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Thromboxane A_2 (TP) receptor in the non-pregnant porcine myometrium and its role in regulation of spontaneous contractile activity

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Abstract

Although there are species-related differences in uterine prostanoid receptor subtypes, functional prostanoid receptors in the porcine uterus are similar with those in the human uterus (FP, TP, EP_1, EP_2, EP_3, DP and IP) except for the TP receptor. These similarities promoted us to determine whether TP receptors are present in the non-pregnant porcine uterus. For this purpose, the effects of TP receptor agonists and antagonists were investigated by a contraction study and by a binding study. 9,11-Dideoxy-9α, 11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid (U46619, 1 nM–10 μM), a stable thromboxane A_2 mimetic, caused tetrodotoxin-resistant contraction in both longitudinal and circular muscles of the uterine cornu. The pEC_{50} value in the longitudinal muscle (6.69) was lower than that in the circular muscle (7.62), but the maximum response in the longitudinal muscle was two times larger than that in the circular muscle. The longitudinal and circular muscles of other regions (corpus and cervix) also responded to U46619, and region-related difference in contractile responses was observed only in the longitudinal muscles. 4(*Z*)-6-(2-*o*-Chlorophenyl-4-*o*-hydroxyphenyl-1,3-dioxan-*cis*-5-yl) hexenoic acid (ICI192605) and 7-[3-[[2-[(phenylamino)carbonyl] hydrazino]methyl]7-oxabicyclo[2.2.1]hept-2-yl]-,[1*S*-[1α,2α(*Z*),3α,4α]]-]5-heptenoic acid (SQ29548) inhibited the contractile responses to U46619 competitively. The longitudinal and circular muscles in the cornu contained a single class of [^{3}H]SQ29548 binding site with similar K_d values (30 nM), but B_{max} in the circular muscle (90.9 ± 8.6 fmol/mg protein) was two times higher than that in the longitudinal muscle (58.2 ± 8.6 fmol/mg protein). The ranking order of competition by TP receptor agonists and antagonists (with pK_i values in parentheses) was [1*S*-[1,2(*Z*),3(1*E*,3*S**),4]]-7-[3-[3-Hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (I-BOP, 7.70)>SQ29548 (7.39)>7-[3-(3-Hydroxy-1-octenyl)bicycle[3.1.1]hept-2-yl]-,[2*S*-[2α(*Z*),3β(1*E*,3*R**)]]-5-heptenoic acid (CTA_2, 6.55)>7-[3-(3-hydroxy-1-octenyl)-6,6-dimethylbicyclo[3.1.1]hept-2-yl-,[1*S*-[1α,2β(*Z*),3α(1*E*,3*R**),5α]]-5-heptenoic acid (PTA_2, 6.50)>U46619 (6.41)>7-[5-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1] hept-6yl]-,[1*S*-[1α,4α,5α(1*E*,3*R**),6β(*Z*)]]-5-heptenoic acid (U44069, 6.34), and this order is consistent with current TP receptors. Treatment with indomethacin (100 nM) and *N*-*tert*-butyl-*N*¢-[(2-cyclohexylamino-5-nitrobenzene) sulfonyl] urea (BM-531, 10 μM) inhibited the spontaneous contractile activities of both longitudinal and circular muscles. The present results indicate that contractile TP receptors are present in the non-pregnant porcine uterus. Therefore, the prostanoid receptor subtypes that exist in the porcine uterus (TP, IP, DP, FP, EP_1, EP_2 and EP_3) are the same as those present in the human uterus. The distribution of TP receptors in the porcine uterus differed depending on the type of myometrium (longitudinal and circular muscles) and region of the uterus. The endogenous thromboxane A_2-TP receptor pathway is thought to play a physiological role in regulation of spontaneous contractile activity in the porcine uterus.

Keywords: U46619; Prostanoid TP receptor; Uterus, pig; Thromboxane A_2; Contractility, spontaneous

1. Introduction

Thromboxane A_2 is a biologically potent arachidonic acid metabolite synthesized through cyclooxygenase and thromboxane A_2 synthase pathways. Thromboxane A_2 plays important physiological roles in the regulation of vascular and non-vascular smooth muscle tone, aggregation of platelets, cell proliferation and apoptosis. Vascular contraction and activation of platelets induced by thromboxane A_2 are involved in the pathophysiology of myocardia and cerebral infarction (Sjoberg and Steen, 1989; Mihara et al., 1989; Senchyna and Crankshaw, 1996; Rolin et al.,2001; Gao et al., 2000). These responses to thromboxane A_2 are mediated by the thromboxane A_2 receptor (TP), which belongs to a family of seven membrane-spanning receptors that transduce signals through the G protein (Narumiya et al., 1999;

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Wise et al., 2002). Human, bovine and rat TP receptors have recently been cloned (Wise et al., 2002), and two isoforms (TPα and TPβ) have been identified in the human uterus according to the property of signal transduction pathways (Miggin and Kinsella, 2002; Moore et al., 2002). TPβ receptor activation stimulates the phospholipase C-inositol 1,4,5-trisphosphate-Ca^{2+} pathway and RhoA-associated protein kinase. On the other hand, activation of the TPα receptor increases cytoplasmic cyclic AMP by stimulation of the Gs-adenylate cyclase-cyclic AMP pathway (Kinsella et al., 1997).

In the human uterus, 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5*Z*,13*E*-dien-1-oic acid (U46619), a stable and potent agonist of the TP receptor, has been shown to cause contraction of smooth muscle through activation of myogenic TP receptors, and specific binding sites of [^{125}I] ([1*S*-[1 α,2 α(*Z*),3β(1*E*,3*S**),4α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabi-cyclo[2.2.1]hept-2-yl]5-heptenoic acid) (I-BOP) have been found in the myometrial membrane (Senior et al., 1992; Senchyna and Crankshaw, 1996). The results of a molecular biological study have also revealed that the TP receptor gene is expressed in human uterine smooth muscle (Swanson et al., 1992). It has been reported that the concentrations of thromboxane B_2, a metabolite of thromboxane A_2, increased during pregnancy in the human and rat decidua, placenta, chorion, amnion and myometrium, and reached peaks at the end of pregnancy (Mitchell et al., 1978; Zamecnik et al., 1980). Immunohistochemical studies have shown that thromboxane A_2 synthase is localized in human placenta, endometrial glands, uterine blood vessels and myometrial cells (Wetzka et al., 1993, 1994; Swanson et al., 1992). These findings suggest that thromboxane A_2 plays an important physiological role in regulation of contractility and vascular tone of the uterus during pregnancy and labor.

Isolated myometrial strips are useful for investigation of the characteristics of prostanoid receptors in the uterus, and remarkable species-related variations in mechanical responses (contraction, relaxation or a mixture of both responses) to prostanoids, including thromboxane A_2, have been reported. These species-related variations in mechanical responses are caused by the difference in prostanoid receptor subtype populations in the uterus (Crankshaw, 2001). Functional and molecular biological studies have shown the presence of FP, TP, EP_1, EP_2, EP_3, DP and IP receptors in the human uterus (Senior et al., 1991, 1992; Senchyna and Crankshaw, 1996), EP_1, EP_3, FP, IP and TP receptors in the rat uterus (Crankshaw and Gaspar, 1992; Goureau et al., 1992), FP, EP_1,EP_2, EP_3 and TP receptors in the sheep uterus (Crankshaw and Gaspar, 1995) and EP_1, EP_3 and TP receptors in the guinea-pig uterus (Coleman et al., 1990). In our previous study, we characterized the subtypes of prostanoid receptors in the non-pregnant porcine uterus using naturally occurring prostaglandins and receptor selective-agonists. Contractile FP, EP_1, EP_3 receptors and relaxant DP, IP, EP_2 receptors are present in the porcine uterus, and the distribution of these receptors has been shown to be smooth muscle layer-dependent (longitudinal and circular muscle layers) (Cao et al., 2002). The population of receptor subtypes in the porcine uterus appears to be very similar to that in the human uterus except for the TP receptor. However, the presence of the TP receptor in the porcine myometrium has not been clarified yet.

The aim of the present study was therefore to determine whether TP receptors are present in the porcine myometrium. For this purpose, mechanical responses of TP receptor agonists (U46619, 7-[5-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1]hept-6yl]-,[1*S*-[1α,4α,5α(1*E*,3*R**),6β(*Z*)]] -5-heptenoic acid (U44069) and I-BOP) to uterine contractility and inhibitory effects of TP receptor antagonists (4(*Z*)-6-(2-*o*-chlorophenyl-4-*o*-hydroxyphenyl-1,3-dioxan-*cis*-5-yl) hexenoic acid (ICI192605) and 7-[3-[[2-[(phenylamino)carbonyl] hydrazino]methyl]7-oxabicyclo[2.2.1]-hept-2-yl]-, [1*S*-[1α,2α(*Z*),3α,4α]]-]5-heptenoic acid (SQ29548)) on the response to U46619 were examined in isolated smooth muscles of the porcine uterus. The possible heterogeneous distribution of TP receptors in longitudinal and circular muscle layers was also examined by a radioligand ([^{3}H]SQ29548) binding study. Moreover, in order to find out the role of endogenous thromboxane A_2 in regulation of uterine contractility, changes in spontaneous contraction induced by indomethacin (a cyclooxygenase inhibitor) and *N*-*tert*-butyl-*N*¢-[(2-cyclohexylamino-5-nitrobenzene) sulfonyl]urea (BM-531, a TP receptor antagonist with thromboxane A_2 synthase-inhibiting activity, Dogne et al., 2001) were also examined.

2. Materials and methods

2.1. Tissue preparations

Fresh uteri, with the ovaries intact, from 120 sexually mature crossbred virgin gilts (about 6 months old) were provided by a local abattoir and were used in experiments on the day of slaughter. The pigs were judged to be in proestrus (about day 4 of in the 21-day estrus cycle of the pig) by gross examination of the follicle size (smaller than 2 mm in diameter) and by the appearance of the corpora lutea (McDonald, 1975). Uterine muscle segments (each about 15 mm in length) were isolated surgically from the antimesometrial coat of the adtubal region (10 cm distal from the apex) in either the left or right cornu. After cutting off the endometrium, myometrial strips parallel to the direction of the longitudinal muscle or circular muscle fibers were isolated as described previously (Taneike et al., 1991; Kitazawa et al., 2000). Then the unwanted muscle layer was removed from each muscle strip by meticulously cutting away with fine scissors under a binocular microscope, and the remaining muscle preparations (pure longitudinal or circular muscle) were used in the contraction

and binding experiments. In some experiments, to determine regional differences in response to U46619, longitudinal and circular muscle strips from the uterine corpus and cervical regions were also prepared.

2.2. Contraction study and data analysis

Each muscle strip (10–15 mm in length, approximately 14 mg wet weight) was 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) equilibrated 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) and a computer-aided analysis system (LEG-1000, Nihon Kohden) were used to evaluate the mechanical activity of the myometrium. The muscle strips were loaded with weights of 0.3–0.5 g as initial tension and allowed to equilibrate for 60 min to establish reproducible spontaneous contractions. First, in order to compare the responses to TP receptor agonists in the longitudinal and circular smooth muscles, concentration–response curves of agonists were constructed. As shown in Fig. 1, cumulatively applied U46619 caused phasic contraction (longitudinal muscle) and increased the amplitude of spontaneous contraction (circular muscle) without elevating the resting tension. Therefore, contractile responses to TP receptor agonists were evaluated by motor activity, i.e., the area surrounded by the contraction curve and resting tension (for 5 min), and expressed as a percentage of spontaneous contraction or 50 mM high-K^+-induced area under the curve (for 5 min). In the

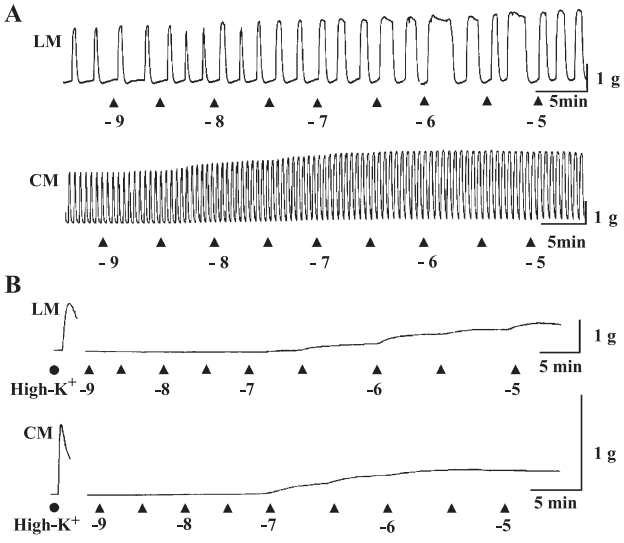

Fig. 1. Representative contractile responses to U46619 in the longitudinal muscle (LM) and circular muscle (CM) of the porcine uterus (cornu). (A) Krebs solution. (B) Kumagai solution. U46619 (1, 3, 10, 30, 100, 300 nM, 1, 3, 10 μM) was cumulatively added to an organ bath at 5-min intervals. The number under each contractile trace indicates the concentration of U46619 (log*M*). The response to high-K^+ (50 mM) in Kumagai solution is also shown.

preparations to which TP receptor agonists were applied, recovery of contractile activity in the circular muscle to resting level took a long time (over 1 h) in spite of every 15 min washing-out. In addition, successive washing-out decreased spontaneous activity of the longitudinal muscle by 20% of the control activity. Therefore, only one concentration–response curve for an agonist was constructed in one myometrial preparation. Contractile responses to TP receptor agonists were also examined in Kumagai solution (Kumagai et al., 1952; mM: NaCl 150.6; KCl 5.4; $CaCl_2$ 0.4; $MgCl_2$ 0.2; $NaHCO_3$ 4.8; $NaHPO_4$ 0.6; KH_2PO_4 0.1 and glucose 2.8 saturated with 95%O_2 + 5%CO_2 at 28 °C). Because of the low Ca^{2+} concentration and low temperature of the nutrient solution, spontaneous contractions of both longitudinal and circular muscle strips almost completely disappeared in Kumagai solution. The mechanical responses were determined as amplitude of contraction and expressed as a percentage of 50 mM high-K^+-induced contraction. The EC_{50} values (concentrations of agonists that caused 50% of maximum response) and the maximum response values were estimated by least-squares non-linear regression analysis of concentration–response curves using Origin software (Ver 7.0; Origin Lab, USA). The EC_{50} value was expressed as the negative logarithm (pEC_{50}).

The effects of TP receptor antagonists on U46619-induced contraction were investigated to characterize contractile responses. Two longitudinal muscles or two circular muscles were isolated from close portion of the cornu, and each muscle preparation was suspended vertically in an organ bath. One preparation was used as a control (of which a vehicle was added) and the other preparation was treated with TP receptor antagonists for 1 h. Then U46619 was applied cumulatively to each preparation, and the EC_{50} values in the absence and presence of antagonists were determined from the concentration–response curves. The apparent dissociation constant (K_b) of each antagonist was determined using the following equation (Van Rossum, 1963): K_b = antagonist concentration/(CR − 1), where CR is the concentration ratio of EC_{50} (EC_{50} of U46619 in the presence of an antagonist divided by EC_{50} of U46619 in the absence of an antagonist). The results were then expressed as the negative logarithm of pK_b ($-\log K_b$).

The effects of pretreatment with indomethacin (a non-selective cyclooxygenase inhibitor) and *N-tert*-butyl-*N*¢-[(2-cyclohexylamino-5-nitrobenzene)sulfonyl]urea (BM-531, a TP receptor antagonist with thromboxane A_2 synthase-inhibiting activity, Dogne et al., 2001) on spontaneous contractility of the uterus were examined to clarify the role of endogenous thromboxane A_2. Two longitudinal muscles or two circular muscles were isolated from the close region of the uterus cornu and suspended in an organ bath. One muscle strip was used as a time-matched control preparation treated with a vehicle, and the other preparation was treated with indomethacin or BM-531. After 1 h, changes in the spontaneous contraction were evaluated by the area under the curve (for 5 min), amplitude and frequency. The effects

of both agents on the responses to high-K^+ (50 mM) were also investigated, and the selectivity of each agent was evaluated.

2.3. Radioligand binding study

To compare the TP receptor distributions between longitudinal muscle and circular muscle of the cornu, a binding study using a TP receptor antagonist, [^{3}H]SQ29548 (NEN Life Science), was carried out. A crude membrane fraction for the binding experiment was prepared by the method described by Chiang and Tai (1998). Isolated longitudinal and circular muscles were cut into small pieces with fine scissors and homogenized in Tris (10 mM)–HCl buffer (pH 7.4) containing 10 μM indomethacin and 50 μg/ml phenylmethyl sulfonyl fluoride. The homogenate was centrifuged at 2500 × *g* for 30 min at 4 °C, and then the supernatant was centrifuged twice at 40,000 × *g* (for 30 min at 4 °C). The resulting pellets were washed twice and suspended in the Tris–HCl buffer and finally used for determination of [^{3}H]SQ29548 binding. Protein in the membrane preparation was measured according to the method of Lowry et al. (1951).

The tissue membrane fraction (100 μg/tube) was incubated with various concentrations of [^{3}H]SQ29548 (1–45 nM) for 45 min at 37 °C to determine the saturation of binding. The binding reaction was terminated by adding 5 ml ice-cold buffer (25 mM Tris–HCl). The incubation mixture was filtered through a Whatman GF/C glass fiber-filter under vacuum, and the membranes to which [^{3}H]SQ29548 had bound were trapped on the filters. After washing out three times, the glass-filters were placed in 20-ml glass vials, and 10 ml scintillation liquid (Scintisol EX-H; Dojin, Japan) was added. Finally, the radioactivity was counted in a liquid scintillation spectrometer (LCS-700; Aloka). The specific binding was calculated as the difference between the total and non-specific binding determined in the presence of 100 μM SQ29548. The concentration of binding sites per mg protein (B_{max}) and the dissociation constant of binding (K_d) were estimated by Scatchard analysis of saturation-binding studies and compared.

A competition study using TP receptor agonists and antagonists was carried out to characterize the [^{3}H]SQ29548 binding sites. [^{3}H]SQ29548 and the crude membrane preparation of the circular muscle were incubated with various concentrations of agonists and antagonists for 45 min at 37 °C. After incubation, [^{3}H]SQ29548 that had bound to membrane receptor was separated by filtration, and the radioactivity remaining on the filters was counted. From the IC_{50} value (concentration of agent that inhibited 50% of the control binding in the absence of a competitor), the inhibition constant (pK_i) was calculated using the following equation: $pK_i = -\log IC_{50}/(1+[L]/K_d)$ (Cheng and Prusoff, 1973), where [L] is the concentration of [^{3}H]SQ29548 (20 nM) in the present competition study.

2.4. Chemicals

The following chemicals were used in the present experiments. 7-[3-(3-Hydroxy-1-octenyl)bicycle[3.1.1]hept-2-yl]-,[2*S*-[2α(*Z*),3β(1*E*,3*R**)]]-5-heptenoic acid (CTA_2), (±)-9α,11α,15*R*-trihydroxy-16-(3-(trifluoromethyl)phenoxy)-17,18,19,20-tetranor-prosta-5*Z*,13E-dien-1-oic acid (fluprostenol), [1*S*-[1,2(*Z*),3(1*E*,3*S**), 4]]-7-[3-[3-Hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (I-BOP), 4(*Z*)-6-(2-*o*-chlorophenyl-4-*o*-hydroxyphenyl-1,3-dioxan-*cis*-5-yl) hexenoic acid (ICI192605), 7-[3-(3-hydroxy-1-octenyl)-6,6-dimethylbicyclo [3.1.1]hept-2-yl-,[1*S*-[1α,2β(*Z*),3α(1*E*,3*R**),5α]]-5-heptenoic acid (PTA_2), 7-[3-[[2-[(phenylamino)carbonyl] hydrazino]methyl]7-oxabicyclo[2.2.1]hept-2-yl]-, [1*S*-[1α,2α(*Z*),3α,4α]]-]5-heptenoic acid (SQ29548), 7-[6-(3-hydroxy-1-ocetenyl)-2-oxabicyclo[2.2.1]hept-5yl]-,[1*R*-[1α,4α,5β(*Z*),6α(1*E*,3*S**)]]-5-heptenoic acid (U46619), 7-[5-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1]hept-6yl]-, [1*S*-[1α,4α,5α(1*E*,3*R**),6β(*Z*)]]-5-heptenoic acid (U44069) were obtained from Cayman Chemical (Ann Arbor, USA). Indomethacin and *N-tert*-butyl-*N*¢-[(2-cyclohexylamino-5-nitrobenzene) sulfonyl]urea (BM-531) were obtained from Sigma-Aldrich (St. Louis, USA). U46619, I-BOP, U44069, PTA_2, CTA_2, fluprostenol and indomethacin were dissolved in absolute ethanol. ICI192605, SQ29548 and BM-531 were dissolved in dimethylsulfoxide. The prepared solutions were stored at −20 °C until use and were diluted with distilled water. The maximum concentrations of dimethylsulfoxide and ethanol in the bathing and binding solution were set below 0.5% and 0.05%, respectively. These concentrations did not change the spontaneous contractile activity of the porcine myometrium and binding of [^{3}H]SQ29548.

2.5. Statistics

The results of the experiments are expressed as means ± S.E.M. of four or more preparations obtained from different pigs. Unpaired Student's *t*-test was used for statistical comparison of the parameters of spontaneous contraction (area under the curve, amplitude and frequency), agonist-induced contractions and binding parameters (K_d and B_{max}) between two groups. A *P* value of 0.05 or less was considered statistically significant.

3. Results

3.1. Effect of U46619 on the mechanical activities of longitudinal and circular muscles isolated from the cornu

As shown in Fig. 1A, the isolated longitudinal muscle and circular muscle of the cornu contracted spontaneously in Krebs solution. The frequency of the contraction in the longitudinal muscle was significantly lower than that in the circular muscle as previously reported (longitudinal muscle,

4.1 ± 0.4/10 min, $n = 18$, circular muscle, 22.8 ± 1.8/10 min, $n = 17$, Kitazawa et al., 1998). U46619 (1 nM–1 μM) applied cumulatively to the organ bath stimulated the contractile activities of both smooth muscle layers without elevating muscle tonus. U46619 induced increases in amplitude and duration of the phasic spontaneous contraction. Therefore stimulating responses to U46619 was evaluated by area under the contractile curve and expressed as a percentage of the spontaneous contractile activity. Fig. 2A shows a comparison of the concentration–response curves of U46619 for the longitudinal muscle and circular muscle. The maximum response of the longitudinal muscle (358 ± 35.3%, $n = 7$) was about two times greater than that of the circular muscle (182 ± 13.8%, $n = 14$). On the other hand, the pEC_{50} value in the longitudinal muscle (6.69 ± 0.10, $n = 7$) was significantly lower than that in the circular muscle (7.62 ± 0.12, $n = 14$). The contractile responses to U46619 were not decreased by tetrodotoxin (1 μM) (pEC_{50} and maximum response: 6.68 ± 0.41 and 347 ± 47.5% in the longitudinal muscle ($n = 5$), and 7.26 ± 0.03 and 204 ± 23.4% in the circular muscle ($n = 6$), respectively), indicating that U46619 had a direct action on smooth muscle cells.

The circular muscle showed a high sensitivity and low magnitude of contractile responses to U46619 in Krebs solution, whereas the longitudinal muscle showed a low sensitivity and high magnitude of contractile response. To confirm these muscle layer-dependent differences in pEC_{50} and maximum contraction, the concentration–response curve was also constructed from data obtained using Kumagai solution. U46619 caused a concentration-dependent increase in muscle tonus (Fig. 1B). The pEC_{50} and maximum contraction values were 6.96 ± 0.12 and 22 ± 2.9% ($n = 7$) in circular muscle and 6.48 ± 0.12 and 44 ± 4.9% ($n = 6$) in longitudinal muscle, respectively (Fig. 2B). Thus, muscle layer-related differences in pEC_{50} and maximum response were also observed in Kumagai solution, and these observations are consistent with those in Krebs solution.

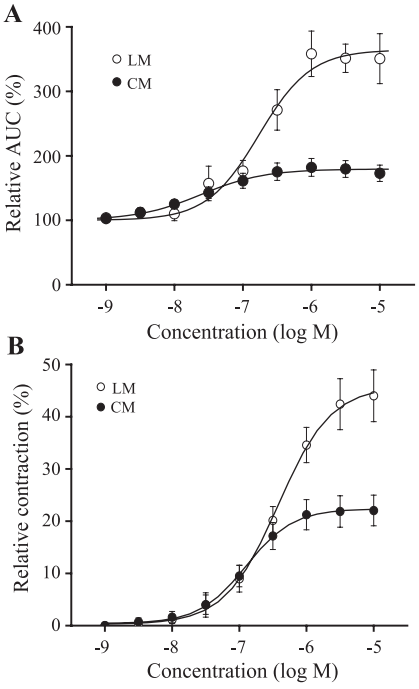

Fig. 2. Comparison of contractile responses to U46619 in the longitudinal muscle (LM) and circular muscle (CM) isolated from the porcine uterus (cornu). Symbols show the concentration–response curves of U46619 in Krebs solution (A) and Kumagai solution (B). Ordinate: contractile responses are expressed as percentages of the area under the curve of spontaneous contractions (A) and as percentages of amplitude of 50 mM high-K^+-induced contraction (B). Abscissa: concentration of U46619 (log*M*). Points represent the means of eight or more experiments with S.E.M shown by vertical lines.

3.2. Responses to TP receptor agonists

TP receptor agonists, U44069 and I-BOP, also induced contraction of the longitudinal muscle of the cornu in a concentration-dependent manner. The maximum responses to U44069 and I-BOP were 244 ± 42.0% ($n = 7$) and 257 ± 28.7% ($n = 5$), which are similar to the maximum response to U46619. The pEC_{50} values were 6.65 ± 0.15 ($n = 7$) for U44069 and 6.59 ± 0.09 ($n = 5$) for I-BOP. U44069 and I-BOP also caused contractile responses in the circular muscle of cornu. The pEC_{50} values and maximum contraction were 7.53 ± 0.06 and 164 ± 7.5% ($n = 10$) for U44069 and 7.30 ± 0.07 and 162 ± 10.8% ($n = 6$) for I-BOP, respectively. The order of potency of TP receptor agonists in the two muscle layers were similar (U46619>U44069>I-BOP).

3.3. Regional differences in contractile responses to U46619

Effects of U46619 on longitudinal and circular muscles isolated from the corpus and cervix were compared with the effects of U46619 on muscle isolated from the cornu to determine region-related differences in responses. U46619 (1 nM–10 μM) also caused contractions of corpus and cervix circular muscles. The maximum contractions of circular muscle isolated from the corpus and cervix were 203 ± 19.8% ($n = 5$) and 233 ± 59.6% ($n = 4$), and the pEC_{50} values were 7.65 ± 0.10 ($n = 5$) and 7.28 ± 0.14, respectively. These values were not significantly different from those in the cornu preparations. Although U46619 caused contractions of longitudinal muscles isolated from the cervix and corpus, there were marked regional differences in contractile response parameters. The maximum contractile responses (corpus, 208 ± 27.9%, $n = 5$; cervix, 233 ± 25.2% $n = 4$; $P < 0.05$ vs. cornu, respectively) were significantly lower than that of the longitudinal muscle isolated from the cornu (358 ± 35.3%, $n = 7$). On the other hand, pEC_{50} values of longitudinal muscle isolated from the corpus and cervix were 7.10 ± 0.11 ($n = 5$, $P < 0.05$ vs. cornu) and 7.46 ± 0.12 ($n = 4$, $P < 0.01$ vs. cornu), and these values were significantly higher than that of the longitudinal muscle isolated from the cornu (6.69 ± 0.10, $n = 7$).

3.4. Effects of ICI192605 and SQ29548 on responses to U46619

ICI192605 (30 nM), a TP receptor antagonist, inhibited the contractile responses to U46619 and shifted the concentration–response curve to the right without affecting the maximum contraction (Fig. 3). The pEC_{50} values in the absence and presence of ICI192605 were 7.01 ± 0.25 ($n=6$) and 6.21 ± 0.08 ($n=6$) in the longitudinal muscle and 7.45 ± 0.19 ($n=4$) and 6.32 ± 0.27 ($n=4$) in the circular muscle, respectively. The maximum contractions in the absence and presence of ICI192605 were 125 ± 15.8% ($n=6$) and 115 ± 17.2% ($n=6$) in the longitudinal muscle and 180 ± 5.5% ($n=4$) and 162 ± 13.4% ($n=4$) in the circular muscle, respectively. The pK_b values were estimated to be 8.17 ± 0.26 ($n=6$) in the longitudinal smooth muscle and 8.62 ± 0.28 ($n=4$) in the circular muscle (Fig. 3A,B). The effects of ICI192605 on the responses to U44069, I-BOP and fluprostenol (a prostanoid FP receptor agonist) were also investigated to assess the specificity of the action by ICI192605. ICI192605 (30 nM) shifted the concentration–response curves for U44069 ($pK_b = 8.48 \pm 0.23$, $n=4$) and I-BOP (8.24 ± 0.13, $n=5$) to the right (Fig. 4A,B). Fluprostenol increased the contractility of the circular muscle in a concentration-dependent manner (increase was significant from 3 μM to 10 μM, $P<0.05$) but these responses were not inhibited by ICI192605 (100 nM) (Fig. 4C). SQ29548 (100 nM), another TP receptor antagonist, also shifted the concentration–response curve for U46619 and decreased the pEC_{50} values (longitudinal muscle, 6.21 ± 0.27, $n=5$; circular muscle, 6.23 ± 0.04, $n=4$). According to the changes in EC_{50} values, pK_b values were calculated to be 7.53 ± 0.20 in the longitudinal muscle ($n=4$) and 8.0 ± 0.17 in the circular muscle ($n=4$) (Fig. 3C,D).

3.5. Effects of indomethacin and BM-531 on spontaneous contractions of longitudinal and circular muscles

Pretreatment with indomethacin (100 nM) for 1 h significantly decreased the spontaneous contractile activities of both muscle layers, and the inhibition was marked on the amplitude (Table 1). Decreases in the area under the curve and the amplitude of spontaneous contraction caused by indomethacin were 65 ± 7.9% ($n=12$) and 28 ± 10.8% ($n=12$) in the longitudinal muscle and 17 ± 3.9% ($n=6$) and 9.2 ± 4.2% ($n=6$) in the circular muscle, respectively. Although indomethacin also decreased the contractile response to high-K^+ (50 mM) in the longitudinal muscle, inhibition was weak compared with spontaneous contraction (area under the curve, 21 ± 4.0% $P<0.01$; amplitude, 23 ± 2.4% $P>0.05$, $n=6$). On the other hand, there was no notable difference between indomethacin-induced inhibition of spontaneous contraction and indomethacin-induced inhibition of high-K^+-induced contraction (area under the curve, 9 ± 3.0%; amplitude, 20 ± 3.4%, $n=5$) in the circular muscle.

BM-531 (1–10 μM) decreased the spontaneous contractile activity (mainly amplitude, similar to indomethacin) of

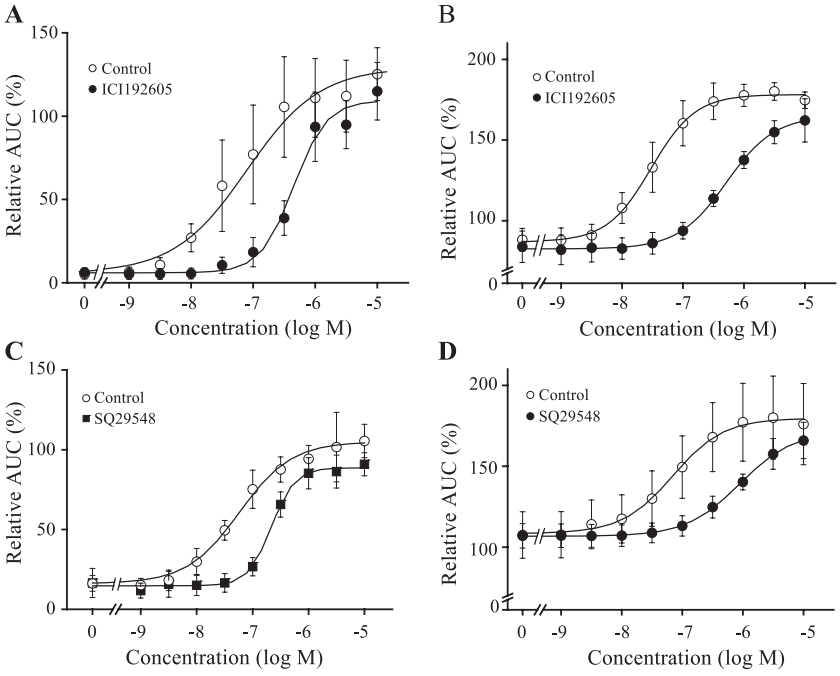

Fig. 3. Effects of TP receptor antagonists on contractile responses to U46619 in the longitudinal and circular muscles of the porcine uterus (cornu). Pretreatment with ICI192605 (A and B, 30 nM) and SQ29548 (C and D, 100 nM) shifted the concentration–response curves of U46619 to the right in the longitudinal (A and C) and circular muscles (B and D). Ordinate: contractile responses are expressed as percentages of the area under the curve of the 50 mM high-K^+-induced contraction. Abscissa: concentration of U46619 (log*M*). Points represent the means of four or more experiments with S.E.M shown by vertical lines.

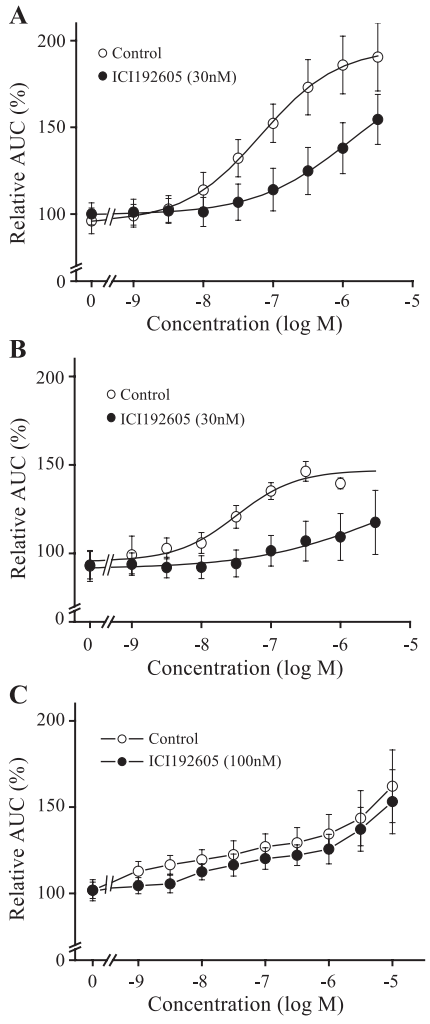

Fig. 4. Effects of ICI192605 on contractile responses to I-BOP, U44069 and fluprostenol in the circular muscle of the porcine uterus (cornu). Symbols indicate the concentration–response curves of I-BOP (A), U44069 (B) and fluprostenol (C) in the absence (control) and presence of ICI192605 (A, B, 30 nM; C, 100 nM). Ordinate: contractile responses are expressed as percentages of the area under the curve of the 50 mM high-K^+-induced contraction. Abscissa: concentration of U46619 (log*M*). Points represent the means of four or more experiments with S.E.M shown by vertical lines.

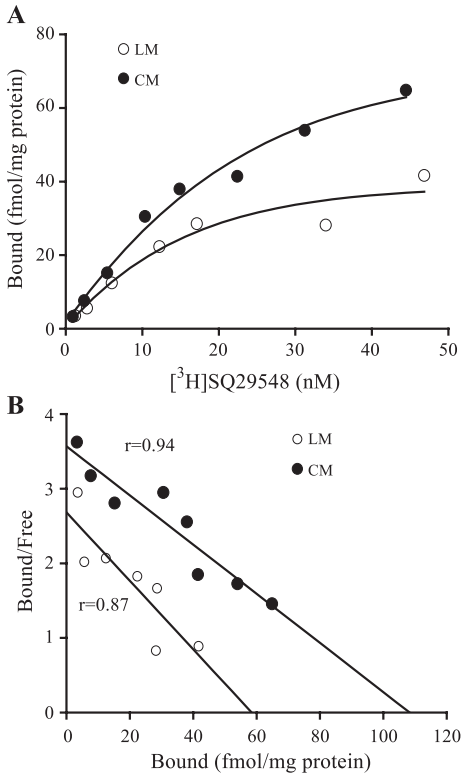

Fig. 5. [^{3}H]SQ29548 binding in the porcine uterus. (A) Crude membrane preparations from longitudinal muscle (LM) and circular muscle (CM) of the cornu were incubated for 45 min at 37 °C with eight increasing concentrations of [^{3}H]SQ29548. Specific binding determined by difference between total binding and non-specific binding was plotted against the concentrations of [^{3}H]SQ29548. (B) Scatchard plots of saturation binding data in both muscle preparations. The lines were determined by linear regression analysis (correlation coefficients are also shown). The points shown are representative data from one of six (circular muscle) and four (longitudinal muscle) similar experiments.

both longitudinal and circular muscles in a concentration-dependent manner, and the inhibition was marked in the longitudinal muscle (Table 1). Decreases in the area under the curve and amplitude of spontaneous contraction by 10 μM BM-531 were 74 ± 10.2% (n = 4) and 46 ± 18.4% in the longitudinal muscle and 20 ± 5.0% (n = 6) and 20 ± 2.5% (n = 6) in the circular muscle, respectively. BM-531 (10 μM) also inhibited the 50 mM high-K^+-induced contraction, but there was a significant difference between the inhibition of spontaneous contraction and inhibition of high-K^+-induced contraction in the longitudinal muscle (area under the curve, 18 ± 8.1%, P < 0.01; amplitude, 16 ± 5.5%, P < 0.05, n = 4). Unlike in the longitudinal muscle, inhibition of the high-K^+-induced contrac-

Table 1
Effects of pretreatment with indomethacin and BM-531 on spontaneous contractions of longitudinal and circular muscles isolated from the porcine uterus

Treatments	Longitudinal muscle			Circular muscle		
	AUC (%)	Amplitude (%)	Frequency (10 min)	AUC (%)	Amplitude (%)	Frequency (10 min)
Control	137 ± 19.3 (n = 20)	110 ± 4.6 (n = 20)	4.1 ± 0.4 (n = 18)	95 ± 2.1 (n = 17)	93 ± 1.1 (n = 17)	22.8 ± 1.8 (n = 17)
Indomethacin (100 nM)	48 ± 10.8b (n = 12)	79 ± 11.8b (n = 12)	3.6 ± 0.9 (n = 12)	79 ± 3.7c (n = 6)	84 ± 3.9b (n = 6)	21.2 ± 4.6 (n = 6)
BM-531 (1μM)	79 ± 15 (n = 4)	95 ± 9.4 (n = 4)	4.5 ± 1.2 (n = 4)	89 ± 4.1 (n = 5)	90 ± 3.1 (n = 5)	27.6 ± 1.3 (n = 5)
BM-531 (10 μM)	35 ± 14a (n = 4)	60 ± 20.3b (n = 4)	3.0 ± 1.3 (n = 4)	76 ± 4.8c (n = 6)	75 ± 2.3c (n = 6)	31.4 ± 3.8a (n = 6)

Values are means ± S.E.M. of 4 to 12 individual experiments. All values of area under the curve (AUC) and amplitude are expressed as percentages of those just before treatment with a vehicle (control), indomethacin or BM531 (for 1 h). a: P < 0.05; b: P < 0.01; c: P < 0.001, significantly different from the time-matched control value.

tion by BM-531 (area under the curve, 22 ± 6.3%, $P>0.05$; amplitude, 25 ± 4.3%, $P>0.05$, $n=5$) was the same as that of spontaneous contraction in the circular muscle.

3.6. [^{3}H]SQ29548 binding

The distributions of TP receptors in longitudinal and circular muscles of the cornu were determined by a binding study using [^{3}H]SQ29548 (a TP receptor antagonist). As shown in Fig. 5A, specific binding of [^{3}H]SQ29548 to the crude membrane increased with increase in free concentration of the ligand (1–20 nM) and almost reached a plateau at 30–40 nM. Scatchard plots of saturation binding parameters of both muscle layers fitted a straight line and revealed the presence of a single class of binding sites in both layers (Fig. 5B). From the regression line, K_d and B_{max} values were calculated to be 29.6 ± 4.6 nM and 58.2 ± 7.4 fmol/mg protein ($n=4$) in the longitudinal muscle and 30.8 ± 2.4 nM and 90.9 ± 8.6 fmol/mg protein ($n=6$) in the circular muscle, respectively. B_{max} of the circular muscle was nearly two times higher than that of the longitudinal muscle, but the K_d values of the longitudinal and circular muscles were almost the same. Hill plots of the binding data were linear with Hill coefficients of 1.03 ± 0.06 ($n=4$) in the longitudinal smooth muscle and 1.06 ± 0.19 ($n=6$) in the circular smooth muscle. These values were not significantly different from unity, indicating that there was no positive or negative cooperativity in binding profiles in the porcine myometrium.

The binding site of [^{3}H]SQ29548 was characterized by carrying out a competition study using TP receptor agonists (I-BOP, CTA_2, U46619 and U44069) and antagonists (SQ29548 and PTA_2). Competition analysis was performed only in the circular muscle, because of the high density of binding sites. All TP receptor agonists and antagonists inhibited [^{3}H]SQ29548 binding in a concentration-dependent manner and the order of competition was as follows (pK_i values in parentheses): I-BOP (7.70 ± 0.33, $n=4$)>SQ29548 (7.39 ± 0.11, $n=5$)>CTA_2 (6.55 ± 0.10, $n=5$)>PTA_2 (6.50 ± 0.14, $n=4$)>U46619 (6.41 ± 0.10, $n=4$)>U44069 (6.34 ± 0.09, $n=4$). The ranking order of potency (I-BOP>SQ29548>CTA_2>U46619>U44069) is consistent with that reported for the TP receptor of human platelets, and a significant correlation was found between pK_i values in the porcine uterus and pK_i values in human platelets ($R=0.96$, $P<0.01$, Theis et al., 1992; Webb et al., 1993) (Fig. 6).

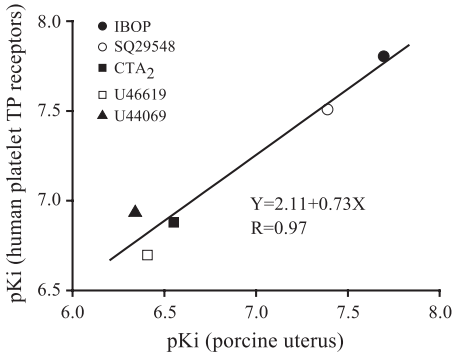

Fig. 6. Correlation of pK_i values in human platelet TP receptor and the porcine uterine [^{3}H]SQ29548 binding sites. Symbols show pK_i values of I-BOP (●), SQ29548 (○), CTA_2(■), U46619(□) and U44069 (▲). A significant ($P<0.01$) correlation was found between both pK_i values. Abscissa scale: pK_i values of respective agents against [^{3}H]SQ29548 binding sites in the porcine uterine circular muscle. Ordinate scale: pK_i values obtained in human platelet TP receptors (Theis et al., 1992; Webb et al., 1993). Equation shows the identity of the regression line.

4. Discussion

We previously reported the muscle layer-dependent heterogeneous distribution of contractile (FP, EP_1, EP_3) and relaxant (IP, DP, EP_2) prostanopid receptors in the non-pregnant porcine myometrium, but the pharmacological effects of thromboxane A_2 in this tissue have not been reported (Cao et al., 2002). Therefore, the main objective of this study was to determine whether the porcine uterus expresses functional TP receptors and to elucidate the muscle layer- and region-related differences in TP receptor distribution. The results of the present study clearly indicated that TP receptors exist in the porcine uterine smooth muscle with a heterogeneous distribution (depending on smooth muscle layers and regions) and that the TP receptor mediates contractile responses to U46619/thromboxane A_2. Although there is a marked species difference in subtypes of prostanoid receptors in the uterus, the population of prostanoid receptors in the porcine uterus is consistent with that reported in the human uterus (Senior et al., 1991, 1992; Crankshaw, 2001). In addition, the findings of decreases in contractile activity of the uterus induced by a cyclooxygenase inhibitor and by a TP receptor antagonist with thromboxane A_2 synthase-inhibiting activity suggest that endogenous thromboxane A_2 plays a physiological role in the regulation of spontaneous contractility in the porcine uterus.

Three findings in this study indicate the presence of TP receptors in the porcine uterus. First, U46619, a potent TP receptor agonist, caused a marked contractile response in the non-pregnant porcine myometrium, and the pEC_{50} values (6.69 and 7.62) are consistent with those in the uterus of the non-pregnant ewe (6.68) and human (6.9). In both tissues U46619 was demonstrated to act on TP receptor (Crankshaw and Gaspar, 1995, Senchyna and Crankshaw, 1996, 1999). Second, the contractile responses to U46619 were competitively antagonized by the TP receptor antagonist ICI192605, and pK_b values (8.6 and 8.2) in the two muscle layers are almost consistent with those in human myometrial (9.2, Senchyna and Crankshaw, 1996), human airway smooth muscle (9.5, Kawikova et al., 1996) and human vascular smooth muscle TP receptors (8.1, Boersma et al.,

1999). Furthermore, the similarity of pK_b values of another TP receptor antagonist, SQ29548 (7.5 and 8.0), in human myometrial (8.2, Senchyna and Crankshaw, 1996), human vascular muscle (7.6, Boersma et al., 1999) and rat vascular muscle TP receptors (8.2, Ko et al., 1995) supports the notion that U46619 causes contraction of the porcine myometrium by activation of TP receptors. Third, [^{3}H]SQ29548, a TP receptor ligand, bound the membrane fraction of the porcine uterus in a saturable manner, and the K_d value (30 nM) was similar with those obtained in porcine vascular muscle (16 nM) (Mihara et al., 1989), human placenta (21 nM) (Wilkes et al., 1994) and rat glomerulus (20 nM) (Wilkes et al., 1992). The K_d values of SQ29548 in saturation binding study were almost equal to the K_b values of this antagonist obtained in the contraction study (10 and 30 nM). In addition, the order of potencies of TP receptor agonists and antagonists (I-BOP>SQ29548>CTA_2>P-TA_2>U46619>U44069) in the competition study is consistent with that for human platelet TP receptor (Theis et al., 1992; Webb et al., 1993) (I-BOP>SQ29548>C-TA_2>U46619>U44069) and showed a good correlation (R = 0.96, Fig. 6). However, the order of potencies of TP receptor agonists in the present contraction study (U46619>U44069>I-BOP) was quite different from that in the present competition binding study (I-BOP>U46619> U44069). Discrepancies have sometimes been found between results of contraction and competition binding studies, and these discrepancies are thought to be due to differences in the experiment conditions. We could only measure the affinity of the drugs combining with the receptor protein in the binding study, and agonists can easily access to the receptor protein compared with muscle strip study. On the other hand, in the muscle strip study, we observed mechanical responses that depend not only on receptor protein−agonist interaction (affinity) but also on its efficacy to stimulate intracellular cascades (increase in enzyme activities and Ca^{2+}concentration) after combining with the receptor. In the porcine uterus, different ranking order of TP receptor agonists in the contraction and binding studies suggests that I-BOP has high affinity for the TP receptor, but low efficacy to stimulate a contraction pathway compared with the higher apparent efficacies of U46619 and U44069.

Muscle layer-related differences in mechanical responses to various contractile agents (acetylcholine, norepinephrine, histamine, oxytocin and endothelin) in the porcine myometrium have already been demonstrated, and the longitudinal muscle showed higher pEC_{50} and maximum contraction values than those for the circular muscle in all cases. Radioligand binding studies have indicated that these differences are due to the heterogeneous distribution of muscarinic M_3 receptor, α_2-adrenoceptor, histamine H_1 receptor, oxytocin and endothelin ET_A receptors in the two muscle layers (longitudinal muscle>circular muscle) (Taneike et al., 1991, 1994, 1995; Kitazawa et al., 1997, 2000, 2001; Isaka et al., 2000). The heterogeneous distribution of receptors and the resulting smooth muscle layer-dependent difference in responsiveness are related to the different physiological roles of the longitudinal and circular muscles in porcine myometrial contractility. Strong contraction of the longitudinal muscle might be required for transport of luminal contents, but relaxation, not contraction, of the circular muscle is required for preservation of luminal contents. In the present study, although pEC_{50} values of agonists in the circular muscle were slightly higher than those of agonists in the longitudinal muscle, the maximum contraction induced by TP receptor agonists (I-BOP, U46619 and U44069) was significantly greater in the longitudinal muscle, indicating that the longitudinal muscle preferential contractile response to TP receptor agonists as it does to other contractile agents. However, the results of the binding study do not agree with those of the contraction study. The K_d values of the two muscle layers were the same, but B_{max}, density of binding sites (TP receptors) was higher in the circular muscle than in the longitudinal muscle. The reason for this discrepancy between maximum contraction and B_{max} is not clear at present, but of the all TP receptors present in the uterus, such as contractile receptors on uterine muscle cells and non-uterine receptors on blood vessels, macrophages and mast cells, could be detected in the binding study, and this might have been the reason for the discrepancy between the results of the contraction study and the binding study. Another possibility is the presence of TP receptor subtypes in the porcine uterus. The existence of two TP receptor isoforms possessing different intracellular signaling pathways, TPα(cAMP-stimulating, relaxant receptor) and TPβ (inositol 1,4,5-trisphosphate, $[Ca^{2+}]_i$ and RhoA-associated kinase-stimulating, contractile receptor), in human myometrium cells has been reported (Moore et al., 2002). If TPα receptors are distributed unequally in the muscle layers (circular muscle>longitudinal muscle), this explains the discrepancy between the results of the present contraction and binding studies.

In addition to muscle layer-dependent differences, we have already reported uterine region-dependent differences in the responsiveness to norepinephrine and oxytocin. Of the responses to norepinephrine, contractile intensity in the longitudinal muscle was greatest in the cornu, slightly weaker in the corpus, and weakest in the cervix. Oxytocin also caused contractions of longitudinal muscle strips from all regions, and the ranking orders of the pEC_{50} and the maximum contraction were cornu>corpus = cervix (pEC_{50}) and cornu = corpus>cervix (maximum contraction). However, both agents were less effective in producing contraction in circular muscle strips from all regions (Taneike et al., 1994; Kitazawa et al., 2001). In the present study, U46619 caused contraction of circular muscle strips from three regions, and there were no significant differences in either pEC_{50} values or maximum contraction. In the case of the longitudinal muscle, on the other hand, muscle strips from different regions showed different intensities of contraction in response to U46619. The pEC_{50} value in the cornu was lower than those in the cervix and corpus,

whereas the maximum response in the cornu was greater than maximum responses in other regions. These results indicate that there is a heterogeneous distribution of TP receptors in the longitudinal muscles of the three uterine regions. Taken together with the results of previous experiments (Taneike et al., 1994; Kitazawa et al., 2001), our results indicate that regional difference in responsiveness to contractile agents is marked in the longitudinal uterine muscle compared with that in the circular uterine muscle and that this might have significant physiological implications for the regulation of uterine motility, such as making a gradient of intra-luminal pressure form the cornu (high) to cervix (low) that is favorable for transportation of uterine luminal contents.

Indomethacin and BM-531 inhibited spontaneous and high-K^+-induced contractions in both muscle layers. However, the inhibition of spontaneous contraction in the longitudinal muscle was significantly stronger than that of 50 mM high-K^+-induced contraction (the difference in the inhibition was not marked in the circular muscle). This conspicuous difference indicates that inhibition of spontaneous contraction by both agents was not due to non-specific actions on uterine contractility. Since indomethacin is a cyclooxygenase inhibitor and reduces endogenous prostanoid concentrations and since BM-531 is a TP receptor antagonist with thromboxane A_2 synthase-inhibiting activity (Dogne et al., 2001), the results suggest tonic effects of the endogenous thromboxane A_2-TP receptor pathway in initiation of spontaneous contractile activity of the porcine uterine longitudinal muscle.

In conclusion, TP receptors are present in smooth muscle of the porcine uterus and are distributed heterogeneously (depending on smooth muscle types and uterine regions), and the TP receptor mediates contractile responses to U46619 and thromboxane A_2. Subtypes of prostanoid receptor in the uterus differ markedly depending on the species, but the results of the present study and our previous study (Cao et al., 2002) indicate that the population of prostanoid receptor subtypes in the porcine uterus is completely consistent with that reported in the human uterus. The porcine uterus might therefore be a useful animal model for assessing the mechanical effects of prostanoid receptor agonists and antagonists on the human uterus. In addition, findings of decreases in contractile activity of the uterine longitudinal muscle induced by a cyclooxygenase inhibitor and by a TP receptor antagonist with thromboxane A_2 synthase-inhibiting activity suggest that endogenous thromboxane A_2-TP receptors are involved in the regulation of spontaneous contraction in the porcine uterus.

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