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Abstract: 5-Hydroxytryptamine (5-HT) receptors mediating excitatory and inhibitory actions of 5-HT on contractility of uterine strips from non-pregnant pigs were characterized. Expression of 5-HT2A and 5-HT7 receptors was examined by molecular biological study. 5-HT-containing cells were observed immunohistochemically. In the spontaneously contracting uterine circular muscle layers, 5-HT caused inhibition of contractile activity. SB269970 (5-HT7 receptor antagonist, 10 nM) shifted the concentration-inhibition curve of 5-HT to the right, but higher concentrations of SB269970 (100 nM -1 ☐ M) changed the monophasic curve to a biphasic curve (mixture of excitatory and inhibitory responses to 5-HT). Addition of ketanserin (5-HT2 receptor antagonist, 10 nM-1 ☐ M) decreased the excitatory effects of 5-HT. 5-HT was less effective in inhibiting the spontaneous contraction in the longitudinal muscles. Ketanserin enhanced the inhibitory responses and SB269970 reversed the inhibitory responses to excitatory responses. In the presence of SB269970, ☐-methyl-5-HT was equipotent to 5-HT in increasing contractility of longitudinal muscle and ketanserin competitively inhibited the responses to ☐-methyl-5-HT (pKd=8.78). Muscle layer-dependent expression of both 5-HT2A receptor and 5-HT7 receptor mRNAs in the porcine uterine muscle

layers was demonstrated by RT-PCR and real-time PCR. 5-HT immunoreactivity was detected only in uterine gland cells, which were localized near the uterine circular muscle layers. In the longitudinal and circular muscle layers with endometrium, compounds 48/80 and ketanserin did not change the spontaneous contractility, but SB269970 significantly increased the contractile activity of the circular muscle. In conclusion, excitatory 5-HT2A and inhibitory 5-HT7 are present in the uterus of non-pregnant pigs. Endogenous 5-HT containing cells are mainly present in uterine glands of the pig. The possible roles of 5-HT and its receptors in regulation of porcine uterine spontaneous contractility are discussed.

Index words: porcine uterus, 5-HT2A receptor, 5-HT7 receptor, 5-HT-immunoreactive cells, uterine contractility.

*** Response to Reviewers**

Letter to Editor EJP-29393R1

Thank you very much for your report concerning our manuscript titled "Excitatory and inhibitory 5-hydroxytryptamine (5-HT) receptors expressed in the isolated porcine uterine muscles".

We are pleased to hear our revised manuscript was acceptable for publication in European Journal of Pharmacology.
According to your stylistic comments, we added "Number headings and subheadings and Supply index words (from 3 to 6) below abstract." in the re-revised version of the manuscript.

We hope the final version is good and will be accepted.

Sincerely yours

Takio Kitazawa PhD

Excitatory and inhibitory 5-hydroxytryptamine (5-HT) receptors expressed in the isolated porcine uterine muscles

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Abstract

5-Hydroxytryptamine (5-HT) receptors mediating excitatory and inhibitory actions of 5-HT on contractility of uterine strips from non-pregnant pigs were characterized. Expression of 5-HT_{2A} and 5-HT_7 receptors was examined by molecular biological study. 5-HT-containing cells were observed immunohistochemically. In the spontaneously contracting uterine circular muscle layers, 5-HT caused inhibition of contractile activity. SB269970 (5-HT_7 receptor antagonist, 10 nM) shifted the concentration-inhibition curve of 5-HT to the right, but higher concentrations of SB269970 (100 nM -1 μM) changed the monophasic curve to a biphasic curve (mixture of excitatory and inhibitory responses to 5-HT). Addition of ketanserin (5-HT_2 receptor antagonist, 10 nM-1 μM) decreased the excitatory effects of 5-HT. 5-HT was less effective in inhibiting the spontaneous contraction in the longitudinal muscles. Ketanserin enhanced the inhibitory responses and SB269970 reversed the inhibitory responses to excitatory responses. In the presence of SB269970, α-methyl-5-HT was equipotent to 5-HT in increasing contractility of longitudinal muscle and ketanserin competitively inhibited the responses to α-methyl-5-HT (pK_d=8.78). Muscle layer-dependent expression of both 5-HT_{2A} receptor and 5-HT_7 receptor mRNAs in the porcine uterine muscle layers was demonstrated by RT-PCR and real-time PCR. 5-HT immunoreactivity was detected only in uterine gland cells, which were localized near the uterine circular muscle layers. In the longitudinal and circular muscle layers with endometrium, compounds 48/80 and ketanserin did not change the spontaneous contractility, but SB269970 significantly increased the contractile activity of the circular muscle. In conclusion, excitatory 5-HT_{2A} and inhibitory 5-HT_7 are present in the uterus of non-pregnant pigs. Endogenous 5-HT containing cells are mainly present in uterine glands

of the pig. The possible roles of 5-HT and its receptors in regulation of porcine uterine spontaneous contractility are discussed.

Index words: porcine uterus, 5-HT_{2A} receptor, 5-HT_7 receptor, 5-HT-immunoreactive cells, uterine contractility.

1. Introduction

5-Hydroxytryptamine (5-HT), a bioactive indoleamine mainly produced by enterochromaffin cells, induces a wide variety of mechanical responses (contraction, relaxation or a mixture of both responses) on vascular and non-vascular smooth muscles (trachea, gastrointestinal tract, uterus) of several mammalian species. The tissue- and species-related variations in 5-HT-induced responses are mainly caused by multiple 5-HT receptor subtypes and their heterogeneous expression in one organ. 5-HT receptors have been classified by ranking order of agonists, antagonists, signal transduction and molecular structure into seven major subtypes (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-ht_5, 5-HT_6 and 5-HT_7). The 5-HT_3 receptor is the only receptor that is coupled with ion channels and is exclusively expressed in neural components. Other subtypes are G-protein- coupled receptors and they couple with three different intracellular signal transduction systems. 5-HT_1 receptors are negatively coupled to adenylate cyclase through $G_{i/o}$ and decrease cytoplasmic cAMP contents. 5-HT_2 receptors couple with $G_{q/11}$ and stimulate the hydrolysis of membrane phospholipids to produce two second messengers (inositol-trisphosphate and diacylglycerol). On the other hand, intracellular signaling pathways of 5-HT_4, 5-HT_6 and 5-HT_7 receptors are common, positively coupling with adenylate cyclase by G_s protein and elevating the level of cytoplasmic cAMP followed by activation of protein kinase A. Signal transduction of 5-ht_5 receptor has not been well defined yet (Hoyer et al., 1994; 2002).

In the uterus of rodents and humans, it was found that 5-HT is contained in mast cells and that the density of mast cells is changed by the estrous cycle and pregnancy (Hine et al., 1985; Padilla et al., 1990; Mori et al., 1997; Rudolph et al., 1998; Garfield et al., 2000;

Bytautience et al., 2002). It has been shown that mast cell degranulation by compound 48/80 modulated uterine muscle contractility, which was antagonized by ketanserin (Bytautiene et al., 2002), and that 5-HT was taken up by mast cells (Rudolph et al., 1998). Based on these findings, 5-HT, one of the chemical mediators contained in mast cells, has been suggested to be an endogenous regulator of myometrial contractility. Besides, the mechanical actions of 5-HT on the myometrium, 5-HT-dependent collagenase induction in myometrial cells of rat has also been reported (Rydelek-Fitzgerald et al., 1993). Contraction studies using isolated uterine smooth muscle strips have indicated the species dependent–different mechanical actions of 5-HT. It has been shown that 5-HT caused contraction of rat uterine strips through activation of the 5-HT_{2A} receptor subtype (Cohen et al., 1986; Minosyan et al., 2007). Isolated human uterine strips were also contracted by 5-HT (Cruz et al., 1989), and a recent study has shown the expression of excitatory 5-HT_{2B} receptor subtype in freshly isolated human uterine muscle cells (Kelly and Sharif, 2006). 5-HT also caused contraction of mouse uterine strips and the involved receptor subtype has not been clarified yet (Rudolph et al., 1992). But bovine uterine smooth muscle was unresponsive to 5-HT (Taneike et al., 1999). In contrast, the effect of 5-HT on contractility of the porcine uterine cornu was quiet different. 5-HT inhibited uterine contractility through activation of the 5-HT_7 receptor coupling with G_s and elevation of cAMP. Due to different expression of 5-HT_7 receptors between uterine muscle layers, the circular muscle was more sensitive to the inhibitory action of 5-HT than was the longitudinal muscle (Kitazawa et al., 1998, 2000). On the other hand, 5-HT increased the contractility of longitudinal muscles in the uterine corpus and cervix, and the excitatory response to 5-HT was marked in the presence of the 5-HT_7 receptor antagonist DR4004, suggesting the presence of excitatory 5-HT receptors in the porcine uterus (Kitazawa et al., 2001). However, pharmacological

profiles of 5-HT receptor subtypes involved in excitatory responses in the porcine uterus have not been clarified yet.

In the present experiments, excitatory 5-HT receptor subtype and its expression in the porcine uterus were characterized using pharmacological (study using selective agonists and antagonists) and molecular biological (RT-PCR and real-time PCR) approaches. The presence of 5-HT-containing cells in the porcine uterus was also examined by an immunohistochemical study. Finally, possible involvement of endogenous 5-HT in the regulation of spontaneous contractility was investigated in isolated uterine strips with endometrium.

2. Materials and methods

2.1. Contraction study

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 pigs were judged to be in proestrus according to the results of gross examination of the follicle size and according to the appearance of the corpora lutea in the ovaries (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. In brief, after removal of the endometrium, each muscle layer was cut through the muscle coat in either the longitudinal muscle direction or circular muscle 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 longitudinal muscle and circular muscle for experimental use. In some experiments for examining the involvement of endogenous 5-HT in regulation of myometrial contractility, the effect of compound 48/80 or 5-HT receptor antagonists on spontaneous contractility of uterine smooth muscle strips with intact endometrium was investigated. The prepared smooth muscle strips (10 mm in length and 1 mm in width) were suspended vertically in an organ bath (5 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-11T, Nihon Kohden), equipped with a pen-writing recorder (Recticorder, Nihon Kohden) and computer-aided analysis system (Mac Lab, Japan Bioresearch Center), 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. After establishing steady spontaneous contractile activity of each muscle preparation, 5-HT and 5-HT receptor agonists were applied cumulatively in an organ bath at 5-min intervals. The area under the curve (AUC) of uterine contraction was used to assess smooth muscle contractility. AUC of spontaneous contraction (5 min) before application of agonists or AUC of 50 mM high-K^+-induced contraction (5 min) was used to normalize the mechanical responses induced by 5-HT receptor agonists. The antagonistic actions of 5-HT_2 and 5-HT_7 receptor antagonists on the responses to 5-HT receptor agonists were characterized through calculation of pK_d values. In brief, concentration-response curves for 5-HT receptor agonists were constructed in the absence and presence of antagonists. After confirming similar maximum responses (E_{max}), concentrations of agonists causing 50% of the maximum response (EC_{50}) were measured and the concentration ratio (CR, EC_{50} in the presence of antagonist/EC_{50} in the absence of antagonist) was calculated. The apparent

dissociation constant (K_d) of each antagonist was determined using the following equation: K_d=antagonist concentration /(CR-1). The results were then expressed as the negative logarithm of K_d (pK_d).

2.2. Analysis of the mRNA expression of 5-HT_{2A} and 5-HT_7 receptors

Isolated porcine uterine longitudinal and circular muscle strips were immediately immersed in RNA Later (Takara) for 12 h and then stored at -30°C until use. Total RNA was extracted by the conventional acid-guanidine-phenol-chloroform method (Trizol reagent, Invitrogen). RNA samples were then used as the template for first-strand cDNA synthesis using Oligo dT primer (Gibco) and reverse transcriptase (SuperScript III, Invitrogen). The reverse-transcriped products were screened for the presence of 5-HT-receptor cDNA by PCR. The PCR-amplified products were subjected to electrophoresis in 2% agarose gels and visualized by ethidium bromide staining. The sample without cDNA was used as a negative control for PCR. The sequences of primers for detection of 5-HT_{2A} and 5-HT_7 receptor mRNAs and the sizes of PCR products were as follows: 5'-GTGAGTGATCTTGGGACACG-3' (forward), 5'-GAGCAACCATAGTGCAGTCG-3' (reverse) and product size of 534bp for 5-HT_{2A} receptor (Gen Bank, NM 214217) and 5'-AGGATTTTGGCTACACGATC-3' (forward), 5'-CTTCCGGTTGATATTCCGGTAC-3' (reverse) and producr size of 523bp for 5-HT_7 receptor (Gen Bank, NM 214085). The sequences of the primers were designed using BLUEPHIN softwear (Biomolecule Design Platform, Sigma).

For quantitative analysis of the mRNA expression of 5-HT_{2A} and 5-HT_7 receptors, real-time PCR was conducted using SYBR Green. Both of the primer pairs used for

real-time RT-PCR were newly custom-synthesized, and their sequences were 5’-AGGTGCTGGGCATAGTCTTTC-3’ (forward) and 5’-GACGGCTGAGGAGAGGTAAC-3’ (reverse) for 5-HT_{2A} receptor 5’-GCAGATCAACTACGGCAGAG-3’ (forward) and 5’-CAGGTA GTTGGAGGGCTGAC-3’ (reverse) for 5-HT_7 receptor. Amplification was carried out in a total volume of 20 μl containing Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The assay was performed using an Opticon Chromo 4 real-time PCR detection system (Bio-Rad), and the thermal program consisted of 2 min at 50°C, 2 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 61.4°C. Plate reading was carried out at 61.4°C. Melting curve analysis following the amplification confirmed the specificity of the primers by detection of a single PCR product in 2% agarose gel. PCR detections were carried out in duplicate from every sample in a 96-well plate. Standard curves were obtained using PCR fragments that were isolated using a PCR purification kit, resuspended in Tris-EDTA buffer (10 mM Tris, pH=8.0), and quantified with spectrophotometer to calculate each mRNA expression level. Standard curves of five points consisted of 10-fold serial dilutions. Sample concentrations calculated from the standard curves were converted into molecules per 1 μg RNA of each receptor mRNA.

2.3. Immunohistochemical study for 5-HT-positive cells

For histological examination, tissue samples from the uterine cornu of three pigs obtained at a slaughter house were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 4 μm, and stained with haematoxylin and eosin. In order to demonstrate the presence of 5-HT, paraffin wax-embedded sections were also examined

immunohistochemically by the streptavidin-biotin-peroxidase complex (SAB) method, employing a rabbit polyclonal antibody to 5-HT (BioGenex) and an SAB kit (Nichirei). Serial sections were cut from paraffin blocks in which the presence of 5-HT-positive cells had been ascertained. Sections adjacent to those with 5-HT immuno-positivity were stained with Giemsa or toluidine blue for identification of mast cells. The adjacent sections were also labeled with rabbit polyclonal antibodies to chromogranin A (Nichirei) and neuron-specific enolase (BioGenex) and with mouse monoclonal antibodies to synaptophysin (Progen), neurofilaments (Dako Corporation), pan-cytokeratin (MNF116) (Dako) and vimentin (Dako) to characterize the cells with 5-HT-positive immunoreactivity.

2.4. Chemicals

The following chemicals were used in the present experiment: compound 48/80 (mast cell degranulator, Sigma), ketanserin hydrochloride (5-HT_2 receptor antagonist, Tocris), (2R)-1-[(3-hydroxyphenyl)sulfonyl]-2-[2-(4-mentyl-1-piperidinyl9ethyl] pyrrolidine hydrochloride (SB269970, 5-HT_7 receptor antagonist, Tocris), 5-hydroxytryptamine creatinine sulfate (5-HT, Sigma), 5-methoxytryptamine hydrochloride (Sigma), α-methyl-5-hydroxytryptamine (α-methyl-5-HT, RBI) and spiperone hydrochloride (Tocris). All compounds were dissolved and diluted in distilled water and applied to the organ directly using a micropipette.

2.5. Statistics

The results of experiments are expressed as means± S.E.M of at least four experiments using uterine muscle strips from different pigs. The significance of differences

between two groups (single comparison) was determined by Student's *t*-test (paired and unpaired). A *P* value <0.05 was considered statistically significant.

3. Results

3.1. Effects of 5-HT on spontaneous contraction of uterine circular muscles

As previously reported (Kitazawa et al., 1998, 2000), in the circular muscle preparations, 5-HT (1 nM-10 μM) applied in the organ bath caused inhibition of spontaneous contraction accompanying a decrease in smooth muscle tonus (Fig. 1). The concentration that induced 50% reduction of contractility (EC_{50}) and the maximum inhibition were 42 ± 17 nM and 93.2 ± 3.5% (n=8), respectively. Pretreatment with SB269970 (10 nM) caused a parallel rightward shift of the concentration-inhibition curve (EC_{50}=242 ± 67 nM, n=5) without affecting the maximum inhibition (99 ± 1%, n=5). The estimated pK_d value was 8.67 (Fig. 2A). In the presence of higher concentrations of SB269970 (100 nM and 1 μM), the inhibitory effect of 5-HT was reduced and the drug (5-HT) caused a slight increase in basal tension (Fig. 1). As shown in Fig. 2A, the concentration-inhibition curve for 5-HT was shifted to the right and also upward by high concentrations of SB269970. Consequently, the monophasic concentration-inhibition curve of 5-HT was changed in the presence of SB269970, into a biphasic concentration-effect curve (Fig. 2A). In the presence of both SB269970 (1 μM) and 5-HT (1 μM), the maximum size of spontaneous contractile response was 120±10% (n=6) of the spontaneous contractile activity in the absence of drugs. The effect of ketanserin (10 nM- 1 μM) on the contractile response to 5-HT in SB269970 (1 μM)-treated preparations was examined to clarify the

receptor subtype (Figs. 1 and 2B). Ketanserin inhibited the excitatory response to 5-HT in a concentration-dependent manner and enhanced the inhibitory responses by 5-HT. The concentration-inhibitory effect curves for 5-HT shifted downward and to the left as shown in Fig. 2B. The effect of ketanserin itself on the inhibition of circular muscle contractility by 5-HT was also examined. The EC_{50} and maximum inhibition in the presence of 10 nM, 100 nM and 1 μM ketanserin were 43 ± 21 nM and 98 ± 1.3% (n=6) , 24 ± 5 nM and 100 ± 0% (n=5) and 29 ± 6.4 nM and 99 ± 1.3% (n=4) , respectively. Ketanserin tended to increase the inhibition by 5-HT (control: EC_{50}=42 ± 17 nM, maximum inhibition= 93.2 ± 3.5%, n=8) in the uterine strips.

In the presence of SB269970 (1 μM), α-methyl-5-HT (1 nM – 10 μM), a 5-HT_2 receptor preferential agonist, was almost equipotent to 5-HT in increasing the contractile activity of circular muscle (E_{max}= 118 ± 1%, n=4), but 5-methoxytryptamine (10 nM – 10 μM) was less effective (E_{max}= 110%) compared with 5-HT and α-methyl-5-HT (5-HT = α-methyl-5-HT > 5-methoxytryptamine). The contractile responses to both α-methyl-5-HT and 5-methoxytryptamine were inhibited by pretreatment with ketanserin (1 μM), similar to the case of 5-HT (data not shown).

3.2 Effects of 5-HT on spontaneous contraction of uterine longitudinal muscles

5-HT, applied cumulatively, decreased the contractile activity in most longitudinal muscle preparations examined (11 out of 14 preparations) (Fig. 3). In the 3 other longitudinal muscle preparations, 5-HT increased the contractility of longitudinal muscles (mean of 3 preparations, 3 nM=109%, 300 nM=130%, 3 μM=160% and 30 μM=174%). Similar to the case in circular muscle strips, SB269970 (1 μM) decreased the inhibitory

responses to 5-HT and changed the inhibition to excitatory mechanical responses with elevation of tonus. Ketanserin (1 μM) inhibited the excitatory responses to 5-HT in the SB269970 treated longitudinal muscles (Figs. 3 and 4). On the other hand, pretreatment with ketanserin (1 μM) itself potentiated the inhibition by 5-HT and shifted the concentration-inhibitory effect curve to downward (Fig. 4).

Although ketanserin is a well-known 5-HT_2 receptor antagonist, this antagonist can discriminate the 5-HT_2 receptor subtype, because the affinities for three 5-HT_2 subtypes were different (pK_i values for 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} being 8.87, 5.49 and 7.32, respectively, Bonhaus et al., 1997). 5-HT-induced excitatory responses in the SB269970-treated longitudinal muscle (EC_{50}=605 ± 161 nM, n=5) was antagonized by ketanserin (10 nM) and ketanserin shifted the concentration-excitatory effect curve to the right (EC_{50}=2106 ± 723 nM, n=4) without affecting the maximum response (control vs. ketanserin, 97 ± 10.1% vs. 70± 9.2%, P=0.21, relative to 50 mM high-K^+). The calculated pK_d value was 8.52. α-Methyl-5-HT also caused contraction of the SB269970-treated longitudinal muscle strips. The EC_{50} value for α-methyl-5-HT was 592 ± 330 nM (n=7), which was comparable with that for 5-HT (Fig. 5). The same concentration of ketanserin (10 nM) shifted the excitatory response curves to α-methyl-5-HT to the right without inhibiting the maximum contraction (Fig. 5). The EC_{50} value of α-methyl-5-HT in the presence of ketanserin was 4184 ± 1016 nM (n=4). Therefore, the pK_d value was estimated to be 8.78. Spiperone is another 5-HT_2 receptor antagonist, which has a different affinity for the three 5-HT_2 receptor subtypes (pK_i values for 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} being 8.85, 5.88 and 6.18, respectively, Bonhaus et al., 1997). Spiperone (10 nM) also inhibited the α-methyl 5-HT-induced contractions and shifted the concentration-excitatory response curves to the right (EC_{50}=7477 ± 1620 nM, n=5). The pK_d value was estimated to be 9.06

(Fig. 5).

3.3 Expression of 5-HT_{2A} and 5-HT_7 receptor mRNAs

To examine the expression of 5-HT_{2A} and 5-HT_7 receptor mRNAs in the porcine uterus, total RNA prepared from uterine cornu longitudinal and circular muscles was subjected to RT-PCR using porcine 5-HT_{2A} and 5-HT_7 receptor-specific primers. In the uterus, 5-HT_{2A} and 5-HT_7 receptor transcripts at predicted sizes (534 bp and 523 bp, respectively) were identified both in longitudinal and circular muscle layers. However, the control lane (polymerase chain reaction without cDNA) did not show any bands (Fig. 6).

Real-time PCR analysis showed that expression of 5-HT_7 receptor mRNA was different in longitudinal muscle (1115 ± 502 copies/μg RNA, n=7) and circular muscle (5639 ± 2427 copies/μg RNA, n=8), suggesting differential expression of 5-HT_7 receptor in the uterine corneal region (longitudinal muscle < circular muscle). In contrast the expression of 5-HT_{2A} receptor mRNA was opposite to that of 5-HT_7 receptor. The number of copies in the longitudinal muscle (24260± 8400 copies/μg RNA, n=5) was higher than that in the circular muscle (7676± 1597 copies/μg RNA, n=4).

3.4. Immunohistochemistry for 5-HT

5-HT was expressed in a few endometrial glands, which were localized near the circular myometrium (Fig. 7A). Most of the positive cells exhibited intense immunolabeling in the nucleus and cytoplasm and were oval in shape. However, few cells were weakly labeled, and were columnar and cytologically similar to surrounding glandular cells. Serial sections

demonstrated the absence of cytoplasmic granules and metachromasia in 5-HT-positive cells (Figs. 7B and C). In addition, coexpression of 5-HT and chromogranin A, neurone-specific enolase or pan-cytokeratin was observed, but synaptophysin, neurofilaments and vimentin were negative in 5-HT-positive cells (data not shown). There were no 5-HT-positive cells in the myometrium. Only a few cells with cytoplasmic granules stained purple with Giemsa (characterization of mast cells) were present beneath the surface epithelium, but the granules were 5-HT-negative and did not show metachromasia.

3.5. Effects of compound 48/80, ketanserin or SB269970 on spontaneous contractility of myometrial strips with endometrium

As demonstrated in the immunohistochemical study, 5-HT-containing cells were located mainly in the uterine glands, scattered in the endometrium. Therefore, longitudinal and circular muscle preparations with the endometriun attached were prepared, and the effects of compound 48/80, ketanserin or SB269970 on spontaneous uterine contractility were observed individually to examine the regulation of uterine motility by endogenous 5-HT. Compound 48/80 (50 μg/ml), a mast cell degranulator, applied in the organ bath did not change spontaneous activity of either longitudinal or circular smooth muscles. Relative magnitudes of spontaneous contractility at 5, 10, 15 and 20 min after application of compound 48/80 were 109±6.5%, 102±4.0%, 125±22.3% and 97.3±7.6% (n=7) in the longitudinal muscles and 99±1.0%, 100±1.6%, 101±2.4% and 101±3.3% (n=6) respectively, in the circular muscles. Treatment with SB269970 (1 μM for 20 min) slightly but significantly increased the contractile activity in the circular muscle (108±2.5%, n=14, P=0.03). The contractility of the longitudinal muscle also tended to be increased by

SB269970 (114±7.5%, n=13, P=0.08). On the other hand, treatment with ketanserin (1 μM for 20 min) decreased both longitudinal muscle contractility (86±11.7%, n=5, P=0.7) and circular muscle contractility (93.2±5.8%, n=14, P=0.22), but the effects were not statistically significant.

4. Discussion

The present experiments revealed the expression of excitatory (contractile) 5-HT_{2A} receptor in the porcine myometrium in addition to the expression of the well-known inhibitory (relaxant) 5-HT_7 receptor demonstrated previously (Kitazawa et al., 1998, 2000). Cells with 5-HT immunoreactivity, different from mast cells, were scattered in uterine glands of the endometrium. Endogenous 5-HT is thought to regulate porcine uterine contractility through activation of inhibitory 5-HT_7 receptor.

As previously reported (Kitazawa et al., 1998, 2000), 5-HT inhibited spontaneous contractile activity of the porcine myometrium in a muscle layer-dependent manner (circular muscle > longitudinal muscle). SB26990, a 5-HT_7 receptor antagonist, decreased the 5-HT-induced inhibition, and the pK_d values (8.4) was consistent with the affinity of SB26990 for 5-HT_7 receptor (pA_2=8.5, Hagan et al., 2000). 5-HT_7 receptor mRNA transcript was also demonstrated to be expressed in the porcine myometrium by RT-PCR (Kitazawa et al., 2000 and present results). These finding confirmed that 5-HT_7 receptors coupled with adenylate cyclase activity positively were present both in longitudinal and circular muscles layers and were involved in the inhibition of porcine uterine muscle contractility by 5-HT. Since 5-HT_7 receptors were preferentially expressed in the circular muscle (Kitazawa et al., 2000 and present results), circular muscle was more sensitive than

longitudinal muscle to the inhibition of muscle contractility by 5-HT. In the presence of high concentrations of SB269970, 5-HT caused biphasic actions in the circular muscles: increase (low concentration) and inhibition (high concentration) of contractile activity, and only contraction in the longitudinal muscles. α-Methyl-5-HT, a 5-HT_2 receptor agonist, was almost equipotent to 5-HT in increasing the uterine contractility, and ketanserin, a 5-HT_2 receptor antagonist, inhibited the excitatory effects of 5-HT and α-methyl-5-HT. These results suggest that 5-HT_2 receptor is an excitatory 5-HT receptor in the porcine uterus. 5-HT_2 receptors are coupled with $G_{q/11}$ and stimulate the hydrolysis of membrane phospholipids to produce two second messengers (inositol-trisphosphate and diacylglycerol). Inositol-trisphosphate-evoked Ca^{2+}-release from the Ca^{2+} store triggers smooth muscle contractions (Hoyer et al., 1994, 2002). At present, 5-HT_2 receptors are classified into three subtypes (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) based on the affinity of agonists and antagonists and based on molecular structures. Among the 5-HT receptor agonists examined, the ranking order in producing excitatory responses is 5-HT = α-methyl-5-HT > 5-methoxytryptamine, which is consistent with that for 5-HT_{2A} receptor (5-HT_{2A}: 5-HT = α-methyl-5-HT > 5-methoxytryptamine, 5-HT_{2B}: 5-methoxytryptamine > 5-HT = α-methyl-5-HT , 5-HT_{2C}: 5-HT > α-methyl-5-HT > 5-methoxytryptamine, Porter et al., 1999). Involvement of 5-HT_{2A} receptor in the excitatory responses was also supported by the results of an antagonist study. Ketanserin decreased both 5-HT- and α-methy-5-HT-induced excitatory responses in a competitive manner, and the estimated pK_d values were 8.52 and 8.78, respectively. These values are similar to the value for 2A type (8.87) but not to the values for 2B type (5.49) and 2C type (7.32). In addition, the pK_d value of spiperone (9.06) was also consistent with that for 5-HT_{2A} receptors (8.87) (Bonhaus et al., 1997). Expression of 5-HT_{2A} receptor mRNA in both longitudinal and

circular muscles was demonstrated by RT-PCR study, and this finding supported the results of the contraction studies. In most mammalian species, increase in uterine contractile activity is a common mechanical response induced by 5-HT, and 5-HT_2 receptors are thought to mediate these excitatory responses. In rats, 5-HT caused contraction of rat uterine strips through activation of 5-HT_{2A} receptor subtype (Cohen et al., 1986; Minosyan et al., 2007). Isolated human uterine strips were also contracted by 5-HT (Cruz et al., 1989), and a recent study showed the expression of 5-HT_{2B} receptor subtype in freshly isolated human uterine smooth muscle cells. Activation of this receptor increases the intracellular Ca^{2+} concentration (Kelly and Sharif, 2006). Taken together, the results indicate that although 5-HT_2 receptor is a common excitatory 5-HT receptor expressed in the mammalian uterus, receptor subtypes involved in 5-HT-induced contractile responses differ from species to species.

The number of mast cells in the uterus has been shown to change depending on the oestrous cycle and pregnancy, and these results suggested that mast cells through mediators such as histamine, 5-hydroxytryptamine and prostaglandins regulate the motility of the uterus (Padilla et al., 1990; Rudolph et al., 1993, 1998, Mori et al., 1997; Bytautine et al., 2002). In uterine smooth muscle of guinea-pigs, contractile responses induced by compound 48/80, a mast cell degranulator, were inhibited by both ketanserin and chlorpheniramine, and these results imply that 5-HT and histamine released from mast cells can influence the uterine contractility (Bytautine et al., 2002). In the mouse uterine horn, mast cells take up and accumulate 5-HT in a saturable manner (Rudolph et al., 1998). Thus, 5-HT is thought to be concentrated in uterine mast cells in rodents. In the present study, in contrast, 5-HT immunoreactivity was found in uterine glands. The positive cells were agranular and cytokeratin-positive, and are thought to be of epithelial origin. The failure of

compound 48/80 to modify the spontaneous contractility of porcine uterine strips supports the notion that 5-HT-positive cells are distinct from mast cells. Because mast cells containing 5-HT varied in number in different oestrus cycles or pregnancy in humans and mice (Padilla et al., 1990; Rudolph et al., 1993, 1998, Mori et al., 1997), it is likely that the number of 5-HT-positive epithelial cells in the uterine glands can change according to pregnancy and oestrous cycle in pigs.

To explore the possible regulatory roles of endogenous 5-HT, the effects of 5-HT_2 receptor or 5-HT_7 receptor antagonist on the contractility of porcine uterine strips with endometrium were assessed. SB26990 increased the AUC of spontaneous contraction in the circular muscles significantly (P=0.03) but not in the case of longitudinal muscles (P=0.08). Although the secretory stimulation to 5-HT-containing cells in uterine glands is unknown at present, the results suggest that endogenous 5-HT and 5-HT_7 receptor mechanisms mediate the inhibition of uterine contractility. Because of the differential expression of inhibitory 5-HT_7 receptor in the muscle layers (circular muscles > longitudinal muscles, Kitazawa et al., 2000 and present results), influence of blockade of 5-HT_7 receptors on uterine contractility might be significant in the circular muscles but not in the longitudinal muscles. On the other hand, ketanserin did not decrease the spontaneous activity of both uterine muscles significantly. Although intensity of 5-HT-induced inhibitory effects was different between muscle layers (circular muscles > longitudinal muscles), inhibition (5-HT_7 receptor-mediated actions) was a dominant effect of 5-HT applied in the organ bath in both muscle layers (Kitazawa et al., 1998 and present results), suggesting that stimulation of 5-HT_2 receptor causes only small influence on spontaneous contractility of porcine uterine strips in the present experimental conditions. Therefore, the effect of blockade of 5-HT_2 receptors by ketanserin was not significant to change the contractility of both uterine

muscle layers.

In conclusion, in addition to inhibitory (relaxant) 5-HT_7 receptors, excitatory (contractile) 5-HT_{2A} receptors are expressed in the uterus of non-pregnant pigs. Endogenous 5-HT-containing cells different from mast cells are present in the uterine glands of the pig. The results suggest possible roles of endogenous 5-HT and its receptors in the regulation of contractility of porcine uterine circular muscles.

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Figure Legends

Fig. 1. Typical effects of 5-HT on spontaneously contracting porcine circular muscle strips in the absence (control) and presence of SB269970 (1 μM) or SB269970 (1 μM) plus ketanserin (1 μM). Increasing concentrations of 5-HT (control: 1, 3, 10, 30, 100, 300 nM, 1 and 3 μM, treatment with antagonist: 1, 3, 10, 30, 100, 300 nM, 1, 3, 10 and 30 μM) was applied cumulatively at 5-min intervals. Dotted lines indicate the base line of spontaneous contractile activity before application of 5-HT.

Fig. 2. Effects of SB269970 and ketanserin on the inhibitory action of 5-HT in porcine myometrial circular muscles. A: Concentration-inhibitory effect curves of 5-HT were established in the absence (control) and presence of three increasing concentrations of SB269970 (10 nM, 100 nM, 1 μM). B: Additional application of ketanserin (Ketan, 10 nM, 100 nM, 1 μM) decreased the excitatory effects and unmasked inhibitory actions of 5-HT in a concentration-dependent manner. Ordinate: contractility of circular muscle strips expressed as a percentage of spontaneous contractility (AUC) before application of 5-HT. Abscissa: concentration of 5-HT (log M). Points represent the means of four or more experiments with SEM shown by vertical lines.

Fig. 3. Typical effects of 5-HT on spontaneously contracting porcine longitudinal muscle strips in the absence (control) and presence of SB269970 (1 μM) or SB269970 (1 μM) plus ketanserin (1 μM). Increasing concentrations of 5-HT (10, 30, 100, 300 nM, 1, 3, 10, 30, 100 μM) were applied cumulatively at 5-min intervals.

Fig. 4. Effects of SB269970 and ketanserin on the mechanical actions of 5-HT in the porcine uterine longitudinal muscles. Concentration-effect curves of 5-HT were established in the absence (control, ○) and presence of SB269970 (1 μM, ●) or ketanserin (1 μM, ■). Additional treatment with ketanserin (1 μM) inhibited the excitatory responses to 5-HT in the SB269970-treated longitudinal muscle preparations (□). Ordinate: contractility of longitudinal muscle strips expressed as a percentage of spontaneous contractility (AUC) before application of 5-HT. Abscissa: concentration of 5-HT (log M). Points represent the means of four or more experiments with SEM shown by vertical lines.

Fig.5. Effects of ketanserin and spiperone on α-methyl-5-HT-induced excitatory responses in the uterine longitudinal muscle strips. Concentration-excitatory effect curves of α-methyl-5-HT in the absence (control, ■) and presence of spiperone (10 nM, ●) or ketanserin (10 nM, ▲) . Ordinate: contractility of longitudinal muscle strips expressed as a percentage of 50 mM high-K^+-induced contraction (AUC). Abscissa: concentration of α-methyl-5-HT (logM). SP indicates spontaneous contraction before application of α-methyl-5-HT. Points represent the means of four or more experiments with SEM shown by vertical lines.

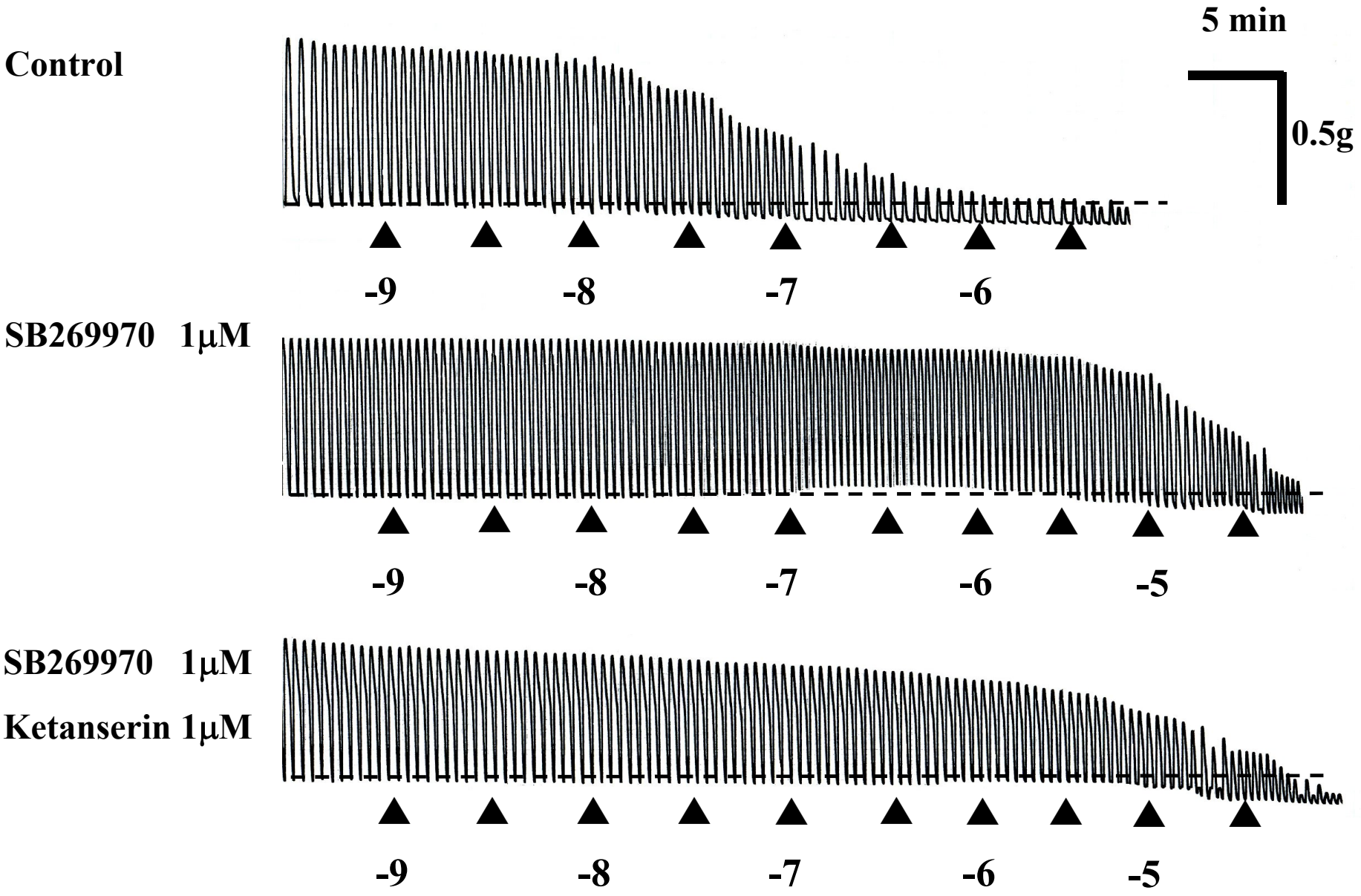
Fig. 6. Expression of 5-HT_{2A} and 5-HT_7 receptor mRNAs in the porcine uterus. RT-PCR was used to amplify signals of 5-HT_{2A} and 5-HT_7 receptor mRNAs isolated from longitudinal (LM) and circular muscles (CM) of the porcine uterus. For a negative control, a solution without cDNA was used for PCR (Cont). Primer sequences and sizes of the PCR

products (5-HT_{2A}=534 bp, 5-HT_7=523 bp) are given in Materials and methods. M : molecular marker.

Fig. 7. Cells showing 5-HT immunoreactivity in the porcine uterus. A: Arrows indicate 5-HT-positive cells. Note that uterine glands with the positive cells lie in the endometrium (Endo) near the uterine circular muscle (bottom, CM). Scale bar: 50 μm. B: Enlargement of a gland in (A). A 5-HT-positive cell (arrow) intervenes among gland-lining cells and is located near the lumen of the gland. Scale bar: 20 μm. C: Adjacent section shows the same cell as in (B), and no granules are visible in the cytoplasm (arrow) by Giemsa staining. Scale bar: 20 μm.

Figure

Fig.1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-8
-7
-6
SB269970 1μM
-9
-8
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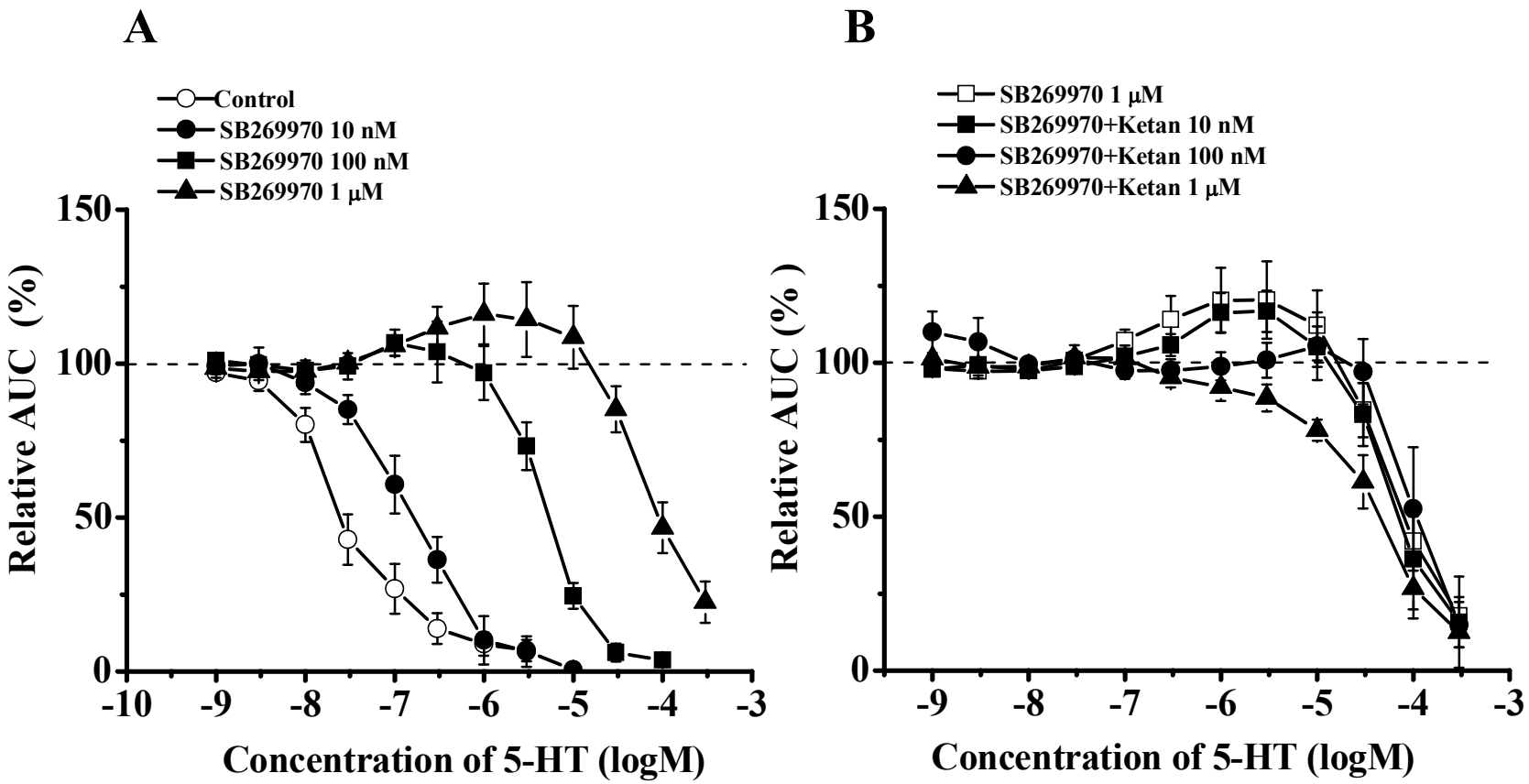
Ketanserin 1μM
-9
-8
-7
-6
-5

Figure

Fig.2


A
Control
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SB269970 100 nM
SB269970 1 μM
Relative AUC (%)
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100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
Concentration of 5-HT (logM)
B
SB269970 1 μM
SB269970+Ketan 10 nM
SB269970+Ketan 100 nM
SB269970+Ketan 1 μM
Relative AUC (%)
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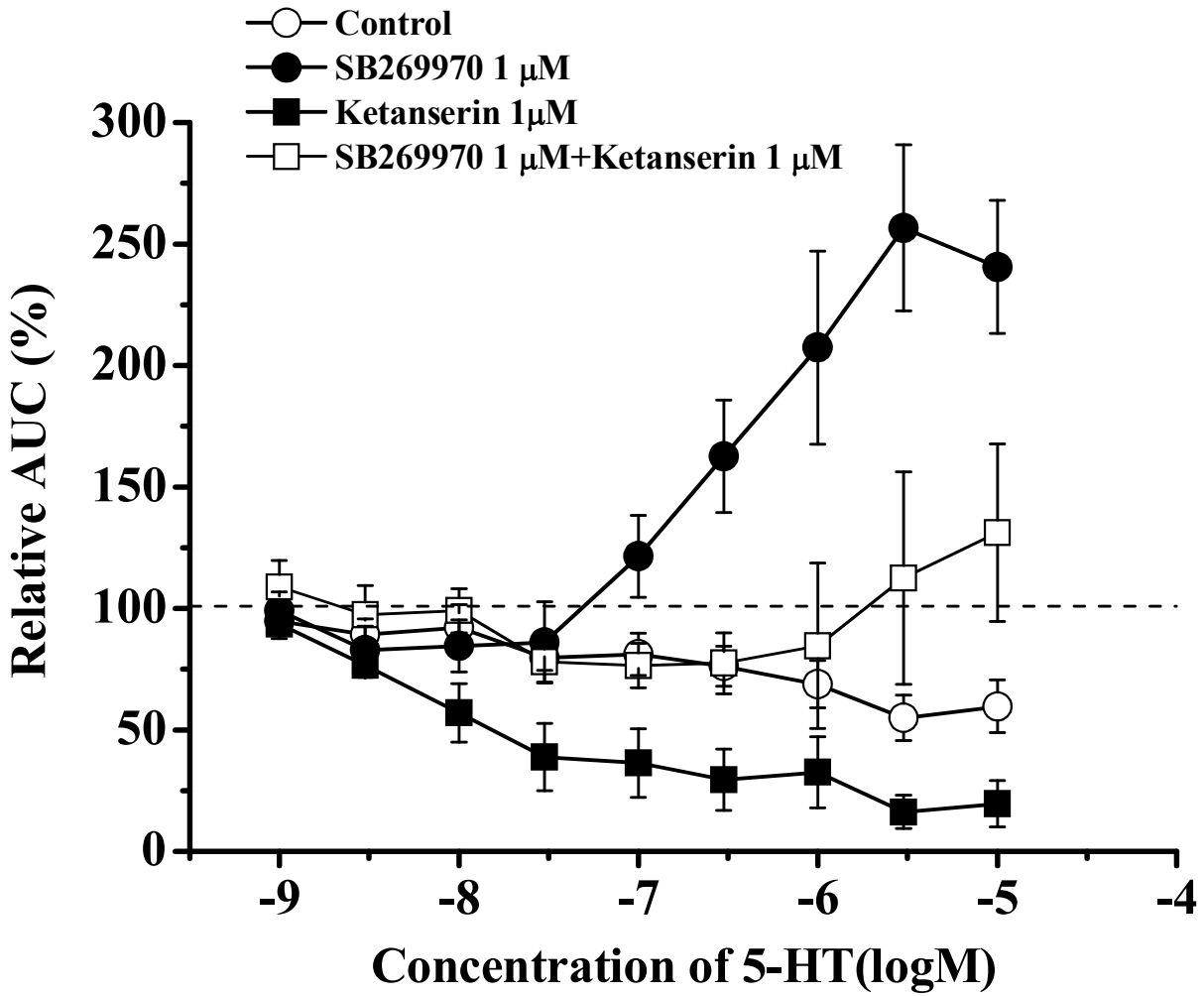
100
50
0
-9
-8
-7
-6
-5
-4
-3
Concentration of 5-HT (logM)

Figure

Fig.4


Control
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Ketanserin 1μM
SB269970 1 μM+Ketanserin 1 μM
Relative AUC (%)
300
250
200
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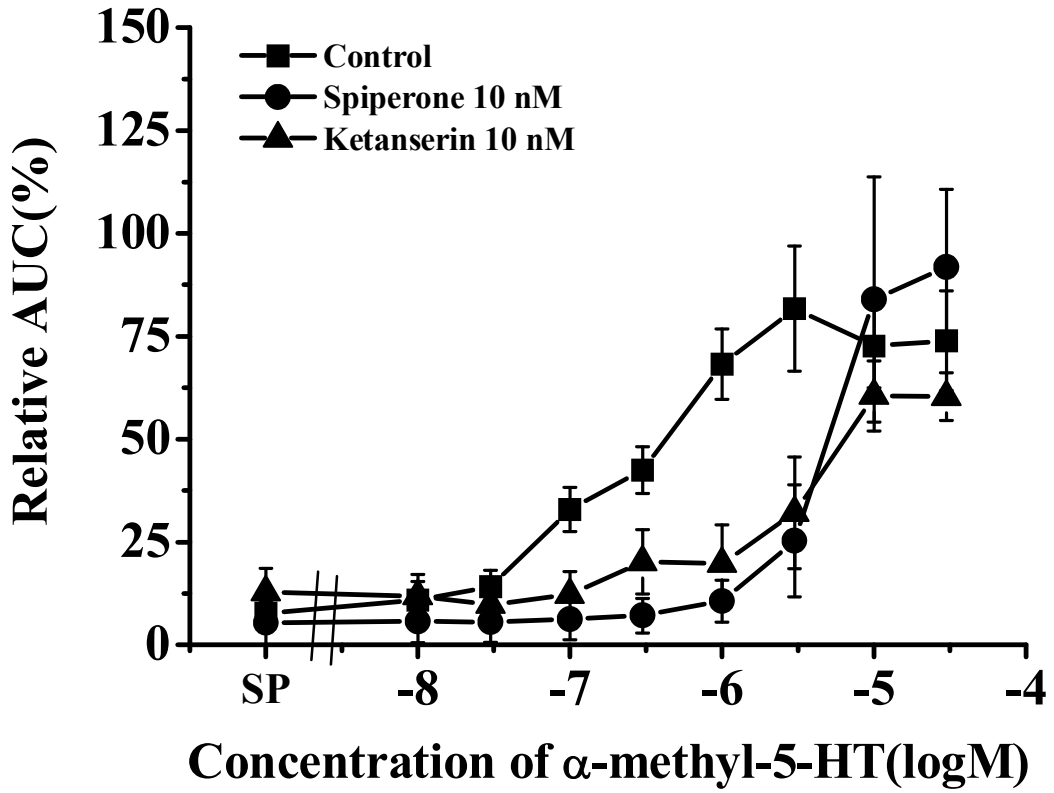
-7
-6
-5
-4
Concentration of 5-HT(logM)

Figure

Fig.5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Ketanserin 10 nM
Relative AUC(%)
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125
100
75
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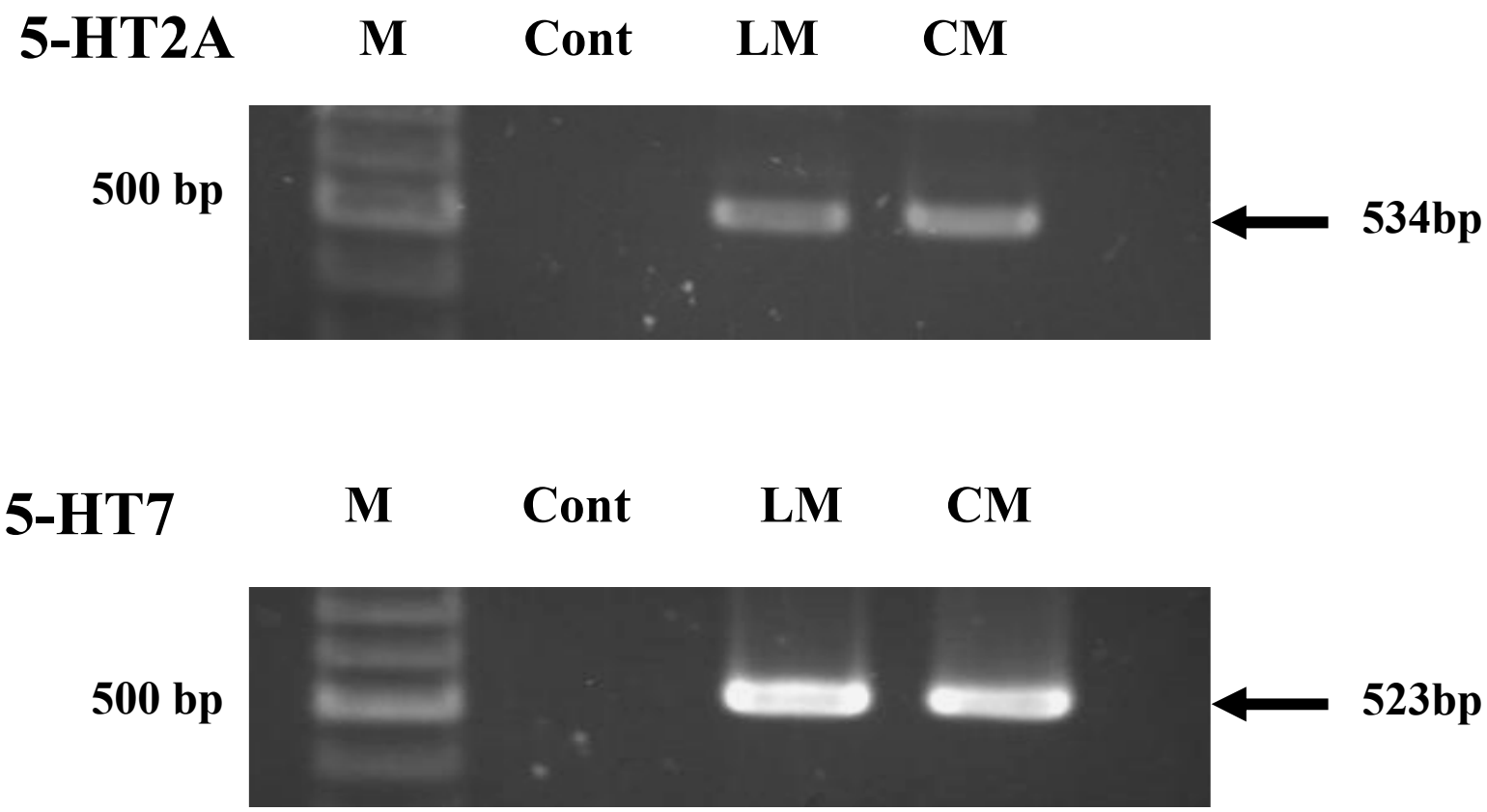
-4
Concentration of α-methyl-5-HT(logM)

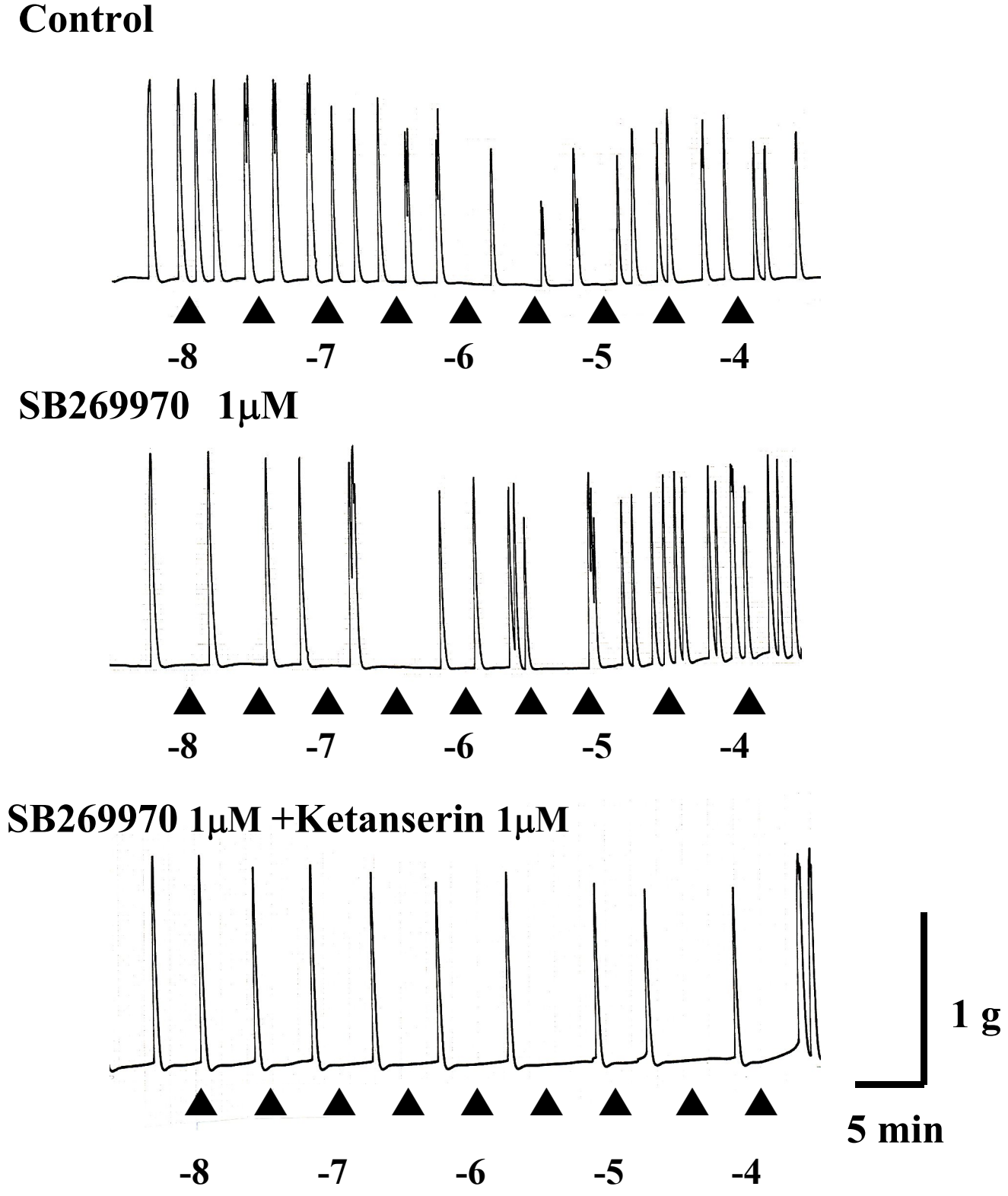
Figure

Fig.6



Figure

Fig.3


Control
-8
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-4
SB269970 1μM
-8
-7
-6
-5
-4
SB269970 1μM +Ketanserin 1μM
-8
-7
-6
-5
-4
1 g
5 min

Figure

Fig.7