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Involvement of 5-hydroxytryptamine$_7$ receptors in inhibition of porcine myometrial contractility by 5-hydroxytryptamine

[1]Takio Kitazawa, Osamu Kubo, Masami Satoh & Tetsuro Taneike

Department of Pharmacology, Faculty of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, 069, Japan

1 5-Hydroxytryptamine (5-HT; 1 nM – 100 μM) concentration-dependently inhibited the amplitude and frequency of spontaneous contractions in longitudinal and circular muscles of the porcine myometrium. The circular muscle (EC_{50}; 68 – 84 nM) was more sensitive than the longitudinal muscle (EC_{50}; 1.3 – 1.44 μM) to 5-HT. To characterize the 5-HT receptor subtype responsible for inhibition of myometrial contractility, the effects of 5-HT receptor agonists on spontaneous contractions and of 5-HT receptor antagonists on inhibition by 5-HT were examined in circular muscle preparations.

2 Pretreatment with tetrodotoxin (1 μM), propranolol (1 μM), atropine (1 μM), guanethidine (10 μM) or L-NAME (100 μM) failed to change the inhibition by 5-HT, indicating that the inhibition was due to a direct action of 5-HT on the smooth muscle cells.

3 5-CT, 5-MeOT and 8-OH-DPAT mimicked the inhibitory response of 5-HT, and the rank order of the potency was 5-CT > 5-HT > 5-MeOT > 8-OH-DPAT. On the other hand, oxymethazoline, α-methyl-5-HT, 2-methyl-5-HT, cisapride, BIMU-1, BIMU-8, ergotamine and dihydroergotamine had almost no effect on spontaneous contractions, even at 10 – 100 μM.

4 Inhibition by 5-HT was not decreased by either pindolol (1 μM), ketanserin (1 μM), tropisetron (10 μM), MDL72222 (1 μM) or GR113808 (10 μM), but was antagonized by the following compounds in a competitive manner (with pA_2 values in parentheses): methiothepin (8.05), methysergide (7.92), metergoline (7.4), mianserin (7.08), clozapine (7.06) and spiperone (6.86).

5 Ro 20-1724 (20 μM) and rolipram (10 μM) significantly enhanced the inhibitory response of 5-HT, but neither zaprinast (10 μM) nor dipyridamole (10 μM) altered the response of 5-HT.

6 5-HT (1 nM – 1 μM) caused a concentration-dependent accumulation of intracellular cyclic AMP in the circular muscle.

7 From the present results, the 5-HT receptor, which is functionally correlated with the 5-HT_7 receptor, mediates the inhibitory effect of 5-HT on porcine myometrial contractility. This inhibitory response is probably due to an increase in intracellular cyclic AMP through the activation of adenylate cyclase that is positively coupled to 5-HT_7 receptors.

Keywords: Porcine myometrium; 5-HT; 5-HT_7 receptor; smooth muscle relaxation; cyclic AMP

Introduction

5-Hydroxytryptamine (5-HT) produces a wide variety of mechanical effects (contraction, relaxation, or both responses) on vascular and non-vascular smooth muscles in several animal species. These tissue- and species-dependent variations of 5-HT-induced responses are caused by the presence of a number of 5-HT receptor subtypes. At present, receptors for 5-HT have been classified by pharmacological, signal transductional and structural criteria into four major subtypes; namely, 5-HT_1 (negatively coupled to adenylate cyclase), 5-HT_2 (positively coupled to phospholipase C), 5-HT_3 (coupled to ligand-gated ion channel) and 5-HT_4 (positively coupled to adenylate cyclase) (Boess & Martin, 1994; Hoyer *et al*., 1994). In addition, molecular cloning studies have indicated the presence of three other less well-defined subtypes (5-HT_5, 5-HT_6, 5-HT_7). Although there is currently a lack of selective antagonists for these receptors, three cloned receptors can be separated pharmacologically by relative agonist potency. For example, lysergic acid diethylamide (LSD) shows relatively high affinity in comparison to 5-HT and 5-carboxamidotryptamine (5-CT) at 5-ht_5 receptors (LSD > 5-CT > 5-HT). At 5-HT_6 receptors, 5-CT is less potent than 5-HT and 5-methoxytryptamine (5-MeOT) (5-MeOT > 5-HT > 5-CT). By contrast, 5-CT is more potent than 5-HT, LSD and 5-MeOT at cloned 5-HT_7 receptors (5-CT > 5-HT $\geqslant$ 5-MeOT > LSD)(Boess & Martin, 1994; Hoyer *et al*., 1994). Since these receptors were initially cloned from central nervous tissues, their physiological functions in the peripheral tissues have not been clearly identified. However, recent pharmacological studies have suggested that 5-HT_7 receptors mediate the relaxation of the guinea-pig ileum (Carter *et al*., 1995), endothelium-independent relaxation of the rabbit femoral vein, monkey jugular vein and dog coronary artery (Martin & Wilson, 1995; Leung *et al*., 1996; Terron, 1996). Therefore, interest has been shown in the 5-HT_7 receptor, as a new 5-HT receptor subtype mediating the inhibition of muscle contractility by 5-HT in the peripheral vascular and non-vascular smooth muscle of various mammalian species. Additionally, a recent molecular biological study has indicated the isoforms and species-dependent diversity of 5-HT_7 receptor due to altered intron-exon organization (Heidmann *et al*., 1997).

Many mast cells are present in the uterus and their density is changed by the oestrous cycle or pregnancy (Hine *et al*., 1985; Padilla *et al*., 1990). From these findings, chemical mediators contained in the mast cells (histamine and 5-HT) are considered to regulate uterine smooth muscle contractility. The effects of histamine have been studied extensively in a variety of animals (man, rat, mouse, rabbit, pig) and histamine has been shown to cause contraction of the myometrium by activation of H_1 histamine receptors, and relaxation through H_2 histamine receptors (Patel *et al*., 1980; Martinez-Mir *et al*., 1992; Rudolph *et al*., 1993; Kitazawa *et al*., 1997). On the other hand, the effect of 5-HT on the myometrium has mainly been

[1] Author for correspondence.

investigated in rats, and it was demonstrated that 5-HT caused contraction of myometrial smooth muscle through activation of 5-HT_2 receptors (Cohen *et al*., 1986; Cohen & Wittenauer, 1987). However, due to the limited number of studies on the effects of 5-HT in the myometrium, species-related variations and mechanism underlying the 5-HT-induced response have not yet been well defined.

While examining the effect of 5-HT on porcine isolated uterine strips, we found that 5-HT decreased spontaneous myometrial contractility, unlike that in the case of the rat myometrium, although the pharmacological profiles of the inhibition by 5-HT have not yet been established. The present study was designed to identify the 5-HT receptor subtype mediating the inhibitory response by using several selective agonists and antagonists, and to determine whether or not 5-HT receptors are positively coupled to adenylate cyclase activity. In the pig uterus, longitudinal and circular muscles can be separated easily, and we can also investigate the smooth muscle layer-dependent difference in responsiveness of 5-HT (Taneike *et al*., 1994; Kitazawa *et al*., 1997).

Methods

Tissue preparations

Fresh uteri, with the ovaries intact, from 100 sexually mature, crossbred virgin gilts (about 6 months old), were provided by a local abattoir and were subjected to experiments on the day of slaughter. According to gross examination of the follicle size and appearance of the corpora lutea, the uteri of gilts were justified as proestrus (McDonald, 1975). Longitudinal and circular muscle layers were isolated surgically from the antimesometrial coat of the adtubal region (10 cm distal from the apex) in either the left or right cornu. As described previously (Taneike *et al*., 1994; Kitazawa *et al*., 1997), after removal of the endometrium, each muscle layer was cut through the muscle coat in either the longitudinal or circular muscle direction. Then, the unwanted muscle layers were removed from each muscle strip by meticulously cutting them away with fine scissors under a binocular microscope, thereby isolating the remaining longitudinal or circular muscle for experimental use. Smooth muscle strips (10×1 mm) were suspended vertically in an organ bath (5 or 20 ml) containing 37°C Krebs solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.5) bubbled with 95% O_2 + 5% CO_2. A force-displacement transducer (SB-1T, Nihon Kohden), equipped with a pen-writing recorder (Recticorder, Nihon Kohden) was used to measure the mechanical activity of the smooth muscle preparations. The muscle strips were loaded at 0.2 g as an initial tension and allowed to equilibrate for 60 min. Each smooth muscle layer showed a spontaneous contraction, the frequency (contractions/5 min) of which was different between longitudinal (5.2 ± 0.2, $n = 17$) and circular muscle (9.8 ± 0.35, $n = 25$). These spontaneous contractile activities were abolished by Ca^{2+}-free (EGTA, 0.5 mM) solution or verapamil (10 μM) treatment.

Experimental protocol and data analysis

After steady spontaneous contractile activity of the preparations had been established, 5-HT and 5-HT receptor agonists were applied cumulatively in the bath at 3 min intervals (maximum response of 5-HT was mostly obtained within 3 min after the application). The amplitude of the minimum spontaneous contraction during each 3 min cycle was determined and expressed as a percentage of the spontaneous contraction obtained before application of agonists. Change in spontaneous contraction frequency (contractions/5 min) by 5-HT was also analysed. The EC_{50} (concentration of agonists that caused half-maximal inhibition) and EC_{100} (concentration of agonists that abolished spontaneous contractile activity) values were determined by least squares non-linear regression analysis of the concentration-response curve.

Antagonistic affinity (pA_2) on the 5-HT-induced inhibition was determined for each antagonist according to the procedure of Arunlakshana & Schild (1959). Concentration-response curves for 5-HT were constructed in the absence and presence (30 min treatment) of three or four increasing concentrations of 5-HT receptor antagonists. The concentration-ratio (CR, EC_{50} in the presence of antagonist/EC_{50} in the absence of antagonist) was determined with the respective antagonists. If the blockade is competitive in manner under equilibrium conditions, then a plot of the logarithm of CR-1 against the negative logarithm of the molar concentration of antagonist should yield a straight line, the slope of which is not different from unity (1.00) and the intercept on the abscissa scale gives the pA_2 value, which is generally considered to be equivalent to the apparent antagonist dissociation constant for receptors ($-\log K_b$).

Measurement of cyclic AMP level

Isolated fresh circular muscle strips weighing approximately 40 mg were used for the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) study. After equilibration in Krebs solution at 37°C for 1 h, the strips were exposed to four increasing concentrations of 5-HT for 5 min. Some strips were used as untreated controls. After incubation, the muscle strips were frozen quickly in liquid nitrogen and homogenized in 6% trichloroacetic acid solution. After centrifugation twice at 3,000 r.p.m., trichloroacetic acid in supernatant was removed by washing with water-saturated ether and cyclic AMP was assayed by use of an enzyme immunoassay kit (Amersham). Tissue cyclic AMP levels are expressed as pmol g^{-1} tissue wet weight.

Chemicals

The following chemicals were used in this experiment: atropine sulphate (Wako), 5-carboxamidotryptamine maleate (5-CT, RBI), clozapine (RBI), cocaine hydrochloride (Sankyo), corticosterone (Sigma), dibutyryl adenosine 3′:5′-cyclic monophosphate (db-cyclic AMP, Sigma), dihydroergotamine tartrate (Tokyo Kasei), dipyridamole (Biomol), ergotamine hydrochloride (Tokyo Kasei), forskolin (Wako), guanethidine hydrochloride (Regis. Chem. Co), 5-hydroxytryptamine creatinine sulphate (5-HT, Wako), 3-isobutyl-1-methylxanthine (Aldrich), MDL72222 (1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate; RBI), metergoline (RBI), methiothepin hydrochloride (RBI), 5-methoxytryptamine hydrochloride (5-MeOT, Sigma), α-methyl-5-hydroxytryptamine (α-methyl-5-HT, RBI), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT, RBI), mianserin hydrochloride (RBI), N^G-nitro-L-arginine-methylester (L-NAME, Sigma), (±)-8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT; RBI), oxymetazoline hydrochloride (RBI), pargyline hydrochloride (RBI), pindolol hydrochloride (RBI), propranolol hydrochloride (Wako), Ro 20-1724 (4-(3-butoxy-4-methoxy-phenyl)methyl-2-imidazolidone; Biomol), rolipram (Biomol), spiperone hydrochloride (RBI), tetrodotoxin (Wako), verapamil hydrochloride (Wako),

and zaprinast (Biomol). The following drugs were kindly donated: BIMU-1 ([endo-N-8-methyl-8-azabicyclo-(3,2,1)oct-3-yl]-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazol-1-carboxamide), BIMU-8 ([endo-N-8-methyl-8-azabicyclo-(3,2,1)oct-3-yl]-2,3-dihydro-(1-methyl)ethyl-2-oxo-1H-benzimidazol-1-carboxamide) (Boehringer Ingelheim, Milano, Italy), GR113808 (Glaxo, Greenford, Middlesex, U.K.), tropisetron (2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino ethyl) ester, methysergide hydrochloride (Novartis AG, Basel, Switzerland) and ketanserin, cisapride (Janssen-Kyowa, Tokyo, Japan). Drugs except for cisapride, clozapine, corticosterone, dihydroergotamine, dipyridamole, forskolin, metergoline, rolipram and zaprinast were dissolved in distilled water and applied directly to an organ bath. Clozapine, corticosterone, dihydroergotamine, dipyridamole, metergoline, rolipram and zaprinast were dissolved in dimethylsulphoxaide (DMSO), and the solution was diluted by distilled water and Krebs solution. Cisapride and forskolin were dissolved in phosphoric acid and ethanol, respectively, and then diluted by distilled water and Krebs solution. The maximum concentration of DMSO or ethanol in the bathing solution was set below 0.2%, and this concentration did not change the spontaneous contractile activity of the pig myometrium.

Statistical analysis

The results of the experiments are expressed as mean $\pm$s.e.mean of more than four experiments. Statistical analysis was performed by paired and unpaired t tests, with $P<0.05$ as the criterion of statistical significance.

Results

Inhibitory effect of 5-HT

Firstly, the effects of 5-HT on spontaneous contraction of the longitudinal and circular muscle strips of the pig uterus were investigated. Of all the circular muscle preparations examined, 5-HT (1 nM – 1 μM) concentration-dependently decreased the amplitude and frequency of the spontaneous contraction and eventually abolished it at a high concentration (300 nM – 1 μM) (Figure 1a). 5-HT-induced inhibition was reproducible with cumulative applications at 45 min intervals, and desensitization was not developed in this muscle preparation. The EC_{50} values of 5-HT for inhibiting the amplitude and frequency were 84 ± 10 nM ($n=25$) and 68 ± 17 nM ($n=25$), respectively, and no significant difference was observed between these EC_{50} values (Figure 1b). On the contrary, the effect of 5-HT on spontaneous contraction of the longitudinal muscle layer varied from preparation to preparation, unlike that in the case of the circular muscle. Of 18 longitudinal muscle preparations examined, 5-HT inhibited the spontaneous contraction and finally abolished it in 7 preparations (10 – 100 μM), caused 10 – 90% inhibition in 7 preparations at 100 μM (Figure 1a, a typical response), and failed to inhibit the spontaneous contraction in 4 preparations (100 μM, inhibition, $<10\%$). Figure 1b shows the concentration-response relationships for 5-HT of 14 longitudinal muscle preparations in which 5-HT caused an inhibitory response. The EC_{50} value of 5-HT was 1.44 ± 0.27 μM ($n=14$) for inhibiting the amplitude and 1.3 ± 0.35 μM ($n=14$) for decreasing the frequency. Maximum inhibition by 5-HT was $68\pm9\%$ ($n=14$) for amplitude and $48\pm7.4\%$ ($n=14$) for frequency. These results indicate that there is a conspicuous muscle layer-dependent difference in responsiveness to 5-HT. Since we were focusing on inhibition by 5-HT, circular muscle preparations were usually used in the following experiments. Also, as there was no significant difference between the EC_{50} values of 5-HT estimated by inhibition of the amplitude and frequency, 5-HT-induced inhibition was evaluated as the decrease in the spontaneous contraction amplitude.

In the next experiments, the effects of uptake inhibitors (cocaine plus corticosterone) and a monoamine oxidase inhibitor (pargyline) on the 5-HT-induced inhibition were investigated in the circular muscles. Cocaine (30 μM) plus corticosterone (30 μM) extended the duration of spontaneous contractions and significantly reduced the frequency (4.9 ± 0.3 5 min^{-1}, $n=11$). The amplitude of spontaneous contraction was also slightly decreased (5 – 10% of the control). However, cocaine (30 μM) plus corticosterone (30 μM) did not affect the inhibitory response of 5-HT. The EC_{50} values in the absence and presence of these uptake inhibitors were 69 ± 16 nM ($n=11$) and 78 ± 19 nM ($n=11$), respectively. Treatment with pargyline (20 μM) also decreased frequency of the spontaneous contraction (7.5 ± 0.6 5 min^{-1}, $n=9$) without inhibiting the amplitude of contraction. Pargyline had no effect on the 5-HT-induced inhibitory response (EC_{50} value, control, 57 ± 13 nM; pargyline, 50 ± 10 nM, $n=9$). As there was no marked effect of these uptake inhibitors or a monoamine oxidase inhibitor, the following experiments, concerning the effects of 5-HT and 5-HT receptor agonists on the myometrial contractility, were carried out in the absence of these inhibitors.

To determine whether inhibitory 5-HT receptors are present on smooth muscle or neural components, the effects of tetrodotoxin (1 μM), atropine (1 μM), propranolol (1 μM) and guanethidine (10 μM) on the inhibition by 5-HT were examined. As indicated in Table 1, these drugs did not change EC_{50} and EC_{100} values of 5-HT. The effect of L-NAME on the response to 5-HT was also investigated to determine the involvement of NO in inhibition. Pretreatment with L-NAME (100 μM) had no effect on spontaneous contractile activity or on inhibition by 5-HT. The EC_{50} values in the absence and presence of L-NAME (100 μM) were 71 ± 14 nM ($n=4$) and 74 ± 10 nM ($n=4$), respectively, while the EC_{100} values were 425 ± 170 nM ($n=4$) and 600 ± 203 nM ($n=4$), respectively (Table 1).

Agonist effects

The effects of several 5-HT receptor agonists on spontaneous contraction were examined and compared with the effect of 5-HT. Oxymethazoline, α-methyl-5-HT, 2-methyl-5-HT, ergotamine and dihydroergotamine did not inhibit spontaneous contraction even at a high concentration (10 – 100 μM). However, 5-CT ($EC_{50}=1.72\pm0.33$ nM, $n=12$), 5-MeOT ($EC_{50}=5.2\pm0.96$ μM, $n=21$) and 8-OH-DPAT ($EC_{50}=27.5\pm6.1$ μM, $n=13$) decreased spontaneous contraction in a concentration-dependent manner. Inhibitions by 5-CT and 5-MeOT were complete in all circular muscle preparations, as was the case with 5-HT, but 8-OH-DPAT caused only a partial inhibition ($75.4\pm4.2\%$, $n=13$) even at 100 μM. On comparison of the EC_{50} values, the rank order of inhibition (with equipotent molar ratio, EC_{50} of respective agonists/EC_{50} of 5-HT) was 5-CT (0.02) > 5-HT (1.0) > 5-MeOT (62.0) > 8-OH-DPAT (327.4) (Figure 2).

Since 5-MeOT is a potent 5-HT_4 receptor agonist (Baxter *et al.*, 1991), the effects of other selective 5-HT_4 receptor agonists on spontaneous contraction were examined. As indicated in Figure 2b, each 5-HT_4 receptor agonist (cisapride, BIMU-1, BIMU-8) was almost ineffective in inhibiting the spontaneous contractile activity (1 nM – 100 μM).

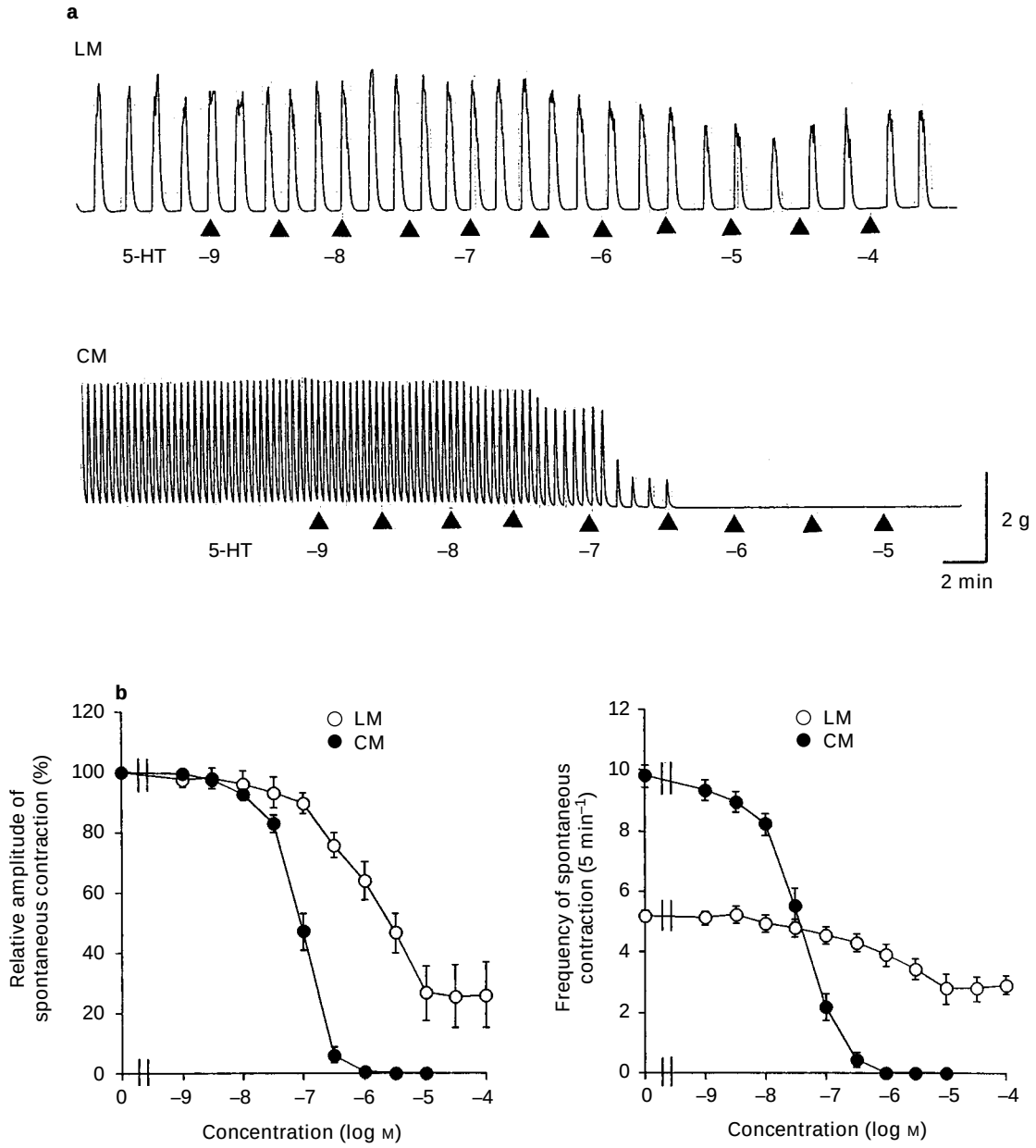

Figure 1 Effect of 5-HT on spontaneous contractile activity of the porcine myometrium. (a) Each trace shows a typical response induced by cumulatively applied 5-HT (1, 3, 10, 30, 100, 300 nM, 1, 3, 10, 30, 100 μM) to the spontaneously contracting longitudinal (LM) and circular muscle layers (CM). Numbers under each curve indicate the concentration of 5-HT (logM). (b) Concentration-response relationships of 5-HT in the longitudinal (LM) and circular muscle layers (CM). 5-HT-induced inhibition was evaluated by change in the amplitude (left panel) and frequency (right panel) of the spontaneous contraction. Ordinate scales: relative amplitude (control = 100%, left panel) and frequency (contractions 5 min^{-1}, right panel) of spontaneous contraction. Abscissa scales: concentration of 5-HT (logM). Points represent the means of 4 or more experiments with s.e.mean shown by vertical lines.

Table 1 Effects of tetrodotoxin, propranolol, atropine, guanethidine and L-NAME on inhibition of spontaneous myometrial contractility by 5-hydroxytryptamine

	Control		*Treatment*	
Drugs	EC_{50} (nM)	EC_{100} (nM)	EC_{50} (nM)	EC_{100} (nM)
Tetrodotoxin (1 μM)	77±33	247±120	61±24	286±115
Propranolol (1 μM)	52±25	188±47	48±20	141±33
Atropine (1 μM)	84±14	220±43	75±15	280±41
Guanethidine (10 μM)	95±20	280±134	83±23	400±140
L-NAME (100 μM)	71±14	425±170	74±10	600±203

Values are means±s.e.mean of 4–7 individual studies. Neither tetrodotoxin, propranolol, atropine, guanethidine nor L-NAME, by themselves, changed the amplitude or frequency of spontaneous contraction in the circular muscle of the pig uterus.

Antagonist studies

For further characterization of the inhibitory 5-HT receptor, the effects of some 5-HT receptor antagonists on 5-HT-induced inhibition were investigated. The EC_{50} and EC_{100} values of 5-HT in the absence (control) and presence of the antagonists are shown in Table 2. Pindolol (1 μM), ketanserin (1 μM), MDL72222 (1 μM), tropisetron (10 μM) and GR113808 (10 μM) did not decrease the inhibition by 5-HT. On the other hand, methiothepin (30–300 nM), metergoline (100 nM–1 μM), methysergide (100 nM–1 μM), clozapine (300 nM–3 μM), spiperone (300 nM–3 μM) and mianserin (300 nM–3 μM) antagonized the inhibitory response of 5-HT in a concentration-dependent manner, and they caused a parallel shift of the concentration-response curve of 5-HT to the right without affecting the maximum response (complete inhibition of spontaneous contraction). None of these antagonists produced a change in spontaneous contraction when they were added to the organ bath. The Schild plot of the inhibitory effect of each antagonist on the response of 5-HT was linear ($r = 0.97–0.99$). The pA_2 value and Schild slope of the antagonists were 8.05 and 0.99 for methiothepin, 7.92 and 0.94 for methysergide, 7.40 and 1.32 for metergoline, 7.08 and 1.18 for mianserin, 7.06 and 1.2 for clozapine, and 6.86 and 1.15 for spiperone. The slope of the Schild plot for each antagonist was not significantly different from unity (Table 3).

The effects of three antagonists (methiothepin, metergoline and mianserin) on the responses of 5-HT receptor agonists were also examined. The inhibitory effects of 5-CT, 5-MeOT and 8-OH-DPAT were concentration-dependently decreased by methiothepin (30–300 nM), metergoline (100 nM–1 μM) or mianserin (300 nM–3 μM) within a similar concentration range as 5-HT ($n = 2–4$, data not shown).

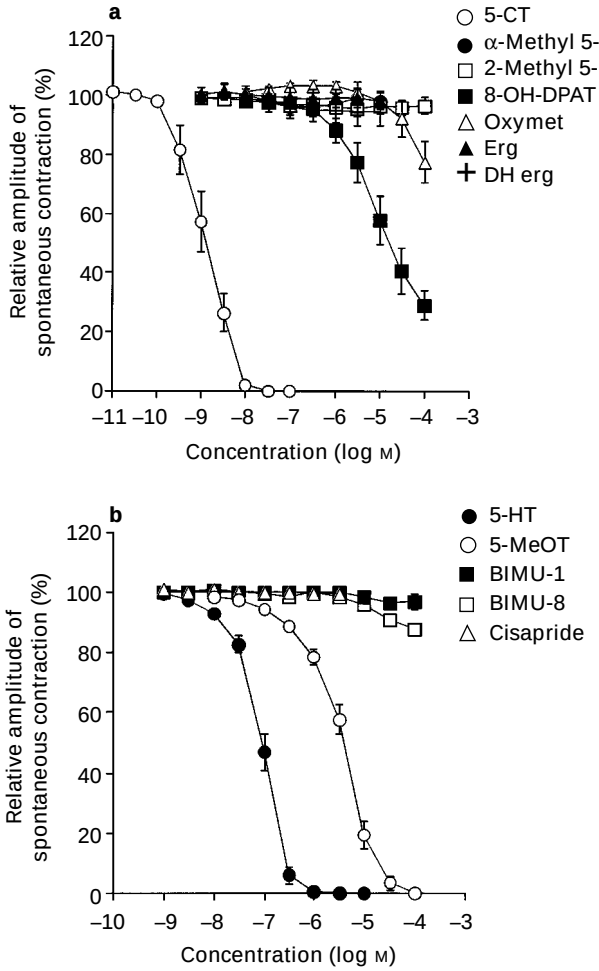

Figure 2 Concentration-response curves of some 5-HT receptor agonists in spontaneously contracting circular muscle of the porcine myometrium. (a) The symbols show the effects of 5-CT, α-methyl 5-HT, 2-methyl 5-HT, 8-OH-DPAT, oxymethazoline (Oxymet), ergotamine (Erg) and dihydroergotamine (DHerg) on amplitude of the spontaneous contraction. (b) Comparison of the inhibitory responses of 5-HT, 5-MeOT and 5-HT_4 receptor agonists (BIMU-1, BIMU-8, and cisapride). Ordinate scales: relative amplitude (control = 100%) of spontaneous contraction. Abscissa scales: concentration of agonists (logM). Points represent the means of 4 or more experiments with s.e.mean shown by vertical lines.

Effect of phosphodiesterase inhibitors

The possible involvement of cyclic AMP or cyclic GMP in the 5-HT-induced inhibition of myometrial contractility was investigated with cyclic AMP-specific phosphodiesterase inhibitors (Ro20-1724, rolipram) or cyclic GMP-specific phosphodiesterase inhibitors (dipyridamole, zaprinast). After the establishment of the control concentration-inhibition relationship of 5-HT, smooth muscle preparations were treated with these inhibitors for 20 min, and then concentration-response curves of 5-HT were determined in the presence of the inhibitors. Pretreatment with rolipram (10 μM) or Ro 20-1724 (20 μM) itself decreased the amplitude of the spontaneous contraction (rolipram, 11 ± 2.3%, $n = 4$; Ro 20-1724, 5 ± 1.1%, $n = 4$). As shown in Figure 3, Ro 20-1724 (20 μM) shifted the concentration-response curve of 5-HT to the left and significantly decreased the EC_{50} value of 5-HT (control, 55 ± 9 nM; Ro 20-1724, 25 ± 2.4 nM, $n = 5$). Similarly, rolipram (10 μM) also potentiated the inhibitory response of 5-HT and shifted the concentration-response curve to the left (EC_{50} value, control, 86 ± 21 nM; rolipram, 40 ± 8.9, $n = 7$). On the other hand, dipyridamole (10 μM) or zaprinast (10 μM) did not change the spontaneous contraction or the concentration-

Table 2 Effects of some 5-hydroxytryptamine receptor antagonists on inhibition of spontaneous myometrial contractility by 5-hydroxytryptamine

		Control		*Treatment*	
Antagonists	*Receptor*	*EC_{50}* (nM)	*EC_{100}* (nM)	*EC_{50}* (nM)	*EC_{100}* (nM)
Pindolol (1 μM)	5-HT_1	49 ± 17	408 ± 160	51 ± 29	246 ± 48
Ketanserin (1 μM)	5-HT_2	116 ± 45	475 ± 150	150 ± 39	650 ± 175
MDL72222 (1 μM)	5-HT_3	60 ± 13	375 ± 184	77 ± 24	200 ± 50
Tropisetron (10 μM)	5-HT_3, 5-HT_4	57 ± 10	220 ± 44	58 ± 14	340 ± 150
GR113808 (10 μM)	5-HT_4	91 ± 23	375 ± 180	98 ± 28	360 ± 148

Values are means ± s.e.mean of 4–6 individual studies. After the inhibitory response of 5-hydroxytryptamine in the normal Krebs solution (control), had been obtained, smooth muscle strips were treated for 30 min with respective antagonists, and then 5-hydroxytryptamine-induced inhibition was examined. Each antagonist itself did not change the spontaneous contraction in the circular muscle of the pig uterus.

response curve of 5-HT. The EC_{50} values for 5-HT in the absence and presence of dipyridamole (10 μM) were 85 ± 19 nM and 86 ± 21 nM ($n = 4$), respectively, and those for 5-HT in the absence and presence of zaprinast (10 μM) were 69 ± 22 nM and 68 ± 28 nM ($n = 4$), respectively (Figure 3).

Table 3 Potency of 5-hydroxytryptamine receptor antagonists in antagonizing the 5-hydroxytryptamine-induced inhibition of the circular muscle of the porcine myometrium

Antagonists	pA_2	*Slope*
Methiothepin	8.05 ± 0.05	0.99 ± 0.12
Methysergide	7.92 ± 0.08	0.94 ± 0.12
Metergoline	7.40 ± 0.06	1.32 ± 0.2
Mianserin	7.08 ± 0.04	1.18 ± 0.29
Clozapine	7.06 ± 0.07	1.20 ± 0.2
Spiperone	6.86 ± 0.11	1.15 ± 0.15

Values are means $\pm$ s.e.mean of 4–8 individual studies. The antagonizing action of each antagonist was estimated by Schild plot analysis (Arunlakshana & Schild, 1959), and pA_2 values and the slope of the linear regression were calculated. The slope of each antagonist was not significantly different from unity.

Effect of 5-HT treatment on cyclic AMP production

The ability of 5-HT to generate cyclic AMP was examined in the porcine myometrial circular muscle. The resting cyclic AMP level in the circular muscle was 367 ± 50 pmol g^{-1} tissue wet weight ($n = 12$). Pretreatment with 5-HT increased tissue cyclic AMP content in a concentration-dependent manner (1 nM, 349 ± 60 pmol, $n = 6$; 10 nM, 470 ± 130 pmol, $n = 6$; 100 nM, 700 ± 158 pmol, $n = 4$; 1 μM, 1174 ± 100 pmol g^{-1} tissue wet weight, $n = 7$). Forskolin (3 μM), an activator of adenylate cyclase also increased the tissue cyclic AMP level (2634 ± 863 pmol g^{-1} tissue wet weight, $n = 5$). A non-selective inhibitor of phosphodiesterase, 3-isobutyl-1-methylxanthine (100 μM), elevated the cyclic AMP content 2.6 fold in the control tissue (966 ± 87 pmol g^{-1} tissue wet weight, $n = 4$). In the presence of 3-isobutyl-1-methylxanthine (100 μM), 5-HT (1 μM) also increased the tissue cyclic AMP content (3187 ± 57 pmol g^{-1} tissue wet weight, $n = 4$) (Figure 4).

Effects of forskolin and db-cyclic AMP

Forskolin (10 nM – 10 μM) applied at 5 min intervals in the organ bath inhibited the amplitude and frequency of

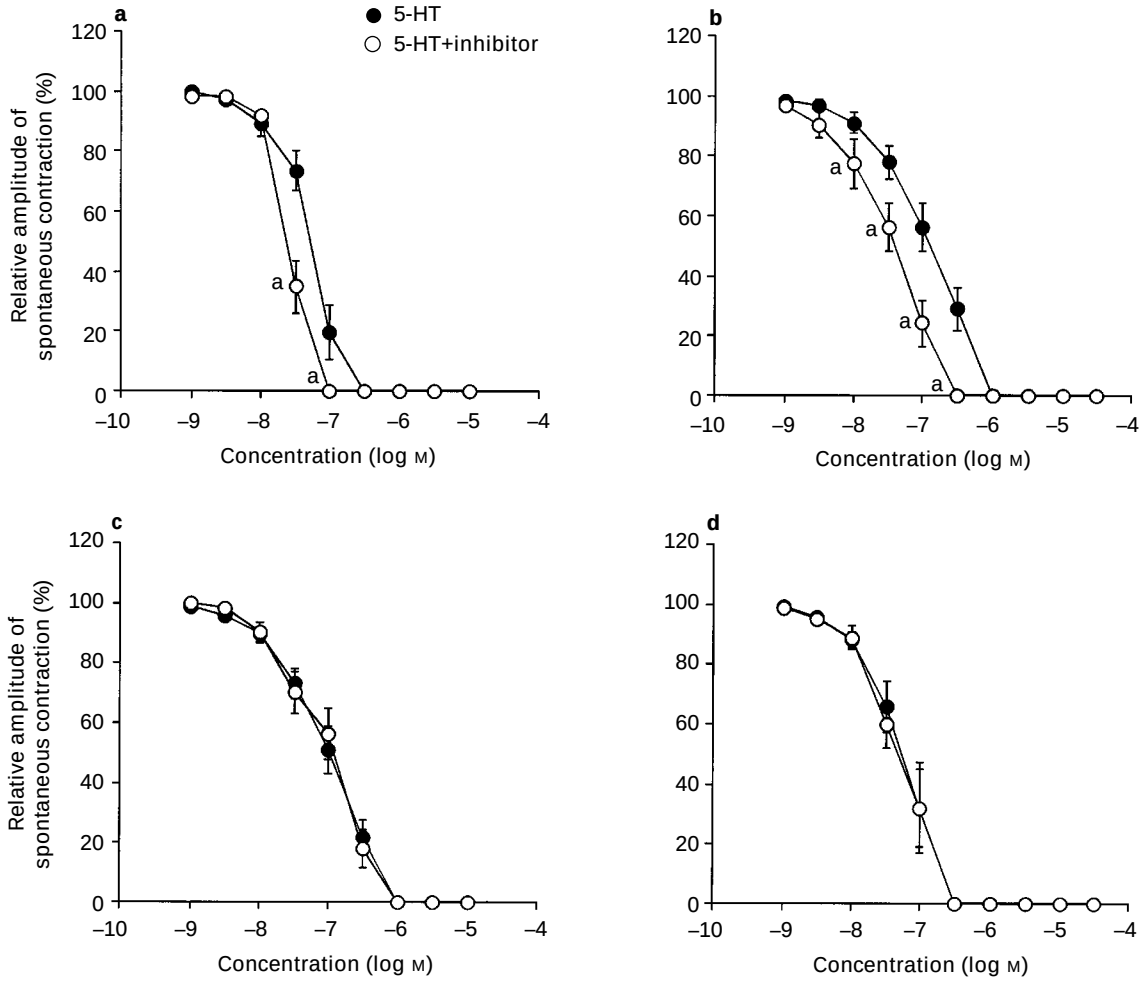

Figure 3 Effects of four phosphodiesterase inhibitors on 5-HT-induced inhibitory response in spontaneously contracting circular muscle of the porcine myometrium. After the control inhibitory response to 5-HT had been obtained, circular muscle preparations were treated with Ro 20-1724 (a, 20 μM), rolipram (b, 10 μM), dipyridamole (c, 10 μM) and zaprinast (d, 10 μM) for 20 min. Then, concentration-response curves for 5-HT in the presence of respective phosphodiesterase inhibitors were established. Ordinate scales: relative amplitude (control = 100%) of spontaneous contraction. Abscissa scales: concentration of 5-HT (logM). Points represent the means of 4 or more experiments with s.e.mean shown by vertical lines. [a]Significantly different from the corresponding control values, $P < 0.05$.

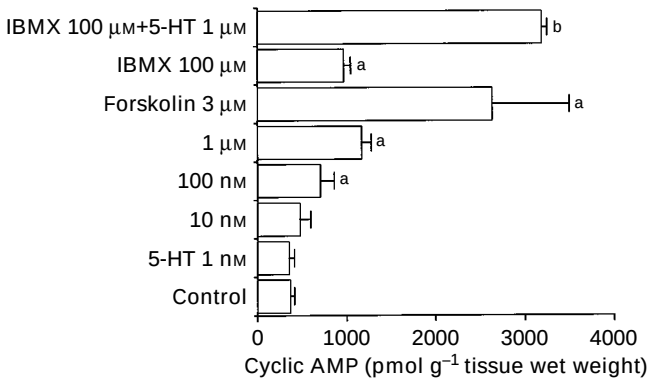

Figure 4 Effects of 5-HT and forskolin on cyclic AMP content of the porcine myometrium. Circular muscle strips were treated with four increasing concentrations of 5-HT (1, 10, 100 nM, 1 μM) or forskolin (3 μM) for 5 min. In some experiments, the effect of 5-HT (1 μM) was examined in the presence of 3-isobutyl-1-methylxanthine (IBMX, 100 μM). Ordinate scale: treatment of muscle preparation. Abscissa scale: cyclic AMP content (pmol g^{-1} tissue wet weight). Columns represent the means of 4 or more experiments with s.e.mean shown by horizontal lines. [a]Significantly different from the control (without 5-HT and IBMX), $P<0.05$. [b]Significantly different from the control with IBMX, $P<0.05$.

spontaneous contraction in the circular muscle in a concentration-dependent manner, and finally abolished the contractile activity completely. The EC_{50} value of forskolin was 2.6 ± 0.6 μM ($n=7$) for inhibition of the amplitude and 1.5 ± 0.2 μM ($n=7$) for decrease in the frequency.

Db-cyclic AMP (100 μM – 1 mM) caused inhibition of the spontaneous contraction (both amplitude and frequency), and the response took a long time (20 – 25 min) to reach maximum inhibition. Inhibition by db-cyclic AMP at 1 mM was $30\pm7.4\%$ ($n=6$) for the contraction amplitude and $57\pm6\%$ ($n=6$)(control, 10.2 ± 0.6 5 min^{-1}; 1 mM db-cyclic AMP, 4.4 ± 0.6 5 min^{-1}, $n=6$) for the contraction frequency.

Discussion

Past studies have shown that 5-HT causes contraction of the myometrium by activation of smooth muscle 5-HT_2 receptors linked to an increase in phosphoinositide turnover and influx of extracellular Ca^{2+} (Cohen *et al*., 1986; Cohen & Wittenauer, 1987). However, the present study in the pig myometrium indicated that 5-HT causes inhibition of the smooth muscle contractile activity and that there is a conspicuous muscle-layer related difference in sensitivity (the longitudinal muscle strip was 17 times less sensitive than the circular muscle to 5-HT). This suggests a species- and muscle layer-dependent difference in the action of 5-HT in the uterine smooth muscle. A muscle-layer related difference in responsiveness has been already demonstrated in the pig myometrium with regard to the responses to acetylcholine (contraction), noradrenaline (contraction), histamine (contraction) and isoprenaline (relaxation) and in cases, the longitudinal muscle always has been shown to have higher sensitivity than that of the circular muscle. Radioligand binding studies indicated that these differences were due to the heterogeneous distribution of muscarinic receptors, α-adrenoceptors, β-adrenoceptors and H_1-histamine receptors between muscle layers (longitudinal muscle > circular muscle, Taneike *et al*., 1991; 1994; 1995; Kitazawa *et al*., 1997). However, in the case of 5-HT, the circular muscle was found to be more sensitive than the longitudinal muscle. Spontaneous contractions of the longitudinal and circular smooth muscles were abolished by Ca^{2+}-free solution or verapamil, suggesting that Ca^{2+} influx through the L-type Ca^{2+} channel is essential for the generation of spontaneous contraction in both muscle layers. Therefore, it is unlikely that the muscle layer-dependent responsiveness of 5-HT is caused by different mechanisms for generation of spontaneous contraction. Clarification of the underlying mechanisms was beyond the scope of the present study. Muscle-layer related differences in the 5-HT receptor subtype (including distribution of receptors) and in the 5-HT uptake or degradation mechanisms might explain the different responsiveness to 5-HT.

Since 5-HT-induced inhibitory response in the circular muscle was not decreased by tetrodotoxin (neurone blocker), atropine (muscarinic receptor blocker), propranolol (β-adrenoceptor blocker) or guanethidine (adrenergic neurone blocker), the inhibitory 5-HT receptor was suggested to be present on smooth muscle cells of the pig myometrium. Recently, NO has been considered to be an inhibitory modulator in the myometrium, because NO gas or NO donor decreases and the NO synthase inhibitors stimulate myometrial contractility (Norman, 1996). However, an NO synthase inhibitor, L-NAME, failed to change the inhibitory response of 5-HT. Hence, the involvement of endogenous NO in 5-HT-induced inhibition can be excluded in the pig myometrium.

Of the 5-HT receptor agonists examined, 5-CT was the most effective agonist at causing an inhibitory response. Among the well-defined 5-HT receptor subtypes (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4), involvement of the 5-HT_1 receptor in the inhibition was first suggested, because 5-CT is known to be more potent than 5-HT at the 5-HT_1 receptor family (Boess & Martin, 1994; Hoyer *et al*., 1994). However, in the present experiment, oxymethazoline (a full agonist for 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors, with a pEC_{50} value of 7.35 – 7.73; a partial agonist for the 5-HT_{1C} receptor, with a pEC_{50} value of 6.57, Schoeffter & Hoyer, 1991) and dihydroergotamine (a potent 5-HT_{1D} receptor agonist, with a pK_i value of 9.87, Weinshank *et al*., 1992) did not cause any inhibitory response, even at 10 μM; and 8-OH-DPAT (a potent 5-HT_{1A} receptor agonist, pEC_{50} value of 8.2, Hoyer *et al*., 1994) was less effective in causing inhibition (pEC_{50} value of 4.56 in the present study). In the antagonist study, pindolol (a 5-HT_{1A} and 5-HT_{1B} receptor antagonist, with pK_b values of 7.9 and 6.8; Hoyer *et al*., 1994) and propranolol (a 5-HT_{1A} and 5-HT_{1B} receptor antagonist, with pK_b values of 6.6 and 6.9; Hoyer *et al*., 1994) had no effect on the inhibitory response of 5-HT at 1 μM. Recently, two newly cloned 5-HT_1 receptors (5-HT_{1E} and 5-HT_{1F}) have been described (Barone *et al*., 1993; Lovenberg *et al*., 1993). But, 5-CT has a lower affinity for 5-HT_{1E} and 5-HT_{1F} receptors (pK_i value, 5.1 – 6.14) than 5-HT does (pK_i value, 6.9 – 8.21) (Boess & Martin, 1994). The above results exclude the possible involvement of the 5-HT_1 receptor family in 5-HT-induced inhibition. Generally, 5-HT_1 receptors are coupled to the inhibition of adenylate cyclase activity and mediate the contraction of smooth muscle, decreasing intracellular cyclic AMP (Boess & Martin, 1994; Hoyer *et al*., 1994). These intracellular signal transductional mechanisms support the results of the present study that showed 5-HT_1 receptors do not mediate 5-HT-induced inhibition.

Neither 5-HT_2 nor 5-HT_3 receptors are involved in 5-HT-induced inhibition in the pig myometrium. This assumption was confirmed with the selective 5-HT_2 receptor (α-methyl-5-HT, pEC_{50} value = 7.3) and the 5-HT_3 receptor agonist (2-methyl-5-HT, pEC_{50} value = 7.7) (Hoyer *et al*., 1994), which had little effect on myometrial contractility, even at 10 –

100 μM. In addition, the results of the antagonist study, which showed that 1 μM ketanserin (a 5-HT_{2A} and 5-HT_{2C} receptor antagonist with pK_b value of 9.3 and 6.5), 1 μM MDL72222 (a 5-HT_3 receptor antagonist, pK_b value = 8.9) and 10 μM tropisetron (a 5-HT_3 receptor antagonist, pK_b value = 10.6) (Hoyer *et al*., 1994) did not change the inhibitory response of 5-HT, support the results of the agonist study.

In the present study, 5-MeOT, a relatively potent 5-HT_4 receptor agonist, caused inhibition and completely abolished spontaneous contractile activity. However, the pEC_{50} of 5-MeOT (5.28) in the pig myometrium was smaller than that of 5-MeOT towards the 5-HT_4 receptor (pEC_{50} value = 7.9, Baxter *et al*., 1991), and 5-HT is more potent than 5-CT on 5-HT_4 receptors (Hoyer *et al*., 1994), unlike in the pig myometrium. Moreover, other 5-HT_4 receptor agonists, cisapride (10 times more potent than 5-MeOT), BIMU-1 (20 times more potent) and BIMU-8 (25 times more potent) (Grossman *et al*., 1993), had almost no effect on spontaneous contraction, and 5-HT_4 receptor antagonist GR113808 (pK_i value = 9.5) and tropisetron (pK_i value = 7.2) (Grossman *et al*., 1993) failed to decrease the inhibitory response of 5-HT, even at 10 μM. These pharmacological findings suggest that the inhibitory 5-HT receptor in the smooth muscle of the pig uterus is not the 5-HT_4 subtype.

In the pig myometrium, 5-HT-induced inhibition was significantly potentiated by Ro 20-1724 and rolipram (cyclic AMP-specific phosphodiesterase inhibitors, Beavo & Reifsnyder, 1990), but not by zaprinast and dipyridamole (cyclic GMP-specific phosphodiesterase inhibitors, Beavo & Reifsnyder, 1990). In addition, 5-HT increased the tissue cyclic AMP level in the myometrium within a similar concentration range to that of the 5-HT-induced inhibition. These results suggest that 5-HT receptors present in the pig myometrium are positively coupled to adenylate cyclase and that stimulation of these receptors increases the intracellular cyclic AMP level. In smooth muscle tissues, cyclic AMP decreases the intracellular Ca^{2+} level through the inhibition of Ca^{2+} influx, acceleration of Ca^{2+} extrusion from the cells, and stimulation of Ca^{2+} sequestration to intracellular stores (Meisheri & Van Breemen, 1982; Abe & Karaki, 1989). Additionally, cyclic AMP causes a decrease in Ca^{2+} sensitivity of the contractile machinery (Nishimura & Van Breemen, 1989) and eventually relaxes the smooth muscle. Forskolin (an activator of adenylate cyclase that increases the tissue cyclic AMP level) and db-cyclic AMP (a membrane permeable cyclic AMP analogue) caused inhibition of the porcine myometrial contractility. Therefore, it is likely that accumulation of intracellular cyclic AMP by 5-HT causes the inhibition of myometrial muscle contractility. Of the 5-HT receptor subtypes, except for 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4, 5-HT_6 and 5-HT_7 receptors are known to be positively coupled with adenylate cyclase (Hoyer *et al*., 1994), and the activation of both receptors causes cyclic AMP accumulation (Bard *et al*., 1993; Plassat *et al*., 1993; Ruat *et al*., 1993a,b; Kohen *et al*., 1996). Although there is a lack of selective antagonists, these two 5-HT receptors can be discriminated by the potency order of 5-HT and 5-CT. The higher potency of 5-CT (pEC_{50} value = 8.76) compared to 5-HT (pEC_{50} value = 7.08) in the present study suggests that this effect is mediated by 5-HT_7 receptors, because 5-HT is more potent than 5-CT in the rat and human cloned 5-HT_6 receptors (displacement of [^{3}H]-5-HT or [^{3}H]-LSD binding; pK_i values, 6.14 for 5-CT and 7.19 for 5-HT in human, 6.6 for 5-CT and 7.3 for 5-HT in the rat 5-HT_6 receptor, Monsma *et al*., 1993; Kohen *et al*., 1996). On the other hand, 5-CT is more potent than 5-HT in the rat, mouse and human cloned 5-HT_7 receptors (displacement of [^{3}H]-5-

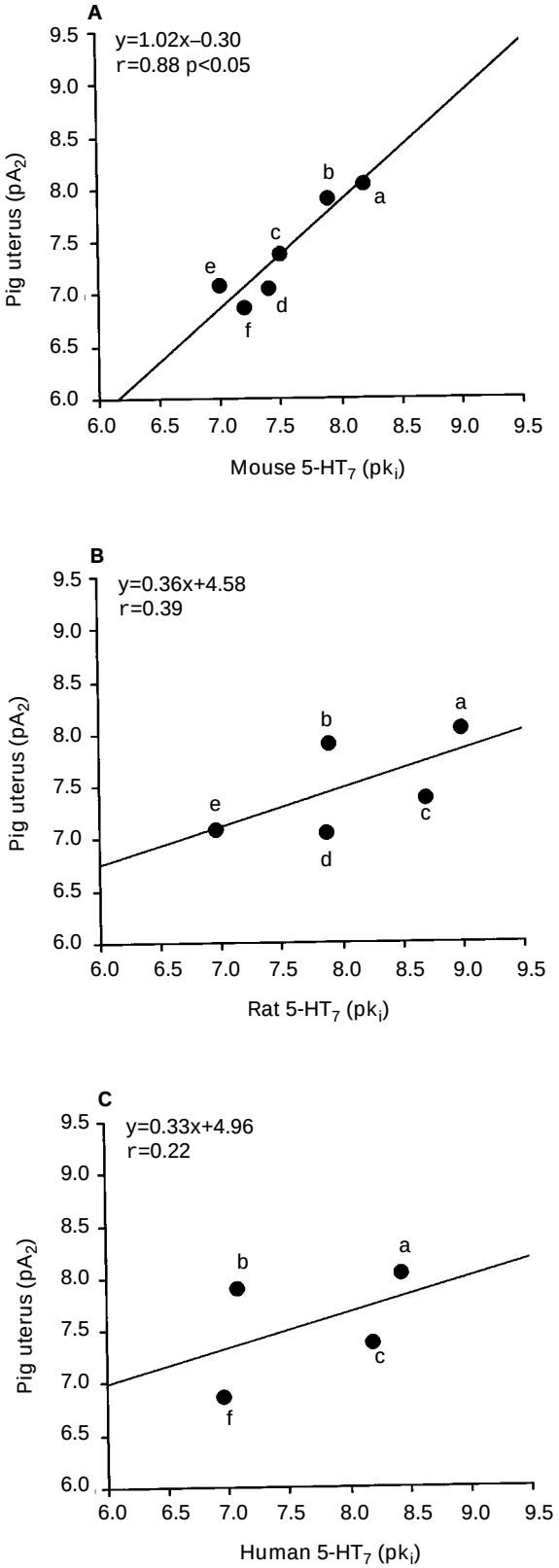

Figure 5 Correlations of antagonist affinity (pA_2 value against 5-HT) at the inhibitory 5-HT receptor in the porcine myometrium and antagonist affinity (pK_i value against [^{3}H]-5-HT or [^{125}I]-LSD binding) at cloned mouse (A), rat (B) and human (C) 5-HT_7 receptors. (a) Methiothepin, (b) methysergide, (c) metergoline, (d) clozapine, (e) mianserin, (f) spiperone. Significant ($P < 0.05$) correlation was found only with cloned mouse 5-HT_7 binding sites. Ordinate scales: pA_2 value of the antagonist against 5-HT-induced inhibition obtained in the present study. Abscissa scales: pK_i value of the antagonist against [^{3}H]-5-HT or [^{125}I]-LSD binding. Each pK_i value was referred by Plassat *et al*. (1993) in (A), Shen *et al*. (1993) in (B) and Bard *et al*. (1993) in (C). Each equation shows identity of the line.

HT binding; pK_i values, 9.0–9.48 for 5-CT and 8.09–8.74 for 5-HT, Bard *et al*., 1993; Ruat *et al*., 1993b; Shen *et al*., 1993). Binding and mechanical studies demonstrated that 5-MeOT and 8-OH-DPAT also act on 5-HT_7 receptors, and that the rank order of agonist potency is 5-CT > 5-HT ≥ 5-MeOT > 8-OH-DPAT (Ruat *et al*., 1993b; Shen *et al*., 1993; Leung *et al*., 1996; Terron, 1996). Although the pEC_{50} value of 5-MeOT (5.28) in the pig myometrium was conspicuously small compared with the pK_i value (8.75, 9.03) of displacement studies (Ruat *et al*., 1993b; Shen *et al*., 1993), the rank order of potency (5-CT > 5-HT > 5-MeOT > 8-OH-DPAT) obtained in the present study is nearly similar to that of the 5-HT_7 receptor. Low responsiveness of 5-MeOT on 5-HT_7 receptors have previously been found in a functional study in smooth muscle strips of the monkey jugular vein (pEC_{50} = 5.7, Leung *et al*., 1996) and the dog coronary artery (pEC_{50} = 4.42, Terron, 1996).

5-ht_5 is another type of 5-HT receptor, but its signal transduction mechanisms have not yet been clearly identified (Hoyer *et al*., 1994). 5-CT is more potent than 5-HT in the cloned mouse 5-ht_5 receptors (displacement of [^{125}I]-LSD binding; pK_i value, 6.6 for 5-HT and 7.4–7.8 for 5-CT, Plassat *et al*., 1992; Matthes *et al*., 1993), as is the case for cloned 5-HT_7 receptors. However, ergotamine, a potent agonist of the 5-ht_5 receptor (pK_i value = 8.4–8.5, Plassat *et al*., 1992; Matthes *et al*., 1993) did not cause inhibition of the pig myometrial contractility, even at 10 μM. Therefore, it is unlikely that 5-ht_5 receptors mediate the inhibitory response of 5-HT.

The conclusion that the 5-HT_7-type receptor mediates the inhibitory response of 5-HT is strongly supported by the results of the experiment with the antagonists. Previous studies in the monkey jugular vein, dog coronary artery and rat cultured astrocytes showed that nanomolar concentration of methiothepin antagonized the 5-HT_7 receptor-mediated relaxation (pK_b = 9.4–9.7, Leung *et al*., 1996; Terron, 1996) and cyclic AMP accumulation (pK_i = 7.98, Hirst *et al*., 1997). Additionally, [^{3}H]-5-HT binding at the cloned 5-HT_7 receptor was also displaced by a low concentration of methiothepin (pK_i = 8.2–9.42, Boess & Martin, 1994). In the present study, methiothepin acted at a nanomolar concentration (30–300 nM) and was the most potent competitive antagonist of the inhibitory response of 5-HT in the pig myometrium (pA_2 = 8.05). Other 5-HT receptor antagonists (methysergide, metergoline, clozapine, mianserin, spiperone) also inhibited 5-HT-induced inhibition in a competitive manner. The rank order of the inhibitory response (pA_2 value), methiothepin (8.05) > methysergide (7.92) > metergoline (7.4) > mianserin (7.08) ≥ clozapine (7.06) > spiperone (6.86), was approximately consistent with that of the pK_b values obtained in the dog coronary artery (Terron, 1996) and the pK_i values for the cloned 5-HT_7 receptor (Plassat *et al*., 1993). But the pA_2 value of methiothepin against 5-HT_7 receptor in the pig myometrium was considerably lower than that in the dog coronary artery (9.7, Terron, 1996), rabbit femoral vein (9.5, Martin & Wilson, 1995) and monkey jugular vein (9.7, Leung *et al*., 1996). A comparison of the pK_i values (displacement of [^{3}H]-5-HT binding) of the cloned 5-HT_7 receptors obtained in the rat (Shen *et al*., 1993), mouse (Plassat *et al*., 1993) and man (Bard *et al*., 1993) shows an interspecies difference in 5-HT_7 receptor pharmacology. Affinities for several compounds such as 5-CT, 5-MeOT and methiothepin were generally higher in the rat cloned 5-HT_7 receptors than in human and mouse cloned receptors, and the pK_i value of methysergide in the mouse 5-HT_7 was higher than that in the human 5-HT_7 receptor. A recent molecular biological study demonstrating the species-dependent diversity of the 5-HT_7 receptor molecule also supported the existence of interspecies variations of 5-HT_7 receptor (Heidmann *et al*., 1997). Therefore, the difference in pA_2 value of methiothepin between the various animal species might be partially due to the different structure of the 5-HT_7 receptor molecule. The correlations of antagonist pK_i values in the cloned rat, mouse and human 5-HT_7 receptors with the pig myometrial 5-HT_7 receptor were investigated to estimate similarity of 5-HT_7 receptor subtype (Figure 5). Significant ($P < 0.05$, $r = 0.88$) correlations were found with 5-HT_7 receptors of mice. The correlations with rat 5-HT_7 ($P > 0.05$, $r = 0.39$) and human 5-HT_7 receptors ($P > 0.05$, $r = 0.22$) were very low and not significant. The present results show the molecular similarity of the pig 5-HT_7 receptor to the mouse 5-HT_7 receptor. To confirm this working hypothesis, molecular cloning and characterization of pig 5-HT_7 receptors is needed.

In conclusion, 5-HT causes muscle-layer-dependent inhibition of the pig myometrial contractility through a direct action on smooth muscle. The circular muscle layer is more sensitive than the longitudinal muscle to 5-HT. The pharmacological profiles of 5-HT receptor agonists and antagonists, and positive coupling of the receptor to adenylate cyclase activity, indicate that the subtype of the 5-HT receptor mediating the inhibition is similar to the cloned 5-HT_7 type. 5-HT is thought to decrease contractility of the myometrium through an increase in tissue cyclic AMP.

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