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5-HT_7 receptor and β_2-adrenoceptor share in the inhibition of porcine uterine contractility in a muscle layer-dependent manner

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Abstract

To compare the inhibition of uterine contractility mediated by β-adrenoceptors and 5-HT_7 receptors, the effects of catecholamines and 5-HT on spontaneous contractions were examined in longitudinal and circular muscles isolated from three different regions (cornu, corpus and cervix) of the non-pregnant proestrus porcine uterus. In addition, the distribution of β-adrenoceptors between muscle layers was characterized by means of adenylate cyclase activity assay, cyclic AMP assay and [^{3}H]dihydroalprenolol binding studies. In the cornu, isoprenaline, adrenaline and noradrenaline inhibited the spontaneous contraction of longitudinal and circular muscles but longitudinal muscle was more sensitive to catecholamines than was circular muscle. The inhibitory response to isoprenaline was antagonized by propranolol (300 nM) or (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol (ICI 118,551; 100 nM). The rank order of potency was isoprenaline ≧ adrenaline>noradrenaline. The β_2-adrenoceptor-selective agonist, clenbuterol, was more potent than xamoterol (β_1-selective) and (±)-4-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid (BRL 37344; β_3-selective) to inhibit the spontaneous contraction of longitudinal muscles. Isoprenaline increased adenylate cyclase activity in both muscle layers, but the activity in the longitudinal muscle was greater than that in the circular muscle. Cyclic AMP production by isoprenaline was also more conspicuous in the longitudinal muscle than in the circular muscle. Although both muscle layers contained a single class of [^{3}H]dihydroalprenolol binding sites with similar K_d values (longitudinal muscle, 3.1 ± 0.94 nM, n = 4; circular muscle, 2.4 ± 0.73 nM, n = 4), B_{max} in the longitudinal muscle (175.7 ± 32.8 fmol/mg protein, n = 4) was significantly higher than that in the circular muscle (53.1 ± 5.1 fmol/mg protein, n = 4). As previously reported [Br. J. Pharmacol. 130 (2000) 79], 5-HT also inhibited the spontaneous contraction of both muscle layers from the cornu and the 5-HT_7 receptor antagonist, 2a-[4-(4-phenyl-1,2,3,6-tetrahydropyridyl)butyl]-2a,3,4,5-tetrahydro-benzo[cd]indol-2(1*H*)-one (DR4004; 100 nM, n = 4) blocked the 5-HT-induced inhibition of spontaneous contractions in the circular muscles, and reversed the less marked inhibition in the longitudinal muscles. In other regions of the uterus (corpus and cervix), 5-HT inhibited the spontaneous contraction of the circular muscles but contracted the longitudinal muscle strips. On the other hand, isoprenaline caused muscle layer-dependent inhibition (longitudinal muscle>circular muscle) in both regions, and the responsiveness tended to increase toward the cervix. In conclusion, β_2-adrenoceptors are present heterogeneously in the porcine uterus (longitudinal muscle>circular muscle) and share the inhibition of uterine contractility with 5-HT_7 receptors in a layer-dependent manner (longitudinal muscle: β_2-adrenoceptors, circular muscle: 5-HT_7 receptors).

Keywords: 5-HT_7 receptor; β_2-Adrenoceptor; Uterus porcine; Contraction spontaneous; Relaxation

1. Introduction

The 5-HT_7 receptor is a new class of 5-HT receptor that couples positively with adenylate cyclase and transduces signals through an intracellular increase in cyclic AMP (Hoyer et al., 1994). Functional studies have indicated that 5-HT_7 receptors mediate the 5-HT-induced relaxation in peripheral visceral smooth muscles, such as the guinea-pig ileum (Carter et al., 1995), monkey jugular vein (Leung et al., 1996), canine coronary artery (Terron, 1996), canine cerebral artery (Terron and Falcon-Neri, 1999), human colonic circular muscle (Prins et al., 1999) and rat jejunum (McLean and Coupar, 1996). Recently, we demonstrated that 5-HT_7 receptors are present in the uterus of non-pregnant proestrus pigs and inhibit myometrial contractility in a muscle layer-dependent manner (circular muscle>lon-

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gitudinal muscle). The smooth muscle layer-dependent difference in inhibition by 5-HT is caused by muscle layer-dependent expression of 5-HT_7 receptors (Kitazawa et al., 1998, 2000).

It is well known that catecholamines influence uterine motility in several mammals (pig, cow, guinea-pig, cat, rat and human) through activation of α (excitatory)-and β (inhibitory)-adrenoceptors (Digges, 1982; Bülbring and Tomita, 1987; Wray, 1993; Taneike et al., 1995, 1999). Although β-adrenoceptors have been subdivided into β_1, β_2 and β_3 subtypes (Bylund et al., 1994), Pennefather and Molenaar (1986) first identified the myometrial β-adrenoceptor as β_2 type and demonstrated that this receptor was distributed homogeneously between the circular and longitudinal muscles of guinea-pig uterus. The presence of β_2-adrenoceptors in the myometrium has also been reported in humans and rats (Breuiller et al., 1987; Maltier and Legrand, 1988). We reported earlier that β-adrenoceptors are present in the porcine uterus and that isoprenaline inhibits the contractility of cornu myometrium in a muscle layer-dependent manner (longitudinal muscle>circular muscle) through activation of these receptors (Taneike et al., 1991). However, the β-adrenoceptor subtype involved in inhibition has not yet been identified. In addition, because the muscle layer-dependent preference of isoprenaline- and 5-HT-induced inhibition was different in the cornu, systemic comparison of the inhibition by both agents in the other regions of the uterus (corpus and cervix) could help to clarify the relationships of the 5-HT_7 receptor and β-adrenoceptor-mediated inhibitory mechanisms in the porcine uterus.

In the present study, first, to identify the β-adrenoceptor subtype, the effects of β-adrenoceptor agonists (catecholamines and subtype-selective agonists) on spontaneous contraction and the effects of β-adrenoceptor antagonists on the response to isoprenaline were investigated in the longitudinal and circular muscles of the uterine cornu. The distribution of β-adrenoceptors between muscle layers was also determined biochemically by assay of adenylate cyclase activity and tissue cyclic AMP, and by [^{3}H]dihydroalprenolol binding. Second, inhibitory responses mediated by β-adrenoceptors were compared with those mediated by 5-HT_7 receptors in three different regions of the porcine uterus (cornu, corpus and cervix).

2. Materials and methods

2.1. Tissue preparations

Fresh uteri, with the ovaries intact, from 100 sexually mature crossbred virgin gilts (about 6 months old) were obtained from a local abattoir and were used for experiments on the day of slaughter. The animals were judged to be in proestrus according to the results of gross examination of follicle size and according to the appearance of the corpora lutea (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., 1991; Kitazawa et al., 2000), after removal of the endometrium, each muscle layer was cut through the muscle coat in either the longitudinal and circular muscle directions. The unwanted muscle layers were then removed from each strip by meticulously cutting them away with fine scissors under a binocular microscope, thereby isolating the remaining muscle strips for experimental use. In some experiments, longitudinal and circular muscle strips from the uterine corpus and cervix regions were also prepared, and the responsiveness to inhibitory agents was compared among regions. Smooth muscle preparations (10×1 mm) were suspended vertically in an organ bath (5 ml) containing 37 °C Krebs solution (NaCl, 118.4 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM and glucose, 11.5 mM) 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 muscle preparations. The muscle strips were loaded at 0.2-g initial tension and allowed to equilibrate for 60 min.

2.2. Contraction study and data analysis

After steady spontaneous contractile activity of cornual muscle preparations was obtained (longitudinal muscle: 6.7 ± 0.7/10 min, $n = 11$; circular muscle: 18 ± 1.4/10 min, $n = 13$), isoprenaline, 5-HT and other agents were applied cumulatively to the organ bath at 5-min intervals with 0.5 log concentration increments. The amplitude of the minimum spontaneous contraction during each 5-min cycle was expressed as a percentage of the spontaneous contraction obtained in the absence of agents, and concentration-response curves were made. The EC_{50} value (concentration of an agonist that caused half-maximum inhibition), EC_{100} value (concentration of an agonist that abolished the spontaneous contraction) and maximum inhibition were determined by least-squares non-linear regression analysis of the concentration-response curves. To determine apparent pA_2 estimates of β-adrenoceptor and 5-HT receptor antagonists, concentration-response curves for isoprenaline or 5-HT were made in the absence and presence of each antagonist. The apparent pA_2 value was then calculated by the Van Rossum equation (Van Rossum, 1963): $pA_2 = -\log$ [antagonist concentration] + log (CR-1), where CR is a ratio of the EC_{50} value (EC_{50} value in the presence of antagonist/EC_{50} value in the absence of antagonist).

To investigate the region-related differences in the responsiveness to inhibitory agents, the effects of 5-HT and isoprenaline on spontaneous contraction were also examined in corpus and cervical preparations. Comparison of cornu preparations showed that the frequencies of spontaneous contraction in the corpus were not different (longitudinal muscle: 7.2 ± 0.6/10 min, $n = 11$; circular muscle:

15.9 ± 1.2/10 min, $n = 9$) but were significantly lower in the cervix (longitudinal muscle: 3.6 ± 0.4/10 min, $n = 12$; circular muscle: 10 ± 0.9/10 min, $n = 12$). Each agent was applied at 5-min intervals with 0.5 log concentration increments, and inhibitory responses were analyzed on the basis of the minimum amplitude of spontaneous contractions during a 5-min cycle and expressed as a percentage of the amplitude of spontaneous contraction before the treatment. In the longitudinal muscles of the corpus and cervix, 5-HT caused an excitatory response and increased the amplitude of spontaneous contractions (Fig. 6). The maximum amplitude during a 5-min cycle was expressed as a percentage of spontaneous contraction in the absence of 5-HT.

2.3. Radioligand binding study

To characterize the difference in β-adrenoceptor density between muscle layers isolated from the cornu, we carried out a receptor binding assay using [^{3}H]dihydroalprenolol (Amersham). The myometrial membrane preparation was prepared using methods described previously (Taneike et al., 1991; Kitazawa et al., 2000). The isolated longitudinal and circular muscle preparations were cut into small pieces and homogenized in 10 volumes of ice-cold Tris-HCl buffer (Tris, 50 mM; $MgCl_2$, 10 mM, pH = 7.4) 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 × g for 20 min at 4 °C, and the pellet was discarded. The supernatant was centrifuged at 80,000 × g for 60 min at 4 °C. The resulting pellets were washed twice and suspended in the buffer and used as a crude membrane preparation for measurement of [^{3}H]dihydroalprenolol binding and adenylate cyclase activity described below. Protein in the membrane preparation was measured by the method of Lowry et al. (1951).

The membrane preparation (200-600 μg protein/0.5 ml) from both muscle layers was incubated with seven increasing concentrations of [^{3}H]dihydroalprenolol (0.3, 0.6, 1.25, 2.5, 5, 10, 20 nM) for 20 min at 25 °C. The binding reaction was stopped by adding ice-cold buffer (4 ml), and the mixture was then filtered through a glass fiber filter (GF/C; Whatman) under 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 with 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 propranolol. The results of four separate saturation studies were used to estimate the maximum number of binding sites per milligram of protein (B_{max}, concentration of receptors) and the equilibrium dissociation binding constant (K_d), after Scatchard analysis. Lines of best fit were calculated using linear regression by the method of least-squares.

The [^{3}H]dihydroalprenolol binding site in the porcine myometrium was characterized in a competition study. [^{3}H]Dihydroalprenolol (5 nM) and crude membrane (150–300 μg protein/tube) of the cornu longitudinal muscle were incubated with eight increasing concentrations of β-adrenoceptor agonists and antagonists (0.1, 1, 10, 100 nM, 1, 10, 100 μM and 1 mM) for 20 min at 25 °C. After incubation, [^{3}H]dihydroalprenolol bound to membrane β-adrenoceptor was separated by filtration through glass fiber filters, and the radioactivity on the filters was measured. Using the IC_{50} value (concentration of an agent that inhibited 50% of the control binding obtained in the absence of displacers) of two or four separate experiments, the inhibition constant (K_i) was calculated with the equation of Cheng and Prusoff (1973): $K_i = IC_{50}/(1+[L]/K_d)$, where $[L]$ is the concentration of [^{3}H]dihydroalprenolol (5 nM) used in the competition study.

2.4. Measurement of adenylate cyclase activity

Adenylate cyclase activity was determined as the enzymatic conversion of [^{32}P]ATP to [^{32}P]cyclic AMP according to the method of Fortier and Krall (1983). The prepared membrane protein (50-100 μg/tube) from each muscle layer of the cornu was incubated for 10 min at 25 °C with various concentrations of isoprenaline in 100 μl of Tris-HCl (25 mM, pH 7.4) buffer containing dithiothreitol, 3 mM; isobutylmethylxanthine, 10 mM; ATP, 25 mM; creatine phosphokinase 5 units; creatine phosphate, 10 mM; Gpp(NH)p, 100 μM; $MgCl_2$, 10 mM and 37 KBq [^{32}P]ATP (Amersham). The reaction was terminated by the addition of 100 μl of Tris-HCl buffer containing ATP, 45 mM; cyclic AMP, 1 mM and sodium dodecyl sulfate, 73 mM, and incubated for 10 min at 90 °C. [^{32}P]Cyclic AMP in the reaction mixture was separated from unconverted [^{32}P]ATP using Dowex (AG 50W-X4, 200 to 400 mesh, Bio-Rad, Richmond, CO) and a sequential neutral alumina column as described by Salomon (1979). To serve as an internal standard and to monitor the efficiency of recovery, [^{3}H]cyclic AMP (15,000 cpm, Amersham) was added to each sample before separation. Then radioactivity (^{3}H or ^{32}P) in the eluted fractions was measured separately using a scintillation counter (Aroka, LSC-700). The radioactivity of [^{32}P]cyclic AMP was corrected for the recovery rate of [^{3}H]cyclic AMP.

2.5. Measurement of tissue cyclic AMP level

Isolated fresh strips of longitudinal and circular muscles from the cornu, weighing approximately 40 mg, were used in the cyclic AMP assay study. After equilibration in Krebs solution at 37 °C for 1 h, the strips were treated with increasing concentrations of isoprenaline 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 cen-

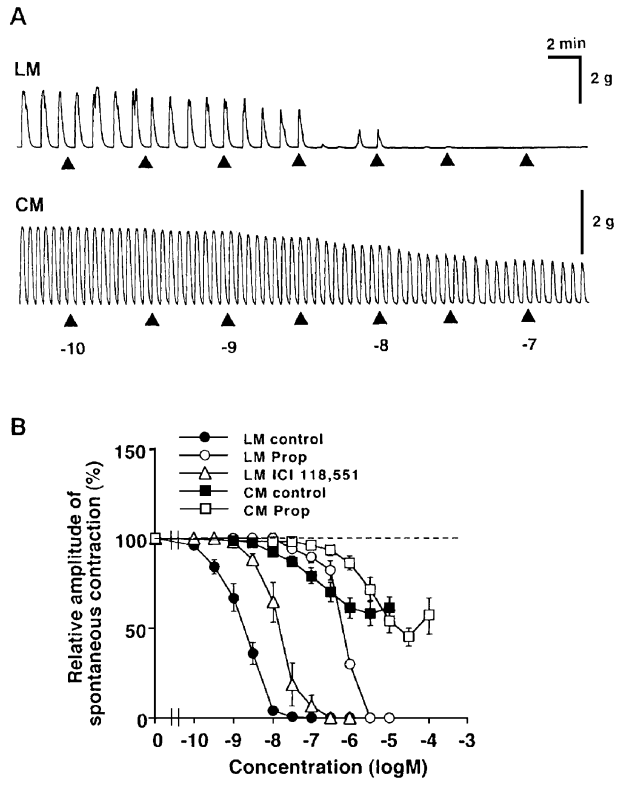

Fig. 1. Effect of isoprenaline on spontaneous contraction of the porcine uterus. (A) Each trace shows a typical response induced by cumulatively applied isoprenaline (0.1, 0.3 1, 3, 10, 30, 100 nM) in spontaneously contracting longitudinal (LM) and circular muscle layers (CM) of the cornu. Numerals under each trace indicate the concentration (logM). (B) Concentration–response curves for isoprenaline in the longitudinal (●) and circular muscle layers (■). Treatment with propranolol (Prop, 300 nM) antagonized the inhibitory response to isoprenaline in both muscle layers (LM, ○; CM, □). In the longitudinal muscle, the effect of ICI 118,551 (100 nM) on the response to isoprenaline was also examined (△). Ordinate: relative amplitude (control = 100%) of spontaneous contraction. Abscissa: concentration of isoprenaline (logM). Points represent the means of four or more experiments with S.E.M. shown by vertical lines.

trifugation twice at 3000 rpm, trichloroacetic acid in the supernatant was removed by washing with water-saturated ether, and cyclic AMP was assayed using a radioimmunoassay kit (Amersham). Tissue cyclic AMP levels are expressed as pmol/mg protein.

2.6. Chemicals

The following chemicals were used in this experiment: (−)-adrenaline bitartrate (Sigma), (−)-alprenolol tartrate (Sigma), atenolol (Sigma), (±)-4-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid (BRL 37344) sodium salt (Tocris), clenbuterol (Tocris), 5-hydroxytryptamine creatinine sulfate (5-HT, Wako), (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol (ICI 118, 551) hydrochloride (Tocris), isobutylmethylxanthine (Aldrich), (−)-isoprenaline hydrochloride (Sigma), (−)-noradrenaline bitartrate (Sigma), phentolamine mesylate (RBI) (-)-propranolol hydrochloride

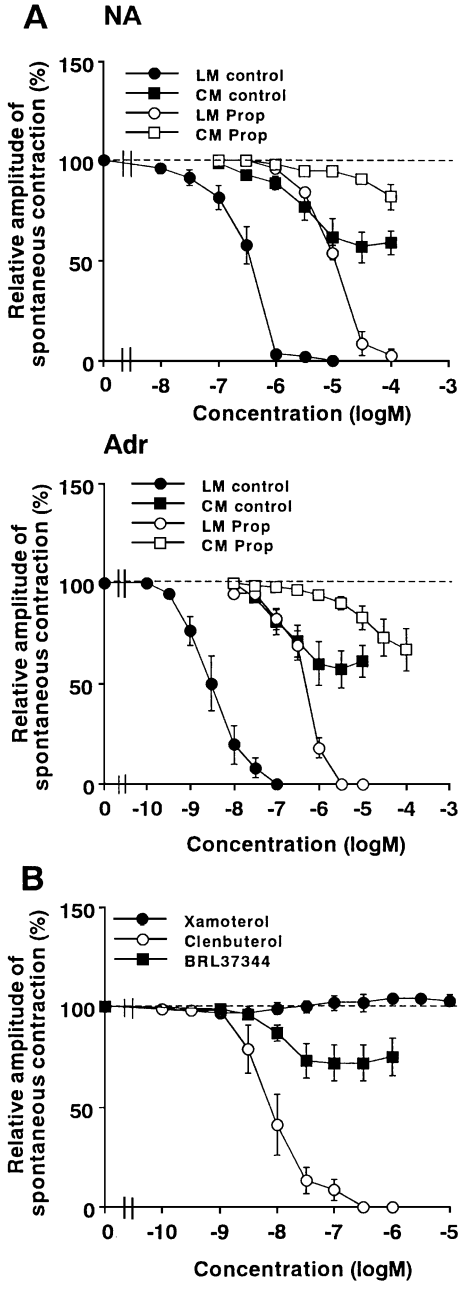

Fig. 2. Effects of noradrenaline, adrenaline, xamoterol, clenbuterol and BRL 37344 on the spontaneous contraction of porcine uterus. (A) Concentration–response curves for noradrenaline (NA) and adrenaline (Adr) in the longitudinal (LM, ●) and circular muscle layers (CM, ■) of the cornu treated with phentolamine (3 μM). Propranolol (Prop, 300 nM) antagonized the inhibitory responses of adrenaline and noradrenaline in both muscle layers (LM, ○; CM, □). (B) Comparison of the inhibitory effects of three selective β-adrenoceptor subtype agonists on the spontaneous contraction of the longitudinal muscle. Symbols show the concentration–response curve for xamoterol (●), clenbuterol (○) and BRL 37344 (■). Ordinate: relative amplitude (control = 100%) of spontaneous contraction. Abscissa: concentration of agonists (logM). Points represent the means of four or more experiments with S.E.M. shown by vertical lines.

(Wako), xamoterol hemifumarate (Tocris). The following drugs were kindly donated: ketanserin (Janssen-Kyowa, Tokyo, Japan) and 2a-[4-(4-phenyl-1,2,3,6-tetrahydropyridyl)butyl]-2a,3,4,5-tetrahydro-benzo[cd]indol-2(1*H*)-one (DR4004, Meiji Seika, Yokohama, Japan). Drugs, except for DR4004 and clenbuterol, were dissolved in distilled water and applied directly to the organ bath. DR4004 was dissolved in dimethylsulfoxide and clenbuterol was dissolved in ethanol, and these solutions were diluted with distilled water or Krebs solution. The maximum concentrations of dimethylsulfoxide and ethanol in the bathing solution were set below 0.2% and 0.1% in the present experiments. Dimethylsulfoxide (0.2%) and ethanol (0.1%) did not affect the amplitude and frequency of spontaneous contractions of both myometrial layers from the cornu ($n=4$).

2.7. Statistical analysis

Most results of the present experiment are expressed as means ± S.E.M. of more than three separate experiments. Student's *t*-test was employed for statistical comparison of paired samples. In multiple comparisons, statistical analysis was performed using a one-way analysis of variance followed by Fisher's protected least significant difference (PLSD) test. Probability values of $P<0.05$ were considered significant.

3. Results

3.1. Inhibition of uterine contractility by β-adrenoceptor agonists in the cornu

The β-adrenoceptor agonist, isoprenaline (0.1 nM-1 μM), inhibited spontaneous contractions of the longitudinal muscle ($EC_{50}=1.8\pm0.4$ nM, $n=11$) in a concentration-dependent manner and abolished them completely in all preparations examined ($EC_{100}=15.4\pm3.8$ nM, $n=11$). However, in the circular muscle preparations, the inhibitory effect of isoprenaline was very weak ($EC_{50}=123\pm43$ nM, $n=7$) and not complete (maximum inhibition = 39 ± 6.2%, $n=7$) (Fig. 1). The inhibition by isoprenaline was decreased by propranolol (300 nM) and concentration–response curves for both muscle layers shifted rightward in parallel, confirming that β-adrenoceptors mediate the inhibitory responses to isoprenaline (Fig. 1B).

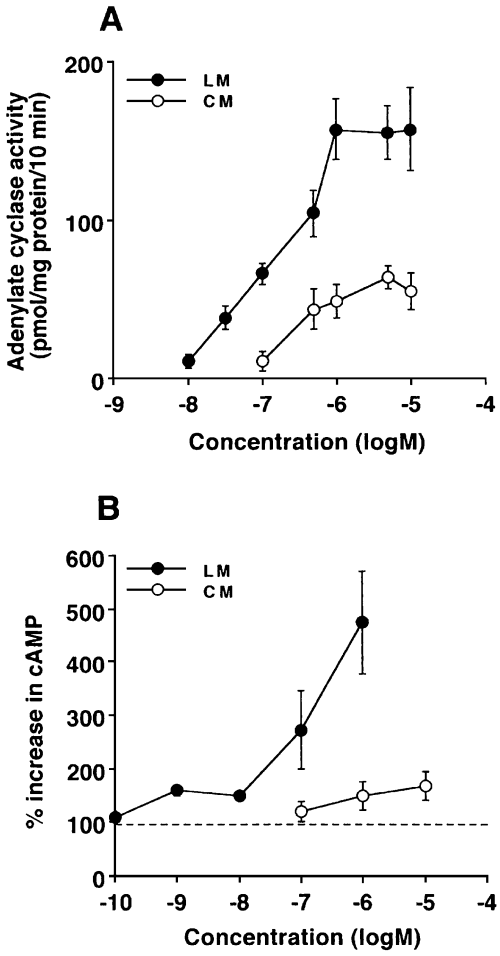

Fig. 3. Comparison of adenylate cyclase activities and cyclic AMP contents in the longitudinal and circular muscles of the porcine uterus stimulated by isoprenaline. Symbols show the concentration–response curves for isoprenaline in the longitudinal (●) and circular muscles (○) in adenylate cyclase activity (A) and cyclic AMP content (B) assay studies. Ordinate: adenylate cyclase activity (pmol/mg protein/10 min) (A) and percent increase in tissue cyclic AMP content (resting = 100%) (B). Resting cyclic AMP content in the longitudinal muscle was not significantly different from that in the circular muscle (see text). Abscissa: concentration of isoprenaline (logM). Points represent the means of three or more experiments with S.E.M. shown by vertical lines.

Table 1
Characteristics of [^{3}H]dihydroalprenolol and [^{3}H]5-carboxamidotryptamine binding sites in crude membrane preparations of the longitudinal and circular muscles isolated from the cornu of porcine uterus

	[^{3}H]Dihydroalprenolol		[^{3}H]5-Carboxamidotryptamine[a]	
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
Longitudinal muscle	3.1 ± 0.94	175.7 ± 32.8 (1.0)	0.24	30.4 (1.0)
Circular muscle	2.4 ± 0.73	53.1 ± 5.1 (0.3)[b]	0.21	120.6 (4.0)

Values are means ± S.E.M. of four separate saturation studies.

[a] Mean K_d and B_{max} values of [^{3}H]5-carboxamidotryptamine binding sites (5-HT$_7$ receptors) were taken from a previous paper (Kitazawa et al., 2000) for comparison.

[b] Significantly different from the B_{max} value of the longitudinal muscle.

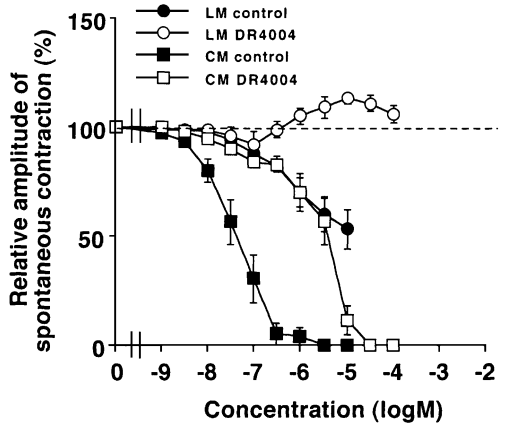

Fig. 4. Effects of 5-HT on spontaneous contraction of the porcine uterus. Concentration–response curves for 5-HT in the longitudinal (●) and circular muscle layers (■) of the cornu. Treatment with DR4004 (100 nM) inhibited the responses to 5-HT and caused a rightward shift of the concentration–response curves. In the longitudinal muscle, 5-HT potentiated the spontaneous contraction in the presence of DR4004 (○). 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.M. shown by vertical lines.

To identify the β-adrenoceptor subtype, first, effects of noradrenaline and adrenaline were compared to those of isoprenaline in the presence of phentolamine (3 μM), because both catecholamines have been shown to be able to act on the α_2-adrenoceptor and cause contraction of the porcine uterus (Taneike et al., 1995). The isoprenaline-induced inhibition was not affected by treatment with phentolamine (3 μM) (data not shown). Adrenaline decreased the amplitude of spontaneous contractions in both muscle layers from the cornu and the longitudinal muscle ($EC_{50} = 3.8 \pm 1.5$ nM; maximum inhibition = 100% $n = 4$) was more sensitive than was the circular muscle ($EC_{50} = 170 \pm 40$ nM; maximum inhibition = 43 ± 9.4%, $n = 4$) as was the case for inhibition by isoprenaline. Compared with adrenaline, noradrenaline was less effective to cause inhibition. The EC_{50} and maximum inhibition were 340 ± 55 nM and 100% ($n = 5$) in the longitudinal muscle and 3.6 ± 0.84 μM and 44 ± 7.7% ($n = 5$) in the circular muscle, respectively (Fig. 2A). Propranolol (300 nM) decreased the noradrenaline- and adrenaline-induced inhibition in both muscle layers and shifted the concentration–response curves to the right almost in parallel. The EC_{50} values of noradrenaline and adrenaline in the propranolol-treated longitudinal muscle were 12 ± 1.2 μM ($n = 4$) and 460 ± 50 nM ($n = 4$), respectively (Fig. 2A). The rank order of inhibition (EC_{50}) was estimated to be isoprenaline ≧ adrenaline > noradrenaline in both muscle layers (Figs. 1B and 2A). Second, the inhibition by xamoterol (β_1-selective), clenbuterol (β_2-selective) and BRL 37344 (β_3-selective) (Bylund et al., 1994; Strosberg and Pietri-Rouxel, 1996) were compared in the longitudinal muscles. Among the three agonists, clenbuterol was the most potent inhibitor ($EC_{50} = 9.6 \pm 2.7$ nM, $n = 8$) and it abolished the spontaneous contraction ($EC_{100} = 110 \pm 43$ nM, $n = 8$) similarly to catecholamines. On the other hand, BRL 37344 was less effective and xamoterol was quite ineffective to inhibit the spontaneous contraction (Fig. 2B). Third, the effect of a selective β_2-adrenoceptor antagonist (ICI 118,551) on the response to isoprenaline was examined in the longitudinal muscle. ICI 118,551 (100 nM) shifted the concentration–response curve for isoprenaline to the right in parallel and increased the EC_{50} value (19 ± 8 nM, $n = 5$) (Fig. 1B). The apparent pA_2 of ICI 118, 551 against isoprenaline was calculated to be 8.0.

3.2. Density of β-adrenoceptors in the longitudinal and circular muscles

The difference in the inhibition of muscle contractility by 5-HT results from the heterogeneous distribution of 5-

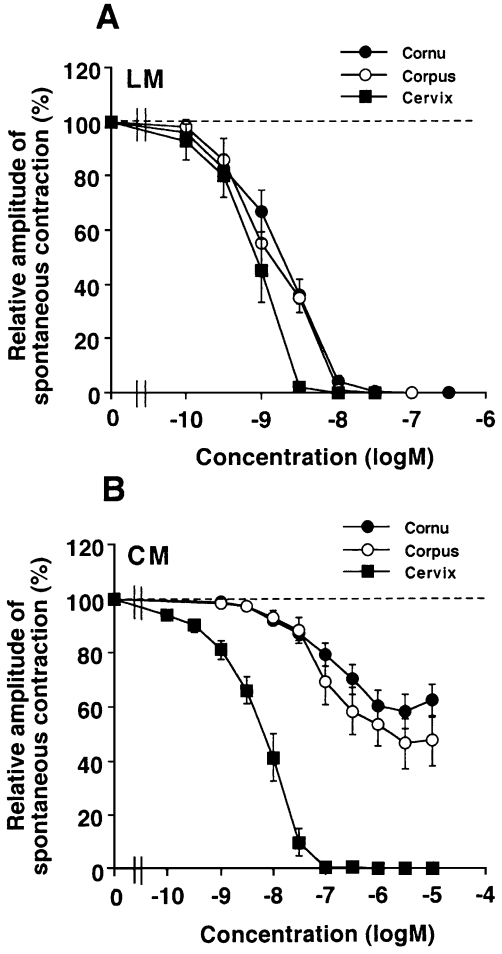

Fig. 5. Comparison of the concentration–response curves for isoprenaline in three different regions of the porcine uterus. Symbols show the inhibitory effects of isoprenaline on the amplitude of spontaneous contractions in longitudinal (A, LM) and circular muscle (B, CM) layers isolated from the cornu, corpus and cervix of the uterus. Ordinate: relative amplitude of the spontaneous contraction (control = 100%). Abscissa: concentration of isoprenaline (logM). Points represent the means of four or more experiments with S.E.M. shown by vertical lines.

HT_7 receptors in the porcine cornu myometrium (Kitazawa et al., 2000, Table 1). Therefore, we compared the density of β-adrenoceptors in the two muscle layers of the cornu. [^{3}H]Dihydroalprenolol (0.3-20 nM) binding to membrane preparations of the longitudinal and circular muscles was saturable. Scatchard analysis of the saturation data showed one straight line in each muscle layer (correlation, r>0.95) and suggested the presence of a single class of binding site in both myometrium. Although there was no significant difference between the apparent dissociation constants (K_d, longitudinal muscle, 3.1 ± 0.94 nM, n = 4; circular muscle, 2.4 ± 0.73 nM, n = 4), B_{max} in the longitudinal muscle (175.7 ± 32.8 fmol/mg protein, n = 4) was significantly higher than that in the circular muscle (53.1 ± 5.1 fmol/mg protein, n = 4) (Table 1). Characterization of the [^{3}H]dihydroalprenolol binding site was carried out in competition experiments. The specific binding of [^{3}H]dihydroalprenolol to the membrane preparation from the cornu longitudinal muscle was inhibited by some β-adrenoceptor agonists and antagonists. The rank order of potency was isoprenaline (K_i, 171 ± 12 nM, n = 4)>adrenaline (1.16 μM, n = 2)>noradrenaline (14.8 μM, n = 2) for agonists and alprenolol (1.8 nM, n=2, non-selective) > propranolol (2.7 nM, n = 2, non-selective)>atenolol (164 μM, n = 2, β_1-selective) for antagonists.

3.3. Comparison of adenylate cyclase activities and cyclic AMP contents in the longitudinal and circular muscles

To determine the muscle layer-dependent difference in β-adrenoceptor-mediated signal transduction pathway, the ability of isoprenaline to generate cyclic AMP was compared in the longitudinal and circular muscle layers of the cornu. As shown in Fig. 3A, isoprenaline caused a concentration-dependent activation of adenylate cyclase in both muscle layers. Maximum activation in the longitudinal muscle was three times greater than that in the circular muscle, whereas half-maximal activation occurred at similar concentrations in both muscle layers (EC_{50}, longitudinal muscle = 290 ± 60 nM, circular muscle = 150 ± 20 nM; n = 4).

In the cyclic AMP content assay study, the resting cyclic AMP level in the longitudinal muscle (4.1 ± 0.9 pmol/mg protein, n = 4) was not significantly different from that in the circular muscle (4.8 ± 0.5 pmol/mg protein, n = 3). Pretreatment with isoprenaline increased the cyclic AMP content of the longitudinal muscle in a concentration-dependent manner. The increases in tissue cyclic AMP with 0.1, 1, 10, 100 nM and 1 μM isoprenaline were calculated to be 1.1, 1.6, 1.5, 2.7 and 4.7-fold, respectively. On the other hand, the circular muscle was less responsive to isoprenaline, and the increases (100 nM, 1.2-fold; 1 μM, 1.5-fold; 10 μM, 1.67-fold) were considerably less than with the longitudinal muscle (Fig. 3B).

3.4. Inhibition of uterine contractility by 5-HT

5-HT also inhibited the spontaneous contractions of both myometrial layers from the cornu. In contrast to the inhibition by β-adrenoceptor agonists, the circular muscle was more sensitive to 5-HT than was the longitudinal muscle, as previously reported (Kitazawa et al., 2000). The EC_{50}

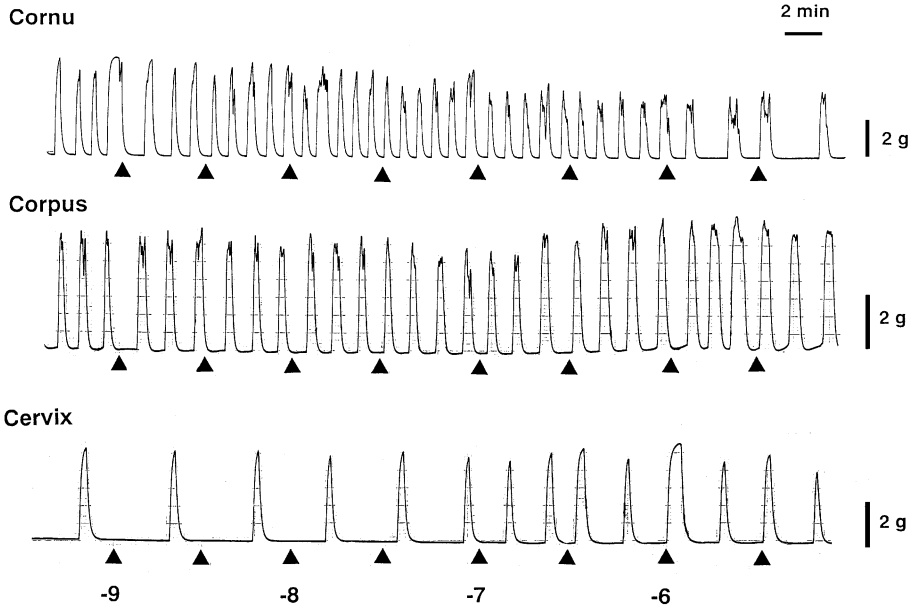

Fig. 6. Typical mechanical responses to 5-HT in spontaneously contracting longitudinal muscle strips obtained from the cornu, corpus and cervix of the porcine uterus. Increasing concentrations of 5-HT (1, 3, 10, 30, 100, 300 nM, 1 and 3 μM) were applied cumulatively in the organ bath at 5-min intervals. Numerals under each trace indicate the concentration of 5-HT (logM).

and EC_{100} values in the circular muscle preparations were 52 ± 11 nM (n = 13) and 360 ± 79 nM (n = 13), respectively. On the other hand, the EC_{50} value in the longitudinal muscle strips was high (460 ± 95 nM, n = 12) and 5-HT failed to abolish the spontaneous contraction (maximum inhibition = 47 ± 9.2%, n = 12) (Fig. 4). The inhibitory responses in both muscle layers were strongly inhibited by DR4004 (100 nM). The concentration-response curve in the circular muscle was shifted to the right in parallel without any effect on the maximum inhibition (100%), and the apparent pA_2 value of DR4004 was calculated to be 8.86. In the case of longitudinal muscle strips, the inhibition by 5-HT was changed into potentiation of spontaneous contraction (Fig. 4). The potentiation by 5-HT in the presence of DR4004 was antagonized by ketanserin (100 nM) (data not shown).

3.5. Regional differences of the isoprenaline- and 5-HT-induced responses

3.5.1. Corpus

In the longitudinal muscle layers, isoprenaline inhibited and finally abolished the spontaneous contraction (EC_{50} = 1 ± 0.22 nM, EC_{100} = 4.4 ± 1.2 nM, n = 5), as with the cornu (Fig. 5A). However, 5-HT caused potentiation of the spontaneous contractions in most of the preparations examined (12 of 15 preparations) as shown in Figs. 6 and 7A. Inhibition by 5-HT was observed in the remaining preparations. Pretreatment with ketanserin (100 nM) antagonized the excitatory responses to 5-HT and changed them into inhibitory responses that were antagonized by 100 nM DR4004. In the circular muscle layers, 5-HT and isoprenaline also caused inhibition of spontaneous contraction. The inhibition by 5-HT (EC_{50} = 50 ± 7.8 nM, EC_{100} = 380 ± 90 nM, n = 14) and that by isoprenaline (EC_{50} = 117 ± 40 nM, maximum inhibition = 54 ± 9.3%, n = 12) were the same as those obtained in the cornu preparations (Figs. 5B and 7B).

3.5.2. Cervix

Isoprenaline concentration-dependently inhibited the spontaneous contraction of the longitudinal muscle strips. The responsiveness (EC_{50} = 0.9 ± 0.18 nM, EC_{100} = 3.7 ± 0.7 nM, n = 10) in the cervix was higher than that in the cornu (EC_{50} = 1.8 ± 0.4 nM, EC_{100} = 15.4 ± 3.8 nM, n = 11) (Fig. 5A). 5-HT did not inhibit the spontaneous contraction but caused a phasic (spontaneous-like) contraction, which was abolished by ketanserin (100 nM) (Figs. 6 and 7A). In the case of the circular muscle layers, both 5-HT and isoprenaline inhibited the spontaneous contractions and finally abolished them. The responsiveness to 5-HT (EC_{50} = 35 ± 10 nM, EC_{100} = 250 ± 80, n = 11) was not significantly different from that in the cornu or corpus. However, inhibition by isoprenaline (EC_{50} = 9 ± 1.7 nM, EC_{100} = 180 ± 95 nM, n = 9) was strong and complete compared with that in the cornu and corpus (Figs. 5B and 7B).

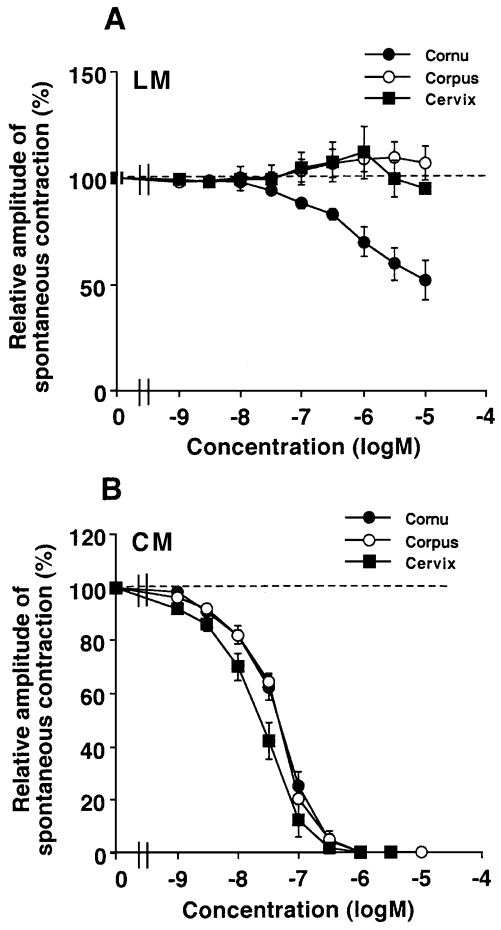

Fig. 7. Comparison of the concentration–response curves for 5-HT in three different regions of the porcine uterus. Symbols show the effects of 5-HT on the amplitude of the spontaneous contractions in longitudinal (A, LM) and circular muscle (B, CM) layers isolated from the cornu, corpus and cervix of the uterus. 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.M. shown by vertical lines.

4. Discussion

The present study characterized the inhibitory response to isoprenaline and distribution of β-adrenoceptors in the porcine uterus. In spontaneously contracting longitudinal and circular muscle preparations from the cornu, three catecholamines (adrenaline, isoprenaline and noradrenaline) caused inhibition of muscle contractility in a muscle layer-dependent manner (longitudinal muscle>circular muscle). The difference in the inhibition of muscle contractility by catecholamines is probably caused by muscle layer-related activity of adenylate cyclase and accumulation of cyclic AMP (longitudinal muscle>circular muscle), due to the smooth muscle layer-dependent heterogeneous distribution (longitudinal muscle: circular muscle = 3.3:1) of [^{3}H]dihy-

droalprenolol binding sites (β-adrenoceptors). The β-adrenoceptors are subdivided into three subtypes according to pharmacological characteristics: β_1, β_2 and β_3 subtypes. Three catecholamines have a differential affinity for these β-adrenoceptor subtypes. A primary distinction between β_1 and β_2 subtypes is the relative potencies of adrenaline and noradrenaline, with the two catecholamines being equipotent for the β_1 subtype and adrenaline having up to a 100-fold greater potency for the β_2 subtype. Conversely, noradrenaline is more potent than adrenaline as a β_3-adrenoceptor agonist (Bylund et al., 1994). In the present study, adrenaline and noradrenaline also caused propranolol-sensitive inhibition of uterine contractility, and the rank order of potency was isoprenaline ≧ adrenaline>noradrenaline. These results suggested that the β_2-adrenoceptor mediates the inhibition of porcine uterine contractility by catecholamines. This result was supported by results of the following studies with selective β-adrenoceptor agonists and antagonists. In a comparison of the effects of xamoterol, clenbuterol and BRL 37344, a selective β_2-adrenoceptor agonist, clenbuterol (Strosberg and Pietri-Rouxel, 1996) was more potent to inhibit uterine contractility than were β_1- and β_3-adrenoceptor agonists. In addition, the inhibition by isoprenaline was antagonized by ICI 118,551, a selective β_2-adrenoceptor antagonist (Bylund et al., 1994) and the apparent pA_2 value (8.0) was close to that for β_2-adrenoceptors (8.42, El Alj et al., 1989). The results of a competition study of [^{3}H]dihydroalprenolol binding also supported the presence of β_2-adrenoceptors in porcine myometrium. That is to say, [^{3}H]dihydroalprenolol binding in the longitudinal muscle preparations was concentration-dependently inhibited by three catecholamines and the rank order of potency (isoprenaline>adrenaline>noradrenaline) was consistent with that of the results of a contraction study and β_2-adrenoceptors (Bylund et al., 1994). Furthermore, the β_1-selective antagonist, atenolol, was 100,000 times less potent than propranolol and alprenolol (non-selective β-adrenoceptor antagonists). Previous studies of myometrial β-adrenoceptor binding sites and of responsiveness to β-adrenoceptor agonists in the uterus of several species (rat, human and guinea-pig) indicated that β_2 is a dominant subtype and is involved in the inhibition of myometrial contractility by β-adrenoceptor agonists as is the case for pigs (Pennefather and Molenaar, 1986; Breuiller et al., 1987; Maltier and Legrand, 1988; El Alj et al., 1989). Therefore, whereas the α-adrenoceptor subtype mediating uterine contraction is different from species to species (α_1 type: rabbit, rat, guinea-pig; α_2 type: cow, pig) (Hoffman et al., 1981; Kaulenas et al., 1991; Haynes and Pennefather, 1993; Taneike et al., 1995, 1999), there are no species differences concerning the subtype of β-adrenoceptor (β_2) mediating relaxation in the uterus. Binding studies with [^{125}I]cyanopindolol indicated that longitudinal and circular muscles of human and guinea-pig uterus have the same density of β_2-adrenoceptors (Pennefather and Molenaar, 1986; Breuiller et al., 1987; Handberg et al., 1988), the present study showed that β_2-adrenoceptor distribution in the porcine uterus was not homogeneous but was smooth muscle layer-dependent (longitudinal muscle>circular muscle). Heterogeneous distribution of receptors for some bioactive agents (acetylcholine, histamine, endothelin, oxytocin) have been demonstrated for the porcine uterus (Taneike et al., 1991; Kitazawa et al., 1997, 2001; Isaka et al., 2000). Although the physiological meaning of these muscle layer-dependent heterogeneous receptor distributions is not clear at present, it might reflect a different functional role of the longitudinal and circular muscles in uterine motility.

5-HT preferentially inhibited the spontaneous contraction of the porcine uterine circular muscle, unlike the case of β_2-adrenoceptor mediated inhibition. The 5-HT-induced inhibition was decreased by a selective 5-HT$_7$ receptor antagonist, DR4004 (Kikuchi et al., 1999), confirming the involvement of 5-HT$_7$ receptors in the 5-HT-induced inhibition as previously demonstrated (Kitazawa et al., 1998, 2000). Since 5-HT$_7$ receptors are distributed heterogeneously (longitudinal muscle/circular muscle: 1:4) and since 5-HT is a potent inhibitor of circular muscle contractility, it is thought that a 5-HT and 5-HT$_7$ receptor-mediated pathway might be more important for regulation of circular muscle contractility than are β_2-adrenoceptor-mediated pathways. In other words, 5-HT$_7$ and β_2-adrenoceptor share the inhibitory regulation of uterine contractility in a smooth muscle layer-dependent manner in the cornual region (5-HT$_7$ for regulation of circular muscle contractility and β_2 for regulation of longitudinal muscle contractility). In other regions of the uterus (corpus and cervix), isoprenaline was also more effective than 5-HT to cause inhibition of the longitudinal muscle, while 5-HT was more effective than isoprenaline to cause inhibition of the circular muscle. Therefore, it is thought that the sharing of muscle layer-dependent inhibition of contractility by 5-HT and isoprenaline applies to all regions of the porcine uterus.

We have reported region-dependent differences in the responsiveness of uterotonic agents for porcine uterus. For example, in the responses to noradrenaline (α_2-adrenoceptor) and oxytocin, contractile intensity in the longitudinal muscle was most potent in the cornu, slightly weaker in the corpus, and weakest in the cervix (Taneike et al., 1994; Kitazawa et al., 2001). In the present study, isoprenaline inhibited the spontaneous contraction of longitudinal and circular muscle strips from the cornu, corpus and cervix, but inhibition was conspicuously strong in the cervix compared with that in the cornu and corpus. The gradient of the responsiveness to isoprenaline (inhibition, cervix>corpus ≧ cornu) and that to noradrenaline and oxytocin (contraction, cornu>corpus>cervix, Taneike et al., 1994; Kitazawa et al., 2001) would produce a pressure gradient within the uterus that could transport uterine contents from the cornu to the cervix. In contrast to that with isoprenaline, the 5-HT-induced inhibition in the circular muscle was not different among the three uterine regions, but the response of 5-HT in the longitudinal muscle changed from inhibition

(cornu) to contraction (corpus, cervix). Since ketanserin decreased the contractile response to 5-HT, the 5-HT_2 receptor was suggested to mediate the contractile response in the corpus and cervix. In the presence of ketanserin, 5-HT caused an inhibition of the spontaneous contraction that was antagonized by DR4004. Conversely, in the cornu, although 5-HT causes inhibition in normal Krebs solution, 5-HT potentiated the spontaneous contraction in the presence of DR4004, and the potentiation was decreased by ketanserin. These results suggest that heterogeneous populations of 5-HT receptor subtypes (5-HT_7 and 5-HT_2 receptors) are present in the porcine uterine longitudinal muscle layers and that the dominant receptor type might be different depending on the region. In the cornu, the 5-HT_7 type is dominant and 5-HT causes inhibition, but, on the other hand, in the corpus and cervix, the 5-HT_2 type is dominant and 5-HT causes contraction of the myometrium. Region-dependent changes in 5-HT-induced responses are unique, but their physiological meaning is not clear at the present time.

In conclusion, β_2-adrenoceptors are present heterogeneously in the porcine uterus (longitudinal muscle>circular muscle) and mediate inhibition of the uterine contractility by catecholamines. This β_2-adrenoceptor-mediated pathway shares the inhibition of uterine contractility with the 5-HT_7 receptor-mediated pathway in a smooth muscle layer-dependent manner (longitudinal muscle: β_2-adrenoceptors, circular muscle: 5-HT_7 receptors). The responsiveness to isoprenaline (both in the longitudinal and circular muscles) differs from region to region (cervix>corpus $\geqq$ cornu), probably because of the gradient of β_2-adrenoceptor density. The 5-HT-induced response in the longitudinal muscle also differs according to region, since the dominant subtype of 5-HT receptors differs from region to region (5-HT_2>5-HT_7, corpus and cervix; 5-HT_7>5-HT_2, cornu).

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