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Investigation into the 5-hydroxytryptamine-induced atropine-resistant neurogenic contraction of guinea-pig proximal colon

[1]Shu-ichi Kojima & Yasuo Shimo

Department of Pharmacology Dokkyo University School of Medicine, Mibu, Tochigi, 321-02, Japan

1 The aim of this study was to characterize the receptors mediating the atropine-resistant neurogenic contraction to 5-hydroxytryptamine (5-HT) in the longitudinal muscle of the guinea-pig proximal colon and to determine the type of tachykinin receptors involved in the contractile response to 5-HT by the use of selective antagonists.

2 In the presence of atropine (0.3 μM), guanethidine (5 μM), hexamethonium (100 μM), ketanserin (0.1 μM) and indomethacin (3 μM), 5-HT (0.01–3 μM) produced concentration-dependent neurogenic contractions of colonic strips and at 0.3 μM produced a maximal effect ($pEC_{50} = 7.39 \pm 0.09$, $n = 18$). The 5-HT_4 receptor stimulant, 5-methoxytryptamine (5-MeOT, 0.03–10 μM) also produced neurogenic contractions with similar maximum effect to those of 5-HT ($pEC_{50} = 6.89 \pm 0.16$).

3 The 5-HT_4 receptor antagonist, DAU 6285 (3 μM) shifted the concentration-response curves to both 5-HT and 5-MeOT to the right without significant depression of the maximum, but the 5-HT_1/5-HT_2 receptor antagonist, metitepine (0.1 μM) and the 5-HT_3 receptor antagonist, ondansetron (0.3 μM) had no effect on the control curves to 5-HT and 5-MeOT.

4 The selective NK_1 receptor antagonist, FK 888 (1 μM) markedly attenuated the contractions to 5-HT and 5-MeOT. In contrast, the selective NK_2 receptor antagonist, SR 48968 (10 nM) and the selective NK_3 receptor antagonist, SR 142801 (10 nM) had no effect on the contractions to 5-HT and 5-MeOT.

5 These results indicate that the 5-HT-induced atropine-resistant neurogenic contraction of guinea-pig proximal colon is due to activation of 5-HT_4 receptors, presumably located on excitatory motor neurones, innervating the longitudinal muscle. The contraction evoked by activation of the 5-HT_4 receptors is mediated primarily via NK_1 receptors but not NK_2 or NK_3, suggesting that the 5-HT_4 receptor-mediated contraction is evoked indirectly via tachykinin release from tachykinin-releasing excitatory neurones.

Keywords: 5-Hydroxytryptamine (5-HT); 5-HT_4 receptors; atropine-resistant contraction; tachykinin receptors; proximal colon (guinea-pig)

Introduction

5-Hydroxytryptamine (5-HT) has been identified as a likely transmitter candidate for the regulation of intestinal motility (Furness & Costa, 1982). However, the precise role of 5-HT in the control of intestinal motility is still not clear.

In isolated preparations of the guinea-pig ileum and proximal colon, 5-HT produces contractions of the longitudinal muscle via an effect on cholinergic and non-cholinergic excitatory neurones (Costa & Furness, 1979). In the ileum, the 5-HT-evoked neurogenic contractions appear to be due to activation of two different receptor subtypes (5-HT_3 and 5-HT_4 receptors) located on the neurones (Buchheit *et al.*, 1985; Craig & Clarke, 1990; Eglen *et al.*, 1990). In the guinea-pig proximal and distal colon, atropine-sensitive contractions induced by 5-HT are mediated by the stimulation of 5-HT_3 and 5-HT_4 receptors, located on cholinergic neurones (Butler *et al.*, 1990; Elswood *et al.*, 1991; Wardle & Sanger, 1993), while the receptor subtypes responsible for atropine-resistant neurogenic contractions remain to be determined.

Substance P (SP) and the structurally related peptides, neurokinin A (NKA) and neurokinin B (NKB), belong to a family of biologically active peptides known as tachykinins, and their actions are mediated through three different types of receptors, NK_1, NK_2 and NK_3 which are preferentially activated by SP, NKA and NKB respectively (Mussap *et al.*, 1993, for review). Several lines of evidence converge to indicate tachykinins, SP and NKA, as the major excitatory transmitters responsible for atropine-resistant transmission to the longitudinal muscle of the mammalian gut (Bartho & Holzer, 1985, for review).

Therefore, in this study we have examined the effects of selective 5-HT receptor antagonists in order to characterize the receptor(s) responsible for the 5-HT-induced atropine-resistant neurogenic contraction of the longitudinal muscle strips of the guinea-pig proximal colon. Additionally, we have investigated a role for tachykinin receptors in the contractile response to 5-HT using recently available, selective tachykinin receptor antagonists.

Methods

Tissue preparation

Male Dunkin Hartley guinea-pigs, weighing 250–500 g, were anaesthetized with halothane and bled. A 10 cm segment of the proximal colon starting 2 cm from the caecum was removed. Strips of mucosa-free longitudinal muscle from the proximal colon were prepared as described in a previous study (Kojima & Shimo, 1995). The strips were suspended in longitudinal direction under a 0.5 g load in 15 ml tissue baths filled with a modified Tyrode solution (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) (pH 7.4) at 37°C and bubbled with 5% CO_2-95% O_2. The modified Tyrode solution always contained atropine (0.3 μM) and guanethidine (5 μM) for the elimination of cholinergic and adrenergic responses and in-

[1] Author for correspondence.

domethacin (3 μM), to minimize endogenous prostanoid biosynthesis in response to agonists. Hexamethonium (100 μM), was present to ensure that the responses recorded were the result of stimulation of postganglionic nerves. After setup, the strips were allowed to equilibrate for at least 60 min with renewal of the bathing solution every 15 min. Changes in mechanical activity of the tissue were recorded isotonically (isotonic transducer, Nihon Kohden, TD-112S; Nihon Kohden recticoder, RJG-4128).

Establishing concentration-response curves

In order to exclude an interference of the 5-HT_2 receptors mediating a contraction in the smooth muscle (Kojima, 1991), all the experiments designed to estimate 5-HT-induced NANC contractions were carried out in the presence of ketanserin (0.1 μM), a 5-HT_{2A} receptor antagonist. In the presence of ketanserin, noncumulative concentration-response curves for 5-HT or 5-methoxytryptamine (5-MeOT) within the range of 0.01–10 μM were established by adding increasing concentrations of the agonists to the tissue bath at intervals of at least 45 min. Each concentration was left in contact with the tissue for 1–2 min. In most cases, one of four preparations served as control and the three others were for the study of agonists in the presence of a set concentration of antagonist. Antagonists were allowed to equilibrate for 30–60 min prior to addition of agonists. The size of the contractions induced by agonists was expressed as the percentage of the response obtained with substance P (30 nM) in each preparation. Substance P was added at the first stage of the experimental protocol. The percent contractions were plotted as mean values to obtain log concentration-response curves.

Analysis of data

E_{max} (percentage of the effect of substance P, 30 nM) was the maximum contractile effect and pEC_{50} (or pEC_{30}) was the negative logarithm of the molar concentration of agonist causing 50% (or 30%) of the maximal contraction. pEC_{50} (or pEC_{30}) values were calculated according to Van Rossum (1963). Means ± s.e. mean of n experiments are given throughout the paper. The significance of the difference between mean values was assessed by Student's unpaired t test, or by Dunnet's multicomparison test when appropriate. Results were considered significant if $P<0.05$.

Drugs

The following drugs were used: atropine sulphate, hexamethonium chloride dihydrate (Wako, Osaka, Japan); guanethidine sulphate (Ciba, Basel, Switzerland); tetrodotoxin, ondansetron hydrochloride (Sankyo, Tokyo, Japan); substance P (Peptide Institute, Osaka, Japan); 5-HT creatine sulphate (Merck, Darmstadt, Germany); indomethacin, 5-MeOT hydrochloride (Sigma, St. Louis, MO, U.S.A.); DAU 6285 hydrochloride (endo-6-methoxy-8-methyl-8-azabicyclo [3.2.1] oct 3-yl-2, 3-dihydro-2-oxo-1H-benzimidazole-1 carboxylate hydrochloride, gift from Dr C.A. Rizzi, Boehringer Ingelheim, Milano, Italy); FK 888 ((2-(N-Me)indolil)-co-Hyp-Nal-NMe-Bzl, gift from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan); SR 48968 ((S)-N-methyl-N-[4-acetylamino-4-phenyl-piperidino)-2-(3,4-dichlorophenyl)butyl] benzamide), SR 142801 ((S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3- yl) propyl)-4-phenylpiperidin-4-yl)-N-methylacetamide, gift from Dr X. Emonds-Alt, Sanofi Recherche, Montpellier, France); ketanserin tartrate, metitepine maleate (Research Biochemicals Inc, Natick, U.S.A.).

All drugs were initially dissolved in saline with the following exceptions: indomethacin (100 μM) was dissolved in distilled water containing equimolar concentrations of Na_2CO_3, SR 48968 (100 μM) and SR 142801 (100 μM) in 100% dimethylsulphoxide, 5-HT (100 μM) and 5-MeOT (100 μM) in saline containing ascorbic acid (0.12 mM). All subsequent dilutions of the drugs were made with saline, except for the 5-HT or 5-MeOT solutions, which always contained ascorbic acid (0.12 mM). The vehicles had no effects on 5-HT-induced contractions. The reported concentrations are the calculated final concentrations in the bath solution.

Results

Effects of 5-HT and 5-MeOT

In the presence of atropine (0.3 μM), guanethidine (5 μM), hexamethonium (100 μM), indomethacin (3 μM) and ketanserin (0.1 μM), 5-HT (0.01–3 μM) caused a phasic contraction in a concentration-dependent manner to a maximum effect at 0.3 μM, amounting to 59.7 ± 3.4% of the effect of substance P (30 nM) and the pEC_{50} value was 7.39 ± 0.09 ($n=18$) (Figures 1a and 2a). 5-Methoxytryptamine (5-MeOT, 0.03–10 μM) mimicked the 5-HT-induced contraction with a pEC_{50} value of 6.89 ± 0.16 and a maximum effect at 3 μM, amounting to 59.0 ± 3.2% ($n=12$) of the effect of substance P (30 nM) (Figures 1b and 2b).

The contractions induced by 5-HT and 5-MeOT faded quickly in the continued presence of agonists (Figure 1). The responses to both agonists were almost abolished by tetrodotoxin (300 nM, Figure 2).

Effects of antagonists

Several antagonists with some degree of selectivity for the different receptor subtypes were tested against the 5-HT or 5-MeOT-induced contraction of guinea-pig proximal colon and these results are summarised in Table 1. None of the antagonists investigated had a significant influence on basal tone. Neither metitepine (0.1 μM), a 5-HT_1/5-HT_2 receptor antago-

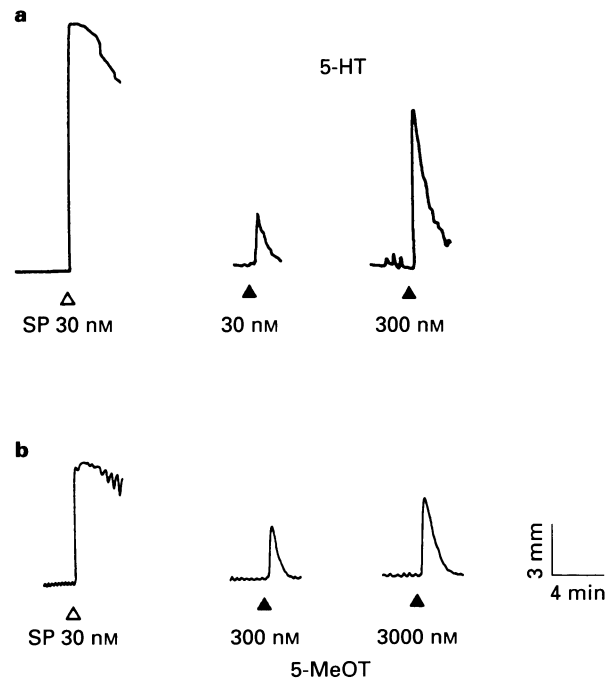

Figure 1 Typical tracings showing the contractile responses in the mucosa-free longitudinal muscle of guinea-pig isolated proximal colon to: (a) 5-hydroxytryptamine (5-HT); (b) 5-methoxytryptamine (5-MeOT). Arrows indicate the addition of agonists. Vertical calibration shows 3 mm shortening of the tissue, horizontal calibration shows 4 min. Atropine (0.3 μM), guanethidine (5 μM), hexamethonium (100 μM), indomethacin (3 μM) and ketanserin (0.1 μM) were present throughout the experiments.

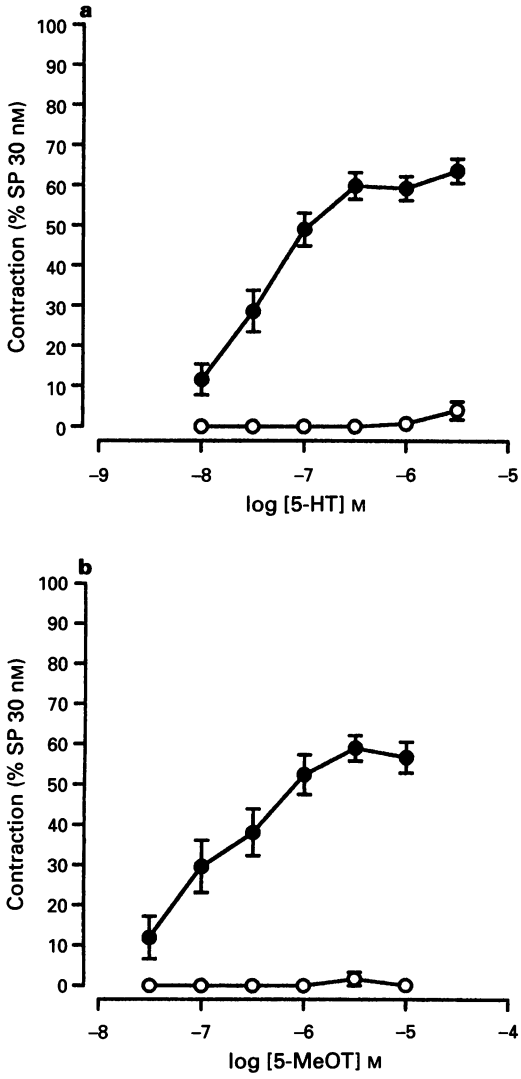

Figure 2 Concentration-response curves for 5-HT (a) and 5-MeOT (b)-induced contraction of guinea-pig proximal colon in the absence (●) and in the presence (○) of 300 nM tetrodotoxin. Each point represents the mean ± s.e. mean of 6–18 preparations and is expressed as a percentage of the contractile response to 30 nM substance P. Atropine (0.3 μM), guanethidine (5 μM), hexamethonium (100 μM), indomethacin (3 μM) and ketanserin (0.1 μM) were present throughout the experiments.

nist nor ondansetron (0.3 μM), a 5-HT_3 receptor antagonist, caused any significant alteration in the concentration-response curves to 5-HT or 5-MeOT (Figure 3, Table 1, $P>0.05$, Dunnet's test). DAU 6285 (3 μM), a 5-HT_4 receptor antagonist displaced the concentration-response curves to both 5-HT and 5-MeOT to the right without significant depression of the maximum (Figure 3, Table 1). Furthermore, to evaluate the participation of tachykinin receptors in the contractile response to 5-HT, tachykinin receptor antagonists were studied. The contractile responses to both 5-HT and 5-MeOT were markedly depressed by FK 888 (1 μM), a highly selective tachykinin NK_1 receptor antagonist (Figure 4, Table 1). The contraction evoked by substance P (3 nM, the amplitude of which was 72.9 ± 5.4% of 30 nM substance P ($n=4$)-induced contraction) was also blocked by preincubation of the tissue with FK 888 (1 μM) (2.7 ± 2.0% of 30 nM substance P, $n=4$, $P<0.001$). Neither SR 48968 (10 nM), a highly selective tachykinin NK_2 receptor antagonist nor SR 142801 (10 nM), a highly selective tachykinin NK_3 receptor antagonist, affected the concentration-response curves to 5-HT or 5-MeOT (Figure 4, Table 1, $P>0.05$, Dunnet's test).

Discussion

The present study has shown that 5-HT produces concentration-dependent and reproducible contractions of guinea-pig proximal colon in the presence of atropine, guanethidine, hexamethonium, ketanserin and indomethacin. Since tetrodotoxin almost totally abolished the contractions, this response is due to stimulation of postganglionic neurones, and is not due to activation of mechanisms involving the release of acetylcholine, noradrenaline or prostanoids.

The aims of this study were two fold. First, to characterize the receptor mediating the neurogenic contractions to 5-HT in the guinea-pig proximal colon and secondly to determine the type of tachykinin receptors involved in the contractile responses to 5-HT.

According to the current classification, four main types of functional 5-HT receptors, termed 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 can be distinguished (Hoyer *et al.*, 1994). Studies with 5-HT receptor antagonists have given some insight into the types of 5-HT receptor responsible for the 5-HT-induced contraction. Metitepine, a 5-HT_1/5-HT_2 receptor antagonist, failed to block the 5-HT-induced contraction, suggesting that neither the 5-HT_1 nor 5-HT_2 receptor class makes a contribution to the contractile response to 5-HT.

It has repeatedly been shown that 5-HT_3 and 5-HT_4 receptors are on excitatory motor neurones which supply the longitudinal muscle of the guinea-pig ileum, proximal colon and distal colon (Eglen *et al.*, 1990; Butler *et al.*, 1990; Wardle & Sanger, 1994). However, in the present study using long-

Table 1 The potencies of 5-hydroxytryptamine (5-HT) and 5-methoxytryptamine (5-MeOT) in guinea-pig proximal colon in the absence and presence of various antagonists

	5-HT		*5-MeOT*	
Antagonists	*pEC_{50} (or pEC_{30})*	*E_{max} (%)*	*pEC_{50} (or pEC_{30})*	*E_{max} (%)*
Control	7.39 ± 0.09 (7.62 ± 0.09)[a]	59.7 ± 3.4	6.89 ± 0.16 (7.18 ± 0.16)[a]	59.0 ± 3.2
Metitepine (0.1 μM)	7.23 ± 0.15	65.5 ± 7.1	6.86 ± 0.12	59.4 ± 4.3
Ondansetron (0.3 μM)	7.28 ± 0.04	61.6 ± 6.0	6.74 ± 0.12	59.4 ± 6.3
DAU 6285 (3 μM)	6.53 ± 0.21[a]*	50.8 ± 8.8	6.07 ± 0.23[a]*	39.6 ± 9.4
FK 888 (1 μM)		4.3 ± 1.6**		14.5 ± 7.2**
SR 48968 (10 nM)	7.37 ± 0.08	49.6 ± 1.9	6.76 ± 0.11	65.0 ± 6.8
SR 142801 (10 nM)	7.15 ± 0.21	64.3 ± 5.2	6.65 ± 0.19	52.1 ± 5.7

Listed are pEC_{50} values (or pEC_{30}[a]) and E_{max} values (percentage of the effect of substance P, 30 nM). The data are means ± s.e.mean from 6–18 preparations. Atropine (0.3 μM), guanethidine (5 μM), hexamethonium (100 μM), indomethacin (3 μM) and ketanserin (0.1 μM) were present throughout experiments. *$P<0.001$ versus control pEC_{30} (Student's t test). **$P<0.01$ versus control E_{max} (Dunnet's test).

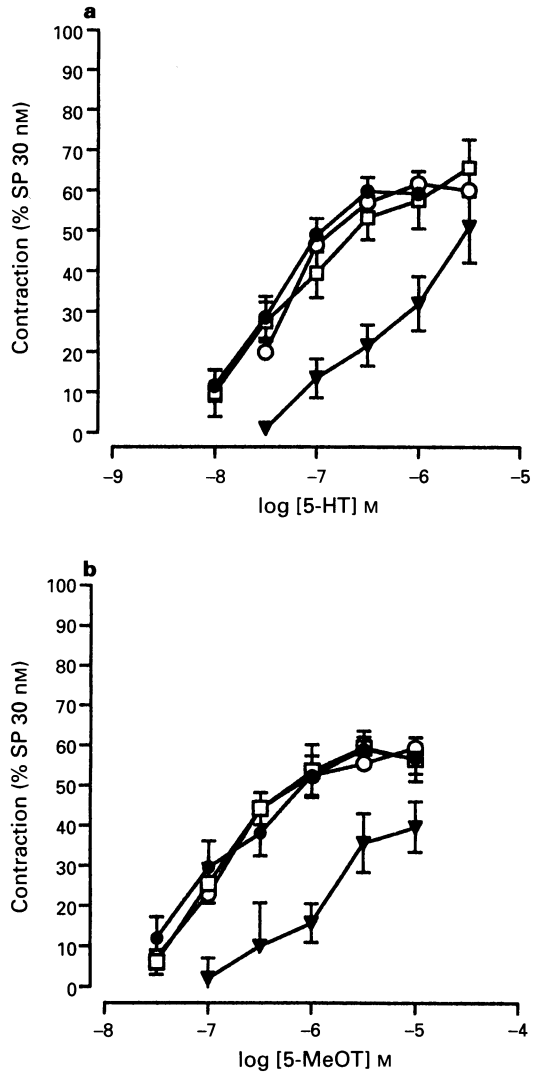

Figure 3 Concentration-response curves for 5-HT (a) and 5-MeOT (b)-induced contraction of guinea-pig proximal colon in the absence (●) and in the presence of 0.1 μM metitepine (□); 0.3 μM ondansetron (○); 3 μM DAU 6285 (▼). Each point represents the mean ± s.e. mean of 6–18 preparations and is expressed as a percentage of the contractile response to 30 nM substance P.

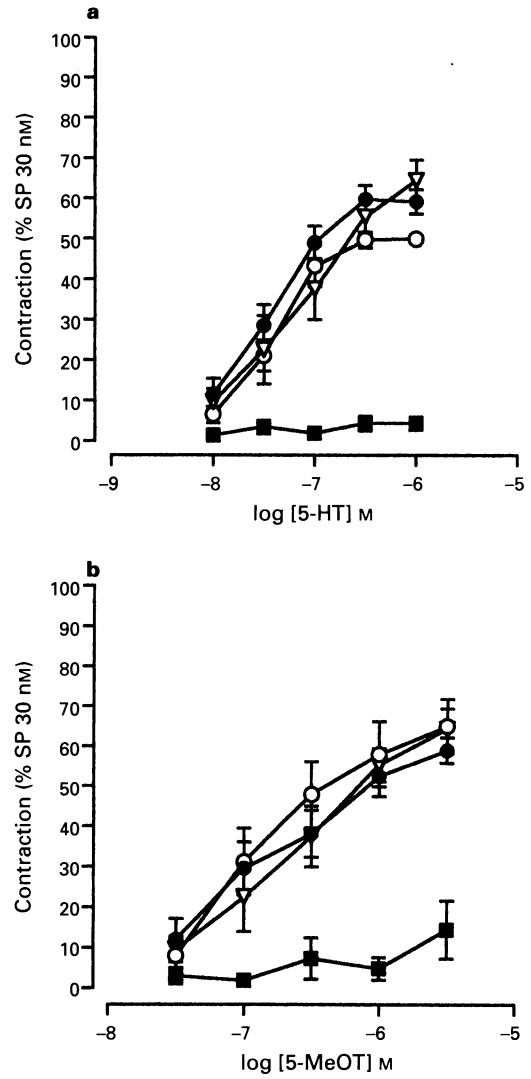

Figure 4 Concentration-response curves for 5-HT (a) and 5-MeOT (b)-induced contraction of guinea-pig proximal colon in the absence (●) and in the presence of 1 μM FK 888 (■); 10 nM SR 48968 (○); 10 nM SR 142801 (▽). Each point represents the mean ± s.e. mean of 6–18 preparations and is expressed as a percentage of the contractile response to 30 nM substance P.

itudinal strips of proximal colon, we have found no evidence for the involvement of 5-HT_3 receptors in mediating contractile responses to 5-HT because ondansetron, a 5-HT_3 receptor antagonist, failed to modify the 5-HT concentration-response curve. There are two possible reasons for this discrepancy. Firstly, 5-HT_3 receptor-mediated responses only become significant from 1 μM and above (Eglen *et al.*, 1990; Butler *et al.*, 1990; Wardle & Sanger, 1994), whereas we tested 5-HT only up to 3 μM and 5-MeOT is not an agonist at 5-HT_3 receptors. Secondly, differences in effective 5-HT_3 receptor reserves (leading to tachykinin release) in different regions of the intestine may be involved, since the 5-HT_3 receptor-mediated contraction of guinea-pig ileum was inhibited by D-Pro^4, D-$Trp^{7,9}$ substance P (4-11), a tachykinin receptor antagonist (Buchheit *et al.*, 1985), whereas spantide, a tachykinin receptor antagonist caused only a slight inhibition of 5-HT_3 receptor-mediated contraction of guinea-pig distal colon (Woollard *et al.*, 1994). On the other hand, in the present study, DAU 6285, a 5-HT_4 receptor antagonist was effective in antagonizing the contractile effect of 5-HT, suggesting an involvement of the 5-HT_4 receptor subtype in the contractile response to 5-HT. Additional support for this contention is provided by results of experiments where the effects of the 5-HT_4 receptor stimulant, 5-MeOT were examined. We found that 5-MeOT mimicked the 5-HT-induced contraction and that the 5-MeOT-induced contractions were antagonized by DAU 6285. Thus, these results suggest that the 5-HT-induced atropine-resistant contraction is due to activation of 5-HT_4 receptors. We have also examined the effects of selective NK_1 and NK_2 receptor antagonists in order to gain information on the nature of NK receptor types involved in the atropine-resistant contraction induced by 5-HT. FK 888 has been characterized as a highly selective NK_1 receptor antagonist ($pK_B = 7.13$), while SR 48968 is a potent and highly selective NK_2 receptor antagonist ($pK_B = 9.41$) in the guinea-pig proximal colon (Maggi *et al.*, 1994). The present results show that the NK_1, but not the NK_2 receptor antagonist markedly attenuated the contractions induced by 5-HT or 5-MeOT, suggesting that NK_1 receptors are the primary tachykinin receptors which mediate the contractions upon 5-HT_4 receptor activation. This result is also compatible with previous work indicating a major role for NK_1 receptors in mediating non-cholinergic excitatory junction potentials and contractions evoked by electrical field stimulation in the guinea-pig proximal colon (Zagorodnyuk *et al.*, 1993) or in mediating substance P-induced contractions of guinea-pig proximal colon (Briejer *et al.*, 1993). Furthermore, we have made use of the recently described NK_3 receptor antagonist, SR 142801 (Emonds-Alt *et al.*, 1995) to define a role

for NK_3 receptors because previous work indicated that the activation of NK_3 receptors on myenteric plexus neurones results in acetylcholine and tachykinin release (Guard & Watson, 1987). However, the possibility that NK_3 receptors play a role in the contractile response to 5-HT is unlikely, because 10 nM SR 142801, a potent and highly selective NK_3 receptor antagonist ($pA_2 = 9.15$ in the guinea-pig ileum) (Emonds-Alt *et al.*, 1995) had no effect upon the contractile responses to 5-HT or 5-MeOT. Thus, these results suggest that the contractile responses to 5-HT_4 receptor activation are evoked indirectly via tachykinin release from tachykinin-releasing neurones. The exact nature of the tachykinin responsible for the 5-HT-induced atropine-resistant contraction has not yet been determined. However, since substance P and neurokinin A have been shown to be co-localized in myenteric plexus neurones (Deacon *et al.*, 1987), and further both these tachykinins can stimulate NK_1 receptors with differing affinity and efficacy (Mussap *et al.*, 1993), both these tachykinins may be involved in the atropine-resistant contraction. On the other hand, coexistence of acetylcholine with tachykinins has been repeatedly described in excitatory enteric neurones (Furness *et al.*, 1992), and it is thus to be expected that, if 5-HT_4 receptor stimulation leads to acetylcholine release, the release of the tachykinins is also enhanced. Indeed, the present study is congruent with the idea that the 5-HT_4 receptor-mediated contraction is due to the co-release of acetylcholine and tachykinins from the same population of enteric neurones, but coexistence of 5-HT with tachykinins has also been described in myenteric neurones of guinea-pig colon (Legay *et al.*, 1984). Thus, it is also possible that 5-HT_4 receptor activation causes the release of tachykinins from a separate population of non-cholinergic neurones.

In conclusion, these results indicate that the 5-HT-induced atropine-resistant contraction is mediated by the stimulation of 5-HT_4 receptors, located on excitatory motor neurones which supply the longitudinal muscle of guinea-pig proximal colon. The contraction evoked by activation of the 5-HT_4 receptors is mediated primarily via NK_1 receptors but not NK_2 or NK_3 receptors, suggesting that the 5-HT_4 receptor-mediated contraction is evoked via endogenously released tachykinins. Thus, our data support our previous hypothesis indicating the presence of prejunctional 5-HT_4 receptors localized on tachykinin-releasing neurones in the guinea-pig proximal colon (Kojima & Shimo, 1995). However, the exact physiological importance of the 5-HT_4 receptors described still needs to be determined.

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