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# Mechanism of trypsin-induced contraction in the rat myometrium: the possible involvement of a novel member of protease-activated receptor

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**1** The mechanism of trypsin-induced contraction in the rat myometrium was investigated using front-surface fluorimetry on fura-PE3-loaded strips. The expression of protease-activated receptors (PARs) in the rat myometrium was determined by reverse transcription polymerase chain reaction (RT–PCR).

**2** In non-pregnant rats, 10 $\mu$M trypsin developed a force of up to 30.5±5.1% of that obtained during the 40 mM $K^+$-depolarization-induced contraction. In pregnant rats, the maximal level of the cytosolic $Ca^{2+}$ concentration and tension obtained with 3 $\mu$M trypsin was 143.2±6.0% and 63.2±7.9%, respectively. The depletion of the extracellular $Ca^{2+}$ abolished the trypsin-induced contraction.

**3** Trypsin-induced contraction was abolished by the pre-treatment of a serine protease inhibitor. PAR1-activating peptide (PAR1-AP) caused a potent contraction of the myometrium, while neither PAR2-AP nor PAR4-AP induced any contraction.

**4** RT–PCR analysis detected the expression of PAR1 mRNA. However, neither PAR2 nor PAR4 mRNA was detected in the rat myometrium.

**5** Once the strips were stimulated with thrombin, the subsequent application of thrombin failed to induce any contraction, while trypsin induced a contraction similar to that observed without the pre-stimulation with thrombin. Once the strip was stimulated with trypsin, neither trypsin nor thrombin induced any contraction. The response to PAR1-AP remained after the pre-stimulation with thrombin and trypsin.

**6** In conclusion, PAR1 was the only known receptor for trypsin expressed in the rat myometrium, but it was suggested to be cleaved and inactivated by trypsin. Trypsin was thus suggested to contract the rat myometrium *via* a novel type of PAR, which might be upregulated during pregnancy.
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**Keywords:** Trypsin; myometrial contraction; rat; protease-activated receptor

**Abbreviations:** $[Ca^{2+}]_i$, the intracellular $Ca^{2+}$ concentration; cyclic AMP, adenosine 3′,5′-cyclic phosphate; EGTA, ethyleneglycol-bis($\beta$-aminoethylether)-N,N,N′,N′-tetraacetic acid; G protein, GTP-binding protein; $IP_3$, inositol 1,4,5-trisphosphate; PAR, protease-activated receptor; PAR-AP, PAR-activating peptide; PGs, prostaglandins; PLC$\beta$, $\beta$ isoform of phospholipase C; PSS, physiological saline solution; RT–PCR, reverse transcription-polymerase chain reaction; VOC, voltage-operated $Ca^{2+}$ channel

## Introduction

Proteases such as thrombin and trypsin are known to exert a cellular effect by activating protease-activated receptors (PARs), which belong to a family of the G-protein coupled receptors (Dery *et al*., 1998). Four members of PARs have so far been identified; PAR1, PAR2, PAR3 and PAR4 (Ishihara *et al*., 1997; Kahn *et al*., 1998; Nystedt *et al*., 1994; Vu *et al*., 1991; Xu *et al*., 1998). PAR1, PAR3 and PAR4 can be activated by thrombin, while PAR1, PAR2 and PAR4 can be activated by trypsin (Cocks & Moffatt, 2000). We have recently demonstrated that thrombin induced myometrial contraction in the rat, and that this contractile effect was mainly mediated by PAR1 (Shintani *et al*., 2000a). The activation of PARs is unique in that the proteolytic cleavage of the receptor initiates the activation process. The cleavage of the $NH_2$-terminal extracellular domain of PAR unmasks a new $NH_2$-terminus, which in turn acts as a tethered ligand and then initiates the intracellular signalling to provoke the cellular response (Dery *et al*., 1998). The synthetic peptides corresponding to the tethered ligand sequences were shown to activate PARs, except for PAR3 (Ishihara *et al*., 1997). Since the activation of PARs by PAR-activating peptides (PAR-APs) is independent of proteolysis, PAR-APs are thus considered to be a useful tool to investigate the regulation of PARs.

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There is evidence that protease may play some role in myometrial contraction. Aprotinin, an inhibitor of proteases such as kallikrein and trypsin, was shown to decrease the contractility of the uterus in the pregnant rat (Whalley & Riley, 1979). Other reports showed that the oxytocic response to kallikrein was unaffected by kininase and kinin antagonist, thus suggesting the direct contractile effect of kallikrein on the myometrium (Chao *et al*., 1981; Orce *et al*., 1994). Moreover, the hormone-stimulated trypsin-like protease activity was suggested to exist in the rat uterus (Katz *et al*., 1976). The proteases may thus contribute either directly or indirectly to myometrial contraction under physiological conditions. However, the identity of proteases remains to be elucidated.

Trypsin is a member of serine protease and has been shown to induce contraction in vascular and gastric smooth muscle (Cocks *et al*., 1999b; Saifeddine *et al*., 1996) as well as endothelium-dependent vasorelaxation (Al-Ani *et al*., 1995; Hamilton *et al*., 1998; Saifeddine *et al*., 1996) and epithelium-dependent tracheal relaxation (Cocks *et al*., 1999a). Only a limited number of reports have shown the effect of trypsin on the contraction of the uterine smooth muscle (Nustad & Pierce, 1974; Orce *et al*., 1989; 1994). Trypsin has been reported to induce myometrial contraction in the rat, which was inhibited by angiotensin II receptor antagonist and angiotensin converting enzyme inhibitor (Orce *et al*., 1994). However, the mechanism of trypsin-induced myometrial contraction and the receptor mediating the contractile effect of trypsin has yet to be identified. PAR2 serves as a specific receptor for trypsin (Bohm *et al*., 1996a, b). However, several reports have demonstrated that the effects of trypsin were not mediated by PAR2 in the trypsin-induced endothelium-dependent vasorelaxation of the human coronary artery (Hamilton *et al*., 1998), intestinal ion transport in the rat jejunum (Vergnolle *et al*., 1998) or the activation of mitogen-activated protein kinase in mouse fibroblasts (Belham *et al*., 1996). The type of PARs involved in the trypsin-induced myometrial contraction thus remains to be elucidated.

In the present study, we examined the effect of trypsin on the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and contraction of the rat myometrium, by using front-surface fluorimetry on fura-PE3-loaded strips (Kanaide, 1999), and thus investigated the $Ca^{2+}$ signalling in the trypsin-induced myometrial contraction. We further investigated the involvement of the known members of PAR in trypsin-induced myometrial contraction, by analysing the expression of mRNA in the rat myometrium and by examining the effect of the desensitization of PARs by the preceding stimulation with thrombin, trypsin and PAR1-AP on the subsequent applications of these agonists. We herein demonstrate that trypsin induced myometrial contraction in the rat through a novel PAR, and that none of the known members of PARs played a major role in this trypsin-induced contraction.

## Methods

### *Tissue preparation*

The study protocol was approved by the Animal Care Committee of Faculty of Medicine, Kyushu University. Myometrial strips were prepared from non-pregnant and day 19 pregnant Wistar-Kyoto rats as described previously (Shintani *et al*., 2000b). 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. The wet weight of the strips were $1.08 \pm 0.04$ mg (non-pregnant myometrium) and $1.30 \pm 0.04$ mg (pregnant myometrium) ($n = 7$).

### *Tension measurement of intact myometrial strips*

All measurements were performed in physiological salt solution (PSS) at 37°C. The myometrial strips were mounted vertically in a quartz organ bath and connected to a force transducer (TB-612T, 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 analysed by the area under the tension trace for the first 10 min of contraction as previously described (Shintani *et al*., 2000a). The values obtained with 40 mM $K^+$ depolarization were designated to be 100%.

### *Front-surface fluorimetry of $[Ca^{2+}]_i$ in the myometrial strips of the pregnant rats*

The myometrial strips of the pregnant rats were loaded with fura-PE3 in the form of acetoxymethyl ester (fura-PE3/AM) as previously described (Shintani *et al*., 2000b). The changes in the fluorescence ratio and tension development were simultaneously monitored using a front-surface fluorimeter CAM-OF-1 (Jasco, Tokyo, Japan) as previously reported (Shintani *et al*., 2000b). The fluorescence (500 nm) intensities at alternating (400 Hz) excitation (340 and 380 nm) and the ratio (F340/F380) were continuously measured. The data were stored in a Macintosh computer using a data acquisition system (MacLab; Analog Digital Instruments, Australia). The fluorescence ratio was expressed as a percentage, assigning the values in normal (5.9 mM $K^+$) and those obtained with 40 mM $K^+$ PSS to be 0 and 100%, respectively.

### *Reverse transcription-polymerase chain reaction (RT–PCR) analysis*

The total RNAs of the rat myometrium and placenta were isolated as previously described (Chomczynski & Sacchi, 1987), and treated with RNase-free DNase to remove any possible contamination by genomic DNA. The primers used in a 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 a RT–PCR analysis of rat PAR2 (Saifeddine *et al*., 1996) were 5′-CAA AGT AGT AGA CAA AGG GG-3′ for the RT reaction, 5′-CCA GGA AGA GGG CCA ACA T-3′ for the upper primer in PCR reaction, and 5′-ACG GTG CGG ACG CTT CGG CA-3′ for the lower primer in PCR reaction. The primers used in a RT–PCR analysis of PAR4 (Kahn *et al*., 1998) were 5′-GAC ACA TAG TAG TAG ATG AA-3′ for the RT reaction, 5′-TGG TTC AGT GTT 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. One $\mu$g of the total RNA was used for the RT reaction using Moloney murine leukaemia virus reverse transcriptase in a total volume of 20 $\mu$l. One $\mu$l aliquot of RT product was subjected to PCR amplification. The thermal cycle profile used for the amplification of PAR1, PAR2 and PAR4 fragments was composed of the initial denaturation at 94°C, for 2 min and the following 35–40 cycle amplification step consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. The expression of $\beta$-actin was analysed as a control as previously described (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 analysed by a NIH image (the National Institutes of Health, Bethesola, MD, U.S.A.) after obtaining the fluorescence image with a CCD camera (ATTO, Tokyo, Japan).

### *Drugs and solutions*

The composition of PSS used in the present study was (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, gassed with 95% $O_2$ and 5% $CO_2$. High-$K^+$ PSS was made by an equimolar substitution of KCl for NaCl. Heparin (MW3000), thrombin (bovine plasma), trypsin (bovine pancreas) and 4-aminidophenylmethane-sulphonyl fluoride (p-APMSF) were purchased from Sigma (St. Louis, MO, U.S.A.). Human PAR1-AP (SFLLRNP) and rat PAR2-AP (SLIGRL) were from BACHEM (Bubendorf, Switzerland). PAR3-AP (SFNGGPQ) and PAR4-AP (GYPGKFC) were synthesized based on the mouse sequence (Kahn *et al*., 1998) by Hokkaido System Science (Sapporo, Japan). Moloney murine leukaemia virus reverse transcriptase was purchased from Life Technologies (Rockville, MD, U.S.A.). Fura-PE3/AM was purchased from the Texas Fluorescence Laboratory (Austin, TX, U.S.A.). Ethyleneglycol-bis($\beta$-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) was obtained from Dojindo Laboratories (Kumamoto, Japan). The receptor antagonists used in the present study are as follows; $AT_1$ angiotensin II receptor antagonists, 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5-ylphenyl) benzyl] imidazole-5-methanol monopotassium salt (Losartan) was donated by Merck & Co., Inc. (Rahway, NJ, U.S.A.); 2-ethoxy-1-[[2′-(1H-tetrazol-5-yl) biphenyl-4-yl] methyl]-1H-benzimidazole-7-carboxylic acid (Candesartan) was from Takeda Chemical Industries Ltd. (Osaka, Japan); $AT_2$ angiotensin II receptor antagonist, S(+)-1-[[4-(Dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl) -4,5,6,7,-tetrahydro-1H-imidazo[4,5-c] pyridine-6-carboxylic acid ditrifluoroacetate (PD-123319) was purchased from Sigma; $NK_1$ receptor antagonist, (S)-(+)-1-(2-(3-(3,4-dichlorophenyl)-1- (3-isopropoxyphenylacetyl) piperidin-3-yl)-ethyl)-4-phenyl-1-azoniabicyclo (2.2.2) octane, chloride (SR140333), $NK_2$ receptor antagonist, (S)-(−)-N-methyl-N-[4-(4-acetylamino-4-phenyl)piperidino-2-(3,4-dichlorophenyl)-butyl]-benzamide, hydrochloride (SR48968) and $NK_3$ receptor antagonist, (R)-N-(1-(3-(1-benzol-3-(3,4-dichlorophenyl)-piperidin-3-yl) propyl) -4-phenylpiperidin-4-yl)-N-methylacetamide, hydrochloride (SR-142801) were gifted from Sanofi Recherche (Montpellier, France).

### *Statistical analysis*

All data are expressed as the mean ± s.e.mean. The unpaired Student's *t*-test was used to determine significant difference 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 trypsin on tension and $[Ca^{2+}]_i$ in the rat myometrium*

In the non-pregnant myometrium, 3 $\mu$M trypsin induced a small contraction (Figure 1a). The extent of the force development induced by 3 and 10 $\mu$M trypsin was 21.5 ± 6.0% and 30.5 ± 5.1% ($n=7$) of that obtained during the 40 mM $K^+$-depolarization-induced contraction in the non-pregnant myometrium (Figure 1c), respectively. On the other hand, in the 19-day pregnant rat myometrium, 3 $\mu$M trypsin caused a rapid and transient increase in $[Ca^{2+}]_i$ and tension, followed by a quick decline to the baseline within about 5 min (Figure 1b). The spontaneous oscillatory contractions were frequently superimposed on the declining phase of the trypsin-induced contraction (Figure 1b). Trypsin induced contractions at 1 nM and higher concentrations in the pregnant myometrium (Figure 1c). The level of the peak $[Ca^{2+}]_i$ elevation obtained with 3 $\mu$M trypsin was 143.2 ± 6.0% ($n=7$) of that obtained during the sustained phase of the contraction induced by 40 mM $K^+$-depolarization. The maximal force development was observed at concentrations between 0.3 and 3 $\mu$M, and the level of force obtained with 3 $\mu$M trypsin was 63.2 ± 7.9% ($n=9$). When normalized by the 40 mM $K^+$-induced contraction, the contraction induced by 3 $\mu$M trypsin in the pregnant rat myometrium was enhanced by 3 fold compared to that obtained in the non-pregnant rat. The $EC_{50}$ value (28.0 ± 6.6 nM, $n=9$) obtained in the pregnant rat myometrium was significantly ($P<0.001$) lower than that obtained in the non-pregnant rat myometrium (2.4 ± 1.0 $\mu$M, $n=7$) (Figure 1c). Since the contractile response to trypsin was significant and large enough to be statistically evaluated, the mechanism of trypsin-induced contraction was thereafter determined in the day 19 pregnant rat myometrium.

The contribution of the $Ca^{2+}$ influx to the trypsin-induced myometrial contraction was determined by examining the contractile response in the absence of extracellular $Ca^{2+}$ and in the presence of 3 mM $Ni^{2+}$, an inorganic $Ca^{2+}$ channel blocker (Chaib *et al*., 1998) (Figure 2). The exposure of the strips to the $Ca^{2+}$-free PSS caused a gradual decline of the resting $[Ca^{2+}]_i$, but had no effect on the resting level of tension (Figure 2a). The subsequent stimulation with trypsin induced only a residual increase, if any, in $[Ca^{2+}]_i$ but no contraction (Figure 2a). The exposure of the strips to 3 mM $Ni^{2+}$ also caused a gradual decline of the resting $[Ca^{2+}]_i$, but had no effect on the resting level of tension (Figure 2b). The subsequent stimulation with trypsin induced a residual small elevation of $[Ca^{2+}]_i$ and tension development.

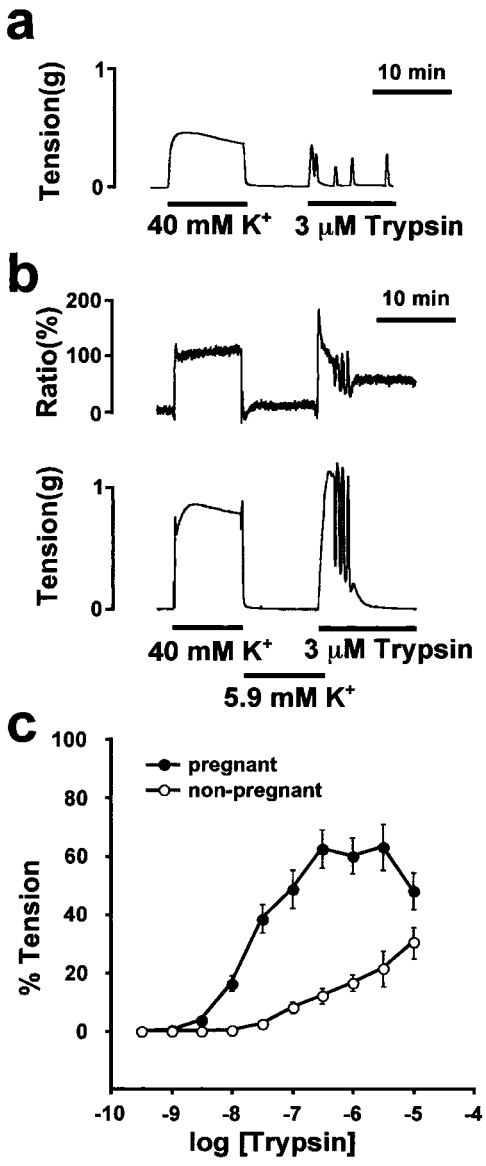


**Figure 1** The effect of trypsin on the rat myometrium. (a and b), Representative recordings showing the effect of 3 $\mu$M trypsin on the tension of the non-pregnant rat myometrium without loading fura-PE3 (a) and on the $[Ca^{2+}]i$ (upper trace) and tension (lower trace) of the day 19 pregnant rat myometrium loaded with fura-PE3 (b). (c) The concentration-response curves of the trypsin-induced contraction in the non-pregnant and pregnant rat myometrium. The contractile response was analysed by the area under the curve of the tension trace for the initial 10 min of contraction. The developed tension was expressed as a percentage, while assigning the values obtained in normal PSS and those obtained with 40 mM $K^+$ PSS to be 0 and 100%, respectively. Data are the mean $\pm$ s.e.mean ($n=7-9$).

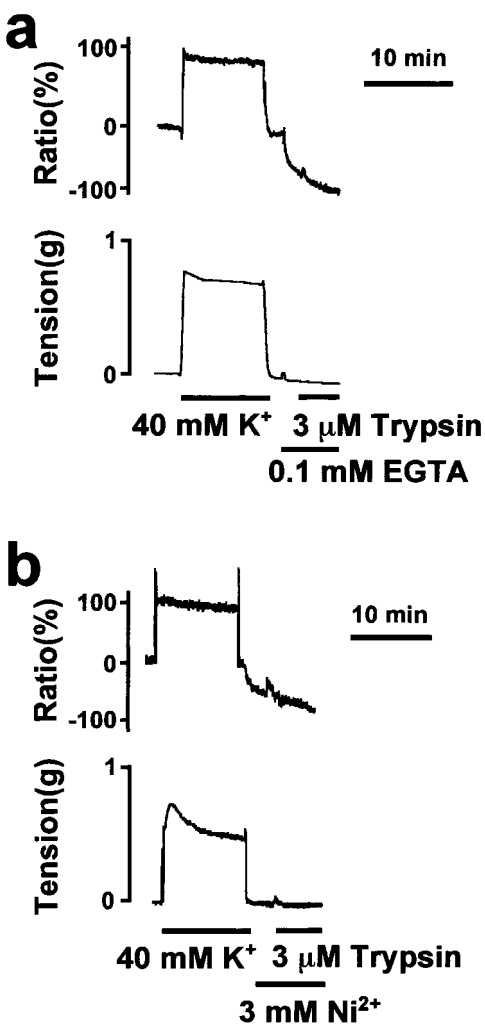


**Figure 2** Effect of trypsin on the mobilization of $Ca^{2+}$ and tension development in the pregnant rat myometrium. Representative traces of the trypsin-induced changes in $[Ca^{2+}]i$ (upper trace) and tension (lower trace) in the rat pregnant myometrium observed in the $Ca^{2+}$-free PSS containing 0.1 mM EGTA (a), and in the normal PSS containing 3 mM $Ni^{2+}$ (b). The strips were exposed to the $Ca^{2+}$-free PSS and $Ni^{2+}$-containing PSS 10 min prior to and during stimulation with trypsin. Similar results were obtained in three independent experiments.

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

When trypsin was pre-treated with 10 $\mu$M p-APMSF, a serine protease inhibitor, for 10 min, and then applied to the strip of rat myometrium at a final concentration of 3 $\mu$M, no contractile response was evoked (Figure 3a). After extensive washing to remove p-APMSF-treated trypsin in the normal PSS and recording the control response to 40 mM $K^+$, the subsequent stimulation with untreated trypsin induced a contraction similar to that observed without the preceding application of the p-APMSF-treated trypsin. This observation thus indicated that the contractile effect of trypsin required proteolytic activity, and therefore PAR1, PAR2 and PAR4 (Cocks & Moffatt, 2000) are thus suggested to be candidate receptors which mediate the trypsin-induced contractile response in the rat myometrium.

On the other hand, trypsin-induced myometrial contraction was reported to be inhibited by angiotensin II antagonists, thus suggesting the involvement of angiotensin II receptor (Orce *et al.*, 1994). We examined this possibility by investigating the effect of angiotensin II antagonists on the trypsin-induced contraction. Losartan, Candesartan ($AT_1$ receptor antagonists) and PD-123319 ($AT_2$ receptor antagonist) had no effect on the trypsin-

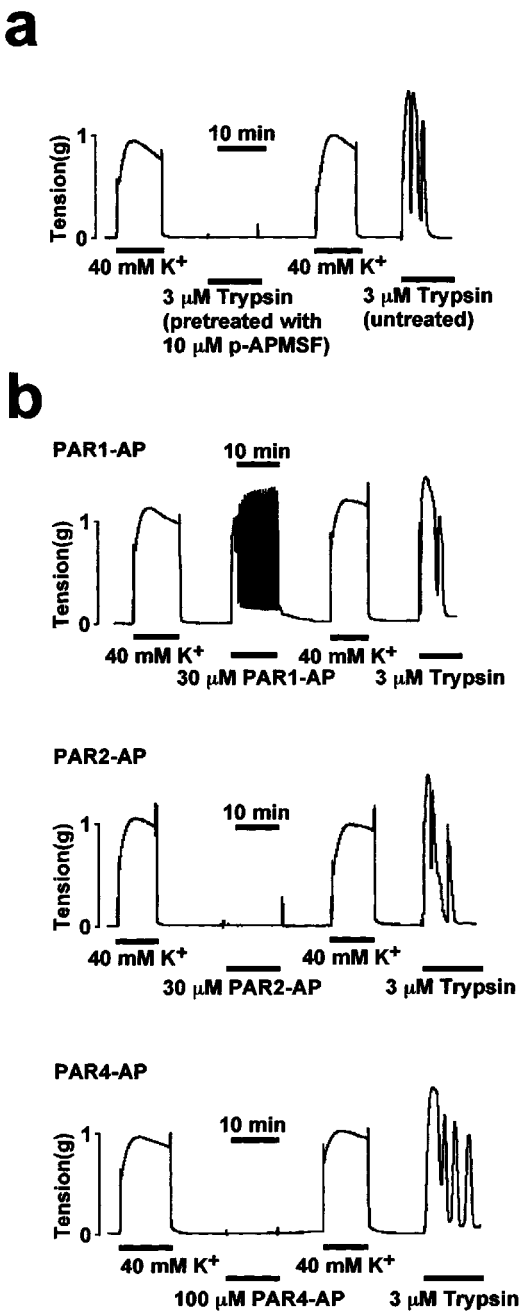


**Figure 3** Effects of 4-aminido-phenylmethane-sulphonyl fluoride (p-APMSF) and PARs-activating peptides (PARs-AP) on the trypsin-induced myometrial contraction in the pregnant rat. (a) Representative traces showing the inhibitory effects of 4-aminido-phenylmethane-sulphonyl fluoride (p-APMSF) on the trypsin-induced contraction of the pregnant rat myometrium. Trypsin (18 nmol) was pretreated with 0.6 nmol p-APMSF in a 60 $\mu$l volume, and then was applied to the strips in the presence of 10 $\mu$M p-APMSF, and the final contractions of trypsin and p-APMSF in the organ bath were observed to be 3 $\mu$M and 10 $\mu$M, respectively. After recording the effects of p-APMSF-treated trypsin, the bath was extensively washed with the buffer, and then the control response to 40 mM $K^+$ and the responsiveness to 3 $\mu$M trypsin (untreated) were determined. Similar results were obtained in three independent experiments. (b) Representative traces showing the effects of 30 $\mu$M PAR1-activating peptide (PAR1-AP), 30 $\mu$M PAR2-AP and 100 $\mu$M PAR4-AP on the tension in the pregnant rat myometrium. After recording the effects of PARs-AP and the control response to the subsequent stimulation with 40 mM $K^+$, the responsiveness to 3 $\mu$M trypsin was examined. Similar results were obtained in three independent experiments.

induced contraction ($P>0.05$ between with and without antagonist). Moreover, 1 $\mu$M angiotensin II did not induce any contraction of the rat myometrium. Furthermore, kallikrein was shown to induce contraction in the rat myometrium by activating the formation of kinins (Orce *et al*., 1989), and we reported neurokinin A to induce myometrial contraction (Shintani *et al*., 2000a,b). However, SR140333 (NK1 receptor antagonist), SR48968 (NK2 receptor antagonist), and SR142801 (NK3 receptor antagonist) all had no effect on the trypsin-induced contraction ($P>0.05$ between with and without antagonist).

To determine the type of receptor involved in the trypsin-induced contraction, we first examined the effects of PAR1-AP, PAR2-AP and PAR4-AP on the contraction in the rat myometrium (Figure 3b). The human PAR1-AP (SFLLRNP) at 30 $\mu$M induced a contraction to an extent similar to that obtained with 3 $\mu$M trypsin in the pregnant rat myometrium. The extent of tension developed by 30 $\mu$M PAR1-AP was $80.0\pm7.7\%$ ($n=5$) in the pregnant myometrium. The level of contraction induced by 100 $\mu$M PAR1-AP was $23.8\pm4.1\%$ ($n=5$) in the non-pregnant and $102.0\pm3.7\%$ ($n=7$) in the pregnant myometrium (4 fold increase). On the other hand, the rat PAR2-AP (Figure 3b) and the mouse PAR4-AP (Figure 3b) failed to induce any contraction in the rat myometrium (Figure 3b), while the subsequent application of 3 $\mu$M trypsin did induce contractions. The same PAR2-AP as well as trypsin induced a transient $[Ca^{2+}]_i$ elevation in the endothelial cells of the porcine aortic valve (data not shown).

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

The expression of PAR1, PAR2, and PAR4 mRNA in the rat myometrium was examined by a RT–PCR analysis (Figure 4). PAR1 mRNA was slightly detected in the non-pregnant myometrium, and its level increased approximately 10 fold in the myometrium during both pregnancy and the post-parturition period compared to that in the non-pregnant

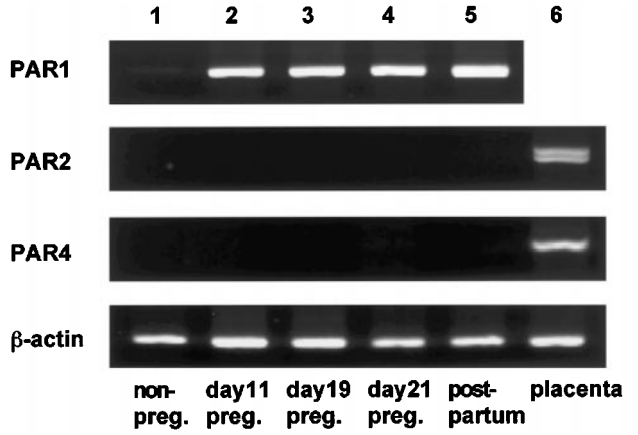


**Figure 4** Expression of PAR1, PAR2 and PAR4 mRNA in the rat myometrium. Representative photographs showing the RT–PCR products for PAR1 (545 bp), PAR2 (489 bp), PAR4 (477 bp) and $\beta$-actin (224 bp) mRNA in the myometrium of non-pregnant (lane 1), pregnant day 11 (lane 2), pregnant day 19 (lane 3), pregnant day 21 (lane 4) and postpartum rats (lane 5), and in the rat placenta (lane 6). The photos are representative of three independent experiments.

myometrium (Figure 4). The level of PAR1 mRNA did not significantly differ with the day of pregnancy. The level of $\beta$-actin mRNA slightly increased (1.5 fold) in the pregnant myometrium (Figure 4). The expression of PAR2 mRNA was hardly detected in both the non-pregnant and the pregnant rat myometrium by using the set of primers based on the sequence of rat PAR2 (Saifeddine *et al*., 1996), while the expression of PAR2 was detected in the rat placenta (Figure 4, panel 2). The expression of PAR4 mRNA also was scarcely detected in both the non-pregnant and pregnant rat myometrium using the set of primers based on the sequence of mouse PAR4 (Kahn *et al*., 1998), while the expression of PAR4 mRNA was detected in the rat placenta (Figure 4, panel 3). The nucleotide sequences of the PCR product for PAR1 and PAR2 were found to be identical to the published rat sequences (Saifeddine *et al*., 1996; Zhong *et al*., 1992), and that for PAR4 were determined to be highly similar (94% similarity) to the published mouse sequences (Kahn *et al*., 1998). As a result, the absence of an expression of PAR2 and PAR4 mRNA was consistent with the observation that the PAR2-AP and PAR4-AP did not induce any contraction in the rat myometrium.

### *Trypsin-induced non-productive cleavage of PAR1*

To determine the contribution of PAR1 to the trypsin-induced contraction, we examined the effect of the preceding stimulations with thrombin, trypsin and PAR1-AP on the contractile responses to the subsequent stimulations with thrombin, trypsin and PAR1-AP, as shown in Figure 5 and as summarized in Figure 6. In this experiment, myometrial strips were sequentially stimulated for 10 min by various combinations of thrombin, tyrpsin and PAR1-AP at 40 min intervals. The second stimulation was preceded by the stimulation with 40 mM $K^+$ in order to refill the intracellular $Ca^{2+}$ stores (Figure 5). The concentrations of thrombin (1 units $ml^{-1}$), trypsin (3 $\mu$M) and PAR1-AP (30 $\mu$M) used in this experiment were the minimal concentrations required to induce the maximal responses, respectively. As a control, we first determined the contractile response to the first stimulation with thrombin, trypsin and PAR1-AP, without preceding stimulation, at two different intervals, 10 min and 50 min, after the reference contraction induced by the first stimulation with 40 mM $K^+$. As shown in Figure 6a, the extents of tension produced by thrombin (69.0±11.5%,

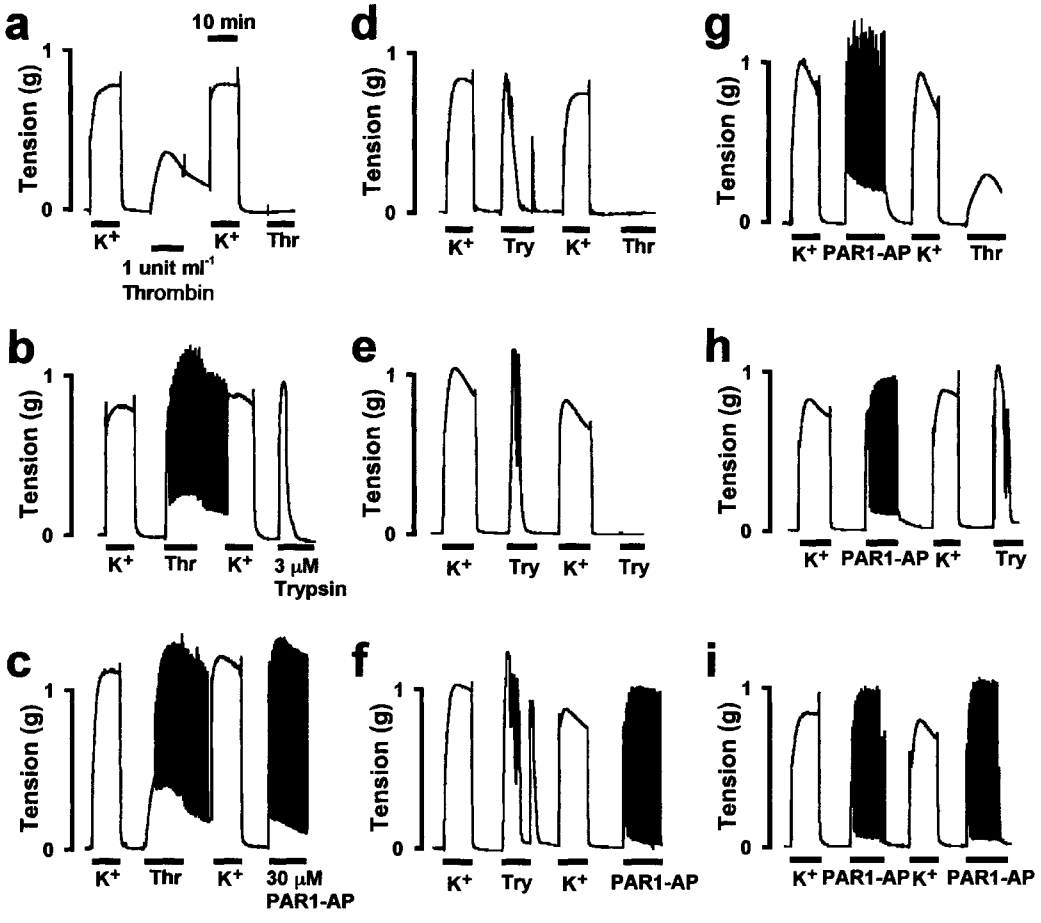


**Figure 5** Effects of the preceding stimulation with thrombin, trypsin and PAR1-AP on the responsiveness to the subsequent stimulation with thrombin, trypsin and PAR1-AP in the myometrium of the pregnant rat. After recording the reference response to 40 mM $K^+$ ($K^+$) for 10 min, the pregnant rat myometrium was incubated in normal PSS for 10 min and sequentially stimulated for 10 min by three agonists (1 unit $ml^{-1}$ thrombin (Thr), 3 $\mu$M trypsin (Try) and 30 $\mu$M PAR1-AP) in the combinations as indicated. The first and second stimulations were separated by 30 min interval (the incubation in normal PSS for 10 min, 40 mM $K^+$ for 10 min, and normal PSS for 10 min). The first and second stimulations were thus applied to the strips 10 min and 50 min after the initial contraction by 40 mM $K^+$.

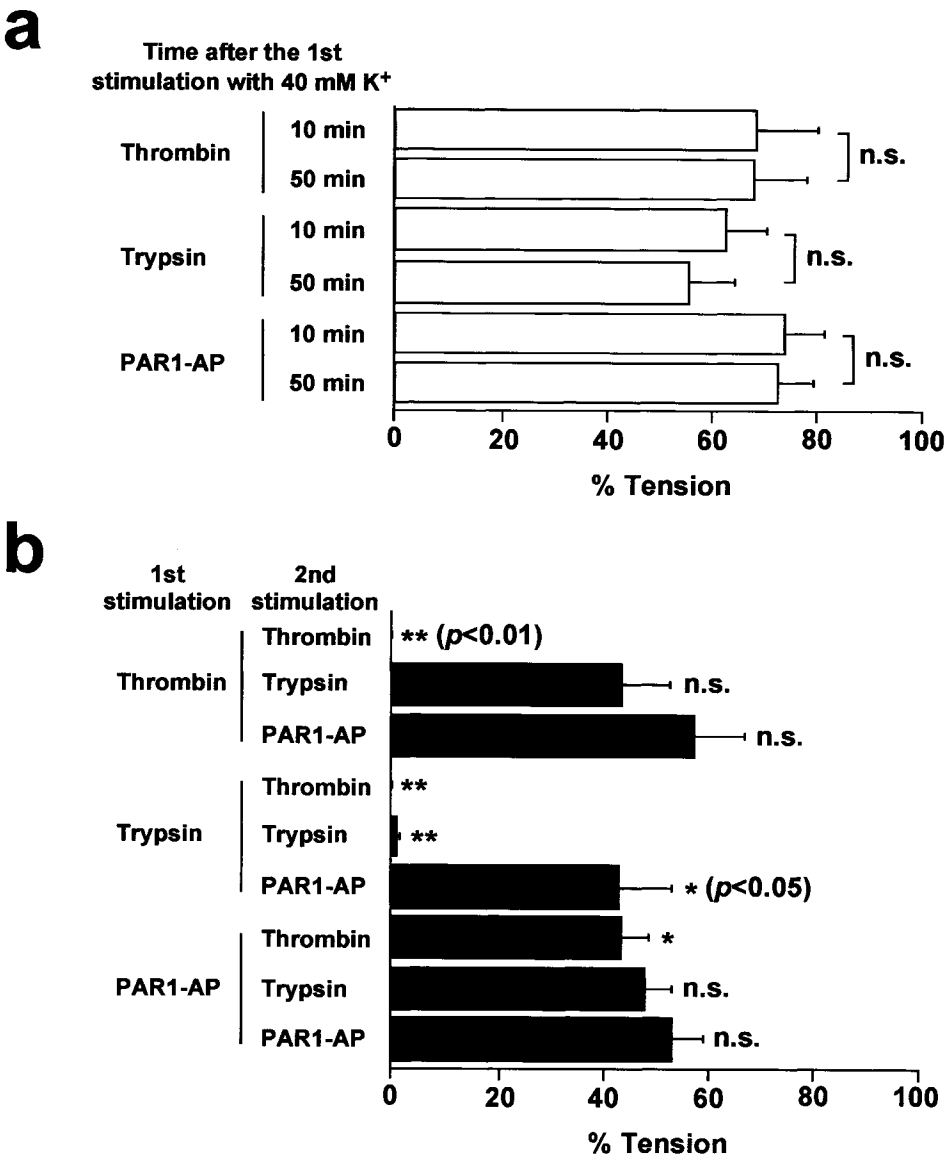


**Figure 6** Summary of the effects of the preceding stimulation on the subsequent responses to thrombin, trypsin and PAR1-AP in the pregnant rat myometrium. Summary of experiments shown in Figure 5. (a) The control responses to 1 unit $ml^{-1}$ thrombin, 3 $\mu$M trypsin and 100 $\mu$M PAR1-AP. The stimulations were applied 10 min and 50 min after the initial contraction by 40 mM $K^+$, n.s., not significant. (b) The responses to the second stimulations after the preceding stimulation as indicated. The developed tension was evaluated by the area under the tension trace for the initial 10 min of contraction, and was expressed as a percentage, assigning the value obtained with normal PSS and that obtained with 40 mM $K^+$ to be 0 and 100%, respectively. All data are the mean$\pm$s.e.mean ($n=5$). *$P<0.05$; **$P<0.01$; n.s., not significant, compared to the time-matched control response to the same agonist without the first stimulation (a, 50 min).

$n=7$), trypsin ($63.2\pm7.9\%$, $n=7$) and PAR1-AP ($74.4\pm7.7\%$, $n=7$) 10 min after the 40 mM $K^+$-induced contraction were same as those obtained 50 min after the 40 mM $K^+$-induced contraction (thrombin, $68.6\pm10.1\%$, $n=5$; trypsin, $56.2\pm8.8\%$, $n=5$; PAR1-AP, $73.2\pm6.8\%$, $n=5$). As a result, the first stimulation induced a consistent response during the 50 min observations after the first stimulation with 40 mM $K^+$.

Once the strips were stimulated with thrombin, the second stimulation with thrombin caused no contraction (Figures 5a and 6b). In contrast, trypsin induced a contraction ($43.3\pm9.3\%$, $n=5$) after the preceding stimulation with thrombin (Figure 5b and 6b), and the extent of this contraction did not significantly differ from that observed without the preceding stimulation by thrombin (Figure 6b). The stimulation with PAR1-AP after the preceding stimulation with thrombin also induced a similar contraction ($57.4\pm9.6\%$, $n=5$) to that obtained with the control PAR1-AP-induced contraction (Figures 5c and 6). On the other hand, once the strips were stimulated with trypsin, the second applications of thrombin or trypsin did not produce any contraction (Figures 5d,e and 6b). PAR1-AP induced the contraction ($43.0\pm10.1\%$, $n=5$, $P<0.05$), but the extent of this contraction was significantly smaller ($\sim$40% reduction) than that obtained without any preceding stimulation by trypsin (Figures 5f and 6). The reduction ($\sim$20%) of the

contractile response to 40 mM $K^+$ (79.70±6.1%, $n=15$, $P<0.05$) was consistently observed after the preceding stimulation with trypsin (Figure 5d,f). When the strips were first stimulated with PAR1-AP, the second stimulations with thrombin, trypsin and PAR1-AP elicited the contractions to 43.3±5.4% ($n=5$), 47.9±5.3% ($n=5$) and 53.0±6.3% ($n=5$), respectively. The extent of thrombin-induced contraction was significantly ($P<0.05$) smaller than that obtained without any preceding stimulation by PAR1-AP (Figures 5g and 6), but the other two values did not significantly differ from those obtained without the preceding stimulation by PAR1-AP (Figures 5h,i and 6b).

## Discussion

We herein demonstrated that trypsin, an agonist for PARs, induced a contraction in the rat myometrium. We further investigated the mechanism of the trypsin-induced myometrial contraction, and obtained the following major findings: (1) The trypsin induced myometrial contraction mainly by activating the $Ca^{2+}$ influx from the extracellular space. (2) The trypsin-induced contraction was enhanced in the pregnant rat. (3) The proteolytic activity was required for trypsin to exert its contractile effect. (4) Among the known receptors for trypsin, PAR1 is the only receptor expressed in the rat myometrium. The expression of PAR1 mRNA increased in the rat myometrium. (5) However, even after PAR1 was made unresponsive to the subsequent proteolytic activation by the preceding stimulation with thrombin, trypsin could induce a myometrial contraction to an extent similar to that obtained without the desensitization of PAR1. These findings excluded the contribution of PAR1 to the trypsin-induced myometrial contraction, and thus suggest that trypsin induced myometrial contraction by activating some unidentified member of PAR.

Since the discovery and cloning of PAR1, four members of PAR have so far been identified (Ishihara *et al*., 1997; Nystedt *et al*., 1994; Vu *et al*., 1991; Xu *et al*., 1998). PAR1, PAR2 and PAR4 serve as receptors for trypsin, while PAR1, PAR3 and PAR4 serve as receptors for thrombin (Cocks & Moffatt, 2000; Dery *et al*., 1998). PAR2 is thus specific receptor for trypsin. However, in the present study, the RT–PCR analysis did not detect the expression of PAR2 mRNA in the rat myometrium, however, it did detect the expression of PAR2 mRNA in the rat placenta, thus ruling out the possibility of an inappropriate primer design and condition of RT–PCR analysis. Furthermore, a synthetic peptide, PAR2-AP, failed to induce any contraction in the rat myometrium. These observations indicate the absence of PAR2 in the rat myometrium, and rule out the involvement of PAR2 in the trypsin-induced contraction in the rat myometrium. Similarly, the involvement of PAR4 in the trypsin-induced myometrial contraction was also ruled out. On the other hand, PAR1 expression was detected by a RT–PCR analysis, and its expression level increased in the pregnant rat myometrium. PAR1-AP induced a contraction in the rat myometrium, and the contraction was enhanced in the pregnant rat myometrium (Shintani *et al*., 2000a). The increase in the PAR1 expression level thus apparently correlated with the enhancement of contraction induced by thrombin (Shintani *et al*., 2000a), PAR1-AP (Shintani *et al*., 2000a) and trypsin (the present study). Trypsin was reported to be capable to cleave PAR1 at the thrombin cleavage site and activate PAR1 (Dery *et al*., 1998; Loew *et al*., 2000). As a result, there is a possibility that PAR1 may also mediate trypsin-induced myometrial contraction in the rat.

The involvement of PAR1 in trypsin-induced myometrial contraction was thus further evaluated by utilizing cross-desensitization of the receptors as shown in Figures 5 and 6. Neither thrombin nor trypsin could induce myometrial contraction once the strips were activated with the preceding same stimulation. This observation indicates that both thrombin receptor and trypsin receptor become unresponsive to proteolytic activation, once they are cleaved and activated by thrombin and trypsin, respectively. The desensitization of thrombin receptors have been reported to be mediated not only by proteolytic cleavage of the receptors but also by the phosphorylation of the receptors (Dery *et al*., 1998; Ishii *et al*., 1994; Mizuno *et al*., 2000; Yan *et al*., 1998). However, in the present study, the responsiveness to PAR1-AP remained intact after the preceding stimulation with thrombin. These findings suggest that the thrombin-induced desensitization of thrombin receptors was mainly due to proteolytic cleavage, while the cleavage-independent and probably phosphorylation-mediated desensitization played a negligible role in the thrombin-induced desensitization of thrombin receptors in the rat myometrium. The negligible contribution of the phosphorylation-mediated desensitization is consistent with the observation that the cleavage-independent activation of PAR1 by PAR1-AP did not attenuate the responsiveness to the subsequent stimulation with PAR1-AP. However, the mechanism of a reduction in the contraction induced by thrombin after the preceding stimulation with PAR1-AP remains to be elucidated. When thrombin receptors were desensitized by the preceding stimulation with thrombin, trypsin did induce myometrial contraction. Furthermore, the extent of this contraction was similar to that obtained without the preceding stimulation by thrombin, namely, the desensitization of thrombin receptor. As a result, the receptor mediating the trypsin-induced contraction was suggested to be different from that mediating the thrombin-induced contraction. In the rat myometrium, thrombin induced the contraction mainly through PAR1 activation (Shintani *et al*., 2000a). These findings suggest that PAR1 did not play a major role in the trypsin-induced myometrial contraction in the rat.

As a consequence, none of the three known trypsin receptors, PAR1, PAR2 and PAR4 are suggested to be involved in the trypsin-induced myometrial contraction. Several reports suggested that atypical or novel receptors but not classical PAR2 mediated the effects of trypsin such as the trypsin-induced endothelium-dependent relaxation in the human coronary artery (Hamilton *et al*., 1998), the trypsin-induced intestinal ion transport in the rat jejunum (Vergnolle *et al*., 1998) and the trypsin-induced activation of mitogen-activated protein kinase in the fibroblasts (Belham *et al*., 1996). On the other hand, the angiotensin II receptor was suggested to be involved in the trypsin-induced myometrial contraction (Orce *et al*., 1994). However, the involvement of the angiotensin II and neurokinin receptors was ruled out in the present study. As a consequence, we propose that trypsin activated an as yet unidentified member of PARs or a myometrium-specific subtype of PAR2, and thereby caused a contraction in the rat myometrium. The precise mechanism of

the trypsin-induced contraction, however, remains to be elucidated.

Another important finding of the present study is that the preceding stimulation with trypsin completely inhibited the contractile effect of the subsequent stimulation with thrombin. This inhibition was not due to the proteolytic degradation of thrombin receptor into pieces, because PAR1-AP induced contraction after the preceding stimulation with trypsin. The extent of the PAR1-AP-induced contraction was significantly attenuated (~40%) compared to that obtained without the preceding stimulation by trypsin (Figure 6). However, the contractile responses to 40 mM $K^+$ (Figure 5) and to neurokinin A (data not shown) were similarly attenuated (~20%) after the preceding stimulation with trypsin. It is thus conceivable that the responsiveness of the thrombin receptor to PAR1-AP remained mostly intact after the preceding stimulation with trypsin, although general attenuation of contractile response of the myometrium attributed the attenuation of the PAR1-AP-induced contraction. As a consequence, PAR1 was considered to be cleaved by trypsin and made un-responsive to thrombin. The thrombin site of PAR1 (residues 41,42) is thus not suggested to be the major cleavage site of trypsin. Since the responsiveness to PAR1-AP remained almost intact after the stimulation with trypsin, we propose that trypsin cleaved PAR1 between the thrombin site and the ligand binding site (residues 212–245). Recently, a mass-spectroscopic analysis of the *in vitro* trypsin-cleavage products of the extracellular domain of PAR1 showed that the residues 41,42, 70,71 and 82,83 of PAR1 served as the early cleavage (0 to 5 min) sites, while the residues 47,48 served as the late cleavage sites (5 min to 3 h) (Loew *et al*., 2000). These sites, other than residues 41,42, are thus the candidate sites for the trypsin-induced unproductive cleavage of PAR1 in the rat myometrium. The precise location of the sites responsible for the unproductive cleavage of PAR1 *in vivo*, however, remains to be determined.

In the rat myometrium, the trypsin-induced contraction was shown to be almost completely abolished either by removal of the extracellular $Ca^{2+}$ or by the addition of $Ni^{2+}$ to the medium containing $Ca^{2+}$. We thus concluded that trypsin induced $[Ca^{2+}]_i$ elevation mainly by activating $Ca^{2+}$ influx from the extracellular space and caused the myometrial contraction in the rat. The $Ca^{2+}$ release from the intracellular stores was suggested to play a negligible role, if any, in trypsin-induced myometrial contraction. In the intestinal epithelial cells, trypsin was reported to induce a $[Ca^{2+}]_i$ elevation due to both $Ca^{2+}$ release and $Ca^{2+}$ influx (Bohm *et al*., 1996a). The mechanism of $Ca^{2+}$ mobilization by trypsin thus varies with the type of cell. The difference in the $Ca^{2+}$ mobilization may correlate with the difference in type of receptor responsive to trypsin.

In conclusion, we demonstrated that trypsin induced myometrial contraction in the rat by mainly activating $Ca^{2+}$ influx from the extracellular space. The contraction was enhanced in the myometrium of the pregnant rat. No members of PAR, which are known to serve as trypsin receptors, were suggested to be involved in the trypsin-induced myometrial contraction of the rat. Neither PAR2 nor PAR4 were expressed in the rat myometrium, and PAR1 was suggested to be cleaved by trypsin between the thrombin-cleavage site (residues 41,42) and the ligand-binding site (residues 240–265), and thus was observed to lose responsiveness to thrombin. A novel member of PAR was thus suggested to mediate the contractile response to trypsin in the rat myometrium.

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