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# Enhancement of trypsin-induced contraction by *in vivo* treatment with 17β-estradiol and progesterone in rat myometrium

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**1** We have previously reported that the contractile response to thrombin and trypsin was enhanced in the pregnant rat myometrium. We herein determined whether or not sex hormones contribute to this enhancement and the expression of protease-activated receptors (PARs).

**2** The nonpregnant rats received daily injections of either 17β-estradiol or progesterone, and then the contractile response of the myometrium was examined *ex vivo*. Treatment with either 17β-estradiol or progesterone had almost no significant enhancing effect on the high $K^+$- or oxytocin-induced contraction. On the other hand, both 17β-estradiol and progesterone dose-dependently enhanced the contractile response to trypsin. A maximal enhancement was obtained at 25 and 40 mg kg weight$^{-1}$ day$^{-1}$ for 17β-estradiol and progesterone, respectively. The extent of the enhancement of the trypsin-induced contraction seen in the sex hormone-treated rats in the present study was comparable to that reported in the pregnant rats.

**3** However, the contractile response to thrombin and PAR1/PAR2-AP, SFLLRNP was not enhanced either by progesterone or 17β-estradiol. PAR2-AP and PAR4-AP failed to induce contraction under any conditions.

**4** PAR1 mRNA was scarcely detected in the control myometrium by an RT–PCR analysis, while it slightly increased only in the progesterone-treated rats. Neither PAR2 nor PAR4 mRNA was detected.

**5** We thus conclude that the responsiveness to trypsin, but not thrombin, is controlled by sex hormones. A novel type of receptor, other than PAR1, PAR2 or PAR4, is suggested to mediate the trypsin-induced contraction as in the case of the pregnant rat myometrium.
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**Keywords:** Myometrial contraction; protease-activated receptor; trypsin; estrogen; progesterone; expression; rat

**Abbreviations:** $[Ca^{2+}]_i$, cytosolic $Ca^{2+}$ concentration; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*′,*N*′,*N*′,*N*′-tetra acetic acid; G protein, GTP-binding protein; GTPγS, guanosine 5′–3′ thio-triphosphatase; PSS, physiological saline solution; PAR, protease-activated receptor; PAR-AP, PAR-activating peptide; RT–PCR, reverse transcription–polymerase chain reaction

## Introduction

Thrombin and trypsin activate protease-activated receptors (PARs), which belong to a family of the G-protein-coupled receptors (Dery *et al*., 1998), and thereby induce various cellular effects. Four members of PAR have so far been identified (Vu *et al*., 1991; Nystedt *et al*., 1994; Ishihara *et al*., 1997; Kahn *et al*., 1998; Xu *et al*., 1998). PAR1, PAR2 and PAR4 serve as receptors for trypsin, while PAR1, PAR3 and PAR4 serve as receptors for thrombin (Dery *et al*., 1998; Cocks *et al*., 1999a). The different sets of PARs are expressed in a tissue-specific manner and mediate various cellular responses to thrombin and trypsin. Thrombin has been reported to induce a contraction of vascular, tracheal, gastric or uterine smooth muscle (McNamara *et al*., 1993; Tesfamariam, 1994; Godin *et al*., 1995; Dery *et al*., 1998; Cicala *et al*., 1999; Cocks *et al*., 1999b; Cocks & Moffatt, 2000). Trypsin has also been reported to induce contraction in vascular, gastric and uterine smooth muscle (Al-Ani *et al*., 1995; Saifeddine *et al*., 1996; Hamilton *et al*., 1998; Cocks *et al*., 1999a, b).

We have previously reported that thrombin and trypsin induced an enhanced myometrial contraction in the pregnant rat myometrium (Shintani *et al*., 2000a; 2001). Among the known four members of PARs, PAR1 was the only receptor expressed in the rat myometrium, and it was found to be upregulated in the pregnant rat myometrium (Shintani *et al*., 2000a; 2001). The upregulation of PAR1 was thus considered to contribute to the enhancement of the contractile response to thrombin in the pregnant rat (Shintani *et al*., 2000a). On the other hand, we obtained evidence that PAR1 did not mediate the contractile response to trypsin in the rat myometrium. Our findings thus suggested that some unidentified member of PAR mediated the trypsin-induced contraction. However, the mechanism of the upregulation of PAR1 and a trypsin

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receptor, as well as the enhancement of the contractile responses in the pregnancy remain to be determined. Sex hormones such as estrogen and progesterone were considered to be the primary candidates responsible for such upregulation and enhancement.

In the present study, we tested the hypothesis that sex hormones are responsible for the upregulation of the receptors for thrombin and trypsin and the enhancement of the contractile responses to thrombin and trypsin. For this purpose, we treated nonpregnant rats with 17$\beta$-estradiol and progesterone *in vivo*, and then examined the contractile response to thrombin, trypsin, PAR1-activating peptide (PAR1-AP), PAR2-AP and PAR4-AP in the isolated myometrium as described previously (Shintani *et al*., 2000a; 2001). We also examined the expression of the four known members of PARs by reverse transcription–polymerase chain reaction (RT–PCR).

**Table 1** Effects of the *in vivo* treatment with 17$\beta$-estradiol and progesterone on the contractile response to 40 mM $K^+$ in the isolated rat myometrium

| *Treatment* | *Tension developed by 40* mM $K^+$ [a] (mg) | (n) |
|---|---|---|
| Control | 500 ± 83 | 6 |
| *17β-estradiol* (μg kg weight$^{-1}$ day$^{-1}$) | | |
| 5 | 718 ± 61 | 4 |
| 10 | 643 ± 115 | 4 |
| 25 | 723 ± 59 | 4 |
| 50 | 669 ± 201 | 3 |
| 100 | 430 ± 87 | 3 |
| 200 | 543 ± 109 | 4 |
| *Progesterone* (mg kg weight$^{-1}$ day$^{-1}$) | | |
| 20 | 556 ± 110 | 3 |
| 40 | 469 ± 49 | 4 |
| 60 | 515 ± 106 | 3 |

[a]The tension development induced by 40 mM $K^+$ depolarization in the myometrium isolated from nonpregnant rats treated with the indicated daily dosage of 17$\beta$-estradiol and progesterone for 8 days. All data are the mean ± s.e.m. ($n = 3$–5).

## Methods

### *Tissue preparation*

The study protocol was approved by the Animal Care Committee of Faculty of Medicine, Kyushu University. Virgin Wister–Kyoto rats at the age of 9–11 weeks were treated with daily subcutaneous injection of vehicle (sesame oil), 17$\beta$-estradiol or progesterone for 8 days. The myometrial strips were prepared 24 h after the last injection. Each rat was heparinized with 0.3–0.5 mg heparin (MW3000) and then killed with intraperitoneal injection of 100 mg kg weight$^{-1}$ sodium pentobarbital. Bilateral uterine horns were excised and placed in a physiological saline solution (PSS) consisting of the following compositions (in mM): NaCl 123, KCl 4.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 15.5 and D-glucose 11.5, and gassed with 95% $O_2$ and 5% $CO_2$. The myometrium of the middle part of the horn was excised in a longitudinal direction, cut into strips measuring 1.5 mm in width and 4.5 mm in length.

### *Tension measurement of intact myometrial strips*

All measurements were performed in PSS at 37°C. The strips were mounted vertically in a quartz organ bath and connected to a force transducer (TB612-T, Nihon Koden, Japan). The resting load was adjusted to approximately 50 mg to obtain a maximal response to 40 mM $K^+$ depolarization. The contractile response was analyzed by the area under the tension trace for the first 10 min of contraction, except for Table 1, where the contractile response was evaluated based on the level of the first peak elevation. The values obtained with 40 mM $K^+$ depolarization were designated to be 100%.

### *Front-surface fluorometry of $[Ca^{2+}]_i$*

The myometrial strips were loaded with fura-PE3 in the form of acetoxymethyl ester (fura-PE3/AM) as described previously (Kanaide, 1999; Shintani *et al*., 2000b). The changes in the fluorescence ratio and tension development were simultaneously monitored with a front-surface fluorometer CAM-OF-1 (JASCO, Tokyo, Japan) as reported previously (Niiro *et al*., 1998; Shintani *et al*., 2000b). The fluorescence (500 nm) intensities at alternating (400 Hz) excitation (340 and 380 nm) and their ratios (F340/380) were continuously recorded. The data were stored in a Macintosh computer using a data acquisition system (MacLab; Analog Digital Instruments, Castle Hill, Australia). The fluorescence ratio was expressed as a percentage, assigning the values for normal (5.9 mM $K^+$) and 40 mM $K^+$ PSS to be 0 and 100%, respectively.

### *Tension measurement of α-toxin-permeabilized myometrial strips*

The strips (about 0.5 mm in width and 2 mm in length) were mounted between two tungsten wires. The strips were then permeabilized with 5000 U ml$^{-1}$ *Staphylococcus aureus* α-toxin in a relaxing solution (in mM): potassium methanesulfonate 100, $Na_2ATP$ 2.2, $MgCl_2$ 3.38, ethylene glycol-bis (β-aminoethylether)-*N*′,*N*′,*N*′,*N*′-tetra acetic acid (EGTA) 10, creatine phosphate 10 and tris-maleate 20 (pH 6.8) for 60 min, as described previously (Niiro *et al*., 1998). The activating solution containing the indicated concentration of free $Ca^{2+}$ was made by adding an appropriate amount of $CaCl_2$ to the relaxing solution, using the $Ca^{2+}$-EGTA binding constant of $10^6$/M (Saida & Nonomura, 1978). The tension measurements of the permeabilized tissue were all performed at room temperature. The level of tension in the relaxing solution and maximal tension induced by 10 μM $Ca^{2+}$ was assigned to be 0 and 100%, respectively.

### *RT–PCR analysis*

The total RNA was isolated from the myometrium, liver and lung of the nonpregnant rats ($n = 3$), myometrium of the nonpregnant rats treated with 200 μg 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ ($n = 3$) or 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ for 8 days ($n = 3$), and placenta of the pregnant rats ($n = 3$) by using an RNA isolation kit (RNAqueous™-4PCR, Ambion, Austin, TX, U.S.A.) according to the manufacturer's instructions. The primers used in an RT–PCR analysis of rat PAR1 (Zhong

*et al*., 1992) were 5′-GAG CAG GTA TCC ATC TTA CT-3′ for RT reaction, 5′-TGA CAG TCA TAA GCA TTG AC-3′ for the upper primer in PCR reaction and 5′-GGC ATA GTA GTA AAT CAA GG-3′ for the lower primer in PCR reaction. The primers used in an RT–PCR analysis of rat PAR2 (Saifeddine *et al*., 1996) were 5′-CAA CTC CCA CCT GTC CGT CTC CCG-3′ for RT reaction, 5′-CAA CAG TAA AGG GAG AAG TC-3′ for the upper primer in PCR reaction and 5′-TCG TGC AGC ACT GTC CAC CAC-3′ for the lower primer in PCR reaction. The primers used in an RT–PCR analysis of rat PAR3 (Genbank Accession number AF310076) were 5′-GAC CTT CCC GTT GCC CCT GA-3′ for RT reaction, 5′-AAG CGA AGT GTC CCG AAG AAA GC-3′ for the upper primer in PCR reaction and 5′-CAT ACA CTT CCC TAC CTG GCA CCG-3′ for the lower primer in PCR reaction. The primers used in an RT–PCR analysis of rat PAR4 (Kahn *et al*., 1998; 1999) were 5′-GAC ACA TAG TAG TAG ATG AA-3′ for RT reaction, 5′-TGG TTC AGT GTT GCT GCT GCT GG-3′ for the upper primer in PCR reaction and 5′-TCC ATA GAG ATT GCC CCA GG-3′ for the lower primer in PCR reaction. Total RNA (1 $\mu$g) was used for the RT reaction using Moloney murine leukemia virus reverse transcriptase (Life technologies, Rockville, MD, U.S.A.) in a total volume of 20 $\mu$l. A measure of 1 $\mu$l aliquot of RT product was subjected to PCR amplification using KOD DNA polymerase (Toyobo, Tokyo, Japan). The thermal cycle profile used for the amplification of PAR1, PAR2, PAR3 and PAR4 fragments was composed of the initial denaturation at 94°C, for 2 min and the following 40-cycle amplification step consisting of 15 s denaturation at 94°C, 15 s annealing at 52°C and 30 s extension at 68°C. The expression of $\beta$-actin was determined as a control as described previously (Shintani *et al*., 2000a). The RT–PCR products were separated on 3% agarose gel containing 0.05 $\mu$g ml$^{-1}$ ethidium bromide. The density of the bands was analyzed by an NIH image (the National Institutes of Health, Bethesda, MD, U.S.A.) after obtaining the fluorescence image with a CCD camera (ATTO, Tokyo, Japan).

### *Drugs and solutions*

The composition of the normal physiological salt solution (normal PSS) was described above. High-$K^+$ PSS was made by an equimolar substitution of KCl for NaCl. Thrombin (bovine plasma), trypsin (bovine pancreas), heparin (MW3000), 4-aminidophenyl methane-sulfonyl fluoride (p-APMSF), mifepristone and $\alpha$-toxin were purchased from Sigma (St Louis, MO, U.S.A.). ICI 182,780 was purchased from the Tocris Cookson Ltd (Bristol, U.K.). Fura-PE3/AM was purchased from the Texas Fluorescence Laboratory (Austin, TX, U.S.A.). EGTA was obtained from Dojindo Laboratories (Kumamoto, Japan). A competitive inhibitor of IGF-1 receptor kinase, AG538, was purchased from Calbiochem (La Jolla, CA, U.S.A.). SFLLRNP, TFLLR-$NH_2$, SLIGRL and SLIGRL-$NH_2$ were obtained from Bachem (Budendorf, Switzerland). GYPGKFC was synthesized based on the mouse sequence (Kahn *et al*., 1998). AYPGKF-$NH_2$, FTLLR-$NH_2$, LSIGRL-$NH_2$ and YAPGKF-$NH_2$ were synthesized by Rapid Multiple Peptide Synthesis Service, University of Calgary (Calgary, Alberta, Canada). A PAR1 antagonist, SCH 79797 dihydrochloride, was purchased from the Tocris Cookson Ltd (Bristol, U.K.). The primers used in the RT–PCR analysis were synthesized by Hokkaido System Science (Sapporo, Japan). All other chemicals were of the highest grade commercially available.

### *Statistical analysis*

All data are expressed as the mean ± s.e.m. The unpaired Student's *t*-test was used to determine any significant differences and a one-way analysis of variance followed by Scheffe's test was used for multiple comparisons. *P*-values $<0.05$ were considered to be statistically significant.

## Results

### *Effects of the* in vivo *treatment with 17*β*-estradiol and progesterone on the contractile responses to 40 mM $K^+$ depolarization in the isolated rat myometrium*

We first examined the contractile responses to 40 mM $K^+$ in the myometrial strips isolated from the rats *in vivo* treated with vehicle (control), 17$\beta$-estradiol and progesterone (Table 1). The stimulation with 40 mM $K^+$-depolarization produced 500 ± 83 mg tension in the control. The *in vivo* treatment with 17$\beta$-estradiol and progesterone with the indicated daily dosage for 8 days had no significant effect on the contractile response to 40 mM $K^+$. The response to 40 mM $K^+$ was thus recorded as a reference response at the beginning of the experimental protocol.

### *Enhancement of the myometrial contractile responses to trypsin by* in vivo *treatment with 17*β*-estradiol and progesterone*

In the control myometrium, 3 $\mu$M trypsin induced an initial small contraction, which was frequently followed by oscillatory contractions (Figure 1a). The frequency of the oscillatory contractions was 4.6 ± 1.7 contractile spikes 5 min$^{-1}$ and the peak level was 136 ± 5.4% of that seen with 40 mM $K^+$ ($n=5$). The oscillatory contractions sometimes superimposed to the initial phase of the contraction, thus hindering the evaluation of the peak level of the tension induced by trypsin. Therefore, the contractile response to trypsin was evaluated by the area under the tension trace for the first 10 min. On the other hand, in the myometrium isolated from the rats treated with 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ for 8 days, the initial phase of contractile response to 3 $\mu$M trypsin was augmented (Figure 1b). The oscillatory contractions were frequently superimposed on the declining phase of the trypsin-induced contraction. Although the frequency (6.0 ± 1.1 contractile spikes 5 min$^{-1}$) and peak level (122 ± 2.4% of that obtained with 40 mM $K^+$) of the oscillatory contractions did not significantly differ ($P>0.05$) from those obtained in the control, longstanding oscillatory contractions in the progesterone-treated myometrium were observed. In the myometrium isolated from the rats treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ for 8 days, the contractile response to trypsin was enhanced in a manner similar to that seen with the progesterone-treated myometrium (Figure 1c). Both the initial phase of contraction and the oscillatory contractions were associated with an elevation of the $[Ca^{2+}]_i$ (Figure 1c). The evaluation of the concentration-dependent response to

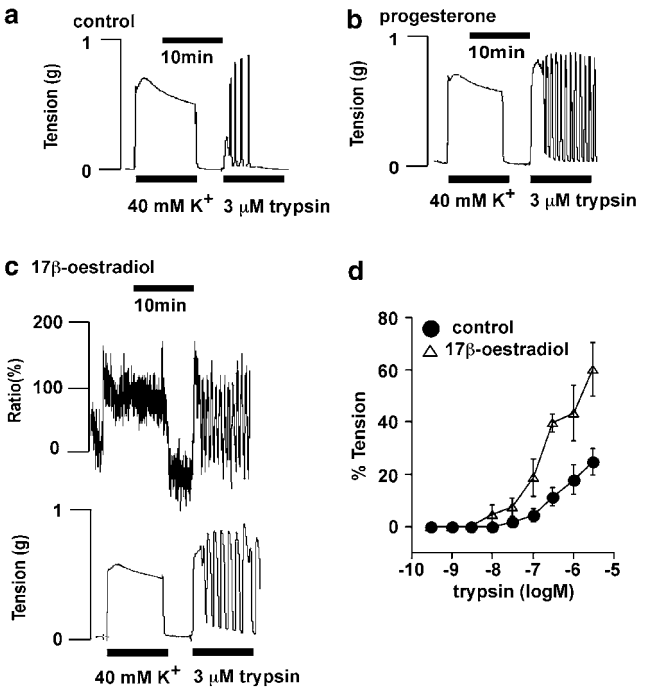


**Figure 1** Effect of *in vivo* treatment with 17$\beta$-estratiol and progesterone on the trypsin-induced contraction in the isolated rat myometrium. (a–c) Representative recordings showing the contractile responses to 3 $\mu$M trypsin in the myometrial strips isolated from rats treated with vehicle (control; a), 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ (b) and 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ (c) for 8 days. (c) also shows the representative recordings of the changes in $[Ca^{2+}]_i$ induced by 3 $\mu$M trypsin. (d) The concentration–response curves of the trypsin-induced contraction in the control myometrium and the myometrium treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ for 8 days. The contractile response was analyzed by the area under the tension trace for the initial 10 min of contraction. The developed tension was expressed as a percentage, while assigning the value obtained in normal PSS and that obtained with 40 mM $K^+$ to be 0 and 100%, respectively. The data are the mean $\pm$ s.e.m. ($n = 3$–6).

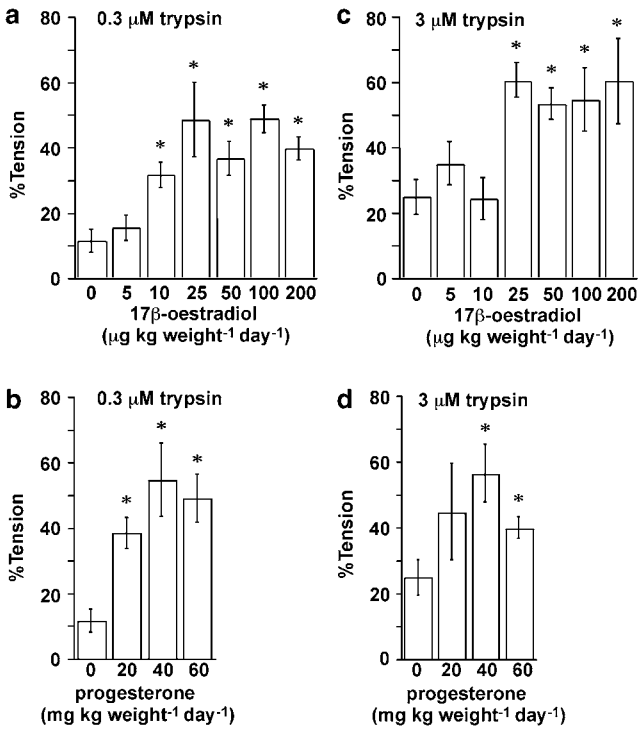


**Figure 2** The dose-dependent effect of 17$\beta$-estradiol and progesterone on the trypsin-induced contraction in the rat myometrium. The rats were *in vivo* treated with the indicated daily dosage of 17$\beta$-estradiol (a, c) and progesterone (b, d) for 8 days. The extent of the contractile responses of the isolated myometrium to 0.3 $\mu$M (a, b) and 3 $\mu$M trypsin (c, d) were evaluated by the area under the tension trace for the initial 10 min of contraction. The developed tension was expressed as a percentage, while assigning the value obtained with 40 mM $K^+$ to be 100%. The data are the mean $\pm$ s.e.m. ($n = 3$–6). *$P < 0.05$ vs the control.

trypsin revealed that the contractile response to trypsin was significantly enhanced ($P < 0.01$) in the myometrium treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$for 8 days at concentrations of trypsin higher than 100 nM (Figure 1d).

### *Dose- and time-dependent enhancement of the contractile responses to trypsin by* in vivo *treatment with 17*$\beta$*-estradiol and progesterone*

The enhancement of the contractile response to trypsin by *in vivo* treatment with 17$\beta$-estradiol and progesterone was quantitatively evaluated (Figure 2). The maximal enhancement of the contractile response to trypsin was obtained with 25 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ (Figure 2a and c). The extent of the contraction induced by 0.3 and 3 $\mu$M trypsin was enhanced from 11.4 $\pm$ 3.5 and 24.7 $\pm$ 5.2% ($n = 6$) to 45.3 $\pm$ 11.3 and 56.4 $\pm$ 8.8% ($n = 4$), respectively, by treatment with 25 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$. On the other hand, the maximal effect of progesterone on the contractile response was observed with 40 mg kg weight$^{-1}$ day$^{-1}$ (Figure 2b and d). Under this condition, the extent of contraction induced by 0.3 and 3 $\mu$M trypsin was 54.5 $\pm$ 11.2 and 60.3 $\pm$ 13.1% ($n = 4$), respectively. The maximal enhancement of the contractile response to trypsin seen with 17$\beta$-estradiol was thus similar to that seen with progesterone, and these enhancements were similar to those reported in the myometrium of the pregnant rats (Shintani *et al.*, 2001).

The cotreatment with 50 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ and 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ for 8 days enhanced the contractile response to an extent similar to that seen with each single treatment (data not shown). The enhancement of the contractile response to trypsin by 50 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ was inhibited by treatment with 1 mg ICI 182,780 kg weight$^{-1}$ day$^{-1}$. The enhancement of the contractile response to trypsin by 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ was inhibited by treatment with 10 mg mifepristone kg weight$^{-1}$ day$^{-1}$. However, mifepristone inhibited the enhancement of the contractile response to trypsin seen with 17$\beta$-estradiol, while ICI 182,780 had no effect on the progesterone-induced enhancement (data not shown).

Figure 3 shows the time course for the enhancement of the contractile response. The treatment with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ for 4 days enhanced the contractile response to trypsin to an extent similar to that seen with the treatment for 8 days (Figure 3a and c). On the other hand, the treatment for 1 day had no significant effect on the contractile response to trypsin (Figure 3a and c). The treatment with 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ for 1 day and 4 days had

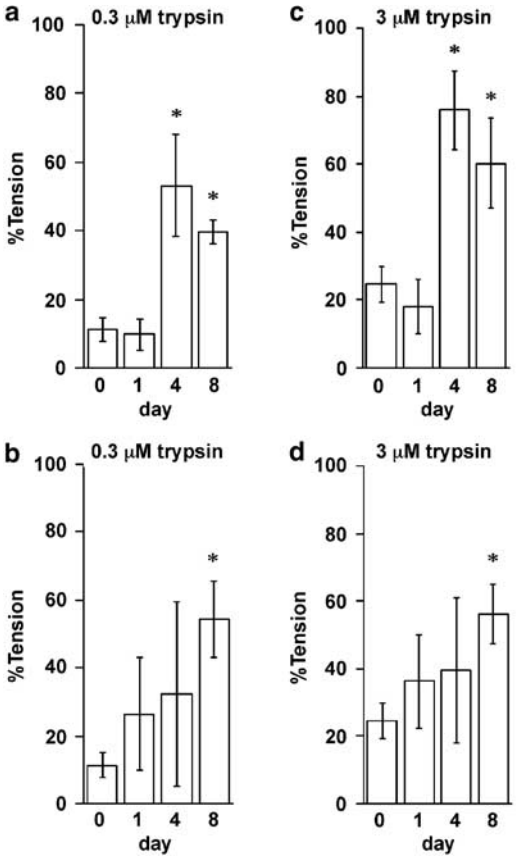


**Figure 3** The time-dependent effect of 17$\beta$-estradiol and progesterone on the trypsin-induced contraction in the rat myometrium. The rats were *in vivo* treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ (a, c) and 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ (b, d) for the indicated days. The extent of the contractile responses of the isolated myometrium to 0.3 $\mu$M (a, b) and 3 $\mu$M trypsin (c, d) were evaluated by the area under the tension trace for the initial 10 min of contraction. The developed tension was expressed as a percentage, while assigning the value obtained with 40 mM $K^+$ to be 100%. The data are the mean $\pm$ s.e.m. ($n = 3$–6). *$P < 0.05$ vs the control.

no significant effect on the contractile response to 3 and 0.3 $\mu$M trypsin (Figure 3b and d).

### *Effects of the* in vivo *treatment with 17*$\beta$*-estradiol and progesterone on the contractile responses to thrombin, SFLLRNP, TFLLR-NH$_2$ and oxytocin in the rat myometrium*

In the control myometrium, the application of 1 U ml$^{-1}$ thrombin did not induce any prompt response of tension development, while the oscillatory contractions were observed (Figure 4a). Similarly, in the myometrium treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ or 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ for 8 days, thrombin did not induce any prompt development of tension, while oscillatory contractions were observed (Figure 4b and c). SFLLRNP induced only oscillatory contractions in the control, 17$\beta$-estradiol-treated or progesterone-treated myometrium (Figure 4d–f). On the other hand, 100 nM oxytocin caused a rapid increase in tension, followed by a slowly declining phase with over-riding oscillatory contractions in the control myometrium. The treatment with 17$\beta$-estradiol or progesterone had no effect on the contractile response to oxytocin (Figure 4g–i). Figure 5 summarizes the dose-dependent effect of the *in vivo* treatment with 17$\beta$-estradiol and progesterone on the contractile response to 1 U ml$^{-1}$ thrombin, 10 $\mu$M SFLLRNP, and 100 nM and 1 $\mu$M oxytocin. The treatment with 17$\beta$-estradiol and progesterone had no significant effect on the response to thrombin, SFLLRNP and oxytocin, except that the response to 100 nM oxytocin was slightly but significantly enhanced from 113.2 $\pm$ 4.2% ($n = 4$) to 134.3 $\pm$ 1.0% ($n = 3$, $P > 0.01$) by the treatment with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$.

We examined the response to TFLLR-$NH_2$, another PAR1-AP (Figure 6), which was reported to be a selective PAR1 agonist in HEK293 cells (Kawabata *et al*., 1999). TFLLR-$NH_2$ induced a contraction in the control strips, and its contractile effect was augmented in the strips treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ for 8 days (Figure 6). However, 50 $\mu$M FTLLR-$NH_2$, an inactive peptide, induced no contraction. A PAR1 antagonist SCH 79797 (1 $\mu$M) had no effect on the TFLLR-$NH_2$-induced contraction (data not shown). The contractile response seen with TFLLR-$NH_2$ was thus similar to that seen with trypsin.

### *Effects of the* in vivo *treatment with 17*$\beta$*-estradiol and progesterone on the $Ca^{2+}$-induced contraction in the $\alpha$-toxin-permeabilized rat myometrium*

In the $\alpha$-toxin-permeabilized myometrial strips obtained from the control rats, a graded increment of $Ca^{2+}$ concentration induced a stepwise increase in tension (Figure 7a). A significant development of tension was seen at the concentrations of $Ca^{2+}$ higher than 300 nM, and the concentration of $Ca^{2+}$ required to induce a half-maximal contraction was 476 nM. In the presence of GTP$\gamma$S, the significant development of tension was observed at concentrations higher than 500 nM, and the concentration of $Ca^{2+}$ required to induce a half-maximal contraction was 572 nM (Figure 7b). The $Ca^{2+}$–tension curves obtained in the strips treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ and 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ for 8 days did not differ from those obtained with the control strips both in the absence and presence of GTP$\gamma$S (Figure 7a and b).

### *Involvement of PAR in the trypsin-induced myometrial contraction and the effects of the PAR-APs on myometrial contraction*

The pretreatment of trypsin with protease inhibitor, 4-aminidophenylmethane sulfonyl fluoride, abolished the contractile response to trypsin (data not shown), thus suggesting the involvement of either PAR1, PAR2 or PAR4 in the trypsin-induced contractile response. However, as shown in Figure 8a, thrombin did not induce any contraction in this series of experiments and it also had no effect on the response to the subsequent stimulation with trypsin in the myometrium treated with 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ for 8 days. A PAR1 antagonist, SCH 79797 had no effect on the trypsin-induced contraction in the myometrium treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ for 8 days (data not shown). Similarly, PAR2-AP (30 $\mu$M, SLIGRL) and PAR4-AP (30 $\mu$M, GYPGKFC) failed to induce any contraction and they also had no effect on the subsequent contractile response to trypsin

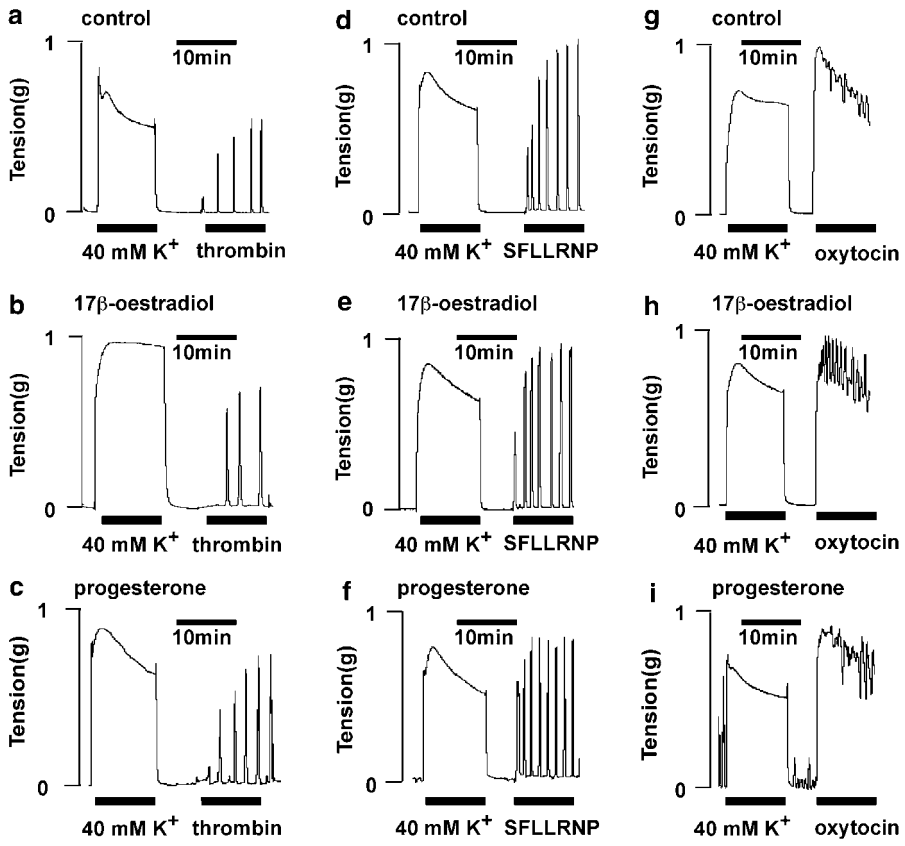


**Figure 4** Effect of the *in vivo* treatment with 17$\beta$-estradiol and progesterone on the contractile response to thrombin, SFLLRNP and oxytocin in the rat myometrium. Representative traces of the changes in tension induced by 1 U ml$^{-1}$ thrombin (a–c), 10 $\mu$M SFLLRNP (d–f) and 100 nM oxytocin (g–i) in the myometrium isolated from the rats treated with vehicle (control; a, d, g), 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ (b, e, h) and 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ (c, f, i) for 8 days.

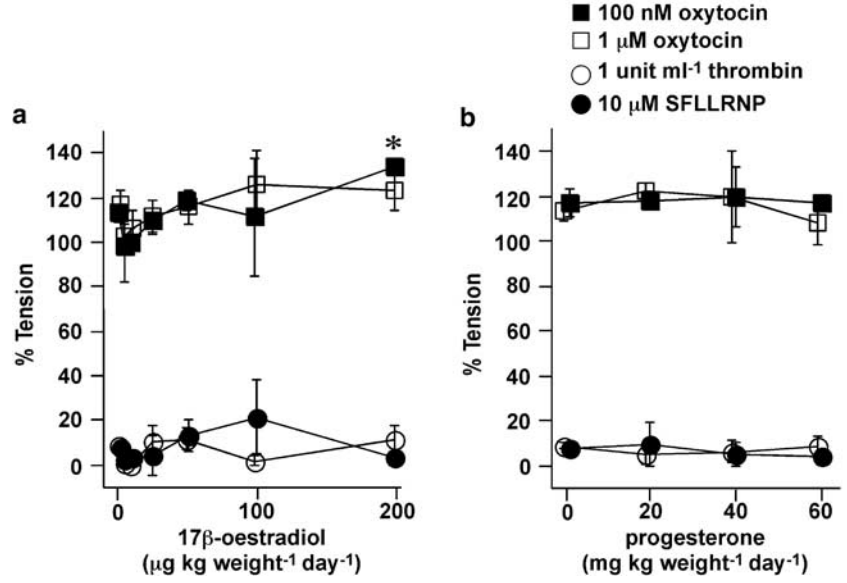


**Figure 5** The dose-dependent effect of 17$\beta$-estradiol (a) and progesterone (b) on the contractile response to oxytocin, thrombin and SFLLRNP in the rat myometrium. The developed tension induced by 100 nM oxytocin, 1 $\mu$M oxytocin, 1 U ml$^{-1}$ thrombin and 10 $\mu$M SFLLRNP were evaluated by the area under the tension trace for the initial 10 min of contraction and expressed as a percentage, while assigning the value obtained with 40 mM $K^+$ PSS to be 100%. The data are the mean $\pm$ s.e.m. ($n = 3$–6). *$P < 0.05$ vs the control.

in the myometrium after progesterone treatment (Figure 8b and c). Similar results were observed in the myometrium treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ (data not shown). Furthermore, in these tissues PAR2-AP (50 $\mu$M, SLIGRL-$NH_2$) and PAR4-AP (200 $\mu$M, AYPGKF-$NH_2$) induced no contraction and had no effect on the response to the subsequent stimulation with trypsin (data not shown). Furthermore, 50 $\mu$M SLIGRL-$NH_2$ and 200 $\mu$M AYPGKF-$NH_2$ induced no contraction and had no effect on the response to the subsequent stimulation with trypsin (data not shown). We also examined the effect of inactive versions of these peptides (50 $\mu$M LSIGRL-$NH_2$ and 200 $\mu$M YAPGKF-$NH_2$) to rule out non-PAR-mediated responses, and no responses were observed (data not shown).

### *Negligible involvement of a trypsin-generated peptide and insulin-like growth factor 1 receptor in the contractile response*

We conducted a bath-transfer experiment (Figure 9) to exclude the possibility that trypsin-generated agonist peptides

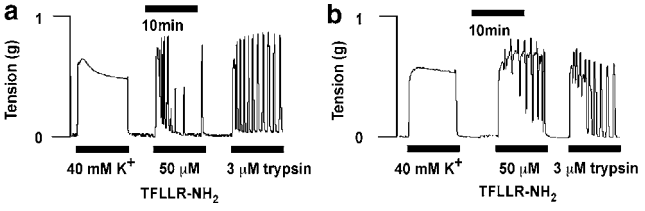


**Figure 6** Effect of TFLLR-$NH_2$ on the myometrial contraction. Representative traces showing the response to 50 $\mu$M TFLLR-$NH_2$ in the myometrial strips isolated from the control rats (a) and the rats treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ for 8 days (b). Similar results were observed in three separate experiments.

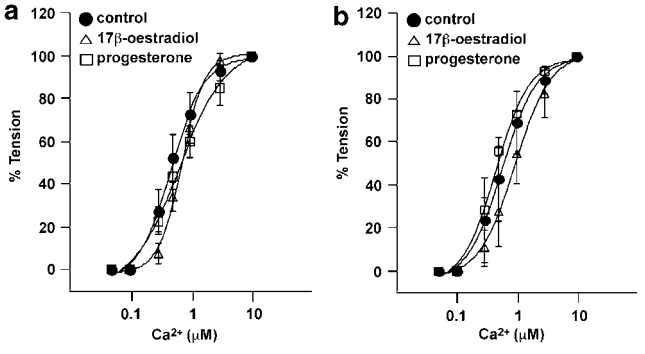


**Figure 7** Effect of the *in vivo* treatment with 17$\beta$-estradiol and progesterone on the $Ca^{2+}$-induced contraction in the $\alpha$-toxin-permeabilized rat myometrium. The concentration–response curves of the $Ca^{2+}$-induced contraction in the absence (a) and presence (b) of GTP$\gamma$S in the $\alpha$-toxin-permeabilized myometrial strips obtained from the rat treated with vehicle, 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ and 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ for 8 days. The contraction was induced by a stepwise increase in the $Ca^{2+}$ concentrations. The developed tension was expressed as a percentage, assigning the values obtained with the relaxing solution and the maximal tension obtained with 10 $\mu$M $Ca^{2+}$ to be 0 and 100%, respectively. The data are the mean $\pm$ s.e.m. ($n = 3$–4).

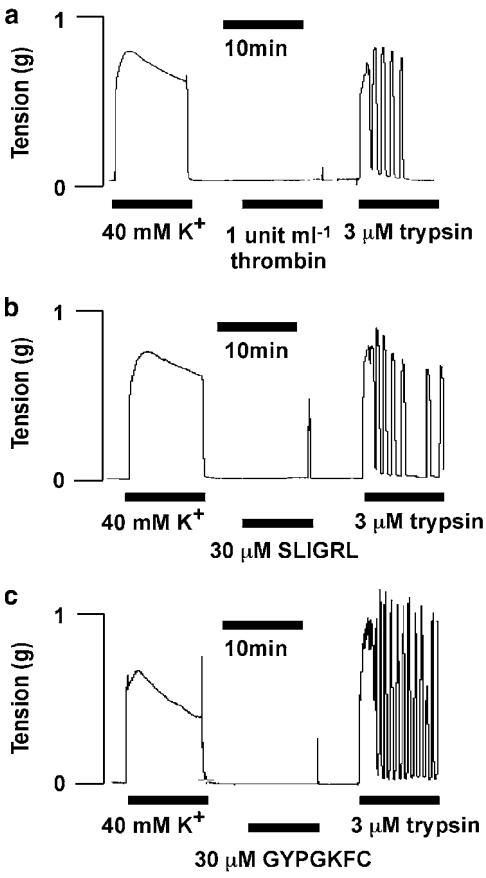


**Figure 8** The contractile response to thrombin, PAR2-AP (SLIGRL) and PAR4-AP (GYPGKFC) in the myometrium isolated from the progesterone-treated rats. Representative traces showing the response to 1 U ml$^{-1}$ thrombin (a), 30 $\mu$M SLIGRL (b) and 30 $\mu$M GYPGKFC (c) in the myometrial strips isolated from the rats treated with 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ for 8 days. The responsiveness to 3 $\mu$M trypsin was examined at the end of each protocol. Similar results were obtained in three independent experiments.

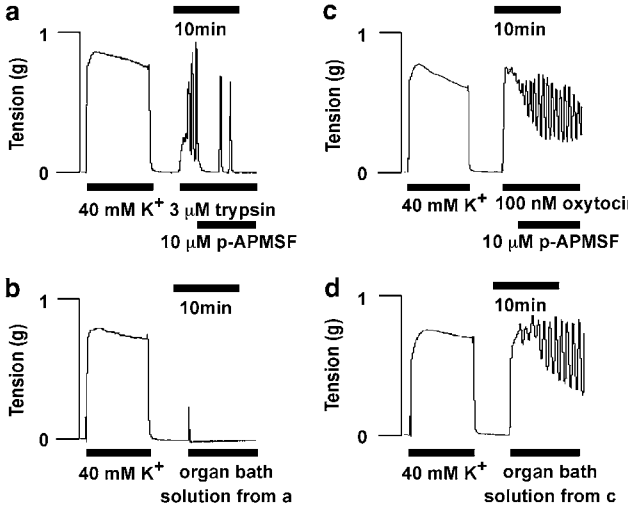


**Figure 9** Bath-transfer experiment. The myometrium isolated from the rat treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ for 8 days was contracted by 3 $\mu$M trypsin (a) or 100 nM oxytocin (c). A serine protease inhibitor, p-APMSF, was added to the bath at the concentration of 10 $\mu$M. After 10 min, the organ bath solution was transferred to the reporter tissue (b, d).

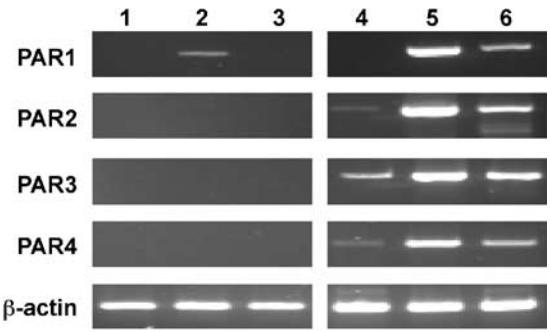


**Figure 10** Effect of the *in vivo* treatment with 17$\beta$-estradiol and progesterone on the expression of PAR1, PAR2, PAR3 and PAR4 mRNA in the rat myometrium. Representative photographs showing the RT–PCR products of PAR1 (545 bp), PAR2 (596 bp), PAR3 (404 bp), PAR4 (336 bp) and $\beta$-actin (224 bp) mRNA in the myometrium obtained from the rats treated with vehicle (lane 1), 40 mg progesterone kg weight$^{-1}$ day$^{-1}$ (lane 2), 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ (lane 3) for 8 days, and in the liver (lane 4), placenta (lane 5) and lung (lane 6) obtained from the control rats. The photos are representative of three independent experiments.

mediated the contractile response to trypsin, as suggested previously (Orce *et al*., 1994). The myometrium isolated from the rat treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ for 8 days was first contracted with 3 $\mu$M trypsin (Figure 9a). A serine protease inhibitor, p-APMSF, was then added to the bath, and 10 min later, the organ bath solution was transferred to the reporter tissue. The transferred bath solution did not induce any contraction (Figure 9b). On the other hand, when the donor tissue was first contracted with 100 nM oxytocin, and then the bath solution was transferred to the reporter tissue, the bath solution induced a contraction similar to that seen in the donor tissue (Figure 9c and d). Trypsin has been reported to exert insulin-like effect (Shoelson *et al*., 1988). However, insulin (10, 1 and 0.1 $\mu$M) induced no contraction and had no effect on the subsequent contractile response to trypsin in the myometrium treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$ for 8 days (data not shown). The treatment of the strips with an inhibitor of IGF-1 receptor kinase AG538 (10 or 1 $\mu$M) 30 min prior to and during stimulation with trypsin had no effect on the trypsin-induced contraction (data not shown).

### *The expression of PAR mRNA in the rat myometrium*

The expression of PAR1, PAR2, PAR3 and PAR4 mRNA in the rat myometrium was examined by an RT–PCR analysis (Figure 10). The expression of PAR1, PAR2, PAR3 and PAR4 mRNA were scarcely detected in the control myometrium, while all of them were detected in the rat liver, placenta and lung, except that PAR1 mRNA was not detected in the liver. The expression of PAR1 mRNA slightly increased in the myometrium treated with 40 mg progesterone kg weight$^{-1}$ day$^{-1}$. However, the expression of PAR1 mRNA was hardly detected in the myometrium treated with 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$. The expression of PAR2, PAR3 and PAR4 mRNA was not detected in the hormone-treated myometrium. The level of $\beta$-actin mRNA did not significantly differ under the different conditions.

## Discussion

The present study demonstrated, for the first time, that the contractile response of the nonpregnant rat myometrium to trypsin was enhanced by the *in vivo* treatment with sex hormones. This enhancement of the contractile response appeared to be specific to trypsin among the contractile stimulants tested in the present study. The high $K^+$ depolarization and oxytocin induced a sustained contraction in the control myometrium, and this contraction remained unaffected by treatment with sex hormones, except for the fact that the contractile response to 100 nM oxytocin was very slightly enhanced by 200 $\mu$g 17$\beta$-estradiol kg weight$^{-1}$ day$^{-1}$. We have previously reported that the contractile responses to thrombin and trypsin were markedly enhanced in the pregnant rat myometrium (Shintani *et al*., 2000a; 2001). The extent of the trypsin-induced contraction seen after *in vivo* treatment with sex hormones (56.4$\pm$8.8% of high $K^+$-induced contraction with 3 $\mu$M trypsin) was comparable to that seen in the pregnant rats (63.2$\pm$7.9%) (Shintani *et al*., 2001). Our findings in the present study thus suggest that sex hormones are responsible for the enhancement of the contractile response to trypsin in the pregnant rat myometrium. On the other hand, thrombin and PAR1/PAR2-AP (SFLLRNP) had little contractile effect in the control rats, and no enhancement of the contractile response to these agonists was induced by treatment with sex hormones in the present study. Therefore, the enhancement of the contractile responses to thrombin and PAR1/PAR2-AP (SFLLRNP) seen in the pregnant rats (Shintani *et al*., 2000a) was suggested to be either independent of sex hormones or it required some additional factors, which are activated during pregnancy.

The changes in the $Ca^{2+}$-sensitivity of the smooth muscle contractile apparatus or intracellular $Ca^{2+}$ handling are not likely to support the enhanced contractile response to trypsin. The direct evaluation of the myofilament $Ca^{2+}$ sensitivity in the $\alpha$-toxin-permeabilized strips demonstrated that the *in vivo* treatment with sex hormones had no effect on the $Ca^{2+}$–tension relationship either in the presence or absence of GTP$\gamma$S. The tension development induced by trypsin in the 17$\beta$-estradiol-treated rat myometrium was accompanied by an increase in $[Ca^{2+}]_i$. These observations thus suggest that the *in vivo* treatment with sex hormones had no significant effect on the $Ca^{2+}$-dependent contractile mechanism or intracellular signal transduction pathways located downstream from the G proteins. On the other hand, the enhancement of the contractile response to trypsin was suggested to be attributable to the enhancement of the responsiveness at the receptor level. This possibility is thus consistent with the observation that the contractile responses to high $K^+$, oxytocin or thrombin were not enhanced by sex hormones. The observation with a bath-transfer experiment is also supportive of the involvement of the trypsin-activated cell surface receptor.

Among the four members of PARs, PAR1, PAR2 and PAR4 are considered to serve as receptors for trypsin (Cocks & Moffatt, 2000). An RT–PCR analysis revealed that progesterone, but not 17$\beta$-estradiol, slightly upregulated the expression of PAR1 mRNA. This observation is consistent with the findings of a previous report (Herkert *et al*., 2001). However, the contractile response to thrombin and SFLLRNP was not enhanced by treatment with either 17$\beta$-estradiol or progesterone. The preceding stimulation with thrombin had no

effect on the subsequent response to trypsin. The contraction induced by trypsin was resistant to the PAR1 antagonist, SCH 79797. These observations suggest that PAR1 played a negligible role in the trypsin-induced contraction. Furthermore, PAR2-AP (SLIGRL, SLIGRL-$NH_2$) and PAR4-AP (GYPGKFC, AYPGKF-$NH_2$) failed to induce any contraction and had no effect on the subsequent response to trypsin. The expression of PAR2 and PAR4 was not detected in the rat myometrium either with or without sex hormone treatment. Collectively, no known PARs could account for the contractile response to trypsin. Furthermore, it is unlikely that the receptors of insulin or IGF-1 are involved in the trypsin-induced contraction, based on the current findings.

The observations with TFLLR-$NH_2$ are apparently inconsistent with the observations that the contractile response to thrombin and SFLLRNP remained unchanged in the 17$\beta$-estradiol-treated rat myometrium, and PAR1 mRNA was not appreciably detected in the control and 17$\beta$-estradiol-treated myometrium. The enhancement of the contractile response to TFLLR-$NH_2$ by *in vivo* treatment with 17$\beta$-estradiol was thus similar to that seen with trypsin. SFLLRNP can activate PAR1 and PAR2 (Hollenberg *et al*., 1997), while TFLLR-$NH_2$ has been reported to activate PAR1, but not PAR2 (Kawabata *et al*., 1999). However, there is a possibility that TFLLR-$NH_2$ acted as an agonist for a putative trypsin-activated receptor in the myometrium, although such possibility remains to be determined.

We previously suggested that the trypsin-induced contraction was mediated by a novel type of PAR based on the following observations (Shintani *et al*., 2001): First, the trypsin-induced contraction was unaffected by the preceding stimulation with thrombin. Second, PAR2 and PAR4 mRNA were not detected in the pregnant rat myometrium, and PAR2-AP and PAR4-AP failed to induce any contraction. Third, the antagonists of angiotensin II receptor and tachykinin receptors had no effect on the trypsin-induced contraction. It is thus conceivable that the receptor mediating the trypsin-induced enhanced contraction in the nonpregnant myometrium treated with sex hormones *in vivo* is similar to that which has been proposed to mediate the enhanced contractile response to trypsin in the pregnant myometrium. The expression of an unidentified trypsin-activated receptor responsible for the trypsin-induced myometrial contraction is thus suggested to be dependent on sex hormones. The present study thus provides the first clue for identifying such receptor.

The *in vivo* treatment with 17$\beta$-estradiol and progesterone required several days to induce an enhancement of the contractile response to trypsin. The antagonists of estrogen and progesterone receptors inhibited the enhancing effect of 17$\beta$-estradiol and progesterone, respectively. These results suggest that the enhancement of the contractile response to trypsin by 17$\beta$-estradiol and progesterone was due to the genomic effect mediated by their specific receptors. It is thus speculated that the expression of a putative trypsin receptor is regulated by sex hormones. However, the precise mechanism for the enhancement of the contractile response to trypsin by 17$\beta$-estradiol and progesterone, and their relationship remain to be elucidated.

In summary, the present study demonstrated, for the first time, that the contractile response of the rat myometrium to trypsin was enhanced by the *in vivo* treatment with 17$\beta$-estradiol and progesterone. None of the members of PARs previously known to serve as receptors for trypsin was suggested to be involved in the enhanced contractile response. Instead, some unidentified member of PAR may be involved, and this receptor is similar to the receptor that has been proposed to be involved in the enhanced contractile response to trypsin in the pregnant rat myometrium. The enhancement of the contractile response to trypsin seen in the pregnant rat is thus suggested to be mediated by sex hormones, and the expression of a putative receptor for trypsin is dependent on sex hormones. On the other hand, the contractile response to thrombin was not enhanced by sex hormone treatment, thus indicating that the enhancement of the contractile response to this agent in the pregnant myometrium may either be independent of sex hormones or that it requires additional factors. Our findings, suggesting that the expression of the trypsin receptor is responsive to sex hormones, will help us to identify and clone such a receptor in a future study.

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