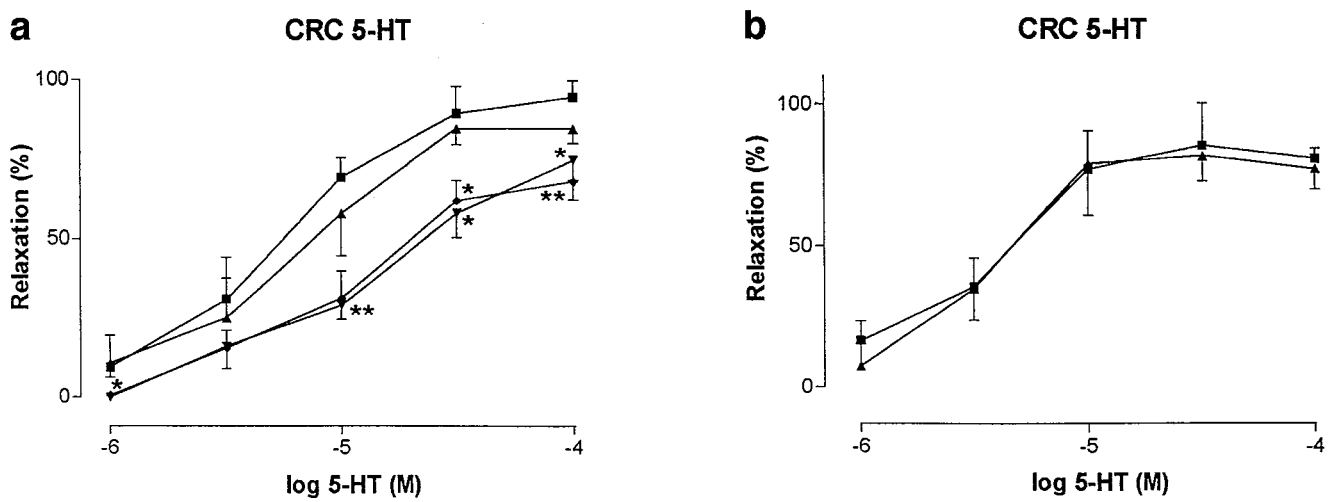

Fig. 3. Concentration-response curves (second series) for 5-HT under control conditions (■), in the presence of the NOS inhibitor L-NOARG at 3.2 μM (▲), 10 μM (▼) or 32 μM (◆) (a) and in the presence of the enantiomer D-NOARG at 32 μM (▲) (b). Values in panel a (all n=6) that are significantly different from control values are marked with an asterisk (*P≤0.05, **P≤0.01). Experimental values in panel b (n=8) were not significantly different from control values. For further details, see Fig. 2.

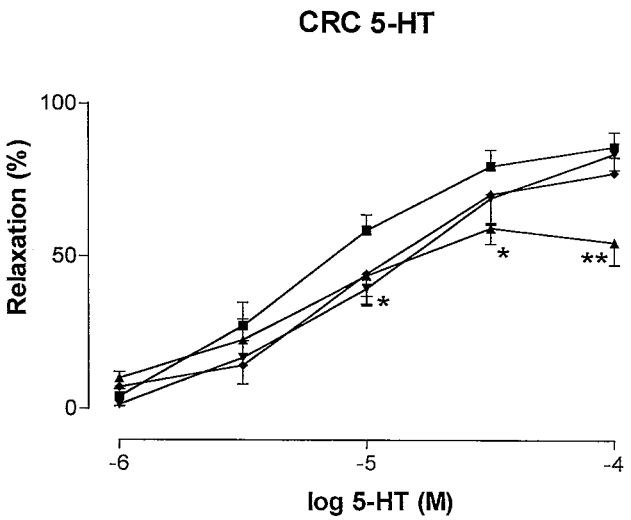

Fig. 4. Concentration-response curves (second series) for 5-HT under control conditions (■), in the presence of the NOS inhibitor L-NOARG at 10 μM (▲) and in the combined presence of L-NOARG at 10 μM plus L-ARG at 100 μM (▼) or 320 μM (◆). Values (all n=12) that are significantly different from control values are marked with an asterisk (*P≤0.05, **P≤0.01). For further details, see Fig. 2.

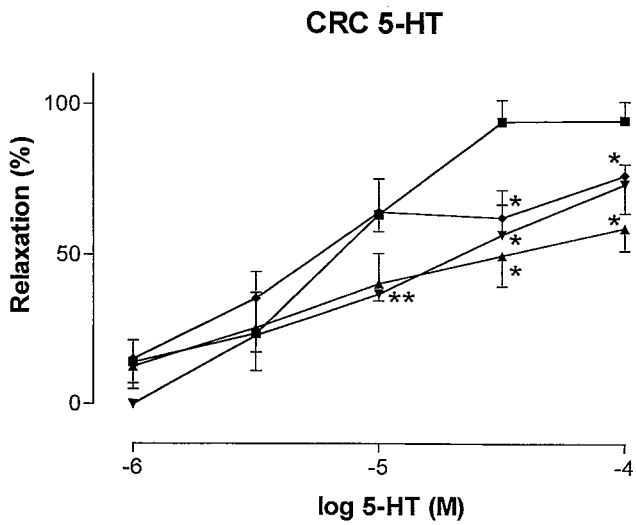

Fig. 5. Concentration-response curves (second series) for 5-HT under control conditions (■), in the presence of the NOS inhibitor L-NOARG at 32 μM (▲) and in the combined presence of L-NOARG at 32 μM plus L-ARG at 100 μM (▼) or 320 μM (◆). Values (all n=6) that are significantly different from control values are marked with an asterisk (*P≤0.05, **P≤0.01). For further details, see Fig. 2.

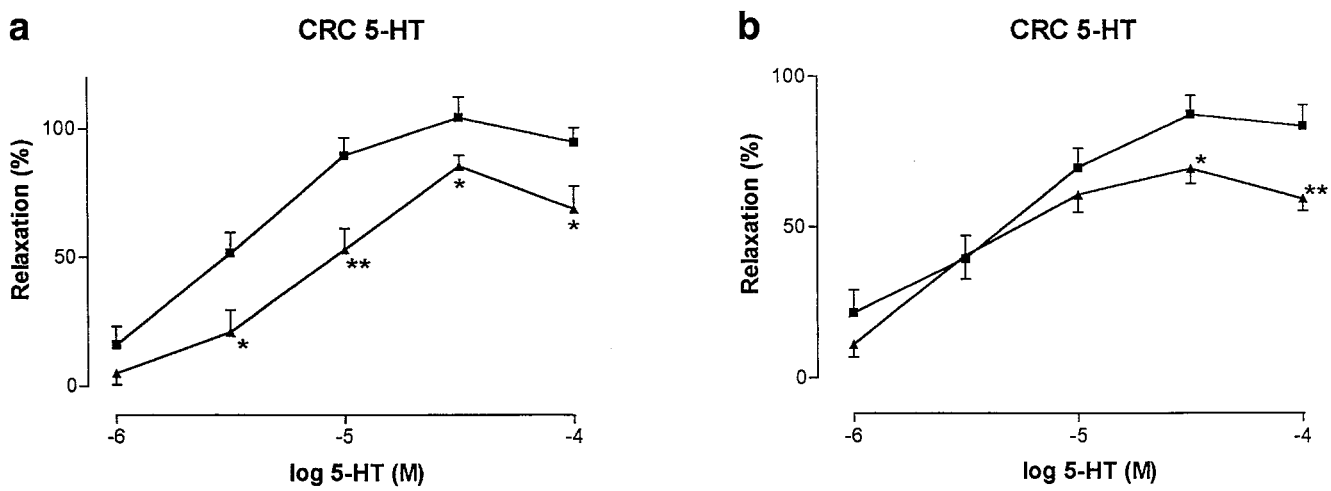

Fig. 6. Concentration-response curves (second series) for 5-HT under control conditions (■), in the presence of hemoglobin (32 μM) (▲) (a) and in the presence of methylene blue (10 μM) (▲) (b). Values (a: n=11, b: n=14) that are significantly different from control values are marked with an asterisk (*P≤0.05, **P≤0.01). For further details, see Fig. 2.

ARG; however, it was not statistically significant (not shown, n=9).

The effect of 5-HT in the presence of other NO-antagonists

Both hemoglobin (32 μM) and methylene blue (10 μM) significantly inhibited 5-HT-evoked relaxations (Fig. 6: a and b). Methylene blue was effective only at higher 5-HT concentrations (32 and 100 μM).

DISCUSSION

The effect of 5-HT in guinea pig proximal colon begins with a fast neurogenic relaxation (6) that is enhanced in the presence of atropine (0.2 μM) (5). It is well established that tetrodotoxin (0.3–0.5 μM) inhibits the 5-HT-induced relaxation (8, 9), providing evidence for its neurogenic origin. Although the subtype of 5-HT receptor responsible for the relaxation is the subject of a current debate, there are several studies supporting the role of the

5-HT_3 subtype. In the guinea pig ileum, the discovery of 5-HT_3 receptors on inhibitory neurons was reported (17). In the guinea pig distal colon, the 5-HT-evoked relaxation was blocked by the 5-HT_3-receptor antagonist granisetron (1 μM) (14). In the guinea pig proximal colon, similar results were obtained in our earlier experiments. 5-HT-evoked relaxation was not antagonized in the presence of the 5-HT_1/5-HT_2-receptor antagonists methiothepin (0.1 μM) and metergoline (0.1 μM), while it was readily antagonized in the presence of the 5-HT_3-receptor antagonists tropisetron (10, 50, 500 nM) and ondansetron (1 μM) (8, 9). The efficacy of both 5-HT_3 antagonists to antagonize the 5-HT-evoked relaxation was confirmed in this study.

The reason for the discrepancies in the attempts to identify the 5-HT receptor subtype responsible for the 5-HT-evoked relaxation is unclear. It seems that an important difference between these studies is the use of preparations held at different (either basal or drug-induced) tone. Kojima and Shimo (5), working with the preparations at basal tone, suggested that the receptor responsible for the neurogenic relaxation might be 5-HT_3-like. However, their next study did not confirm this assumption; instead, the role of 5-HT_1-like receptors has been suggested, although the respective 5-HT_1 receptor subtype could not be determined (7). Since then, contracted preparations pretreated with 5-HT_3 receptor antagonists were preferentially used (11–13). Of course, under the latter condition, a 5-HT_3 receptor-mediated relaxation could not be observed. However, a few studies using preparations at basal tone and not pretreated with 5-HT_3 antagonists (8, 9, 14) supported the role of this receptor type (5). Thus two different relaxant mechanisms possibly utilizing different final mediator(s) might coexist in guinea pig proximal colon. On this background, the final mediator of 5-HT_3-mediated neurogenic relaxation has been investigated.

The 5-HT-evoked relaxation was inhibited in the presence of the L-arginine analogue L-NOARG. This substance is a well-known inhibitor of NOS (18, 19). Since its effect is stereoselective, its enantiomer D-NOARG can be used as a negative control (20, 21). As expected, D-NOARG was entirely ineffective in our experiments. Furthermore, L-ARG, the natural substrate for NOS enzyme, restored the relaxation reduced by L-NOARG. At the highest L-NOARG concentration (32 μM), the restoration was only partial. This is in good agreement with an earlier observation (22) that the effects of L-NOARG could not be readily reversed by washing with L-ARG (22) due to a high affinity of L-NOARG for NOS (23). L-ARG on its own tended to shift the 5-HT-CRCs to the left. Such a shift, although very slight and inconsistent, could be only detected if nothing but L-ARG was present. It is conceivable that the supply of L-ARG was limited under physiological conditions; thus the excessive amount of the substance supplied experimentally might sometimes result in a very slight potentiation of the relaxation. However, another research group could not detect such a change (11).

On the other hand, the slight contraction of the preparations caused by the high concentrations (up to 320 nM) of L-NOARG reported by these authors could not be observed in our experiments. There are at least two reasons for this discrepancy. First, the above mentioned high concentration was not used in our study. Second, it has been reported that the response to NO consists of direct and indirect components, the latter being mediated by the presynaptic NO-mediated inhibition of cholinergic transmission (24). This component would be suppressed also in the presence of NOS inhibitors, resulting in a contraction of the smooth muscle due to disinhibition of acetylcholine release. However, acetylcholine could not exert any muscarinic effect in our experiments because atropine was present.

5-HT_3 relaxations were further inhibited in the presence of hemoglobin or methylene blue. Hemoglobin functions as a NO scavenger (25) and methylene blue inhibits soluble guanylate cyclase, the target of NO (25, 26). These observations are further evidence for an involvement of NO.

Since NO antagonists used in this study could not entirely block the relaxations, the possible existence of another mediator that might be co-released with NO should be considered. In the case of 5-HT_1/5-HT_2 relaxations, a role for ATP as a cotransmitter was recently demonstrated (16).

Based on our data, we conclude that NO plays a role as a final mediator in the fast, transient and neurogenic 5-HT_3-receptor-mediated relaxation in guinea pig proximal colon. NO thus can function as a common denominator subserving both types of 5-HT neurogenic relaxations, i.e., relaxations induced by the activation of either 5-HT_1/5-HT_2 or 5-HT_3 receptors.

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REFERENCES

1 Costa M, Furness JB, Cuello AC, Verhofstad AAJ, Steinbusch HWJ and Elde RP: Neurons with 5-hydroxytryptamine-like immunoreactivity in the enteric nervous system: their visualization and reactions to drug treatment. Neuroscience 7, 351–363 (1982)

2 Furness JB and Costa M: Identification of gastrointestinal neurotransmitters. *In* Mediators and Drugs in Gastrointestinal

Motility I, Edited by Bertaccini G, pp 383–460, Springer-Verlag, Berlin, Heidelberg, New York (1982)
3 Kalkman HO, Engel G and Hoyer D: Inhibition of 5-carboxamidotryptamine-induced relaxation of guinea-pig ileum correlates with [^{125}I]LSD binding. Eur J Pharmacol **129**, 139–145 (1986)
4 Carter D, Champney M, Hwang B and Eglen RM: Characterization of a postjunctional 5-HT receptor mediating relaxation of guinea-pig isolated ileum. Eur J Pharmacol **280**, 243–251 (1995)
5 Kojima S and Shimo Y: The sites of action of 5-hydroxytryptamine in the longitudinal muscle of the guinea pig proximal colon. Asia Pacific J Pharmacol **1**, 111–116 (1986)
6 Costa M and Furness JB: The sites of action of 5-hydroxytryptamine in nerve-muscle preparations from the guinea-pig small intestine and colon. Br J Pharmacol **65**, 237–248 (1979)
7 Kojima S: Characterization of 5-hydroxytryptamine induced relaxations of guinea-pig proximal colon. Arch Int Pharmacodyn Ther **313**, 23–32 (1991)
8 Slánský J, Kadlec O, Mašek K and Gulda O: The effect of immunomodulator muramyl dipeptide on serotoninergic response of isolated neuromuscular preparations. Pharmacology **48**, 11–20 (1994)
9 Ševčík J, Růžička V, Slánský J and Mašek K: Which type of 5-hydroxytryptamine receptor mediates relaxation of the longitudinal muscle of guinea-pig proximal colon in vitro? Methods Find Exp Clin Pharmacol **18**, 421–430 (1996)
10 Martin GR and Humphrey PPA: Classification Review. Receptors for 5-hydroxytryptamine: Current perspectives on classification and nomenclature. Neuropharmacology **33**, 261–273 (1994)
11 Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA and Schuurkes JAJ: Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens in vitro. Br J Pharmacol **107**, 756–761 (1992)
12 Elswood CJ and Bunce KT: Investigation of the 5-HT receptor mediating relaxation in guinea-pig proximal colon. J Pharm Pharmacol **44**, 264–266 (1992)
13 Briejer MR, Akkermans LMA, Lefebvre RA and Schuurkes JAJ: Novel 5-HT$_2$-like receptor mediates neurogenic relaxation of the guinea-pig proximal colon. Eur J Pharmacol **279**, 123–133 (1995)
14 Woollard DJ, Bornstein JC and Furness JB: Characterization of 5-HT receptors mediating contraction and relaxation of the longitudinal muscle of guinea-pig distal colon in vitro. Naunyn Schmiedebergs Arch Pharmacol **349**, 455–462 (1994)
15 Kojima S and Shimo Y: The effect of L-N^G-nitroarginine on non-adrenergic, non-cholinergic (NANC) relaxations in the longitudinal muscle of the guinea pig proximal colon. Nippon Yakugaku Zasshi **99**, 373–379 (1992) (Abstr in English)
16 Briejer MR, Akkermans LMA, Meulemans AL, Lefebvre RA and Schuurkes JAJ: 5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide. Naunyn Schmiedebergs Arch Pharmacol **351**, 126–135 (1995)
17 Gunning SJ and Humphrey PPA: Evidence for 5-HT$_3$ receptor mediated release of an inhibitory transmitter in guinea pig isolated ileum. Br J Pharmacol **91**, 359P (1987)
18 Moore PK, Al-Swayeh OA, Chong NWS, Evans RA and Gibson A: L-N^G-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation in vitro. Br J Pharmacol **99**, 408–412 (1990)
19 Modin A, Weitzberg E, Hokfelt T and Lundberg JM: Nitric oxide synthase in the pig autonomic nervous system in relation to the influence of N^G-nitro-L-arginine on sympathetic and parasympathetic vascular control in vivo. Neuroscience **62**, 189–203 (1994)
20 Chung BH and Chang KC: Photo-induced adequate nitric oxide (PIANO)-mediated relaxation in isolated rabbit corpus cavernosum. Gen Pharmacol **25**, 893–898 (1994)
21 Matthew JD, Wadsworth RM and McPhaden AR: Inhibition of vasodilator neurotransmission in the sheep middle cerebral artery by VIP antiserum. J Auton Pharmacol **17**, 13–19 (1997)
22 Montague PR, Gancayco CD, Winn MJ, Marchase RB and Friedlander MJ: Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex. Science **263**, 973–977 (1994)
23 Dwyer MA, Bredt DS and Snyder SH: Nitric oxide synthase: irreversible inhibition by L-N^G-nitroarginine in brain in vitro and in vivo. Biochem Biophys Res Commun **176**, 1136–1141 (1991)
24 Baccari MC, Iacoviello C and Calamai F: Nitric oxide as modulator of cholinergic neurotransmission in gastric muscle of rabbits. Am J Physiol **273** (Gastrointest Liver Physiol **36**), G456–G463 (1997)
25 Martin W, Villani GM, Jothianandan D and Furchgott RF: Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by haemoglobin and by methylene blue in the rabbit aorta. J Pharmacol Exp Ther **232**, 708–716 (1985)
26 Gruetter CA, Kadowitz PJ and Ignarro LJ: Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerin, sodium nitrite and amyl nitrite. Can J Physiol Pharmacol **59**, 150–156 (1981)