Br. J. Pharmac. (1985), **85**, 529–539

Two types of receptors for 5-hydroxytryptamine on the cholinergic nerves of the guinea-pig myenteric plexus

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1 The effects of 5-hydroxytryptamine (5-HT) on spontaneous and electrically-evoked release of [^{3}H]-acetylcholine (ACh) from guinea-pig myenteric plexus preparations preincubated with [^{3}H]-choline have been investigated in the absence of cholinesterase inhibitors.

2 5-HT caused a transient increase in spontaneous release and an inhibition of the electrically-evoked release of [^{3}H]-ACh. The 5-HT-induced contractions of the longitudinal muscle were clearly related to the increase in spontaneous release. The inhibitory effect was not due to activation of α-adrenoceptors since it was also observed in the presence of tolazoline and on strips from reserpine-pretreated guinea-pigs.

3 After desensitization of the excitatory 5-HT receptors with 5-HT or metoclopramide the effects of 5-HT on spontaneous [^{3}H]-ACh release were largely reduced. A variety of established antagonists at neuronal 5-HT receptors (i.e. metitepine 0.1–1 μM; methysergide 1 μM; ketanserin 0.1–1 μM; MDL 72222 0.1 μM; tropacocaine 1 μM) failed to block the excitation.

4 The inhibition by 5-HT of the electrically evoked [^{3}H]-ACh release was competitively antagonized by metitepine (pA_2 7.6) and methysergide (pA_2 7.0) but not by ketanserin. Tachyphylaxis to the inhibitory action of 5-HT did not occur.

5 The results suggest that the excitatory 5-HT receptor ('M'-receptor) differs in its pharmacological properties from other neuronal 5-HT receptors. The presynaptically located inhibitory receptor may roughly correspond to the 5-HT_1 receptor subtype but probably differs from the 5-HT autoreceptor.

Introduction

It has been suggested that 5-hydroxytryptamine (5-HT) is an enteric neurotransmitter which is involved in the control of gastrointestinal motility (Gershon *et al.*, 1983). Intestinal motility can both be inhibited and stimulated by exogenous 5-HT. Thus, 5-HT contracts the resting ileum and, in addition, inhibits the twitch-like contractions of the ileum induced by electrical stimulation of cholinergic nerves (Gintzler & Musacchio, 1974). As the contractions were partly inhibited by tetrodotoxin or antimuscarinic drugs it was concluded that the contractile action of 5-HT is mediated by release of acetylcholine (ACh) (Gaddum & Picarelli, 1957; Brownlee & Johnson 1963; Costa & Furness, 1979). Direct evidence for an ACh releasing effect of 5-HT was presented by Adam-Vizi & Vizi (1978). When 5-HT is applied to the serosal surface of the small intestine the peristaltic reflex is inhibited but 5-HT stimulates peristalsis when applied to the mucosal surface (see review by Gershon *et al.*, 1983).

Since cholinergic nerves are critically involved in the control of intestinal motility, the above mentioned excitatory and inhibitory actions could be due to differential effects of 5-HT on the release of ACh. The objective of the present study was, therefore, to examine in detail the effects of 5-HT on spontaneous and electrically evoked release of ACh from guinea-pig myenteric neurones. In addition, we have tried to characterize with some established antagonists the 5-HT receptors modulating ACh release. ACh release was measured in the absence of cholinesterase inhibition from preparations that had been preloaded with [^{3}H]-choline. Preliminary reports of these findings have been presented to the German Pharmacological Society (Kilbinger & Pfeuffer-Friederich, 1982).

Methods

Release experiments

Guinea-pigs of either sex weighing 300–400g were stunned by a blow to the head and bled. Unless otherwise stated, longitudinal muscle strips with the

myenteric plexus attached were prepared from the proximal half of the small intestine. Two strips weighing approximately 50 mg were suspended isotonically under a tension of 1 g in a 2 ml organ bath and superfused with Tyrode solution (composition in mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 5.6), that contained in addition 1 μM choline. The solution was gassed with carbogen and warmed to a constant temperature of 37°C. After a 30 min incubation with [^{3}H]-choline (5 μCi ml^{-1}) during which the tissue was stimulated with square wave pulses (0.2 Hz, 1 ms), the strips were superfused (1 ml min^{-1}) with Tyrode solution containing 10 μM hemicholinium-3 to prevent reuptake of choline. After a washout period of 70 min the superfusate was collected in 3 min fractions. The strips were stimulated by field stimulation at frequencies of 0.1 or 1 Hz. Biphasic square wave pulses (1 ms) were applied by 2 platinum electrodes, which were positioned parallel to the strips. The potential drop between the electrodes was 11 V cm^{-1} giving a current of 380 mA. Tritium content of the superfusate samples was measured by liquid scintillation spectrometry and the counting efficiency was determined using external standardization. The outflow of tritium evoked by electrical stimulation or by 5-HT was obtained from the difference of the total tritium outflow and the spontaneous outflow calculated by interpolation from the first 3 and the last 3–5 unstimulated samples as described previously (Kilbinger & Wessler, 1980).

Extraction and separation of radiolabelled choline

Basically, the procedure described by Kilbinger & Wessler (1980) was used. [^{3}H]-choline was extracted from 5 ml of the superfusate into 2 ml allylcyanid containing 10 mg of sodium tetraphenylborate. The organic phase was evaporated to dryness and the residue redissolved in 0.5 ml methanol. The methanol solution was applied to an anion-exchange column (Dowex 1 × 8 Cl$^-$) and eluted with 6 ml methanol. The eluate was again evaporated to dryness and the residue redissolved in 100 μl methanol containing unlabelled carrier choline and ACh (2 μmol each). Ten μl of this solution was spotted on cellulose thin layer chromatography sheets and developed in butan-1-ol: methanol: glacial acetic acid: H_2O (8:2:1:3 by vol.). The choline and ACh bands were detected using Dragendorff's reagent. The thin layer chromatograms were cut into 5 mm sections which were added to vials containing 1 ml of a mixture of methanol and 1 M HCl (19:1 by vol.). The samples were counted in 7 ml of a Triton-toluene-scintillator. Recoveries were determined by adding [^{14}C]-choline (2–10 nCi) as internal standard to each 5 ml superfusate sample. Radioactivity was measured in a Packard Tricarb Model 460 C scintillation spectrometer. External standardization was used to correct for counting efficiency. The recovery for [^{14}C]-choline was 40 ± 2% (n = 22) and the values for [^{3}H]-choline were corrected accordingly.

Contraction experiments

A piece (about 2 cm in length) of longitudinal muscle strip was suspended isometrically under a tension of 0.7 g in a 7 ml organ bath, containing Tyrode solution (37°C, gassed with carbogen). The strips were connected to a force-displacement transducer and the contractions displayed on a Hellige recorder. After 60 min of equilibration, during which the strips were challenged 4 times with 1 μM 5-HT, a non-cumulative concentration-response curve for 5-HT was established. 5-HT was added to the organ bath in increasing concentrations and was left in contact with the tissue for 1 min. Thereafter, the organ bath was washed out 3 times and the next higher concentration applied 15 min later.

In some experiments the strips were stimulated continuously at 0.1 Hz. When the twitch size was constant, 5-HT was added to the bath fluid. The effect of 5-HT on twitch height was expressed as a percentage of the contraction immediately before addition of 5-HT.

Reserpine-pretreatment

Guinea-pigs received reserpine 1.5 mg kg^{-1} subcutaneously and were killed 3 days later. The ileum was homogenized in 15 ml trichloroacetic acid (5%) and the noradrenaline present in the tissue extract determined fluorimetrically with the trihydroxyindole method according to Muscholl (1966).

Statistics

Results are expressed as means ± s.e. means. Student's t test was used to assess the significance of differences between two mean values. If one control group was compared with more than one group of treatments, a one-way analysis of variance was performed followed by Dunnett's test (Dunnett, 1964). To quantify antagonism, apparent pA_2 values were calculated according to: $pA_2 = pA_x + \log$ (dose ratio -1) (Schild, 1947). pD'_2 values were calculated according to van Rossum (1963).

Drugs

The following were used: [methyl-^{3}H]-choline (NEN; 80 Ci mmol^{-1} Dreieich, F.R.G.); choline chloride (Fluka, Neu-Ulm; F.R.G.); hemicholinium-3 (EGA-Chemie, Steinheim, F.R.G.); 5-hydroxytryptamine creatinine sulphate (Merck, Darmstadt, F.R.G.); ketanserin (Janssen, Beerse, Belgium); MDL 72222

(1αH, 3α, 5α H-tropan-3-yl-3,5-dichlorobenzoate) (gift from Dr J.R. Fozard, Merrell, Strasbourg, France); metoclopramide dihydrochloride monohydrate (Dolorgiet, Bad Godesberg, F.R.G.); methysergide hydrogen maleate (Sandoz, Basel, Switzerland); metitepine (Hoffmann-La Roche, Basel, Switzerland); reserpine (Serpasil injection 2.5 mg ml^{-1}, Ciba-Geigy, Basel, Switzerland); tetrodotoxin; tolazoline hydrochloride (both Sigma, St Louis, MO, U.S.A.); tropacocaine (benzoylpseudotropine) hydrochloride (Aldrich, Nettetal, F.R.G.).

Results

Facilitation of spontaneous, and inhibition of electrically evoked outflow of [^{3}H]-acetylcholine

Longitudinal muscle strips which had been preincubated with [^{3}H]-choline were stimulated twice (S1; S2), 5-HT being added to the superfusion medium 30 min before S2. Figure 1 shows that 5-HT (10 μM) caused a transient increase in outflow of tritium whereas the stimulation-evoked outflow was inhibited (by 62 ± 2%; $n = 6$). The reduction by 5-HT of the stimulated outflow might be due to depletion of the available store of tritiated transmitter by the preceding superfusion with 5-HT. In order to test this possibility experiments were performed in which the excitatory effect of 5-HT was prevented by tetrodotoxin. Figure 1 shows that during and after perfusion with tetrodotoxin (300 nM), 5-HT no longer increased the outflow of tritium. Yet, the electrically-evoked outflow was significantly reduced (by 45 ± 11%; $n = 6$) by 5-HT. Thus, the inhibition by 5-HT of the evoked outflow of tritium is independent of the preceding facilitatory action of 5-HT.

In order to identify the nature of the ^{3}H-radioactivity present in the medium before and during the superfusion with 5-HT, choline and ACh were isolated from the Tyrode solution by ion-pair extraction followed by thin layer chromatography. [^{3}H]-ACh was not detected in the samples. The spontaneous outflow

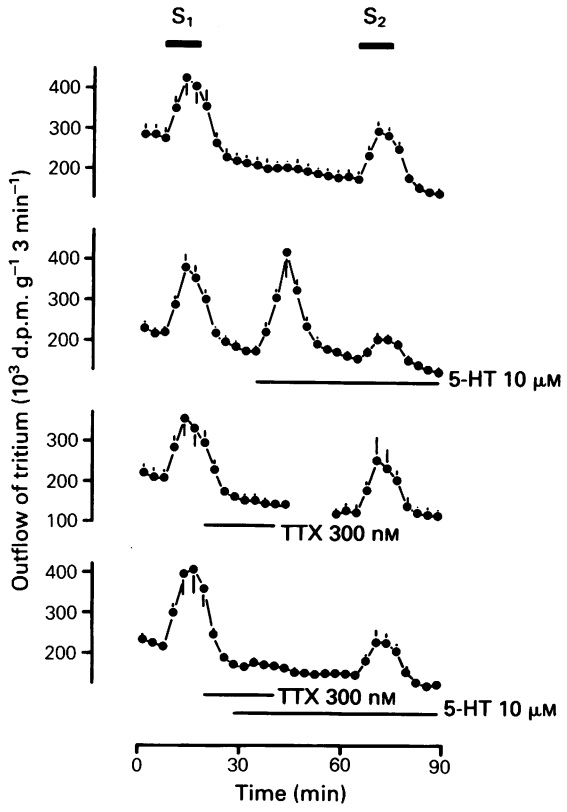

Figure 1 Effects of 5-hydroxytryptamine (5-HT) in the absence and presence of tetrodotoxin on spontaneous and electrically evoked outflow of [^{3}H]-acetylcholine. Strips preincubated with [^{3}H]-choline were subsequently superfused (1 ml min^{-1}) with Tyrode solution containing 10 μM hemicholinium-3. Electrical stimulation (0.1 Hz, 60 pulses) was performed 9 min (S1) and 66 min (S2) after the end of the washout period as indicated at the top of the figure. Abscissa scale starts at the end of the washout period. Horizontal bars indicate superfusion with 5-HT ($n = 6$), tetrodotoxin (TTX, $n = 3$) and TTX plus 5-HT ($n = 6$). Upper panel, control experiments ($n = 6$). Means-± s.e.means are shown in this and subsequent figures.

Table 1 Comparison of the outflow of tritium and [^{3}H]-choline from the myenteric plexus preparation

	Collection period (min)	*Tritium* (10^3 d.p.m. g^{-1})	*[^{3}H]-choline* (% of tritium)	n
Spontaneous	0– 9	609 ± 25	60 ± 8	3
0.1 Hz, 60 pulses	9–27	368 ± 64	101 ± 19	3
Spontaneous	27–36	425 ± 22	57 ± 6	8
5-HT, 10 μM	36–60	780 ± 98	118 ± 15	8

Strips were incubated with [^{3}H]-choline and superfused subsequently (1 ml min^{-1}). At the end of the 60 min washout period (zero time) the superfusate was collected in 3 min intervals. Tritium and [^{3}H]-choline were determined in aliquots of a superfusate collected immediately before, and in aliquots collected during and after stimulation (0.1 Hz) or exposure to 5-HT. The outflow evoked by electrical stimulation or by 5-HT was calculated from the difference of the total outflow and the preceding spontaneous outflow.

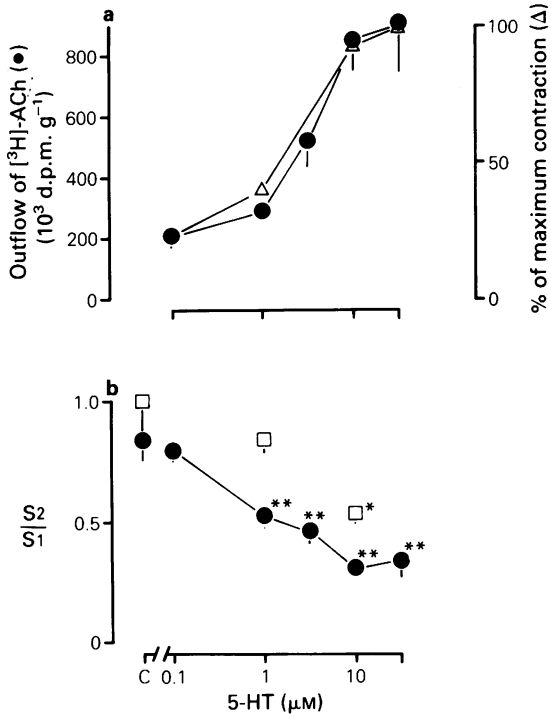

Figure 2 Concentration-response curves for the effects of 5-hydroxytryptamine (5-HT). (a) Effects on spontaneous outflow of [^{3}H]-acetylcholine ([^{3}H]-ACh, left ordinate scale, and (●) n = 4-13), and on isometric tension of the longitudinal muscle (right ordinate scale, and Δ). Contractions were measured in separate experiments (n = 11) and on each strip a complete concentration-response curve to 5-HT was established. (b) Effects on electrically-evoked outflow of [^{3}H]-ACh. The strips were stimulated twice (S1, S2) with trains of 60 pulses. Stimulation frequencies were 0.1 Hz (●; n = 4–6) and 1 Hz (□; n = 4 for each value). 5-HT was added to the medium 30 min before S2. Ordinate scale, ratio of evoked outflow during S2 and S1. C, control experiments. Significance of difference from corresponding control (Dunnett's test): * $P<0.05$; ** $P<0.01$.

of tritium consisted of 50–60% [^{3}H]-choline. The remaining 40–50%, not detected by ion-pair extraction and thin layer chromatography probably represent labelled choline metabolites (Szerb, 1976). The outflow of tritium evoked by electrical stimulation or by 5-HT consisted of 100% tritiated choline (Table 1). Previous experiments (Szerb, 1976; Kilbinger & Wessler, 1980) have shown that the tetrodotoxin-sensitive outflow of tritium from strips preincubated with [^{3}H]-choline represents exclusively release of neuronal [^{3}H]-ACh. We suggest that by analogy, the tetrodotoxin-sensitive outflow of [^{3}H]-choline induced by 5-HT originates from the release of [^{3}H]-ACh that is hydrolyzed by cholinesterases. Subsequently, outflow of [^{3}H]-choline evoked by 5-HT or by electrical stimulation is therefore designated [^{3}H]-ACh outflow.

The excitatory and inhibitory effects of different concentrations of 5-HT are shown in Figure 2. Comparison of the concentration-effect curves indicates that 5-HT is somewhat more potent in inhibiting the electrically-evoked outflow (EC_{50} 0.63 μM) than in facilitating the spontaneous outflow (EC_{50} 2.3 μM). The inhibitory effect of 5-HT depended on the stimulation frequency. At 1 Hz (60 pulses) the reduction by 5-HT of the evoked outflow was smaller than at 0.1 Hz stimulation.

The increase by 5-HT of the spontaneous outflow was paralleled by its effect on longitudinal muscle contraction (Figure 2). The maximal effective concentration and the EC_{50} value for the increase in resting tension (1.27 ± 0.26 μM 5-HT; n = 11) were similar to the corresponding values obtained in release experiments, which suggests that the contractile effect of 5-HT is related to the release of ACh. On the other hand, there was no such clear correlation between the effects of 5-HT on the electrically-evoked outflow of [^{3}H]-ACh and the twitch response to 0.1 Hz stimulation. The twitch height was reduced only by 10 μM 5-HT (by 43 ± 6%; n = 16), whereas 0.1 and 1 μM 5-HT slightly increased the twitch height by 7 ± 1, and 12 ± 2%, respectively (both n = 10).

The stimulation-evoked release of ACh from the guinea-pig myenteric plexus can be inhibited by

Table 2 Effects of 5-hydroxytryptamine (5-HT) on spontaneous and electrically evoked outflow of [^{3}H]-acetylcholine in the presence of 1 μM tolazoline (group A), or from strips of reserpine pretreated guinea-pigs (group B)

Group	*5-HT* (μM)	*S1* (10^3 d.p.m. g^{-1})	*S2/S1*	*5-HT induced outflow* (10^3 d.p.m. g^{-1})	n
A	—	530 ± 98	0.85 ± 0.04	—	4
A	10	775 ± 138	0.44 ± 0.09*	835 ± 121	4
B	—	2861 ± 382	0.67 ± 0.03	—	4
B	10	1166 ± 167	0.21 ± 0.01*	1574 ± 232	4

Strips preloaded with [^{3}H]-choline were stimulated at 0.1 Hz (60 pulses) 9 min (S1) and 66 min (S2) after the end of the washout period. 5-HT was added 30 min before S2, and tolazoline (group A) 30 min before S1. * $P<0.01$.

noradrenaline (Paton & Vizi, 1969). In order to exclude an indirect (noradrenaline releasing) action of 5-HT on the electrically evoked outflow of [^{3}H]-ACh, the effects of 5-HT were investigated in the presence of the α-adrenolytic drug tolazoline. Tolazoline was applied to the superfusion medium 30 min before S1 in a concentration of 1 μM which has been shown to block the effects of α-adrenoceptor agonists in the ileum (Drew, 1978; Görich *et al.*, 1982). Table 2 shows that the facilitatory effect of 10 μM 5-HT was not affected by tolazoline, and that 5-HT significantly reduced (by 49 ± 10%) the evoked outflow of [^{3}H]-ACh in the presence of tolazoline.

In other experiments the actions of 5-HT were studied on strips obtained from four reserpine-pretreated guinea-pigs. Release experiments were performed on strips from the proximal half of the small intestine, whilst the distal half was used for determination of the endogenous noradrenaline content. After reserpine treatment the noradrenaline content of the distal ilea was 0.13 ± 0.03 nmol g^{-1}, which is 4% of the noradrenaline content of distal ilea from four control animals (3.31 ± 0.79 nmol g^{-1}). Electrical stimulation as well as 5-HT caused a much larger outflow of [^{3}H]-ACh from strips from reserpinized guinea-pigs (Table 2) compared to controls (cf. Figure 2). 5-HT significantly depressed the electrically-evoked outflow by 69 ± 1% (Table 2). Thus, it can be concluded that the inhibitory action of 5-HT is not mediated via α-adrenoceptor stimulation.

Costa & Furness (1979) observed that the hyoscine-sensitive contractile effects of 5-HT were more marked in the oral than in the anal parts of the guinea-pig small intestine. In eight experiments we found that the outflow of [^{3}H]-ACh elicited by 1 μM 5-HT was slightly but significantly ($P < 0.05$, paired t test) larger in the proximal part (first 25 cm behind the duodeno-jejunal junction; 325 ± 34 × 10^3 d.p.m. g^{-1}) than in the distal part (last 25 cm before the ileo-caecal junction; 231 ± 27 × 10^3 d.p.m. g^{-1}).

Antagonism of the inhibitory effects of 5-hydroxytryptamine by metitepine and methysergide

Strips were stimulated twice (0.1 Hz, 60 pulses) and metitepine was added to the superfusion medium 30 min before S2. Neither of the two concentrations tested (0.1 and 1 μM) significantly affected the spontaneous outflow or the outflow evoked by electrical stimulation (S2/S1: 0.91 ± 0.13, $n = 4$, and 0.99 ± 0.12, $n = 5$). For interaction experiments metitepine was added to the medium 30 min before S1 and 5-HT 30 min before S2. Figure 3 shows that 1 μM metitepine completely prevented the inhibition by 10 μM 5-HT of the evoked outflow of [^{3}H]-ACh, whilst the facilitatory action of 5-HT remained unaffected. In the presence of 0.1 μM metitepine, the concentration-response curve for the inhibitory effect of 5-HT is shifted in parallel to the right, and there is no statistically significant attenuation of the maximum (Figure 4). The apparent pA_2 value for metitepine calculated from the shift in the curve to 5-HT was 7.6. In the presence of 1 μM metitepine, 5-HT no longer caused any inhibition of the evoked outflow, in contrast, 3 μM 5-HT significantly enhanced evoked outflow of [^{3}H]-ACh (Figure 4). Neither concentration of metitepine (0.1 and 1 μM) significantly affected the increases by 5-HT (0.1–30 μM) of [^{3}H]-ACh outflow.

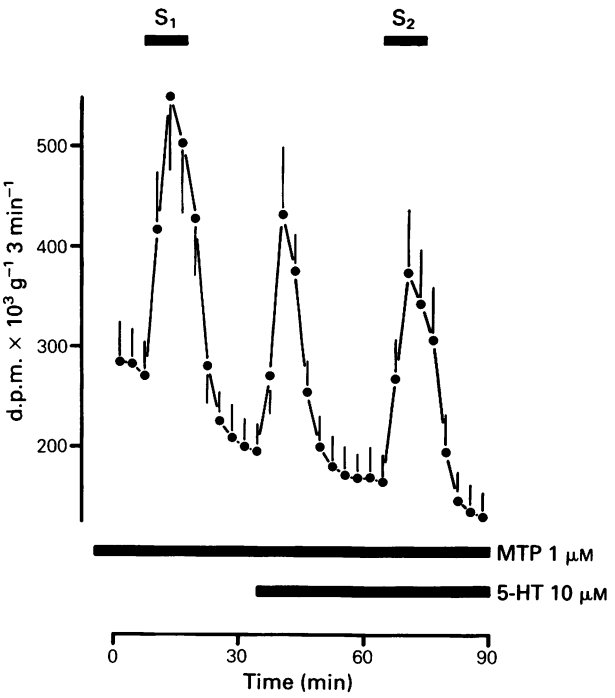

Figure 3 Effects of 5-hydroxytryptamine (5-HT) on spontaneous and electrically evoked (0.1 Hz) outflow of [^{3}H]-acetylcholine in the presence of 1 μM metitepine. Metitepine (MTP) was added to the medium 30 min before S1 in 4 experiments. For further details see legend to Figure 1.

Methysergide alone added to the medium 30 min before S2 significantly reduced the electrically evoked outflow of [^{3}H]-ACh at concentrations of 10 μM (by 32 ± 5%, $n = 4$, $P < 0.05$) and 30 μM (by 54 ± 4%, $n = 4$, $P < 0.01$), but not at a concentration of 1 μM. The spontaneous outflow of tritium was not changed by either concentration of methysergide. For interaction experiments between methysergide and 5-HT the protocol was similar to that shown in Figure 3. Methysergide (1 μM), like metitepine, antagonized the inhibitory action of 5-HT but did not affect the 5-HT-induced increase in [^{3}H]-ACh outflow. The concentra-

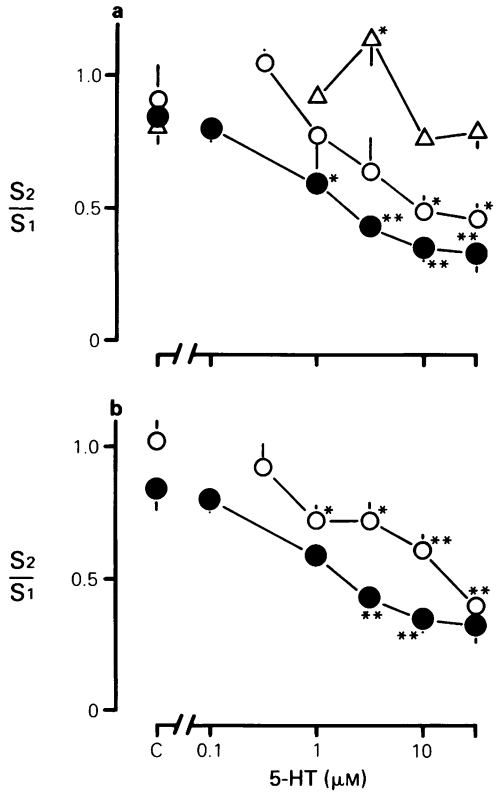

Figure 4 Concentration-response relationship of the inhibitory effect of 5-hydroxytryptamine (5-HT) on the outflow of [^{3}H]-acetylcholine evoked by 0.1 Hz stimulation and the effects of metitepine (a) or methysergide (b). 5-HT was added to the medium 30 min before S2 (●). Metitepine (0.1 μM, ○; 1 μM, △) or methysergide (1 μM, ○) were added 30 min before S1. Each value is the mean of 4–6 experiments, vertical lines show s.e.means. Significance of difference from corresponding control (Dunnetts' test): * $P<0.05$; ** $P<0.01$.

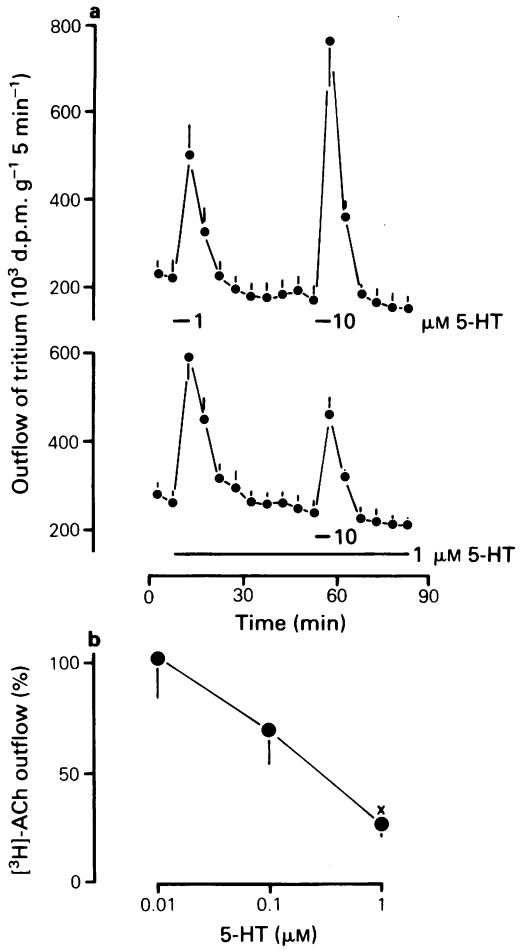

Figure 5 Effects of sustained exposure to 5-hydroxytryptamine (5-HT) on [^{3}H]-acetylcholine ([^{3}H]-ACh) outflow evoked by 10 μM 5-HT. (a) Time course of the experiments. Paired strips from the same small intestine were exposed to 10 μM 5-HT either in the presence of 1 μM 5-HT, or after the effect of 1 μM 5-HT had been washed out (control). Samples were collected in 5 min intervals. $n = 4$ for each experiment. (b) Inhibition by a desensitizing concentration of 5-HT (0.01–1 μM) of the [^{3}H]-ACh outflow evoked by 10 μM 5-HT expressed as percentage of the outflow in corresponding control experiments. Each value is the mean of 4 experiments; vertical lines show s.e. means. Significance of difference from control: * $P<0.01$.

tion-response curve to 5-HT was shifted in parallel to the right and an apparent pA_2 value of 7.0 was calculated for methysergide (Figure 4).

Antagonism of the excitatory effects of 5-HT by 5-HT and metoclopramide

A typical feature observed in contraction experiments on the guinea-pig ileum is that 5-HT produces rapid and reversible tachyphylaxis (see e.g. Huidobro-Toro & Foree, 1980). We have studied the effects of 10 μM 5-HT on [^{3}H]-ACh outflow in the presence of smaller, desensitizing concentrations of 5-HT (Figure 5). In control experiments the response to 10 μM 5-HT was studied after the effects of the desensitizing concentration had been washed out. In the presence of 100 nM 5-HT the excitatory action of 5-HT was slightly but not significantly reduced. However, 1 μM 5-HT inhibited by about 70% the [^{3}H]-ACh releasing effect of 10 μM 5-HT. The release experiments, thus, confirm the previous data indicating that 5-HT desensitizes the receptors after having excited them.

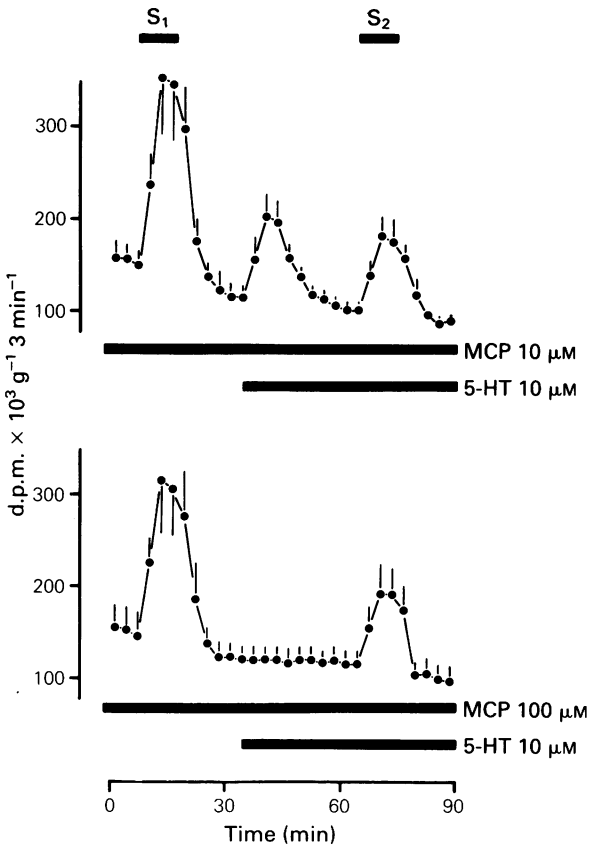

Figure 6 Effects of 5-hydroxytryptamine (5-HT) on spontaneous and electrically (0.1 Hz) evoked outflow of [^{3}H]-acetylcholine in the presence of metoclopramide (MCP). Metoclopramide was added to the superfusion medium 30 min before S1. Means of 3 (MCP 10 μM) and 4 (100 μM) experiments; vertical lines show s.e.means. For further details see legend to Figure 1.

Metoclopramide, like 5-HT, stimulates the excitatory 5-HT receptors of the guinea-pig ileum and causes an increase in spontaneous ACh release (Kilbinger *et al.*, 1982). We have studied whether metoclopramide affects the excitatory or inhibitory action of 5-HT. Strips were stimulated twice at 0.1 Hz (60 pulses) and metoclopramide was added to the medium 30 min before S1. The transient increase in [^{3}H]-ACh outflow occurring immediately after the administration of metoclopramide (Kilbinger *et al.*, 1982) was not studied in these experiments. Figure 6 shows that the excitatory effect of 5-HT (10 μM) on [^{3}H]-ACh outflow was reduced by 66 ± 4 (n = 3), and 94 ± 3 (n = 4) %, respectively, in the presence of 10 and 100 μM metoclopramide. On the other hand, the inhibitory action of 5-HT was not affected in the presence of 10 μM metoclopramide (S2/S1: 0.38 ± 0.01; n = 3). With 100 μM metoclopramide present 5-HT also caused a significant inhibition of the evoked outflow (S2/S1: 0.47 ± 0.02; n = 4) although the degree of inhibition was somewhat less than in control experiments with 10 μM 5-HT alone (S2/S1: 0.35 ± 0.05; n = 4). The ratios S2/S1 in the presence of 10 and 100 μM metoclopramide alone were 0.80 ± 0.02 (n = 4) and 0.83 ± 0.02 (n = 3).

The antagonism by metoclopramide of the facilitatory action of 5-HT was studied in more detail in experiments in which 5-HT was added in increasing concentrations as a bolus into the superfusion stream to give final concentrations of 1–30 μM in the 2 ml organ bath. Metoclopramide shifted the concentration-response curve for 5-HT to the right, but at higher concentrations both the slopes and the maxima of the 5-HT curve were progressively decreased (Figure 7) A pD_2' value of 5.40 ± 0.15 (Ω = 11) was calculated for this effect of metoclopramide.

Effects of ketanserin, MDL 72222 and tropacocaine

Ketanserin The effects of 1 μM 5-HT on spontaneous and electrically-evoked outflow of [^{3}H]-ACh were

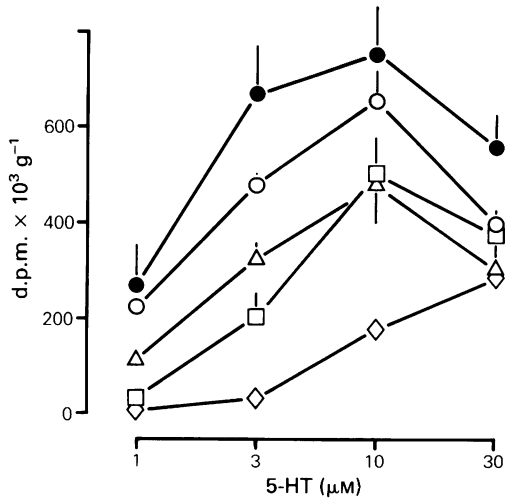

Figure 7 Effects of metoclopramide on [^{3}H]-acetylcholine outflow evoked by 5-hydroxytryptamine (5-HT). Strips preincubated with [^{3}H]-choline were superfused with Tyrode solution. After a 60 min washout period 5-HT was injected (100 μl) four times at 30 min intervals into the superfusion stream to give final concentrations of 1–30 μM. Metoclopramide was superfused from 30 min before the application of the first concentration of 5-HT until the end of the experiment. Cumulative concentration response curves for 5-HT alone (●, n = 4) or in the presence of the following concentrations of metoclopramide (μM): 0.1 (○), 1 (△), 3 (□), (each n = 4), 10 (◇) (n = 3).

Table 3 Effects of 5-hydroxytryptamine (5-HT) on spontaneous and electrically evoked outflow of [^{3}H]-acetylcholine ([^{3}H]-ACh) in the presence of ketanserin

Ketanserin (μM)	*5-HT* (μM)	*S1* (10^3 d.p.m. g^{-1})	*S2/S1*		*5-HT induced outflow* (10^3 d.p.m. g^{-1})	n
—	—	468 ± 64	0.87 ± 0.05	**	—	8
—	1	352 ± 62	0.54 ± 0.05		300 ± 32	6
0.1	—	570 ± 99	0.86 ± 0.10	**	—	4
0.1	1	414 ± 69	0.55 ± 0.05		261 ± 59	8
1.0	—	481 ± 71	0.79 ± 0.06	*	—	6
1.0	1	393 ± 96	0.53 ± 0.03		217 ± 38	4

Ketanserin was added to the medium 30 min before S1. For further details see Table 2. * $P < 0.02$; ** $P < 0.01$.

Table 4 Effects of 5-hydroxytryptamine (5-HT) on spontaneous and electrically-evoked outflow of [^{3}H]-acetylcholine ([^{3}H]-ACh) in the presence of MDL 72222

MDL 72222 (μM)	*5-HT* (μM)	*S1* (10^3 d.p.m. g^{-1})	*S2/S1*		*5-HT induced outflow* (10^3 d.p.m. g^{-1})		n
—	—	450 ± 118	0.78 ± 0.07	**	—	**	6
—	3	486 ± 129	0.44 ± 0.03		516 ± 86		9
0.1	—	534 ± 170	0.73 ± 0.08	*	—	**	4
0.1	3	377 ± 75	0.48 ± 0.08		405 ± 142		6
1.0	—	282 ± 37	0.84 ± 0.05	**	—		4
1.0	3	424 ± 69	0.38 ± 0.05		65 ± 20		5

MDL 72222 was added to the medium 30 min before S1. For further details see Table 2. * $P < 0.05$; ** $P < 0.01$.

tested in the presence of the 5-HT antagonist, ketanserin. Table 3 shows that neither effect of 5-HT was significantly changed in the presence of 0.1 or 1 μM ketanserin. The spontaneous outflow of tritium was not affected by either of the concentrations of ketanserin.

MDL 72222, tropacocaine Both drugs are selective and potent antagonists at neuronal 5-HT receptors of postganglionic sympathetic fibres (Fozard *et al.*, 1979; Fozard, 1984). The effects of MDL 72222 on the facilitatory and inhibitory actions of 5-HT are given in Table 4. MDL 72222 (0.1 and 1 μM) did not antagonize the 5-HT-induced inhibition of [^{3}H]-ACh outflow. On the other hand, 1 μM MDL 72222 markedly reduced the facilitatory effect of 5-HT. The outflow evoked by electrical stimulation during S1 was not significantly changed in the presence of 0.1 or 1 μM MDL 72222. Tropacocaine (1 μM) affected neither the facilitatory nor the inhibitory actions of 3 μM 5-HT (data not shown). Neither MDL 72222 (0.1 and 1 μM) nor tropacocaine changed the spontaneous outflow of tritium.

Discussion

The results demonstrate that 5-HT exerts a dual effect on the cholinergic neurones of the guinea-pig ileum: increase of spontaneous and inhibition of the electrically-evoked release of ACh.

Excitatory effects of 5-hydroxytryptamine

The similarity between concentration-response curves for 5-HT-induced contraction and ACh release suggests that the increase in muscle tension is mainly due to the release of ACh. A similar conclusion was reached by Costa & Furness (1979) who found that in the proximal ileum the indirect action of 5-HT is dominant whereas the direct effects of 5-HT on the longitudinal muscle are almost insignificant.

In order to characterize the excitatory receptor (the so-called 'M' receptor) we have studied the effects of several 5-HT antagonists. Both 5-HT and metoclopramide antagonized the facilitatory action of 5-HT, which probably reflects desensitization after the initial excitation of the receptor. The non-parallel

displacement of the 5-HT curves with depression of the slope and the maximum in the presence of metoclopramide (Figure 7) is typical for a compound having both agonist and antagonist activities (Ariens & Simonis, 1964). Metoclopramide has been shown to act as a competitive antagonist upon excitatory 5-HT receptors on postganglionic sympathetic nerves of the rabbit heart (Fozard & Mobarok Ali, 1978), as have tropacocaine and nanomolar concentrations of MDL 72222 (Fozard *et al.*, 1979; Fozard, 1984). The lack of effect of tropacocaine and 0.1 μM MDL 72222 in the present experiments together with the failure of metoclopramide to antagonize competitively the effects of 5-HT confirms the view (Wallis, 1981; Fozard, 1984) that the excitatory 5-HT receptors on sympathetic nerve terminals are pharmacologically different from those on myenteric cholinergic neurones.

However, micromolar concentrations of MDL 72222 blocked excitatory 5-HT receptors of myenteric nerves (Table 4). On the other hand it is known that similar concentrations fail to antagonize the indirect (i.e. neuronally-mediated), 5-HT contractions of the longitudinal muscle (Fozard, 1984). An explanation for this discrepancy might be the existence of spare receptors for ACh on the longitudinal muscle (Sastry & Cheng, 1972; Birdsall *et al.*, 1978). The amount of ACh liberated by 5-HT in the presence of MDL 72222 may be sufficient to cause a maximal contraction although the release of transmitter is reduced.

Ketanserin is a selective ligand for central 5-HT_2 binding sites (Leysen *et al.*, 1981). In our study ketanserin failed to antagonize the excitatory effects of 5-HT (Table 3). Likewise, metitepine and methysergide (1 μM) which bind to both 5-HT_1 and 5-HT_2 sites (Leysen *et al.*, 1981) did not affect the 5-HT-induced ACh release, suggesting that the excitatory 5-HT receptor does not belong to either the 5-HT_1 or 5-HT_2 subtype.

Inhibitory effects of 5-hydroxytryptamine

5-HT caused a concentration-dependent inhibition of the electrically-evoked release of ACh. As with other agonists that diminish the release of ACh from myenteric nerves (oxotremorine, noradrenaline, morphine; for references see Kilbinger, 1982), the inhibitory effect was more marked at a lower than at a higher stimulation frequency. 5-HT applied to the serosa easily reaches the myenteric plexus (Gershon *et al.*, 1983) and inhibition of the peristaltic reflex is probably due to reduced ACh release, mediated via inhibitory 5-HT receptors. A possible direct or indirect action of 5-HT at inhibitory α-adrenoceptors can be excluded because the inhibition of evoked ACh release was also observed in the presence of tolazoline and after a 96% reduction of the endogenous noradrenaline content by reserpine treatment. It was noted that myenteric plexus preparations from reserpine-treated guinea-pigs responded to electrical stimulation (S1) and to superfusion with 5-HT with a much higher ACh release than control preparations. Such an increased release after catecholamine depletion has also been found by Paton & Vizi (1969) and is probably due to the loss of an endogenous adrenergic inhibition. Thus, both electrically-evoked release and also 5-HT-evoked release of ACh may be modulated through inhibitory α-adrenoceptors. In the presence of tolazoline the electrically-evoked ACh release also appeared to be slightly higher than under control conditions (cf. S1 values of Table 2 with S1 values from Tables 3 and 4), but these differences were not significant because of the large variations of the absolute amounts of ACh released.

Since the inhibitory action of 5-HT was measured after a 30 min superfusion of the tissue with 5-HT, the inhibitory receptor is not readily desensitized in marked contrast to the strong desensitization observed for the excitatory effect of 5-HT.

Electrophysiological studies in which exogenous 5-HT induced membrane depolarization of myenteric neurones (Wood & Mayer, 1979; Johnson *et al.*, 1980), and a reduction of the amplitude of the cholinergic fast e.p.s.p. (North *et al.*, 1980) may aid in the interpretation of our results. The studies of North *et al.* (1980) indicate that 5-HT acts presynaptically at the terminals of cholinergic interneurones. From our results it seems unlikely that the cholinergic nerves endowed with excitatory 5-HT receptors also bear inhibitory 5-HT receptors. If this were so, one would expect 5-HT-evoked ACh release to be enhanced when the inhibitory response is blocked by metitepine or methysergide, whereas it was not affected. The inhibition of the electrically-evoked ACh released by 5-HT was not paralleled by a simultaneous reduction of the twitch contraction. The twitch height was significantly reduced only by 10 μM 5-HT, whilst 0.1 and 1 μM 5-HT enhanced the contractions. It is known that 5-HT increases the longitudinal muscle contraction to exogenous ACh or nicotine (Bülbring & Crema, 1958). Thus, only when large concentrations of 5-HT are used is the twitch response reduced through reduction of the evoked ACh release.

Metitepine and methysergide, but not ketanserin antagonized the inhibition by 5-HT of electrically-evoked ACh release. This suggests that the inhibitory 5-HT receptor may roughly correspond to the 5-HT_1 subtype. In the presence of 100 nM metitepine or 1 μM methysergide the concentration-response curve was shifted in parallel to the right and there was no depression of the maximum response, characteristic for an interaction between an agonist and a competitive antagonist. With 1 μM metitepine a reduction by 5-HT of the electrically-evoked ACh release was not observed, but whether this is indicative of a non-

competitive mode of interaction only additional studies will reveal. From the shift of the 5-HT concentration-response curve in the presence of 100 nM metitepine an apparent pA_2 value of 7.6 was calculated. This is considerably higher than pA_2 values obtained for metitepine at central 5-HT autoreceptors (6.6, Schlicker & Göthert, 1981; 7.0, Engel *et al.*, 1983; 6.9, Middlemiss, 1984). Since methysergide was also an antagonist in the present experiments (pA_2 7.0) and in electrophysiological studies (North *et al.*, 1980) but is inactive at the central 5-HT autoreceptor (Cerrito & Raiteri, 1979; Baumann & Waldmeier, 1981; Martin & Sanders-Bush, 1982), we assume that the 5-HT receptor inhibiting ACh release from myenteric neurones differs in its pharmacological properties from the 5-HT autoreceptor.

In conclusion, we have demonstrated that 5-HT increases spontaneous and inhibits electrically-evoked release of ACh from myenteric nerves. Both effects are mediated through stimulation of specific 5-HT receptors which differ in their pharmacological properties.

We thank Miss Doris Wolf for competent technical assistance and the pharmaceutical companies for donation of drugs. This work was supported by the Deutsche Forschungsgemeinschaft. The paper contains part of the Dr. rer. nat. Thesis of I.P.-F., Naturwissenschaftliche Fachbereiche der Universität Mainz.

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(Received November 30, 1984.
Revised January 25, 1985.
Accepted February 6, 1985.)