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European Journal of Pharmacology 355 (1998) 51–55

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Facilitation by endogenous acetylcholine and nitric oxide of luminal serotonin release from the guinea-pig colon

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Received 19 February 1998; revised 18 June 1998; accepted 19 June 1998

Abstract

The present study was designed to determine the influence of endogenous acetylcholine and nitric oxide (NO) on spontaneous luminal serotonin (5-hydroxytryptamine, 5-HT) release in the luminally perfused isolated guinea-pig proximal colon in vitro. 5-HT was determined by high-performance liquid chromatography with electro-chemical detection. The luminal outflow of 5-HT was significantly reduced by atropine (0.2 μM), hexamethonium (100 μM), the NO synthase inhibitor N^G-nitro-L-arginine (L-NNA, 10 μM) and the NO-trapping agent 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO, 30 μM). Addition of excess L-arginine (300 μM) reversed the inhibitory effect of L-NNA on the 5-HT outflow. Physostigmine (1 μM) caused a great increase (atropine-sensitive) in 5-HT outflow. The enhancing action of physostigmine on 5-HT outflow was partially inhibited by L-NNA (100 μM) or carboxy-PTIO (30 μM), but was unaffected by the muscarinic M_1 receptor antagonist pirenzepine (0.2 μM) or a muscarinic M_3 receptor antagonist 4-diphenyl-acetoxy-*N*-methyl-piperidine methiodide (0.2 μM). These results suggest that 5-HT release from luminally perfused proximal colon of the guinea pig is stimulated via a NO pathway and cholinergic pathways which utilize muscarinic synapses and nicotinic synapses. Further, an intrinsic cholinergic–NO link appears to play a role in the stimulation of luminal 5-HT release, which may reflect the release of 5-HT from entero-chromaffin cells.

Keywords: 5-HT (5-hydroxytryptamine, serotonin) release; Acetyl-choline, endogenous; Nitric oxide (NO); Colon, guinea-pig

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) has been identified as a transmitter candidate for the regulation of intestinal motility (Goyal and Hirano, 1996). Most intestinal 5-HT is localized in mucosal entero-chromaffin cells (Erspamer and Asero, 1952) from which this amine is released into both the intestinal lumen and the portal circulation. The release of 5-HT into the gut lumen has been characterized physiologically and pharmacologically in vivo or in vitro studies. Various stimuli, such as increased intraluminal pressure (Bülbring and Crema, 1959), vagal stimulation (Ahlman and Dahlström, 1983) or cholera toxin (Beubler and Horina, 1990), enhance the release of 5-HT into the gut lumen. Very recently, the morphological characteristics of the luminal release of 5-HT were examined in the isolated luminally perfused rat intestine (Fujimiya et al., 1997). However, the physiological importance of endoluminally released 5-HT and the precise mechanism controlling the release of 5-HT into the intestinal lumen remain unclear.

In isolated guinea-pig proximal colon, 5-HT can prejunctionally affect the cholinergic or nitrergic motor control of colonic smooth muscle (Elswood et al., 1991; Briejer et al., 1992). However, the reverse relationship, that is, modulation of intraluminal 5-HT release from enterochromaffin cells by acetylcholine or nitric oxide (NO) released from intramural neurons, has not yet fully investigated in the colon.

Therefore, we designed the present study to determine the influence of endogenous acetylcholine released from intrinsic cholinergic neurons and a NO-generating system on intraluminal spontaneous 5-HT release from the luminally perfused proximal colon of the guinea pig in vitro.

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0014-2999/98/$19.00

2. Materials and methods

2.1. Experimental set-up

Male Dunkin–Hartley guinea pigs, weighing 250–550 g, were anaesthetized with enflurane and bled. A 5.5-cm segment of the proximal colon, starting 4 cm from the caecum, was removed and the luminal contents were removed by washing with a physiological salt solution (PSS, mM; NaCl 136.8, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, glucose 5.56, ascorbic acid 0.12, Na_2EDTA 0.03, pH 7.4). The oral end of the colon was tied to an inflow cannula which permitted the continuous infusion of prewarmed, oxygenated PSS at a flow rate of 0.7 ml/min. The aboral end was connected to an outflow cannula to allow the collection of the fluid expelled. The colonic segment was then suspended in a longitudinal direction under a 3.2-g load in a 25-ml tissue bath filled with PSS at 37°C and oxygenated with 5% CO_2–95% O_2. After set up, the segment was allowed to equilibrate for 70 min with renewal of the bathing solution every 14 min.

2.2. Measurement of intraluminal 5-HT and 5-HIAA

After equilibration for 70 min, the experiments were carried out by collecting the luminal fluid every 3 min. The collected luminal fluid was lyophilized, dissolved in 0.4 M perchloric acid (200 μl) and passed through a 0.45-μm filter (chromatodisc 4A, GL Sciences). 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in the filtrate were measured by high-performance liquid chromatography with electro-chemical detection (LC-4B, BAS) as described previously (Forsberg and Miller, 1983). Known concentrations of 5-HT and 5-HIAA (Sigma, St. Louis, MO, USA) were used as standards. The separation of 5-HT and 5-HIAA was achieved by a reverse-phase capillary column (length 150 mm, inner diameter 1.0 mm, C-18, 5 μm, BAS), using a mobile phase consisting of 0.1 M monochloroacetic acid, 1 mM Na_2EDTA, 60 mg/l sodium octylsulfate and 8–10% acetonitrile (pH 3.2) at a flow rate of 0.3 ml/min. At the end of the experiments, the colonic segments were blotted on tissue paper and weighed; in some experiments the 5-HT/5-HIAA content was also determined. In this case, the underlying mucosa sheets (muscle-free) were homogenized in 0.4 M perchloric acid and the supernatant was analyzed as described above.

2.3. Drugs

The following drugs were used: atropine sulphate, 2-(4-carboxy-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO), hexamethonium bromide, physostigmine sulphate (Wako, Osaka); L-arginine (Nacalai, Kyoto); N^G-nitro-L-arginine (L-NNA, Sigma); 4-diphenyl-acetoxy-*N*-methylpiperidine methiodide (4-DAMP), pirenzepine dihydrochloride (Funakoshi, Tokyo). The reported concentrations are the calculated final concentrations in the bath medium (atropine, physostigmine) or in the perfusion medium (carboxy-PTIO, 4-DAMP, hexamethonium, L-arginine, L-NNA, pirenzepine).

2.4. Statistical analysis

The intraluminal outflow of 5-HT and 5-HIAA is expressed as pmol per 100 mg wet weight of tissue and per collection period (pmol per 100 mg tissue per 3 min). Data are presented as means ± standard error of the mean (S.E.M.) from 4 to 10 experiments with proximal colons from different animals. The significance of differences was evaluated by analysis of variance followed by Student's *t*-test. Probability values < 0.05 were considered significant.

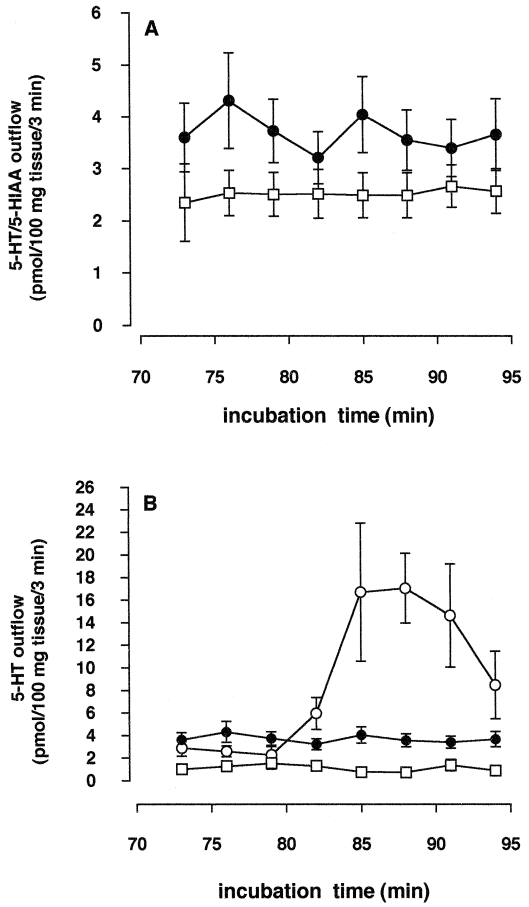

Fig. 1. (A) The spontaneous luminal outflow of 5-HT (●) and 5-HIAA (□) from isolated luminally perfused proximal colon of the guinea-pig. (B) Effects of atropine (0.2 μM, □) and physostigmine (1 μM, ○) on the luminal 5-HT outflow from the luminally perfused proximal colon (control, ●). Physostigmine was present from 76 to 95 min of incubation. Ordinate scale: outflow of 5-HT or 5-HIAA, expressed as pmol per 100 mg tissue per 3 min. Each point represents the mean ± S.E.M (vertical bars) from 6–10 experiments.

3. Results

3.1. Spontaneous outflow of 5-HT and 5-HIAA

The total content of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in the proximal colon mucosa sheets was 1358 ± 248 pmol/100 mg tissue and 269 ± 42 pmol/100 mg tissue ($n = 6$), respectively. The mean spontaneous luminal outflow in the absence of drugs (measured between 70 and 94 min of incubation) was 3.7 ± 0.6 pmol per 100 mg tissue per 3 min for 5-HT and 2.4 ± 0.4 pmol per 100 mg tissue per 3 min for 5-HIAA ($n = 10$). In control experiments, the luminal outflow of 5-HT and 5-HIAA remained constant during the observation period (Fig. 1A).

3.2. 5-HT outflow in the presence of drugs

The luminal outflow of 5-HT was significantly reduced when atropine (0.2 μM, $n = 6$) was present from the start of incubation (Fig. 1B and Fig. 2). The outflow of 5-HT was also reduced when hexamethonium (100 μM, $n = 4$), N^G-nitro-L-arginine (L-NNA, 10 μM, $n = 6$), a NO synthase inhibitor, or 2-(4-carboxy-phenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO, 30 μM, $n = 4$), a NO scavenger, was present from 10 min to 95 min of incubation (Fig. 2). Addition of L-arginine (300 μM, $n = 5$, from the onset of incubation) to the perfusion medium reversed the inhibitory effect of L-NNA (10 μM) on 5-HT outflow (Fig. 2). The combination of atropine (0.2 μM), hexamethonium (100 μM) and L-NNA (10 μM) caused a maximal reduction of the mean 5-HT outflow (0.2 ± 0.2 pmol per 100 mg tissue per 3 min, $n = 4$), an effect which was significantly different from the inhibition exerted by atropine or hexamethonium alone ($P < 0.05$). Addition of physostigmine (1 μM) to the bath medium caused a clear increase in the outflow of 5-HT (Fig. 1B).

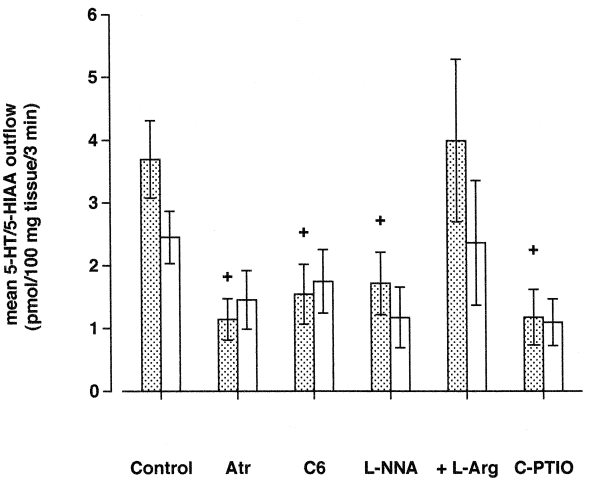

Fig. 2. The spontaneous luminal outflow of 5-HT (dotted columns) and 5-HIAA (open columns) in the absence (Control) and in the presence of atropine (0.2 μM, Atr), hexamethonium (100 μM, C6), L-NNA (10 μM), L-NNA (10 μM) plus L-Arg (300 μM) (+L-Arg) or carboxy-PTIO (30 μM, C-PTIO). Height of columns: outflow of 5-HT and 5-HIAA, expressed as the mean outflow (pmol per 100 mg tissue per 3 min) between 70 and 82 min of incubation in individual experiments; mean ± S.E.M (vertical bars) from 4–10 experiments. + $P < 0.05$ vs. the control 5-HT outflow.

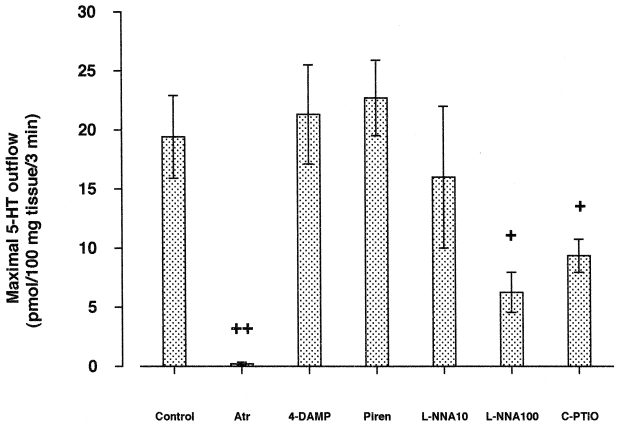

Fig. 3. Physostigmine (1 μM)-evoked maximal 5-HT outflow in the absence (Control) and in the presence of atropine (0.2 μM, Atr), 4-DAMP (0.2 μM), pirenzepine (0.2 μM, Piren), L-NNA (10 or 100 μM) or carboxy-PTIO (30 μM, C-PTIO). Height of columns: physostigmine-evoked peak 5-HT outflow (pmol per 100 mg tissue per 3 min), mean ± S.E.M. (vertical bars) from 4–6 experiments. + $P < 0.05$, + + $P < 0.01$ vs. the control outflow.

3.3. The enhancing action of physostigmine on luminal 5-HT outflow

Neither 4-diphenyl-acetoxy-*N*-methylpiperidine methiodide (0.2 μM, $n = 4$), a muscarinic M_3 receptor antagonist, nor pirenzepine (0.2 μM, $n = 4$), a muscarinic M_1 receptor antagonist (from the start of incubation), affected the enhancing action of physostigmine (1 μM) on 5-HT outflow, while the enhancing effect of physostigmine was abolished in the presence of atropine (0.2 μM, $n = 4$, from the start of incubation) (Fig. 3). In further experiments, the effects of L-NNA or carboxy-PTIO on the physostigmine (1 μM)-evoked 5-HT outflow were investigated. The physostigmine-evoked 5-HT outflow was not significantly altered by L-NNA (10 μM, $n = 7$, from 10 min to 95 min of incubation). However, in the presence of L-NNA (100 μM, $n = 4$) or carboxy-PTIO (30 μM, $n = 5$) (from 10 min to 95 min of incubation), the physostigmine-evoked 5-HT outflow was significantly reduced by 32% and 48%, respectively (Fig. 3).

4. Discussion

The isolated luminally perfused intestine has recently been shown to be a useful preparation to study the luminal

release of serotonin (5-HT) from enterochromaffin cells (Fujimiya et al., 1997). We used the luminally perfused intestinal preparation of guinea pig to investigate whether endogenous acetylcholine has a stimulatory effect on the luminal outflow of 5-HT similar to the stimulatory effect of exogenous acetylcholine on the release of 5-HT from enterochromaffin cells in dog intestine (Burks and Long, 1966; Burks and Long, 1967). This objective was achieved by pretreating the luminally perfused colon with pharmacologic agents that blocked or enhanced the action of acetylcholine. In the present study, the spontaneous luminal outflow of 5-HT was inhibited by atropine or hexamethonium, suggesting that the luminal release of 5-HT is facilitated via muscarinic and nicotinic synapses. These findings are consistent with the observation that in dog, rabbit and cat intestine, the release of 5-HT from enterochromaffin cells into the portal circulation is facilitated by activation of muscarinic and nicotinic receptors (Racké and Schwörer, 1991; Racké et al., 1996). Furthermore, the finding that the anticholinesterase drug, physostigmine, produced a great increase in 5-HT outflow suggests that luminal 5-HT release is facilitated by endogenous acetylcholine released by spontaneous neuronal activity. However, in the light of recent findings demonstrating non-neuronal acetylcholine in the mucosa of the large intestine, (Klapproth et al., 1997), it is also possible that the physostigmine-evoked 5-HT outflow is the result of a prolonged/enhanced action of non-neuronal acetylcholine. We also examined the effects of selective muscarinic M_1 and M_3 receptor antagonist on the physostigmine-evoked 5-HT outflow to define the role of muscarinic M_1 and M_3 receptors because previous studies indicated that muscarinic M_1 and M_3 receptors have a role in the control of 5-HT release from enterochromaffin cells (Schwörer et al., 1989; Reimann et al., 1993). However, the possibility that muscarinic M_1 or M_3 receptors play a role in the physostigmine-evoked 5-HT outflow is unlikely, because neither 0.2 μM pirenzepine, a selective muscarinic M_1 receptor antagonist ($pA_2 = 8.5$ in the guinea-pig ileum) (Kilbinger and Nafziger, 1985), nor 0.2 μM 4-diphenylacetoxy-*N*-methylpiperidine methiodide, a selective muscarinic M_3 receptor antagonist ($pA_2 = 8.83$ in the guinea-pig ileum) (Eltze et al., 1993), inhibited the physostigmine-evoked 5-HT outflow. More detailed pharmacological studies will be necessary for a clear characterization of the present muscarinic receptor. The presence of nitric oxide (NO)-synthesizing neurons in the submucous plexus of the guinea-pig colon (McConalogue and Furness, 1993) raises the obvious question of its physiological role in this tissue. In the present study, the spontaneous luminal outflow of 5-HT was inhibited by a NO synthase inhibitor, N^G-nitro-L-arginine (L-NNA), and the inhibitory effect of L-NNA on 5-HT outflow was reversed by the addition of excess L-arginine, the substrate of NO synthase. In addition, a NO-trapping agent, 2-(4-carboxy-phenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl 3-oxide (carboxy-PTIO) (Akaike et al., 1993), also reduced luminal 5-HT outflow. Taken together, these findings are consistent with the involvement of a NO pathway that leads to an increase in 5-HT outflow. In our study, the involvement of a NO pathway in physostigmine-evoked 5-HT outflow was further examined by using two approaches. The inhibition of endogenous NO production by L-NNA or the inactivation of endogenously released NO by carboxy-PTIO partially inhibited the enhancing action of physostigmine on 5-HT outflow. These results indicate that a part of the stimulatory effect of endogenous acetylcholine that is amplified by physostigmine is mediated through the release of endogenous NO.

In conclusion, these results showed that 5-HT release from luminally perfused proximal colon of the guinea-pig is stimulated via a NO pathway and cholinergic pathways which utilize muscarinic and nicotinic synapses. The present results also raise the possibility that an intrinsic cholinergic–NO link plays a role in the stimulation of luminal 5-HT release, which may reflect the release of 5-HT from entero-chromaffin cells.

Acknowledgements

This work was supported by the grant provided by the Ichiro Kanehara Foundation.

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