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Smooth muscle layer-dependent distribution of 5-hydroxytryptamine$_7$ receptor in the porcine myometrium

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1 To analyse the mechanisms of muscle layer-dependent inhibition of porcine myometrial contractility by 5-hydroxytryptamine (5-HT), the effects of 5-HT, 5-carboxamidotryptamine(5-CT), 5-methoxytryptamine (5-MeOT), forskolin and cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) analogues on spontaneous and stimulant-induced contractions were examined in longitudinal (LM) and circular muscles (CM). In addition, accumulation of cyclic AMP by 5-HT and distribution of 5-HT$_7$ receptors in LM and CM layers were compared using biochemical and molecular approaches.

2 5-HT receptor agonists inhibited the spontaneous contractions of LM and CM (5-CT > 5-HT > 5-MeOT), but CM was more sensitive than was LM. The inhibition by the agonists was antagonized by methiothepin (100 nM).

3 Carbachol-, high-K^+-, histamine- and Ca^{2+}-induced contractions were inhibited by 5-HT with different responsivenesses (CM > LM). Even in the presence of 3-isobutyl-1-methylxanthine (IBMX), the inhibition by 5-HT in the CM was still more conspicuous than that in the LM.

4 Compared with the CM, the inhibition of spontaneous contraction by forskolin, dibutyryl-cyclic AMP and 8-bromo-cyclic AMP was marked in the LM.

5 5-HT (1 nM – 1 μM) increased the cyclic AMP in both muscle layers, but the increment in the CM was higher than that in the LM whether IBMX was present or not.

6 LM and CM layers contained a single class of [^{3}H]-5-CT binding sites with a similar K_d value (0.21 – 0.24 nM). However, B_{max} (5-HT$_7$ receptor concentration) in the CM (120.6 fmol mg^{-1} protein) was higher than that in the LM (30.4 fmol mg^{-1} protein).

7 The molecular study (reverse transcription polymerase chain reaction) demonstrated the expression of 5-HT$_7$ receptor mRNA in the CM was higher than that in the LM.

8 These results suggest that the muscle layer-dependent difference in inhibition by 5-HT is not restricted to spontaneous contraction but applies to various contractions in the porcine myometrium. Different inhibition of the contractility by 5-HT is caused by muscle layer-related accumulation of cyclic AMP (CM > LM), due to smooth muscle-layer dependent distribution (CM > LM) of 5-HT$_7$ receptors.

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Abbreviations: CM, circular muscle; 5-CT, 5-carboxamidotryptamine; cyclic AMP, cyclic adenosine, 3′, 5′-monophosphate; db-cyclic AMP, dibutyryl cyclic adenosine 3′, 5′-monophosphate; dNTP, deoxyribonucleoside 5′-triphosphate; 5-HT, 5-hydroxytryptamine; IBMX, 3-isobutyl-1-methylxanthine; LM, longitudinal muscle; 5-MeOT, 5-methoxytryptamine; 8-OH-DPAT, (±)-8-hydroxy-2-(di-n-propylamino)tetralin; PDE, phosphodiesterase; RT–PCR, reverse transcription polymerase chain reaction

Introduction

The 5-hydroxytryptamine (5-HT)$_7$ receptor is a new class of 5-HT receptor subtype, which was initially cloned from the central nervous system (Hoyer *et al*., 1994), and at present the 5-HT$_7$ receptor has been shown to mediate 5-HT-induced relaxation in peripheral visceral smooth muscles, such as the guinea-pig ileum (Carter *et al*., 1995), rabbit femoral vein (Martin & Wilson, 1995), monkey jugular vein (Leung *et al*., 1996), dog coronary artery (Terron, 1996), canine cerebral artery (Terron & Falcon-Neri, 1999) and human colonic circular muscle (Prins *et al*., 1999). In the porcine uterus, on the basis of pharmacological profiles of 5-HT receptor agonists and antagonists (ranking order of activity), it was demonstrated that the 5-HT$_7$ type receptor mediates the inhibition of myometrial contractility by 5-HT (Kitazawa *et al*., 1998). The 5-HT$_7$ receptor is thought to positively couple with adenylate cyclase by Gs (Boess & Martin, 1994; Hoyer *et al*., 1994), and it has been indicated that the activation of 5-HT$_7$ receptors increased cytoplasmic cyclic adenosine, 3′, 5′-monophosphate (cyclic AMP) production in cultured rat astrocytes (Hirst *et al*., 1997), cultured human vascular smooth muscle cells (Schoeffter *et al*., 1996) and isolated porcine myometrial strips (Kitazawa *et al*., 1998; 1999a). An increase in cyclic AMP in the smooth muscle cells decreased the cytosolic Ca^{2+} level and the Ca^{2+} sensitivity of the contractile elements, and eventually caused inhibition of the porcine myometrial contractility (Kitazawa *et al*., 1999a).

We previously investigated the autonomic innervation of the porcine uterus using longitudinal (LM) and circular muscle (CM) strips, and we found that the LM is mainly innervated by adrenergic nerves, whereas the CM is exclusively regulated by cholinergic nerves. Additionally, smooth muscle-layer related

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differences in responsiveness have been also demonstrated with regard to the mechanical responses of acetylcholine (contraction), noradrenaline (contraction), histamine (contraction) and isoproterenol (relaxation), and in all cases, LM is more sensitive to bioactive substances than is CM. Radioligand binding studies using respective radioligands indicated that these differences were due to the heterogeneous distributions of muscarinic, α_2-adrenaline, β-adrenaline and H_1-histamine receptors in the two muscle layers (LM > CM, Taneike *et al*., 1991; 1994; 1995; Kitazawa *et al*., 1997). These muscle layer-dependent variations in autonomic innervation and drug responsiveness were considered to reflect the inherent properties deriving from foetal-stage differentiation of the two muscle layers at the time of organogenesis and the different fuctional roles of LM and CM layers in uterine motility. In a recent study, although we found that the 5-HT$_7$ receptor mediated inhibition of the porcine myometrial contractility, we also demonstrated that there was a marked muscle-layer related difference in the effects of 5-HT on spontaneous contraction (CM was 17 times more sensitive to 5-HT than was LM, and the inhibition was almost complete in the CM; Kitazawa *et al*., 1998). The preference of 5-HT-induced inhibition in the CM layer is quite unique, different from acetylcholine, noradrenaline, isoproterenol and histamine. Since spontaneous contractions of both LM and CM were abolished by a Ca^{2+}-free solution or by verapamil, it is unlikely that the muscle layer-dependent responsiveness of 5-HT is caused by different mechanisms for generation of spontaneous contraction. Although the underlying mechanisms have not been clarified yet, several hypothesis, such as muscle-layer related differences in metabolic breakdown of 5-HT, in the 5-HT$_7$ receptors distribution or in the responsiveness of cyclic AMP (second messenger linked to 5-HT$_7$ receptor activation) might explain the different inhibitions by 5-HT in the LM and CM layers.

In the present experiments, we first characterized the muscle layer-related inhibition by 5-HT and next analysed the mechanisms underlying different inhibitions of contractility in the two muscle layers. For this purpose, LM and CM strips were isolated from nonpregnant porcine uteri and used in *in vitro* experiments to: (1) compare the effects of 5-carboxamidotryptamine (5-CT) and 5-methoxytryptamine (5-MeOT) on the spontaneous contraction of LM and CM strips; (2) determine the effects of 5-HT on high-K^+-, carbachol-, histamine- and Ca^{2+}-induced contractions; (3) determine the effects of 5-HT uptake blockers and pargyline on the inhibition by 5-HT; (4) determine the modification of 5-HT-induced inhibition in the presence of 3-isobutyl-1-methylxanthine (IBMX); (5) determine the effects of forskolin and membrane-permeable cyclic AMP analogues on the myometrial contractility; and (6) compare the effects of 5-HT on cytoplasmic cyclic AMP production in the LM and CM. The 5-HT$_7$ receptor distributions in the LM and CM were investigated using [^{3}H]-5-CT binding study and reverse transcription polymerase chain reaction (RT–PCR) for detecting the 5-HT$_7$ receptor-coding gene.

Methods

Tissue preparations

Fresh uteri, with the ovaries intact, from 120 sexually matured crossbred virgin gilts (about 6 months old) were obtained from a local abattoir and were used for experiments on the day of slaughter. The uteri were judged to be in proestrus according to the results of gross examination of the follicle size and to the appearance of the corpora lutca (McDonald, 1975). LM and CM 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 (Kitazawa *et al*., 1997; 1998), after removal of the endometrium, each muscle layer was cut through the muscle coat in either the LM and CM direction. The unwanted muscle layers were then removed from each muscle strip by meticulously cutting them away with fine scissors under a binocular microscope, thereby isolating the remaining LM and CM for experimental use. Both smooth muscle strips (10×1 mm) were suspended vertically in an organ bath (5 or 20 ml) containing 37°C Krebs solution (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 $95\%O_2 + 5\%CO_2$ (pH = 7.4). 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.

Experimental protocol and data analysis

After establishing steady spontaneous contractile activity of each muscle preparation (number of contractions 5 min^{-1}: LM, 4.8 ± 0.3, $n = 25$; CM, 10.2 ± 0.4, $n = 30$), 5-HT and 5-HT receptor agonists (5-MeOT, 5-CT) were applied cumulatively in an organ bath at 3-min intervals. The amplitude of the minimum spontaneous contraction during each 3-min cycle was expressed as a percentage of the spontaneous contraction obtained in the absence of agonists, and concentration-response curves were constructed. The EC_{50} value (concentration of agonists that caused half-maximum inhibition), EC_{100} value (concentration of agonists that abolished the spontaneous contractile activity), and the maximum inhibition were determined by least-squares non-linear regression analysis of the concentration-response curves.

The effect of 5-HT on the contraction induced by high-K^+, carbachol and histamine in both muscle layers were examined to clarify whether the muscle layer-dependent difference in the inhibition by 5-HT is restricted to spontaneous contraction of the porcine myometrium. After observing the reproducible control contractile responses of high-K^+ (50 mM), carbachol (1 μM) or histamine (10 μM), myometerial preparations were treated with increasing concentrations of 5-HT (1 nM–100 μM) for 5 min, and then the contractions induced by three different stimulants were tested. In some experiments, the effect of 5-HT on the Ca^{2+}-induced contraction was also investigated. In Ca^{2+}-free Krebs solution (EGTA, 1 mM) containing a high concentration of KCl (50 mM), application of Ca^{2+} (2.5 mM) caused a sustained contraction that developed slowly in both the LM and CM preparations. Relaxation of the Ca^{2+}-induced contraction by cumulatively applied 5-HT was first examined and compared in the LM and CM. Next, the effect of pretreatment with 5-HT on the Ca^{2+}-induced contraction was tested, and the muscle-layer dependent difference in inhibition by 5-HT was evaluated. A 50 mM high-K^+ solution was made by substituting an equimolar concentration of KCl for NaCl.

To compare the responsivenesses of cyclic AMP in the LM and CM layers, the effects of forskolin (an activator of adenylate cyclase), dibutyryl-cyclic AMP (db-cyclic AMP) and 8-bromo-cyclic AMP (membrane-permeable cyclic AMP analogues) on the spontaneous contractions were investigated. In these experiments, forskolin was applied at 5-min intervals, and cyclic AMP analogues were applied at 30-min intervals to obtain a sufficient inhibitory response.

Measurement of cyclic AMP level

Isolated fresh LM and CM strips weighing approximately 20–30 mg were used in the cyclic AMP study. After equilibration in warmed (37°C) and gassed (95% O_2 + 5% CO_2) Krebs solution for 1 h, the myometrial strips were exposed to increasing concentrations of 5-HT for 5-min. After incubation, the smooth muscle strips were quickly frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid solution with a Polytron. The homogenate was centrifuged at $2000 \times g$ for 20 min (two times) and the resulting supernatant was collected. After removing trichloroacetic acid in the supernatant by washing three times with water-saturated ether, cyclic AMP in the extract was assayed using an enzyme immunoassay kit (Amersham). In some experiments, the effects of 5-HT on tissue cyclic AMP production were examined in the presence of a non-selective phosphodiesterase (PDE) inhibitor, IBMX (100 μM). Tissue cyclic AMP levels were expressed as pmol g^{-1} tissue wet weight.

Radioligand binding study

To characterize the 5-HT$_7$ receptors in the porcine myometrium, we carried out a receptor binding assay using [^{3}H]-5-CT (37 MBq ml^{-1}, NEN Life Science Products Inc.). [^{3}H]-5-CT has been used previously to label 5-HT$_7$ receptors in transfected cells (To *et al*., 1995; Jasper *et al*., 1997) and has been shown to be a very potent 5-HT receptor agonist in the inhibition of porcine myometrial contractility (Kitazawa *et al*., 1998). The myometrial membrane of the porcine uterus was prepared by methods described previously (Kitazawa *et al*., 1997; 1999b). The isolated LM and CM preparations were cut into small pieces and homogenized in 10 volumes of ice-cold Tris-EDTA buffer solution (mM: Tris, 50; Na_2EDTA, 0.5; $MgSO_4$, 10; $CaCl_2$, 2; pargyline, 0.01, ascorbic acid, 0.1%, neutralized with HCl to pH 7.4 at 4°C) with the use of a Polytron. The homogenate was filtered through a single layer of nylon-mesh (pore size, 250 μm) and centrifuged at $1200 \times g$ for 20 min at 4°C, and the pellet was discarded. The supernatant was centrifuged at $80{,}000 \times g$ for 60 min at 4°C. The resulting pellets were washed twice and suspended in the Tris-EDTA buffer and used as a crude membrane preparation for determination of [^{3}H]-5-CT binding. Protein in the membrane preparation was measured according to the method of Lowry *et al*. (1951).

The membrane preparation (CM: 200 μg protein $tube^{-1}$) LM: 400 μg protein $tube^{-1}$) was incubated with increasing concentrations of [^{3}H]-5-CT (0.023–5.5 nM) in 500 μl of Tris-EDTA buffer (at 37°C for 60 min). The binding reaction was stopped by adding ice-cold Tris-EDTA buffer (4 ml), and the mixture was then filtered through a 0.3% polyethylenimine-presoaked glass fibre filter (GF/B; Whatman) under a vacuum to trap the crude membrane. The filter was then rapidly washed four times with 3 ml of ice-cold incubation buffer and placed in 20-ml glass scintillation vials with scintillation fluid (Scintisol EX-H, Dojin). Radioactivity trapped on the filter paper was measured by a liquid scintillation spectrometer (LCS-700; Aloka). Specific binding was calculated by subtracting non-specific binding from total binding. Non-specific binding was determined in the presence of 10 μM 5-HT. The maximum number of binding sites mg^{-1} protein (B_{max}, concentration of receptors) and the equilibrium dissociation binding constant (K_d) were estimated by Scatchard analysis. Lines of the best fit were calculated using linear regression by the method of least squares.

To characterize the [^{3}H]-5-CT binding site in the porcine myometrium, a displacement study was carried out using several 5-HT receptor agonists and antagonists. [^{3}H]-5-CT (0.25 nM) and crude membrane (CM: 200 μg protein, LM: 400 μg protein) were incubated with various concentrations of 5-HT receptor agonists (5-HT, 5-CT, 5-MeOT, (±)-8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)) and antagonists (clozapine, metergoline, methiothepin, methysergide, mesulergine, mianserin, spiperone) for 60 min at 37°C. After incubation, [^{3}H]-5-CT bound on membrane 5-HT receptors was separated by filtration through 0.3% polyethylenimine-presoaked glass fibre filters, and the radioactivity on the filters was measured. From the IC_{50} value (concentration of agents that inhibited 50% of the control binding obtained in the absence of displacers), the inhibition constant (K_i) was calculated by the equation of Cheng and Prusoff (1973): $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the concentration of [^{3}H]-5-CT (0.25 nM) used in the displacement study.

Measurement of 5-HT$_7$ receptor mRNA expression by RT–PCR

Fresh uterine LM and CM layers obtained from gilts at a local abattoir were quickly frozen in liquid nitrogen and stored at −80°C until use. Frozen tissues were scraped off with a cold fine razor, and small tissue fragments were collected in a polyethylene tube filled with 1.5 ml of TRIzolTM Reagent (Total RNA isolation Reagent, GIBCO BRL) and homogenized by a Polytron. Total RNA was then prepared from the homogenates each of the porcine uterine LM and CM. Reverse transcription was performed according to the following procedure. First, the total RNA (5 μg) and 200 ng of random hexamer in RNase-free H_2O were heated for 10 min at 70°C and incubated for 10 min at 25°C for annealing. This sample was incubated for 60 min at 42°C using 150 units of SUPERSCRIPT II RNase H-reverse transcriptase (GIBCO BRL) in a solution of a final volume of 20 μl that contained Tris-HCl (pH 8.3) (mM) 50, KCl 40, $MgCl_2$ 6, dithiothreitol 1, and individual deoxyribonucleoside 5′-triphosphate (dNTP) 1. After this reverse transcription procedure, the reaction mixture was added to 80 μl of distilled water and used for PCR. The primer sequence was designed on the basis of the conserved regions between human and rat 5-HT$_7$ receptor sequences (Bard *et al*., 1993; Ruat *et al*., 1993; Shen *et al*., 1993; Ullmer *et al*., 1995). The 5-HT$_7$ receptor primers were as follows: sense primer, 5′-AGGATTTTGGCTACACGATC-3′, corresponding to rat 5-HT$_7$ receptor nucleotides 713–732; and antisense primer, 5′-CTTCCGGTTGATATTCCGGTA-3′, corresponding to rat 5-HT$_7$ receptor nucleotides 1215–1236. The reverse transcription cDNA products were amplified using GeneAmp 2400 (Perkin Elmer) in a total volume of 20 μl of a solution containing dNTPs (mM) 250, $MgCl_2$ 2, Tris-HCl (pH 8.8) 10, 5 pmol μl^{-1} each of the sense and antisense primers, and 0.25 units of Taq DNA polymerase (TaKaRa Taq: TaKaRa and AmpliTaq Gold: ABI). The thermal cycler program used for PCR amplification was denaturing for 60 s at 94°C, annealing for 60 s at 57°C, extending for 70 s at 72°C. The amplification was performed for 32 cycles. Thereafter, reaction mixtures were heated at 72°C for 7 min. Amplified products were separated on 8 M urea-denatured-5% polyacryamide gels in Tris-borate/EDTA buffer at 300 V for 90 min, visualized with 1 μg ml^{-1} ethidium bromide, and imaged by a FluoroImager 595. After confirming the presence of the 5-HT$_7$ receptor-coding gene in LM and CM preparations of the porcine myometrium, expression of the mRNA coding 5-HT$_7$ receptor was estimated using a quantitative PCR procedure. First, each

cDNA obtained from the LM and CM preparations was diluted gradedly (2, 4, 8, 16 and 32 fold), and then PCR amplification was performed using 5 pmol μl^{-1} of porcine β-actin primers (sense primer, 5′-GTGCGGGACATCAAGGAGAA-3′; antisense primer, 5′-TGTCCACGTCGCACTTCAT-3′) to estimate the amount of cDNA. The PCR products were separated by electrophoresis on a 2% agarose gel and analysed using ethidium bromide staining. Based on the band intensities photographed by Kodak Digital Science (Kodak), the relative amount of cDNAs in each muscle layer sample was determined. Second, for comparison of the expressions of 5-HT$_7$ receptors in the two muscle layers, PCR was performed using the same amounts of cDNAs, which were prepared on the basis of the expression of β-actin. In this study, the PCR reaction was stopped at 22 to 34 cycles (every two cycles), and the relationships between the PCR cycle and intensity of PCR products were compared in the LM and CM layers.

Chemicals

The following chemicals were used in this experiment: 8-bromo cyclic adenosine, 3′, 5′-monophosphate (8-bromo-cyclic AMP, Sigma), 5-carboxamidotryptamine maleate (5-CT, RBI), clozapine (RBI), cocaine hydrochloride (Takeda), corticosterone (Sigma), dibutyryl cyclic adenosine 3′, 5′-monophosphate (db-cyclic AMP, Sigma), forskolin (Wako), 5-hydroxytryptamine creatinine sulphate (5-HT, Wako), (±)-8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, RBI), 3-isobutyl-1-methylxanthine (IBMX, Aldrich), mesulergine hydrochloride (RBI), metergoline (RBI), methiothepin mesylate (RBI), 5-methoxytryptamine hydrochloride (5-MeOT, Sigma), methysergide hydrochloride (RBI), mianserin hydrochloride (RBI), pargyline hydrochloride (RBI), spiperone hydrochloride (RBI) and verapamil hydrochloride (Wako). Drugs except for 8-bromo-cyclic AMP, clozapine, corticosterone, metergoline, forskolin and spiperone were dissolved in distilled water. Corticosterone, 8-bromo-cyclic AMP, metergoline and clozapine were dissolved in dimethylsulphoxide, and forskolin and spiperone were dissolved in ethanol and diluted by Krebs solution or Tris-EDTA buffer. The maximum concentrations of dimethylsulphoxide and ethanol in the organ bath solution or incubation buffer was set below 0.2 and 0.1%, respectively, and these concentrations did not change the spontaneous contracting activity, muscle tonus of the porcine myometrium, or [^{3}H]-5-CT binding.

Statistical analysis

The results of the experiments are expressed as means ±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

Comparison the effects of 5-HT, 5-MeOT and 5-CT on spontaneous contraction in the LM and CM

As previously reported (Kitazawa *et al*., 1998), 5-HT (10 nM–1 μM), 5-MeOT (100 nM–100 μM) and 5-CT (0.1–10 nM) concentration-dependently inhibited the amplitude of spontaneous contraction in the CM layers. The EC_{50} and EC_{100} values of 5-HT, 5-MeOT and 5-CT were 76±20 and 232±42 nM ($n=6$), 6.7±1.7 and 45±11 μM ($n=14$), 2.2±0.5 and 20±5.8 nM ($n=14$), respectively. In spontaneously contracting LM strips, 5-HT also caused inhibition of the contractility, but the inhibition was obviously weaker ($EC_{50}=2.7\pm0.6$ μM; maximum inhibition = 57±7%, $n=25$) than that in the CM (Figure 1A). Similar to the case of 5-HT, 5-MeOT and 5-CT inhibited the spontaneous contraction of the LM strips, but the inhibition was considerably weaker than that observed in the CM preparations. The EC_{50} values and the maximum inhibition of 5-MeOT and 5-CT were 16±6 μM and

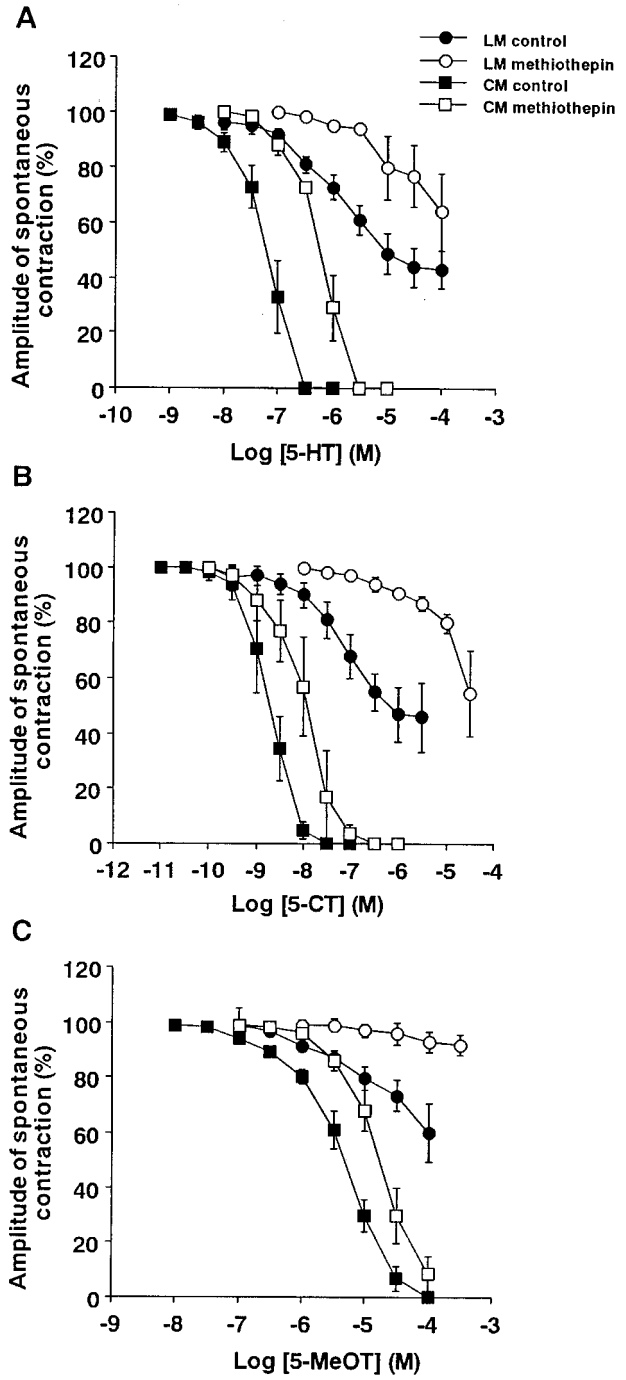

Figure 1 Effects of 5-HT, 5-CT and 5-MeOT on spontaneous contractile activity of the porcine uterus. The concentration-response curves for 5-HT (A), 5-CT (B) and 5-MeOT (C) in the LM and CM. Treatment with methiothepin (100 nM) inhibited the responses of 5-HT, 5-CT and 5-MeOT and caused a rightward shift of the concentration-response curves. Ordinate: relative amplitude of the spontaneous contraction (control = 100%). Abscissa: concentration of agonists (logM). Points represent the means of four or more experiments with s.e.mean shown by vertical lines.

40±10% (n=8), and 150±60 nM and 54±13% (n=9), respectively (Figure 1B,C). As indicated in Figure 1, the inhibitory responses of 5-HT, 5-MeOT and 5-CT were antagonized by methiothepin (100 nM) in a competitive manner, and the concentration-response curves shifted rightward in both LM and CM preparations. The pA_2 value of methiothepin against the respective 5-HT receptor agonists was estimated to be 7.8–8.1 in the CM and 8.0–8.6 in the LM from the equation of Van Rossum (1963).

One possible explanation for the different inhibitory actions by 5-HT in the LM and CM layers is smooth muscle-dependent differences in 5-HT uptake mechanisms, because the autonomic innervation in the LM and CM layers is different (Taneike *et al*., 1994). Therefore, the effects of 5-HT on the spontaneous contraction were examined in the presence of cocaine (30 μM), a neural uptake inhibitor, and corticosterone (30 μM), a non-neural uptake inhibitor. Treatments with both uptake inhibitors did not markedly change the inhibition by 5-HT in either muscle layer (Figure 2). We also investigated the responses of 5-HT in the absence and presence of pargyline (30 μM), a monoamine oxidase inhibitor. As indicated in Figure 2, pargyline did not modify the inhibition by 5-HT in either muscle layer. The above results indicated that the CM layer was still more sensitive to 5-HT than LM was in spite of the presence of uptake and monoamine oxidase inhibitors.

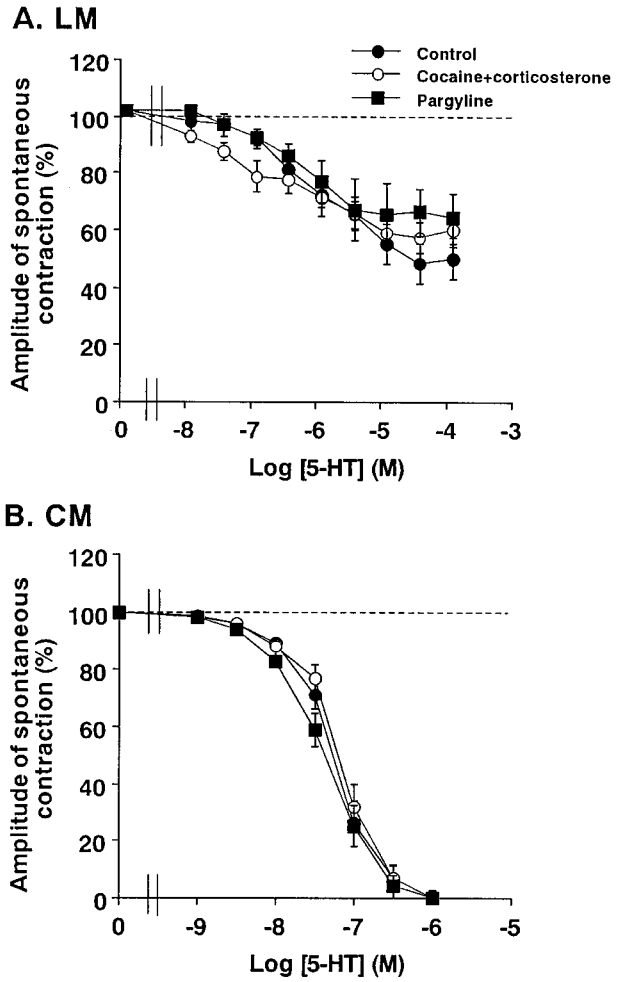

Figure 2 Effects of 5-HT uptake inhibitors and pargyline on the 5-HT-induced inhibition of spontaneous contraction in the porcine uterus. The symbols show the concentration-response curves for 5-HT in the absence (control) and presence of cocaine (30 μM) plus corticosterone (30 μM) or pargyline (30 μM) in the LM (A) and CM (B). Ordinate: relative amplitude of the spontaneous contraction (control=100%). Abscissa: concentration of 5-HT (logM). Points represent the means of four or more experiments with s.e.mean shown by vertical lines.

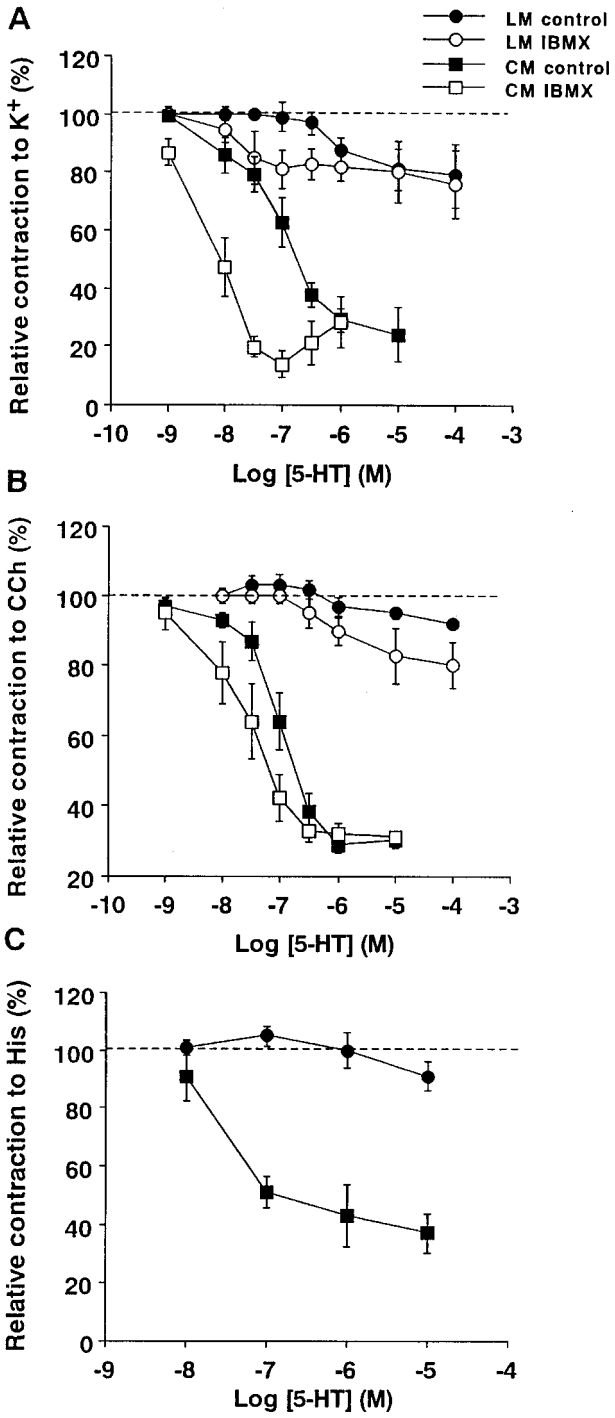

Figure 3 Inhibition of high-K^+-, carbachol- and histamine-induced contractions by 5-HT in the porcine uterus. The symbols show the concentration-response curves for the effects of 5-HT on high-K^+ (A, 50 mM), carbachol (B, 1 μM) and histamine (C, 10 μM)-induced contractions in the LM (LM control) and CM preparations (CM control). In the presence of IBMX (100 μM), the concentration-response curves for 5-HT were also made in both muscle layers (LM IBMX, CM IBMX). Ordinate: relative amplitude of contractions (control=100%). Abscissa: concentration of 5-HT (logM). Points represent the means of four or more experiments with s.e.mean shown by vertical lines.

Effects of 5-HT on high-K^+-, carbachol-, histamine- and Ca^{2+}-induced contractions

Pretreatment with 5-HT (for 5 min) inhibited the high-K^+-induced contraction in a concentration dependent manner, but the inhibition was conspicuously stronger in the CM than in the LM. The EC_{50} values and maximum inhibition by 5-HT were 91 ± 25 nM and $76 \pm 9.2\%$ ($n=7$) in the CM, and 540 ± 190 nM and $23 \pm 5.8\%$ ($n=9$) in the LM, respectively (Figure 3A). Carbachol (1 μM) causes contraction of the myometrium through activation of muscarinic M_3 receptors on smooth muscle cells (Kitazawa *et al*., 1999b). The inhibitory effect of 5-HT on the contraction induced by carbachol was also marked in the CM but was considerably weak in the LM (inhibition by 100 μM 5-HT, $6 \pm 1.4\%$, $n=4$) (Figure 3B). Histamine is another contractile stimulant in the porcine myometrium (Kitazawa *et al*., 1997). Therefore, inhibition of histamine (10 μM)-induced contraction by 5-HT was also tested. Relative amplitudes of the histamine-induced contraction in the presence of 10 nM, 100 nM, 1 μM and 10 μM 5-HT (for 5 min) were 101 ± 2.6, 105 ± 3.4, 100 ± 6.0 and $91 \pm 5.2\%$ ($n=4$) in the LM, and 91 ± 8.8, 51 ± 5.3, 43 ± 10.7 and $37 \pm 6.6\%$ ($n=4$) in the CM, respectively (Figure 3C).

Application of Ca^{2+} (2.5 mM) caused a sustained contraction of both LM and CM strips in the Ca^{2+}-free solution containing a high concentration of KCl (50 mM). These contractions were abolished by verapamil (10 μM), indicating the involvement of a L-type Ca^{2+} channel in this contraction. First, the relaxations of the precontracted LM and CM strips by 5-HT were compared. As indicated in Figure 4A, 5-HT relaxed both smooth muscle layers, but the relaxation by 5-HT was stronger in the CM than in the LM. The EC_{50} values and the maximum relaxation (percentage of Ca^{2+}-induced contraction just before application of 5-HT) were 8.8 ± 1.2 nM and $103 \pm 6.2\%$ ($n=5$) in the CM, and 400 ± 160 nM and $32 \pm 10\%$ ($n=5$) in the LM, respectively. Next, we examined the effects of pretreament with 5-HT on the Ca^{2+}-induced contraction in both muscle layers. In the CM strips, 5-HT inhibited the Ca^{2+}-induced contractions ($EC_{50}=16 \pm 6.4$ nM, $n=5$) and finally abolished them completely at 300 nM – 1 μM ($EC_{100}=500 \pm 210$ nM, $n=5$). On the other hand, the Ca^{2+}-induced contraction in the LM preparation was only slightly

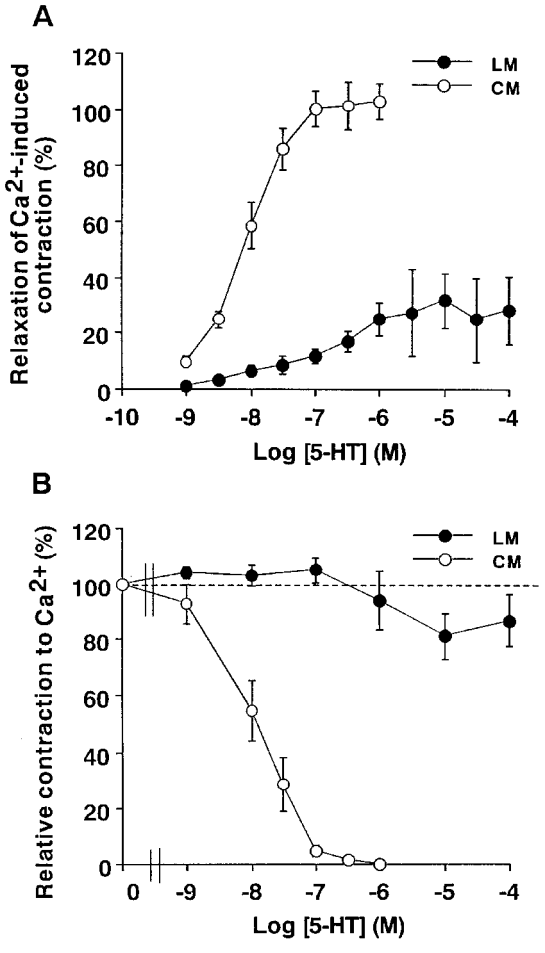

Figure 4 Inhibition of Ca^{2+}-induced contraction by 5-HT in the porcine uterus. (A) In Ca^{2+}-free Krebs solution (EGTA, 1 mM) containing a high concentration of KCl (50 mM), Ca^{2+} (2.5 mM) caused a sustained contraction in both muscle layers. Relaxation by cumulatively applied 5-HT was observed both in the LM and CM. Ordinate: relaxation is expressed as a percentage of the contraction just before application of 5-HT. (B) Effects of pretreatment with 5-HT on Ca^{2+}-induced contraction in the LM and CM. Ordinate: relative amplitude of Ca^{2+}-induced contraction (control = 100%). Abscissa: concentration of 5-HT (logM). Points represent the means of four or more experiments with s.e.mean shown by vertical lines.

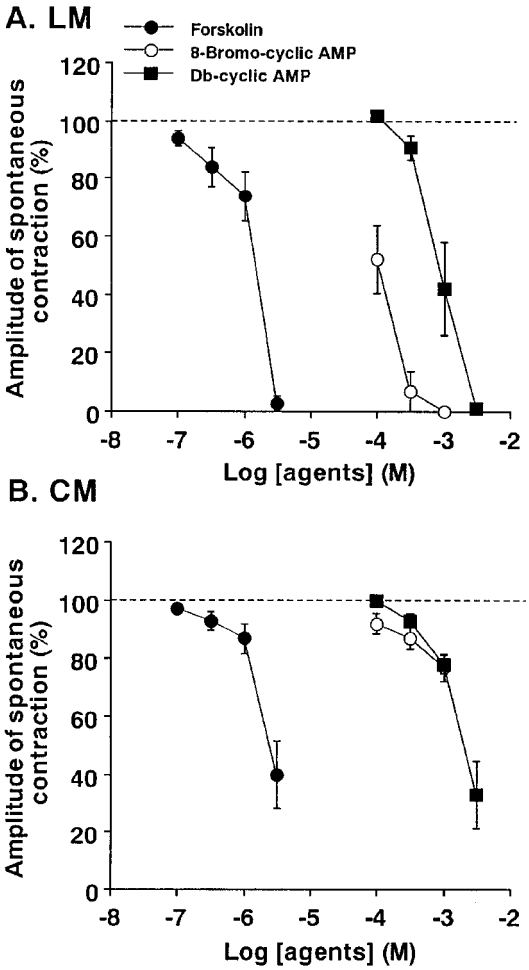

Figure 5 Effects of forskolin and cyclic AMP analogues on spontaneous contractile activity of the porcine uterus. The concentration-response curves for forskolin, 8-bromo-cyclic AMP and db-cyclic AMP in the LM (A) and CM (B). After establishing the control spontaneous contraction, each muscle preparation was treated with forskolin (for 5 min), 8-bromo-cyclic AMP (for 30 min) or db-cyclic AMP (for 30 min), respectively. Ordinate: relative amplitude of the spontaneous contraction (control = 100%). Abscissa: concentration of agents (logM). Points represent the means of four or more experiments with s.e.mean shown by vertical lines.

decreased by 5-HT. The maximum inhibition was 20±8.2% (n=5) at 10 μM 5-HT (Figure 4B).

Comparison of the inhibitory effects of forskolin, db-cyclic AMP and 8-bromo-cyclic AMP

In our previous study, we demonstrated that activation of the 5-HT$_7$ receptor in the porcine myometrium stimulated cytoplasmic cyclic AMP production and that this second messenger mediated the inhibitory response of 5-HT (Kitazawa *et al*., 1999a). Therefore, the difference between the responsivenesses of cyclic AMP in different muscle layers could explain the muscle layer-dependent inhibition by 5-HT. We compared the inhibitory effects of forskolin, db-cyclic AMP and 8-bromo-cyclic AMP on the spontaneous contractions in the LM and CM layers. As indicated in Figure 5, in contrast with the 5-HT-induced inhibition, the inhibitory responses of forskolin, db-cyclic AMP and 8-bromo-cyclic AMP were more conspicuous in the LM than in the CM.

Inhibitory effect of 5-HT in IBMX-treated myometrial strips

Since the effect of cyclic AMP as a second messenger of 5-HT$_7$ receptor activation is terminated by its hydrolysis to 5′-AMP through the action of PDE, it is likely that muscle layer-dependent inhibitory effects of 5-HT are due to the difference of PDE activities in the two muscle layers. Therefore, we examined the effect of 5-HT on the high-K^+-induced contraction in the presence of IBMX, a non-selective PDE inhibitor. IBMX (100 μM) shifted the concentration-response curves of 5-HT to the left without conspicuous change in the maximum inhibition in both muscle layers. However, in spite of the presence of IBMX, CM was more sensitive to the inhibition by 5-HT than was LM (EC_{50}: LM, 20±4 nM, n=5; CM, 8±2.1 nM, n=4; maximum inhibition: LM, 24±11.8%, n=5; CM, 72±8.7%, n=4) (Figure 3A). In the case of carbachol-induced contraction, IBMX also potentiated the inhibition by 5-HT in both muscle layers, but the inhibition by 5-HT was still marked in the CM strips (Figure 3B).

Effect of 5-HT on cytoplasmic cyclic AMP level

The resting cyclic AMP level in the LM was 450±41 pmol g^{-1} tissue wet weight (n=16), which was not significantly different from that in the CM (427±36 pmol g^{-1} tissue wet weight, n=13). 5-HT (1 nM–1 μM for 5 min) concentration-dependently increased the tissue cyclic AMP level of the CM (1 nM, 410±46 pmol, n=7; 10 nM, 559±122 pmol, n=7; 100 nM, 770±69 pmol, n=8; 1 μM, 1330±180 pmol, n=11) (Figure 6). The increments in tissue cyclic AMP by 1, 10, 100 nM and 1 μM 5-HT were calculated to be 0.96, 1.31, 1.8 and 3.11, respectively. On the other hand, although 5-HT also stimulated tissue cyclic AMP production in the LM (10 nM, 528±83 pmol, n=6; 100 nM, 597±50 pmol, n=8; 1 μM, 633±50 pmol, n=11), the increments by 5-HT in the LM (10 nM, 1.17; 100 nM, 1.33; 1 μM, 1.41) were considerably small compared to those in the CM (Figure 6).

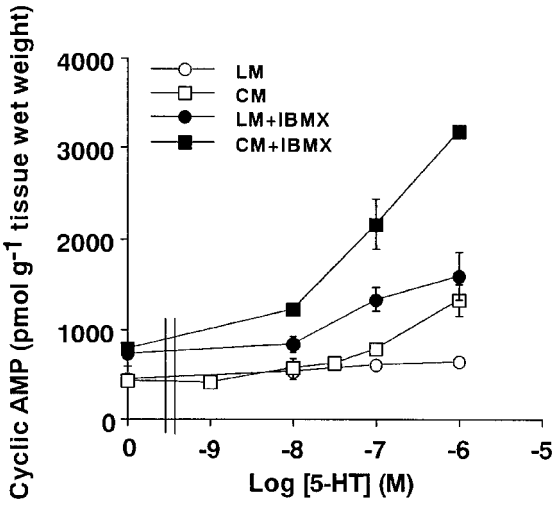

Figure 6 Comparison of the cyclic AMP production by 5-HT in the LM and CM of the porcine uterus. The symbols show the control concentration-response curves for 5-HT in the LM and CM. Using 3-isobutyl-methylxanthine (IBMX, 100 μM)-treated uterine strips, the concentration-response relationship for 5-HT were also examined (LM+IBMX, CM+IBMX). Ordinate: cyclic AMP (pmol g^{-1} tissue wet weight). Abscissa: concentration of 5-HT (logM). Points represent the means of four or more experiments with s.e.mean shown by vertical lines.

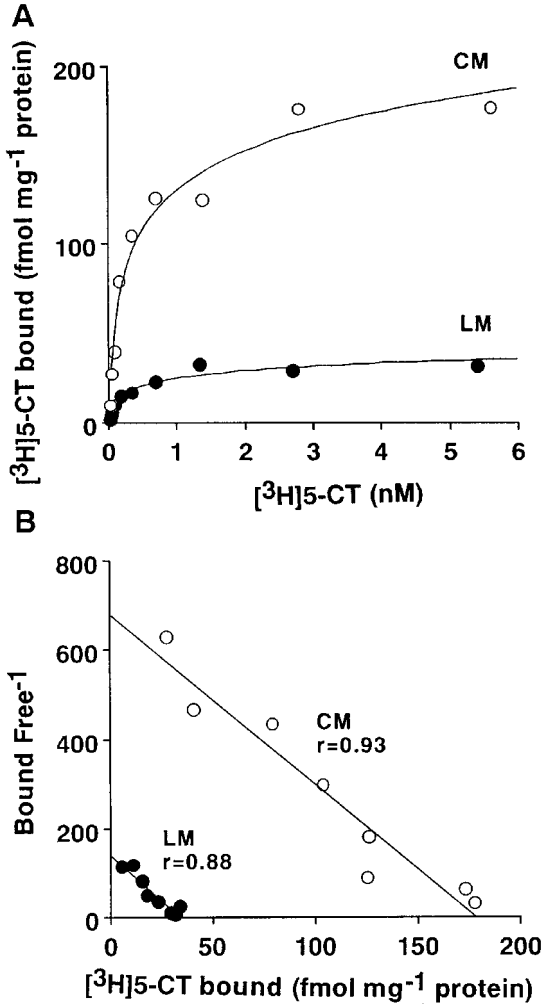

Figure 7 [^{3}H]-5-CT binding to porcine uterine LM and CM. (A) Crude membrane preparations obtained from LM and CM were incubated with increasing concentrations of [^{3}H]-5-CT for 60 min at 37°C. Specific binding was determined as the difference between total and non-specific bindings obtained in the presence of 10 μM 5-HT. Abscissa: [^{3}H]-5-CT concentration (nM). Ordinate: specific [^{3}H]-5-CT bound (fmol mg^{-1} protein). (B) Scatchard plot of the binding data in the LM and CM. The line was determined by linear regression analysis. The points shown are from one of six (CM) or eight (LM) similar experiments.

Table 1 Pharmacological characterization of [^{3}H]-5-CT binding sites in the longitudinal (LM) and circular muscles (CM) of the porcine uterus

			pK$_i$ value			
	LM	*CM*	*Mouse 5-HT$_7$*	*Rat 5-HT$_7$*	*Human 5-HT$_7$*	*Guniea-pig 5-HT$_7$*
Host cell			*COS-7*	*COS-7*	*COS-7*	*CHO-K1*
Radioligand			*[^{3}H]-5-HT*	*[^{3}H]-5-HT*	*[^{3}H]-5-HT*	*[^{3}H]-5-CT*
Reference			*Plassat* et al. *(1993)*	*Shen* et al. *(1993)*	*Bard* et al. *(1993)*	*To* et al. *(1995)*
Agonists						
5-HT	8.68±0.05	8.74±0.09	8.3	8.82	8.09	9.6
5-CT	9.57±0.22	9.70±0.07	9.0	9.8	9.03	9.7
5-MeOT	8.54±0.08	8.64±0.06	8.2	9.24	8.3	9.3
8-OH-DPAT	7.16±0.06	7.26±0.21	6.6	7.46	6.33	7.4
Antagonists						
Methiothepin	8.53±0.37	8.38±0.37	8.2	9.42	8.43	8.4
Metergoline	7.40±0.13	7.30±0.21	7.5	8.21	8.19	8.2
Methysergide	7.62±0.11	7.85±0.08	7.9	7.87	7.08	7.7
Mesulergine	7.75±0.13	7.85±0.13	7.6	7.68	7.74	7.8
Clozapine	7.50±0.07	7.17±0.13	7.4	7.4	–	7.3
Mianserin	7.25±0.22	7.26±0.20	7.0	7.43	–	7.0
Spiperone	7.30±0.18	6.95±0.07	7.2	–	6.96	7.3
Correlation		0.96	0.89	0.86	0.71	0.83
Slope		1.07	0.88	1.07	0.89	1.07
Significance coefficient		$P<0.001$	$P<0.001$	$P<0.01$	$P<0.05$	$P<0.01$

Data are the means±s.e.mean of four or more seperate experiments or mean values from radioligand displacement studies with recombinant 5-HT$_7$ receptors expressed in mammalian cells. Correlations to the pK$_i$ of the LM were determined and the coefficients, slopes of regression lines and significance are indicated.

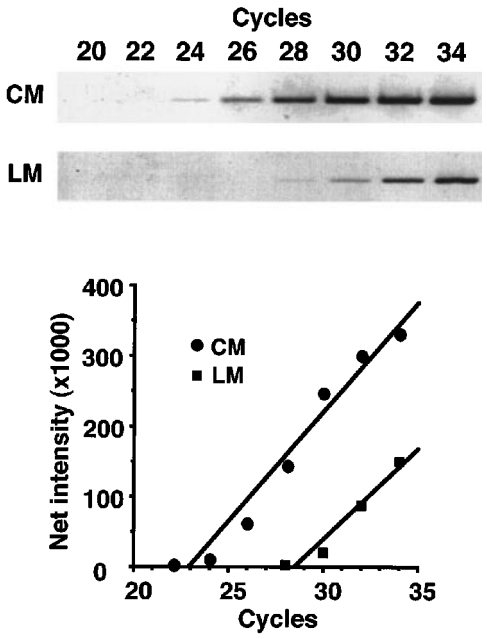

Figure 8 Comparison of 5-HT$_7$ receptor expression levels in the LM and CM of the porcine uterus. The upper part shows a typical electrophoresis pattern of PCR products amplified with primers specific for the 5-HT$_7$ receptor. The reaction was terminated at 20–34 cycles (every two cycles). The PCR amplified products possessing the expected size (523 base pairs) were detected in both muscle layers (30–34 cycles). The lower part indicates the relationships between net intensity and PCR cycles (see Methods). Compared to PCR products (as a density) at a linear amplification, a marked difference was observed (CM > LM). Ordinate: net intensity. Abscissa: Number of PCR cycles.

The effects of 5-HT on the cytoplasmic cyclic AMP production were also investigated in the presence of IBMX (100 μM) to determine whether the difference in cyclic AMP level is due to different ability of 5-HT for cyclic AMP production. Treatment with IBMX (100 μM) itself increased the cyclic AMP level in both smooth muscles (LM, 712±193 pmol g^{-1} tissue wet weight, $n=5$; CM; 843±99 pmol g^{-1} tissue wet weight, $n=6$). In the presence of IBMX, 5-HT also increased the tissue cyclic AMP level in both muscles, but the increments in the CM (10 nM, 1.57; 100 nM, 2.76; 1 μM, 4.05) were conspicuously higher than those in the LM (10 nM, 1.15; 100 nM, 1.7; 1 μM, 2.2) (Figure 6).

[^{3}H]-5-CT binding sites in the LM and CM

A [^{3}H]-5-CT binding study (saturation and displacement) was performed to examine the distributions of 5-HT$_7$ receptors in the LM and CM layers. As indicated in Figure 7A, specific binding of [^{3}H]-5-CT to the crude membrane increased upon increment of the free concentration of the ligand and reached a plateau at 3–5 nM. Scatchard plots of saturation binding parameters of both muscle layers fitted one straight line (correlation of regression line, $r=0.88-0.93$) and revealed the presence of a single class of binding sites. From the regression line, K_d and B_{max} values were estimated to be 0.24±0.03 nM and 30.4±2.9 fmol mg^{-1} protein ($n=8$) in the LM, and 0.21±0.02 nM and 120.6±14.8 fmol mg^{-1} protein ($n=6$) in the CM, respectively (Figure 7B). Specific binding for [^{3}H]-5-CT at a concentration close to the K_d value (0.3 nM) was 75–80% of the total binding in the LM and 90–95% of total binding in the CM, respectively. Hill plots of the binding data were linear with Hill coefficients of 1.03±0.05 ($n=8$) in the LM and 0.99±0.01 ($n=6$) in the CM. These values were not significantly different from unity, indicating that there was no positive or negative cooperativity in binding profiles.

Inhibitory effects of four 5-HT receptor agonists and seven 5-HT receptor antagonists on the [^{3}H]-5-CT binding were examined to confirm the identity of the binding site. In the membrane preparation obtained from the LM, all agonists and antagonists concentration-dependently inhibited the specific [^{3}H]-5-CT binding and finally completely displaced it. The ranking order of potency (pK$_i$) for agonists in competition for the specific binding was 5-CT (9.57) > 5-HT (8.68) > 5-MeOT

(8.54) > 8-OH-DPAT (7.16). In the case of antagonists, the ranking order was methiothepin (pK_i, 8.53) > mesulergine (7.75) > methysergide (7.62) > clozapine (7.5) > metergoline (7.4) > spiperone (7.3) > mianserin (7.25) (Table 1). We also examined the displacement effects of these agents on specific [^{3}H]-5-CT binding in the CM membrane preparations and compared them with those in the LM membrane. 5-HT receptor agonists and antagonists also concentration-dependently inhibited the specific binding in the CM, and the pK_i value for each agent was comparable with that obtained in the LM preparations. The correlation between pK_i values of 11 agents in the LM and that in the CM was 0.96 (significant, $P < 0.001$), and the slope of the regression line was 1.07, which was not significantly different from 1.0 (Table 1). The pK_i values obtained in the porcine uterus were compared with those of agents obtained at recombinant 5-HT_7 receptors of different species. The affinity (pK_i) data generated in the present study for the porcine uterus were significantly close to those values for the rat (Shen *et al*., 1993), mouse (Plassat *et al*., 1993), human (Bard *et al*., 1993) and guinea-pig (To *et al*., 1995) 5-HT_7 receptors (Table 1).

5-HT_7 receptor-coding gene expression in the LM and CM

RT–PCR was performed with total RNA extracted from the porcine uterus (LM and CM) and the specific primers for the porcine 5-HT_7 receptor. Agarose gel electrophoresis of the PCR amplified products indicated the band of expected size (523 base pairs) in both muscle layers (see cycles 32 and 34, Figure 8). The amplified products were extracted, cloned, and sequenced, and the identity of the cDNA was confirmed. The results indicate that the mRNA of 5-HT_7 receptor is expressed in both the LM and the CM of the porcine myometrium. Next, PCR was performed using the same amounts of cDNA obtained from the LM and CM, and the reaction was stopped from 20 to 34 cycles (every two cycles) to compare relative levels of the 5-HT_7 receptor in the LM and CM. Figure 8 shows the typical results of the agarose gel electrophoresis of PCR products. The PCR amplified products could be detected from 26 cycles when CM-derived cDNA was used. However, on the other hand, the amplified products could be detected from 30 cycles in the case of LM-derived cDNA. The band intensities of 5-HT_7 receptors in both muscle layers were plotted against the PCR cycles, and the regression line was obtained. The lines with similar slopes of the product-cycle relationships suggested that the amplification of the 5-HT_7 receptor occurred with similar efficacy between 30–34 cycles in the LM and CM preparations. These relationships clearly indicated that the expression of the 5-HT_7 receptor-coding gene is higher in the CM than in the LM of the porcine uterus (Figure 8).

Discussion

The 5-HT_7 receptor, initially cloned from the central nervous system (Hoyer *et al*., 1994), is a new class of 5-HT receptor subtype and has been shown to mediate the 5-HT-induced relaxation of the guinea-pig ileum (Carter *et al*., 1995), rabbit femoral vein (Martin & Wilson, 1995), monkey jugular vein (Leung *et al*., 1996), dog coronary artery (Terron, 1996), canine cerebral artery (Terron & Falcon-Neri, 1999) and human colonic circular muscle (Prins *et al*., 1999). Recently, we demonstrated that the 5-HT_7 receptor mediates the inhibition of the porcine myometrial contractility through stimulation of cyclic AMP production (Kitazawa *et al*., 1998; 1999a). However, there was a marked muscle layer-related difference in the inhibitory effects of 5-HT on spontaneous contraction (CM > LM, Kitazawa *et al*., 1998). To clarify the mechanisms of muscle layer-dependent inhibition by 5-HT, several possibilities (smooth muscle layer-dependent differences in the metabolic breakdown of 5-HT, in the 5-HT_7 receptors distribution, in the responsiveness of cyclic AMP and in the PDE activity) were analysed. The results of the present study demonstrated that (1) the muscle layer-dependent inhibition by 5-HT is not restricted to spontaneous contraction but applies to various contractions (high-K^+, carbachol, histamine and Ca^{2+}), and that (2) the muscle layer-dependent susceptibility of myometrial contractility to 5-HT is caused by muscle layer-related synthesis of cyclic AMP, reflecting the heterogeneous distribution (LM < CM) of the 5-HT_7 receptor positively coupled with adenylate cyclase.

Although we reported weak inhibition of spontaneous contractions by 5-HT in the LM layers, the 5-HT receptor subtype has not been clearly characterized yet (Kitazawa *et al*., 1998). We first investigated the inhibitory effects of other 5-HT receptor agonists and the antagonistic action of methiothepin in the LM. 5-CT and 5-MeOT also inhibited the spontaneous contraction of the LM, but the maximal inhibition was only about 50%, similar to that of the 5-HT-induced inhibition. The rank order of three agonists in the LM (5-CT > 5-HT > 5-MeOT) was consistent with that observed in the CM (Kitazawa *et al*., 1998, and the present study), and similar to that reported in 5-HT_7 receptor-mediated action (Leung *et al*., 1996; Terron, 1996). Methiothepin is a potent antagonist of 5-HT_7 receptors in the monkey jugular vein, dog coronary artery, rat cultured astrocytes and porcine uterine CM (Leung *et al*., 1996; Terron, 1996; Hirst *et al*., 1997; Kitazawa *et al*., 1998). Methiothepin antagonized the inhibition by 5-HT, 5-CT, 5-MeOT in the porcine uterine LM with a similar pA_2 (8.0–8.6) to that obtained in the CM (7.8–8.1). These pharmacological results support the involvement of the 5-HT_7 receptor in the inhibitory response of 5-HT in the LM, as that in the CM.

In general, 5-HT is taken up by both neural and non-neural components and is mainly metabolized by a monoamine oxidase. Therefore, one plausible cause for the different inhibitory effects of 5-HT in the LM and CM layers is different degradation ability of 5-HT in smooth muscle layers. However, the concentration-response curve of 5-HT in both smooth muscle layers was not changed by the treatment of uptake inhibitors or pargyline, indicating that the different inhibitory responses of 5-HT in the LM and CM layers could not be explained by the muscle-layer dependent different degradation ability of 5-HT.

Analysis of the inhibitory mechanisms of 5-HT suggests a following cascade of events in the porcine myometrium: 5-HT_7 receptor activation→activation of Gs→increase in adenylate cyclase activity→increase in cytoplasmic cyclic AMP→decrease in intracellular Ca^{2+} concentration and Ca^{2+}-sensitivity of the contractile elements→inhibition of smooth muscle contractility (Kitazawa *et al*., 1998; 1999a). Therefore, the muscle layer-related differences in the cyclic AMP responsiveness or in the PDE activity might explain the high responsiveness of the CM to 5-HT. However, inhibition by forskolin and membrane-permeable cyclic AMP analogues (8-bromo-cyclic AMP, db-cyclic AMP) in the CM was weak compared with that in the LM. Because the muscle layer-related difference in the response of cyclic AMP was not consistent with that of 5-HT, it is difficult to explain the muscle layer-related inhibition of 5-HT from the different responsive-

nesses of cyclic AMP in the two muscle layers. Our previous study indicated that muscle layers had different PDE activities in the porcine myometrium (LM > CM, Nakagoshi *et al*., 1991). It is thought that the weak inhibitory response of 5-HT is due to a high activity of PDE in the LM. In the present experiment, IBMX, a non-selective PDE inhibitor, potentiated the inhibition by 5-HT in both muscle layers, but the CM was still more sensitive to 5-HT than was the LM. Therefore, the muscle layer-dependent inhibition by 5-HT would not be caused by different activities of PDE in the two muscle layers.

Muscle layer-related difference in the responsiveness to some bioactive agents (acetylcholine, noradrenaline, histamine and isoproterenol) has already been demonstrated in the porcine myometrium, and in all cases, the LM has been shown to have higher sensitivity than that of the CM. Radioligand binding studies indicated that these differences were due to the heterogeneous distributions of muscarinic, α_2-adrenaline, β-adrenaline and H_1-histamine receptors in the two muscle layers (LM > CM, Taneike *et al*., 1991; 1995; Kitazawa *et al*., 1997; 1999b). [^{3}H]-5-CT binding study was carried out using crude membrane preparations of the LM and CM to clarify the distribution of 5-HT$_7$ receptors. This ligand exhibits nanomolar affinity at 5-HT$_1$, 5-HT$_2$, 5-ht$_5$ and 5-HT$_7$ receptors (Boess & Martin, 1994; Zifa & Fillion, 1992). However, in the present study, a comparison of pK_i values and rank order of affinities for 5-HT receptor agonists (5-CT, 5-HT, 5-MeOT and 8-OH-DPAT) and antagonists (methiothepin, mesulergine, methysergide, metergoline, mianserin, clozapine and spiperone) in the porcine myometrium with those reported by Bard *et al*. (1993), Plassat *et al*. (1993), Shen *et al*. (1993) and To *et al*. (1995) for [^{3}H]-5-HT or [^{3}H]-5-CT binding to several species of 5-HT$_7$ receptor-transfected cells indicated highly significant correlations (Table 1). These results demonstrated that [^{3}H]-5-CT labels the 5-HT$_7$ receptor in the porcine myometrial membrane. [^{3}H]-5-CT binds to a single site in membrame preparations of both LM and CM in a saturable manner, and its K_d values (CM, 0.21 nM; LM, 0.24 nM) are consistent with that demonstrated in 5-HT$_7$ receptors of the guinea-pig brain (0.76 nM, To *et al*., 1995) and 5-HT$_7$ receptor-expressed cultured cells (0.28 nM, Jasper *et al*., 1997). Although the K_d values in the LM and CM were almost the same, there was a significant muscle layer-related difference in the B_{max} of 5-CT binding sites (CM, 120.6 fmol mg^{-1} protein; LM, 30.4 fmol mg^{-1} protein). This result clearly indicated the heterogeneous distribution of 5-HT$_7$ receptors (LM: CM = 1 : 4) in the porcine myometrium. The RT – PCR studies demonstrated the presence of 5-HT$_7$ receptor mRNA and confirmed that the porcine myometrium has the capacity to express this receptor subtype. The results of quantitative PCR analysis indicated the smooth muscle-dependent expression of 5-HT$_7$ receptor-coding mRNA in the porcine myometrium and supported the results of functional and [^{3}H]-5-CT binding studies.

In the cyclic AMP study, 5-HT increased cytoplasmic cyclic AMP accumulation in a concentration-dependent manner in both muscle layers. However, there was a conspicuous muscle layer-dependent difference in the production of cyclic AMP (LM < CM) in response to 5-HT regardless of the presence of IBMX. The heterogeneous distribution of 5-HT$_7$ receptors might explain the different ability of the muscle layers to produce cyclic AMP in response to 5-HT. Although the responsiveness of cyclic AMP was slightly higher in the LM than in the CM, owing to the high production of cyclic AMP in the CM, 5-HT caused a marked inhibition in the CM compared to that in the LM.

In conclusion, the present results suggest that the muscle layer-dependent difference in inhibition by 5-HT is not restricted to spontaneous contraction but applies to various types of contraction in the porcine myometrium. Different inhibition of the contractility by 5-HT is caused by muscle layer-related accumulation of cyclic AMP (CM > LM), probably due to smooth muscle layer-dependent heterogeneous distribution (CM > LM) of 5-HT$_7$ receptors. This heterogeneous distribution of 5-HT$_7$ receptors is quite interesting and different from the distributions of other receptors (muscarine M_3 receptor, histamine H_1 receptor, β and α_2-adrenaline receptors) in the porcine uterus. Therefore, 5-HT and 5-HT$_7$ receptors might play an important physiological role in the regulation of CM motility in the porcine uterus.

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