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Unproductive cleavage and the inactivation of protease-activated receptor-1 by trypsin in vascular endothelial cells

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1 Using fura-2 fluorometry of $[Ca^{2+}]_i$ in response to thrombin, trypsin and protease-activated receptor activating peptides (PAR-APs), we determined whether trypsin cleaves protease-activated receptor 1 (PAR1) and activates it in the endothelial cells of the porcine aortic valves and human umbilical vein.

2 Once stimulated with thrombin, the subsequent application of trypsin induced a $[Ca^{2+}]_i$ elevation similar to that obtained without the preceding stimulation with thrombin in the valvular endothelial cells. However, the preceding stimulation with trypsin abolished the subsequent response to thrombin, but not to bradykinin or substance P.

3 The response to PAR1-AP (SFLLRNP) was significantly ($P<0.05$) reduced by the preceding stimulation with thrombin and PAR1-AP in the valvular endothelial cells, while, importantly, it remained unaffected by the preceding stimulation with either trypsin or PAR2-AP (SLIGRL). The response to PAR2-AP was reduced by the preceding stimulation with trypsin and PAP2-AP. PAR1-AP attenuated the subsequent responses not only to thrombin and PAR1-AP but also to trypsin and PAR2-AP, while PAR2-AP specifically attenuated the subsequent responses to trypsin and PAR2-AP.

4 In human umbilical vein endothelial cells, a higher affinity PAR1-AP (haPAR1-AP) (Ala-pF-Arg-Cha-HArg-Tyr-NH_2) specifically attenuated the responses to thrombin but not trypsin. On the other hand, the response to haPAR1-AP was significantly ($P<0.05$) attenuated by the preceding stimulation with thrombin but not trypsin.

5 In conclusion, trypsin cleaved PAR1 but did not activate it in the endothelial cells. Moreover, the trypsin-cleaved PAR1 was no longer responsive to thrombin.
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Keywords: Protease-activated receptor; thrombin; trypsin; endothelial cells

Abbreviations: p-APMSF, 4-aminidophenylmethane sulphonyl fluoride; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentrations; DMEM, Dulbecco's modified Eagle medium; HUVEC, human umbilical vein endothelial cells; PAR, protease-activated receptor; PAR-APs, PAR-activating peptides; PAR1-AP, PAR1-activating peptide; PAR2-AP, PAR2-activating peptide; haPAR1-AP, high affinity PAR1-AP; PSS, physiological salt solution

Introduction

Proteases such as thrombin and trypsin are known to activate protease-activated receptors (PARs), and thereby exert cellular effects such as platelet aggregation, endothelium-dependent relaxation and myometrial contraction (Dery *et al*., 1998; Hamilton *et al*., 1998; Nakayama *et al*., 2001; Shintani *et al*., 2001). PARs belong to a family of the G-protein coupled receptors (Dery *et al*., 1998; Macfarlane *et al*., 2001), and four members of PARs, PAR1, PAR2, PAR3 and PAR4, have so far been identified (Kahn *et al*., 1998; Nystedt *et al*., 1994; Vu *et al*., 1991; Xu *et al*., 1998). PAR1, PAR3 and PAR4 are considered to serve as receptors for thrombin, while PAR1, PAR2 and PAR4 are considered to serve as receptors for trypsin (Cocks & Moffatt, 2000; Dery *et al*., 1998; Macfarlane *et al*., 2001). The activation of PARs is unique in that the proteolytic cleavage of the receptor triggers the activation process. The cleavage of the NH_2-terminal extracellular domain of PAR at the specific site unmasks a new NH_2-terminus, which in turn acts as a tethered ligand and then initiates intracellular signalling to provoke a cellular response (Dery *et al*., 1998). The activating cleavage sites of PAR1, PAR2, PAR3 and PAR4 are the residues 41–42, 36–37, 38–39 and 47–48 in humans (Dery *et al*., 1998; Macfarlane *et al*., 2001). The synthetic peptides corresponding to the tethered ligand sequences has been 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 may thus be a useful tool for investigating the regulation of PARs.

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Trypsin, a specific agonist for PAR2 (Bohm *et al*., 1996a, b), has been reported to cleave PAR1 at the activating cleavage site (Loew *et al*., 2000), and is thus considered to activate PAR1 (Macfarlane *et al*., 2001). However, the evidence for the activation of PAR1 by trypsin appears to be weak and indirect (Hamilton *et al*., 1998; 2001). Moreover, trypsin has also been reported to cleave PAR1 at residues 70–71 and 82–83 by means of similar kinetics to that observed with the activating cleavage of residues 41–42 (Loew *et al*., 2000). Collectively, it remains to be clarified as to whether or not trypsin activates PAR1 by cleaving the residues 41–42, especially *in vivo*. In the rat myometrium, we previously reported that thrombin and trypsin induced contraction (Shintani *et al*., 2000; 2001). The cross-desensitization experiments revealed that the preceding stimulation with trypsin completely abolished the myometrial contraction induced by the subsequent stimulation with thrombin, while the preceding stimulation with thrombin had no effect on the subsequent response to trypsin (Shintani *et al*., 2001). We thus suggested that trypsin cleaves PAR1, but does not activate it. Moreover, we proposed that the trypsin-cleaved PAR1 is no longer responsive to thrombin. However, the rat myometrium was found to be defective in PAR2 (Shintani *et al*., 2001). The effects of trypsin on the PAR1 activity remain to be examined in the cells, which express both PAR1 and PAR2. We previously reported that trypsin activates PAR2 and evokes Ca^{2+} signal in the endothelial cells of the porcine aortic valve, and that trypsin induces endothelium-dependent relaxation in the porcine coronary artery (Nakayama *et al*., 2001). We also reported that thrombin activated PAR1 in the porcine aortic valvular strips, and induced endothelium-dependent relaxation in the porcine coronary artery (Mizuno *et al*., 1998).

In the present study, we aimed to determine whether trypsin cleaves PAR1 and activates it in vascular endothelial cells. For this purpose, we used front-surface fura-2 fluorimetry (Kanaide, 1999) to examine the $[Ca^{2+}]_i$ elevations in response to thrombin, trypsin and PAR-APs and in the cross-desensitization among these stimulations. We demonstrated that trypsin cleaved PAR1 but did not activate it in the vascular endothelial cells. Moreover, we demonstrated that trypsin cleavage made PAR1 unresponsive to thrombin.

Methods

Preparation of strips of the porcine aortic valve

Porcine aortic valves were obtained at a local slaughterhouse. The valve leaflets were cut into strips in an axial direction (approximately 3 mm wide, 5 mm long and 0.18 mm thick) as previously described (Nakayama *et al*., 2001). Care was taken to avoid damaging the endothelial lining. These strips were then used to monitor the changes in $[Ca^{2+}]_i$ of *in situ* endothelial cells.

Cell culture of human umbilical vein endothelial cells (HUVECs)

The human umbilical cords were kindly provided from the Fukuoka Municipal Hospital. HUVECs were isolated by treatment with trypsin in the EDTA-containing PBS as previously described (Jaffe *et al*., 1973), and plated in 35 mm culture dishes and cultured to confluence in EBM-2 media supplemented with EGM-2 Bred kit (Sankyo Junyaku, Tokyo, Japan). After achieving a confluent monolayer of endothelial cells, the medium was changed to RPMI-1640 media (Life Technologies, Tokyo, Japan) containing 10% foetal bovine serum, and the cells were further incubated for 48 h until experimental use. The cells were used in the experiment within the second passage. When the cells were subcultured, they were harvested by treatment with trypsin. The HUVECs at confluence showed typical cobblestone monolayer morphology under a phase contrast microscope.

Measurement of $[Ca^{2+}]_i$ in endothelial cells

The valvular strips were loaded with fura-2 by incubating them in Dulbecco's modified Eagle medium (DMEM) containing 50 μM fura-2/AM (an acetoxymethyl ester form of fura-2), 1 mM probenecid, 5% foetal bovine serum for 90 min at 37°C as previously described (Nakayama *et al*., 2001). After loading with fura-2, the strips were washed and equilibrated in normal physiological salt solution (PSS) for at least 1 h at room temperature. The strips were then mounted vertically in a quartz organ bath filled with PSS, and the measurements were started. HUVECs were loaded with fura-2 by incubating them in DMEM containing 10 μM fura-2/AM for 1 h at 37°C (Hirano *et al*., 1993). After loading with fura-2, the cells were washed and equilibrated in HEPES-buffered saline (HBS) for at least 30 min at room temperature before starting the measurements. The changes in $[Ca^{2+}]_i$ in endothelial cells of the valvular strips and cultured HUVECs were monitored using a front-surface fluorometer as previously described (Hirano *et al*., 1993; Nakayama *et al*., 2001). Fluorometry was performed at 25°C to prevent any leakage of fura-2 (Kuroiwa *et al*., 1995). The 500 nm fluorescence intensities at 340 nm and 380 nm excitation and their ratio were continuously monitored. The fluorescence ratio data were expressed as a percentage, while assigning the values at rest and at the peak $[Ca^{2+}]_i$ elevation induced by 10 μM ATP to be 0% and 100%, respectively. All data were collected at a sampling rate of 17 Hz using a computerized data acquisition system (MacLab, Analog Digital Instruments, Australia; Macintosh, Apple computer, U.S.A.).

Preparation of strips of the porcine coronary artery and measurement of tension

The segments of the left circumflex arteries (2–3 cm from the origin) were excised from the porcine hearts at a local slaughterhouse immediately after the animals had been slaughtered. After removing the adventitia, the circular strips (approximately 1 mm wide, 5 mm long, and 0.1 mm thick) were prepared as described previously (Nakayama *et al*., 2001). Care was taken to avoid damaging the endothelial cells. The strips were mounted vertically to a force transducer, TB-612T (Nihon Koden, Japan), in a quartz organ bath (37°C) filled with normal PSS, and the changes in tension were monitored as previously described (Nakayama *et al*., 2001). The data were expressed as a percentage, assigning the values in normal PSS (5.9 mM K^+) and those in

118 mM K^+-PSS to be 0% and 100%, respectively. All measurements of tension were carried out at 37°C.

Drugs and solutions

The composition of PSS was 123 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 15.5 mM $NaHCO_3$ and 11.5 mM D-glucose, gassed with 95% O_2 and 5% CO_2. The composition of HBS was (in mM): HEPES 10 (pH 7.4), NaCl 135, KCl 5, $CaCl_2$ 1.0, $MgCl_2$ 1.0 and D-glucose 5.5. Thrombin (bovine plasma, 1880 NIH units mg^{-1} protein; 1 units $ml^{-1} = \sim 10$ nM), trypsin (bovine pancreas, 10900 units mg^{-1} protein) 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 purchased from Bachem (Bubendorf, Switzerland). A high affinity PAR1-AP (haP-AR1-AP: Ala-pF-Arg-Cha-HArg-Tyr-NH_2) was purchased from Neosystem (Strasbourg, France). Substance P and bradykinin were purchased from the Peptide Inc. (Osaka, Japan). U46619 (9, 11-dideoxy9α, 11α-methanoepoxy prostaglandin F2α) was purchased from Funakoshi (Tokyo, Japan).

Data analysis

The data are the mean ± s.e.mean of the indicated number of experiments. The presence of significant differences was statistically analysed by the unpaired Student's *t*-test and by an analysis of variance (ANOVA). A P value of less than 0.05 was considered to be statistically significant.

Results

PAR agonists-induced elevations of $[Ca^{2+}]_i$ in the in situ *endothelial cells of the porcine aortic valve*

Thrombin, trypsin PAR1-AP, PAR2-AP all induced a rapid and transient increase in $[Ca^{2+}]_i$ in the valvular strips in a concentration-dependent manner (Figure 1). Thrombin induced a significant $[Ca^{2+}]_i$ elevation at 0.3 units ml^{-1} (equivalent to ~3 nM) and higher concentrations, and the maximal $[Ca^{2+}]_i$ elevation (33.0 ± 2.5%, $n = 5$) was obtained at 6 units ml^{-1} (equivalent to ~60 nM) (Figure 1b). Trypsin induced a significant $[Ca^{2+}]_i$ elevation at 1 nM and higher concentrations, and the maximal $[Ca^{2+}]_i$ elevation (48.0 ± 3.5%, $n = 5$) was obtained at 100 nM (Figure 1b). PAR1-AP induced a significant $[Ca^{2+}]_i$ elevation at 1 μM and higher concentrations, and the maximal $[Ca^{2+}]_i$ elevation (98.0 ± 5.5%, $n = 5$) was obtained at 30 μM (Figure 1b). PAR2-AP induced a significant $[Ca^{2+}]_i$ elevation at 1 μM and higher concentrations, and the maximal $[Ca^{2+}]_i$ elevation (33.0 ± 3.5%, $n = 5$) was obtained at 30 μM (Figure 1b). The maximal $[Ca^{2+}]_i$ elevation obtained with PAR1-AP was approximately 3 fold as high as that obtained with thrombin. On the other hand, the maximal $[Ca^{2+}]_i$ elevation obtained with PAR2-AP was slightly smaller than that obtained with trypsin. The $[Ca^{2+}]_i$ elevations induced by thrombin and trypsin, but not those induced by the activating peptides, were completely abolished by pretreatment with a serine protease inhibitor, p-APMSF (data not shown). The

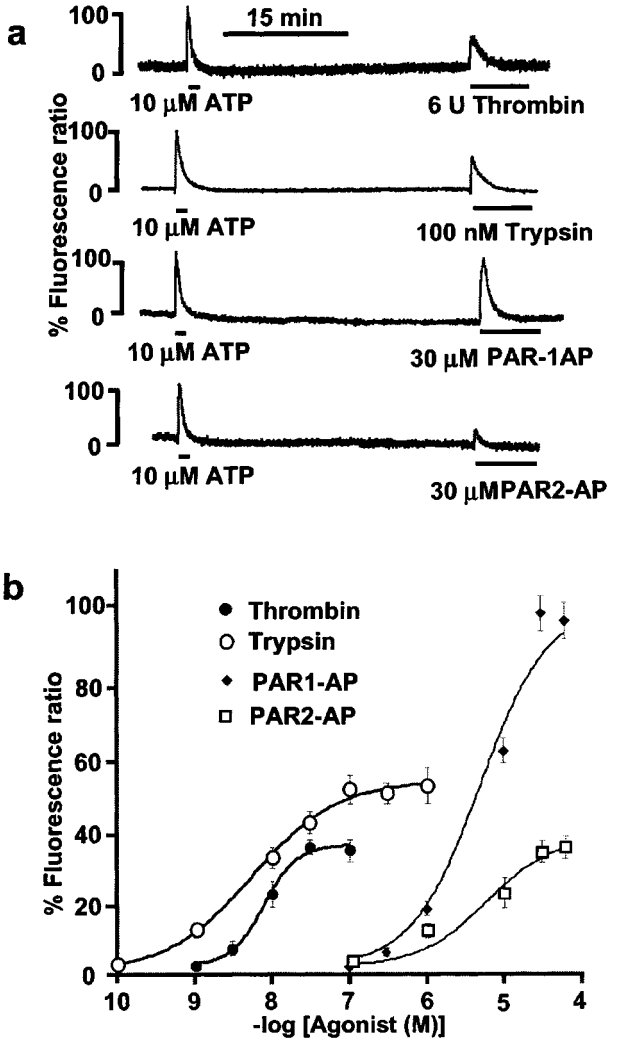

Figure 1 $[Ca^{2+}]_i$ elevations induced by thrombin, trypsin, PAR1-AP and PAR2-AP in the *in situ* endothelial cells of the porcine aortic valve. (a) Representative recordings of the changes in $[Ca^{2+}]_i$ induced by 6 units ml^{-1} thrombin, 100 nM trypsin, 30 μM PAR1-AP and 30 μM PAR2-AP in the strips of the porcine aortic valve. The reference response to 10 μM ATP was recorded at the beginning of each measurement. The levels of $[Ca^{2+}]_i$ at rest and at the peak response to 10 μM ATP were assigned to be 0% and 100%, respectively. (b) The concentration–response curves for the peak elevation of $[Ca^{2+}]_i$ induced by thrombin, trypsin, PAR1-AP and PAP2-AP. The data are the mean ± s.e.mean ($n = 5$). The concentration of thrombin was estimated based on the proteolytic activity, while assigning 1 unit ml^{-1} to be 10 nM.

concentrations required to induce a maximal response were used in the following experiments; 6 units ml^{-1} thrombin, 100 nM trypsin, 30 μM PAR1-AP and 30 μM PAR2-1AP.

Cross desensitization of thrombin and trypsin

The strips were sequentially stimulated in various combinations of thrombin and trypsin at intervals of 15 min (Figure 2). The desensitization of the response to thrombin or trypsin was evaluated by comparing the extent of $[Ca^{2+}]_i$ elevation obtained with the preceding stimulation to that obtained by the same stimulation without the preceding stimulation. Once the endothelial cells were stimulated with thrombin or

trypsin, the subsequent application of thrombin after 15 min induced no $[Ca^{2+}]_i$ elevation (Figure 2a,c). On the other hand, trypsin produced no $[Ca^{2+}]_i$ elevation after the preceding stimulation with trypsin (Figure 2b,c). However, after the preceding stimulation with thrombin, the subsequent application of trypsin produced a $[Ca^{2+}]_i$ elevation ($45.6 \pm 2.8\%$, $n=5$) similar ($P>0.05$) to that obtained without the preceding stimulation with thrombin ($48.8 \pm 3.6\%$, $n=5$) (Figure 2b,c). When the strips were stimulated with trypsin in the presence of 10 μM p-APMSF, the subsequent stimulation with thrombin and trypsin did induce a response similar to that seen without any preceding stimulation.

Trypsin inhibited the response to subsequent stimulation with thrombin in a concentration-dependent manner (Figure 2). A significant inhibition was observed at concentrations higher than 0.1 nM. Slightly lower concentrations of trypsin were required to inhibit the response to thrombin than those required to induce an elevation of $[Ca^{2+}]_i$ by activating PAR2 (Figure 1).

It is possible that the inhibition of the response to thrombin by the preceding stimulation with trypsin was due to the non-specific digestion of the membrane proteins. We therefore examined the responsiveness to the agonists for other receptors, bradykinin and substance P. The responses to these stimulations remained intact after the preceding stimulation with trypsin (Figure 3).

The response to PAR-APs after receptor cleavage

The valvular strips were sequentially stimulated in various combinations of thrombin or trypsin and PAR1-AP or PAR2-AP at 15 min intervals. Once the endothelial cells were stimulated with thrombin, the subsequent application of PAR1-AP after 15 min induced a $[Ca^{2+}]_i$ elevation ($46.0 \pm 6.5\%$, $n=5$) significantly ($P<0.05$) smaller than that

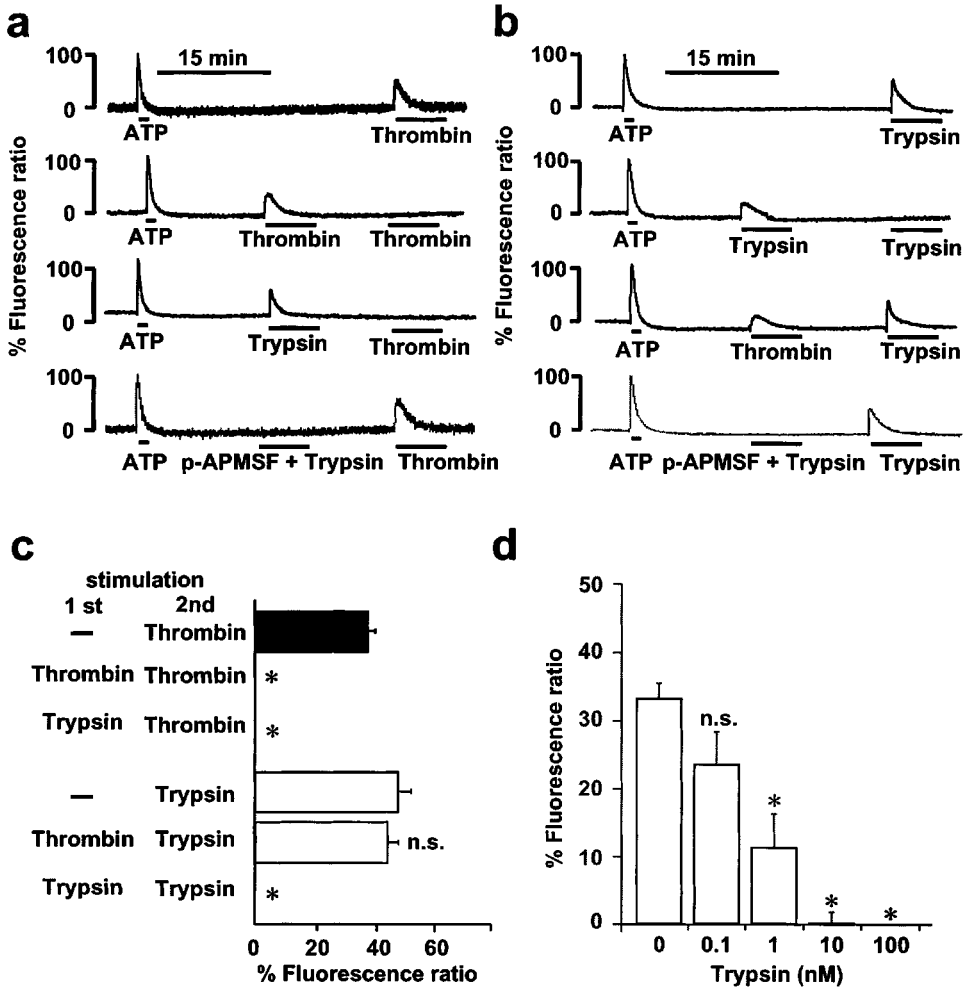

Figure 2 Cross-desensitization of the responses to thrombin and trypsin in the *in situ* endothelial cells of the porcine aortic valve. (**a**,**b**) Representative recordings showing the changes in $[Ca^{2+}]_i$ induced by 6 units ml^{-1} thrombin (a) and 100 nM trypsin (b) after the preceding stimulation with 6 units ml^{-1} thrombin and 100 nM trypsin, either in the presence or absence of 10 μM p-APMSF. The reference response to 10 μM ATP was recorded at the beginning of each measurement. The strips were sequentially stimulated at 15 min intervals in various combinations of thrombin and trypsin. The levels of $[Ca^{2+}]_i$ at rest and at peak response to 10 μM ATP were assigned to be 0% and 100% respectively. (c) Summary of five independent measurements. The data are the mean $\pm$ s.e.mean. $^*P<0.05$; n.s., not significant ($P>0.05$). (d) The concentration-dependent inhibition of the thrombin response by trypsin. The concentration of trypsin of the preceding stimulation was varied, and the response to the subsequent stimulation with thrombin was evaluated. The data are the mean $\pm$ s.e.mean. $^*P<0.05$; n.s., not significant ($P>0.05$).

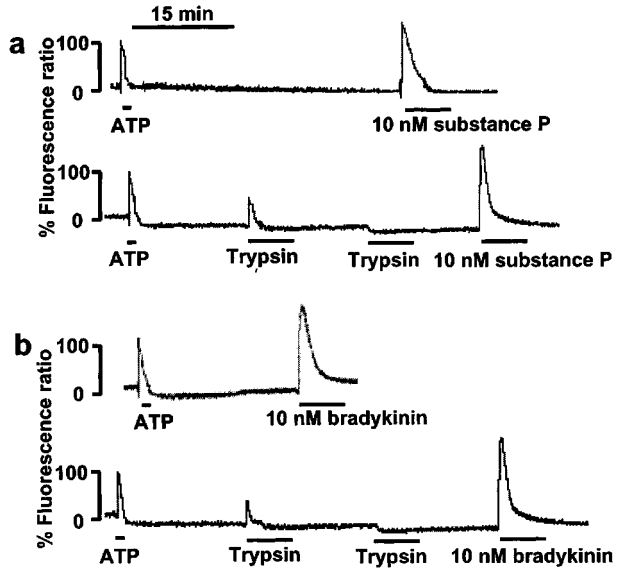

Figure 3 Responsiveness to bradykinin and substance P after the preceding stimulation with trypsin in the *in situ* endothelial cells of the porcine aortic valve. (a,b) Representative traces showing the response to 10 nM bradykinin (a) and 10 nM substance P (b) with and without the preceding stimulations with 100 nM trypsin. The levels of $[Ca^{2+}]_i$ at rest and at peak response to 10 μM ATP were assigned to be 0% and 100%, respectively. The traces are representative of three independent experiments.

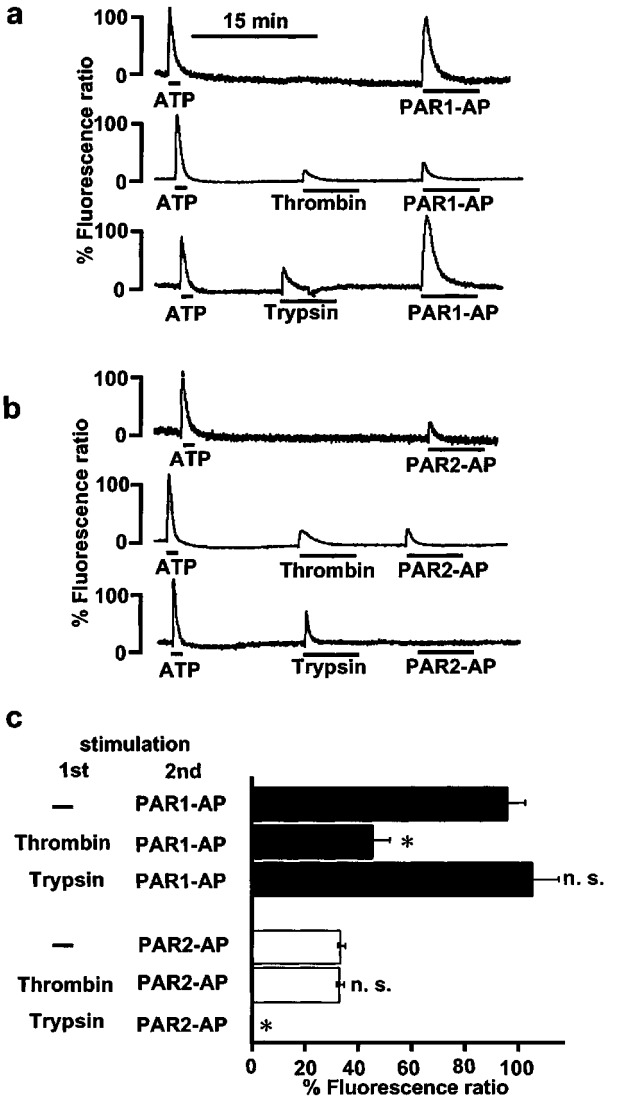

Figure 4 Response to the activating peptides, PAR1-AP and PAR2-AP, after the receptor cleavage by thrombin and trypsin in the *in situ* endothelial cells of the porcine aortic valve. (a,b) Representative traces showing the responses to 30 μM PAR1-AP (a) and 30 μM PAR2-AP (b) with and without the preceding stimulations with 6 units ml^{-1} thrombin or 100 nM trypsin. The levels of $[Ca^{2+}]_i$ at rest and at peak response to 10 μM ATP were assigned to be 0% and 100%, respectively. (c) Summary of five independent measurements. The data are the mean$\pm$s.e.mean. $^*P<0.05$, n.s., not significant ($P>0.05$).

obtained without the preceding stimulation with thrombin ($98.0\pm5.9\%$, $n=5$) (Figure 4a,c). On the other hand, the $[Ca^{2+}]_i$ elevation ($107.0\pm9.8\%$, $n=5$) obtained with PAR1-AP after the preceding stimulation with trypsin did not significantly ($P>0.05$) differ from that obtained without the preceding stimulation (Figure 4a). Similarly, after the preceding stimulation with thrombin, PAR2-AP induced the $[Ca^{2+}]_i$ elevation ($34.0\pm1.2\%$, $n=5$) similar to that obtained without the preceding stimulation ($33.0\pm1.3\%$, $n=5$) (Figure 4b,c). However, once stimulated with trypsin, the subsequent application of PAR2-AP produced no $[Ca^{2+}]_i$ elevation (Figure 4b,c). Thrombin and trypsin thus specifically desensitized the responsiveness to PAR1-AP and PAR2-AP, respectively. The most critical observation was that the responsiveness to PAR1-AP remained intact after the preceding stimulation with trypsin, in contrast to the observation that the responsiveness to thrombin was completely abolished in the same situation (Figure 2).

The responsiveness to proteolytic activation and non-proteolytic activation of PARs after the preceding stimulation with activating peptides

The valvular strips were stimulated with thrombin, trypsin, PAR1-AP and PAR2-AP 15 min after the preceding stimulation with PAR1-AP or PAR2-AP (Figure 5). The preceding stimulation with PAR1-AP but not PAR2-AP attenuated the response to thrombin (Figure 5a). The $[Ca^{2+}]_i$ elevation obtained with 6 units ml^{-1} thrombin after the stimulations with PAR1-AP ($6.0\pm0.7\%$, $n=5$) was significantly ($P<0.05$) smaller than that obtained without any preceding stimulation ($35.0\pm2.8\%$, $n=5$), while that obtained after the stimulation with PAR2-AP ($32.5\pm1.7\%$, $n=5$) did not differ significantly ($P>0.05$) from the control value (Figure 5e). On the other hand, the response to trypsin was attenuated by the preceding stimulations with both PAR1-AP and PAR2-AP (Figure 5b). The $[Ca^{2+}]_i$ elevations obtained with 100 nM trypsin after the stimulations with PAR1-AP ($9.0\pm1.0\%$, $n=5$) and PAR2-AP ($9.6\pm1.8\%$, $n=5$) were significantly ($P<0.05$) smaller than those obtained without any preceding stimulation ($45.8\pm3.6\%$, $n=5$) (Figure 5e). The response to PAR1-AP was attenuated by the preceding stimulation with PAR1-AP but not PAR2-AP (Figure 5c). The $[Ca^{2+}]_i$ elevations obtained with 30 μM PAR1-AP after the stimulations with PAR1-AP ($8.7\pm1.8\%$, $n=5$) was significantly ($P<0.05$) smaller than that obtained

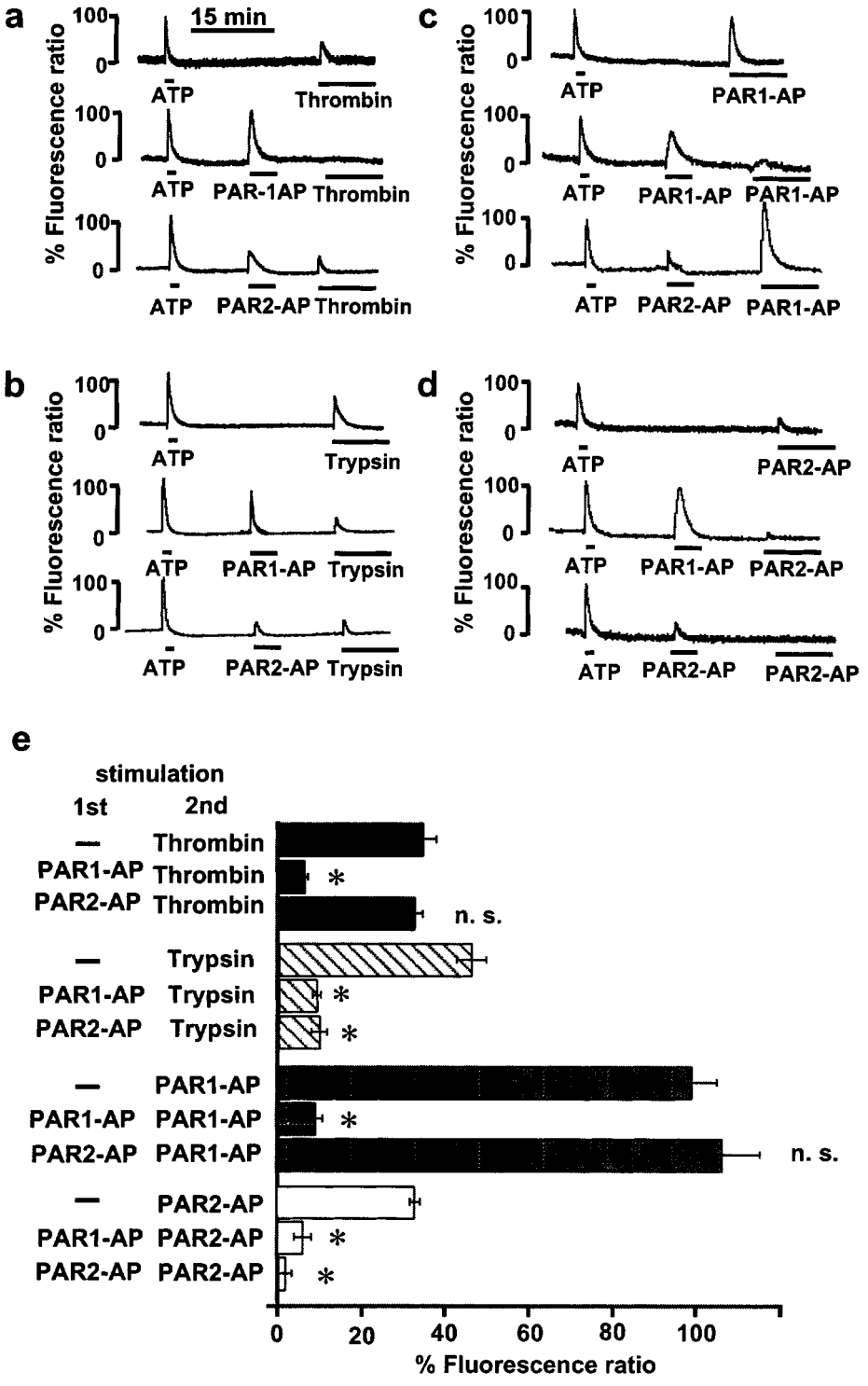

Figure 5 Responses to proteolytic activation and non-proteolytic activation after the preceding stimulation with activating peptides in the *in situ* endothelial cells of the porcine aortic valve. (a–d) Representative traces showing the responses to 6 units ml^{-1} thrombin (a), 100 nM trypsin (b), 30 μM PAR1-AP (c) and 30 μM PAR2-AP (d) after the preceding stimulations with PAR1-AP or PAR2-AP. The levels of $[Ca^{2+}]_i$ at rest and at peak response to 10 μM ATP were assigned to be 0% and 100%, respectively. (e) Summary of five independent measurements. The data are the mean ± s.e.mean. *$P<0.05$, n.s., not significant ($P>0.05$).

without the preceding stimulation (96.8 ± 5.9%, $n=5$), while that obtained after the stimulation with PAR2-AP (106.0 ± 8.9%, $n=5$) did not significantly ($P>0.05$) differ from that obtained without pretreatment (Figure 5e). On the other hand, the response to PAR2-AP was attenuated by the preceding stimulation with both PAR1-AP and PAR2-AP (Figure 5d). The $[Ca^{2+}]_i$ elevations obtained with 30 μM PAR2-AP after the stimulations with PAR1-AP (6.0 ± 1.9%, $n=5$) and PAR2-AP (1.6 ± 1.9%, $n=5$) were significantly ($P<0.05$) smaller than those obtained without any preceding

stimulation (33.0±1.3%, $n=5$) (Figure 5e). Collectively, PAR2-AP specifically attenuated the response to trypsin and PAR2-AP, while PAR1-AP attenuated not only the responses to thrombin and PAR1-AP but also the responses to trypsin and PAR2-AP. There was a possibility that PAR1-AP activated not only PAR1 but also PAR2 in the endothelial cells as reported in *Xenopus* oocyte cells expressing PAR1 and PAR2 (Blackhart *et al*., 1996; Lerner *et al*., 1996).

Effects of a high affinity PAR1-AP (haPAR1-AP) on the response to thrombin and trypsin in HUVEC

To determine whether the PAR1-AP-induced attenuation of the responses to trypsin and PAR2-AP in the porcine aortic valve was due to the activation of PAR2 by PAR1-AP or the heterologous desensitization following the activation of PAR1 by PAR1-AP, we examined the effects of haPAR1-AP, an agonist peptide with a higher affinity and specificity towards PAR1 than PAR1-AP (Ahn *et al*., 1997), on the response to trypsin and *vice versa*. However, haPAR1-AP induced a very slight elevation of $Ca^{2+}{}_i$ in the porcine aortic valve (10% of the response to 10 μM ATP), probably due to the difference in the amino acid sequences of the tethered ligand region of PAR1 between human (Macfarlane *et al*., 2001) and porcine (T. Nakayama, K. Hirano, J. Nishimura, & H. Kanaide, unpublished observation). We therefore examined the effect of haPAR1-AP in HUVECs. The haPAR1-AP induced a transient $[Ca^{2+}]_i$ elevation in a concentration-dependent manner, while inducing the maximal response (230±21%, $n=5$) at 10 μM. Thrombin and trypsin induced a rapid and transient increase in $[Ca^{2+}]_i$ in the HUVECs (Figure 6a), and the maximal $[Ca^{2+}]_i$ elevation was obtained with 6 units ml^{-1} thrombin (288.5±25%, $n=5$) and 100 nM trypsin (240.5±22%, $n=5$) in HUVECs.

As we observed in the porcine aortic valve, PAR1-AP attenuated the response to both thrombin and trypsin in HUVEC (data not shown). Both $[Ca^{2+}]_i$ elevations induced by 6 units ml^{-1} thrombin and 100 nM trypsin was significantly ($P<0.05$) attenuated by the preceding stimulation with 10 μM PAR1-AP (110.7±38% and 120.5±45%, respectively, $n=5$). We also observed that the preceding stimulation with trypsin abolished the response to thrombin and that the preceding stimulation with thrombin had no effect on the subsequent response to trypsin in HUVEC as observed in the porcine aortic valves (data not shown). On the contrary, the preceding stimulation with 10 μM haPAR1-AP attenuated the subsequent response to thrombin, while it had no significant ($P>0.05$) effect on the subsequent response to 100 nM trypsin (Figure 6a,c). On the other hand, the preceding stimulation with 6 units ml^{-1} thrombin significantly ($P<0.05$) attenuated the subsequent response to haPAR1-AP (104±14% vs 230±21% with and without the preceding stimulation by thrombin, respectively, $n=5$), while the preceding stimulation with trypsin had no significant ($P>0.05$) effect on it (235±23%, with the preceding stimulation by trypsin, $n=5$).

Inhibition of the thrombin-induced endothelium-dependent relaxation by the preceding stimulation with trypsin

To examine the functional significance of the inhibition of the thrombin-induced $[Ca^{2+}]_i$ elevation by the preceding stimulation with trypsin in the endothelial cells, we investigated the effect of trypsin on the thrombin-induced endothelium-dependent relaxation, using the strips of the porcine coronary artery. We previously reported that both thrombin and trypsin induced an endothelium-dependent relaxation in the porcine coronary artery and had no direct effect on the smooth muscle contraction (Mizuno *et al*., 1998; Nakayama *et al*., 2001). As shown in Figure 7, both thrombin and

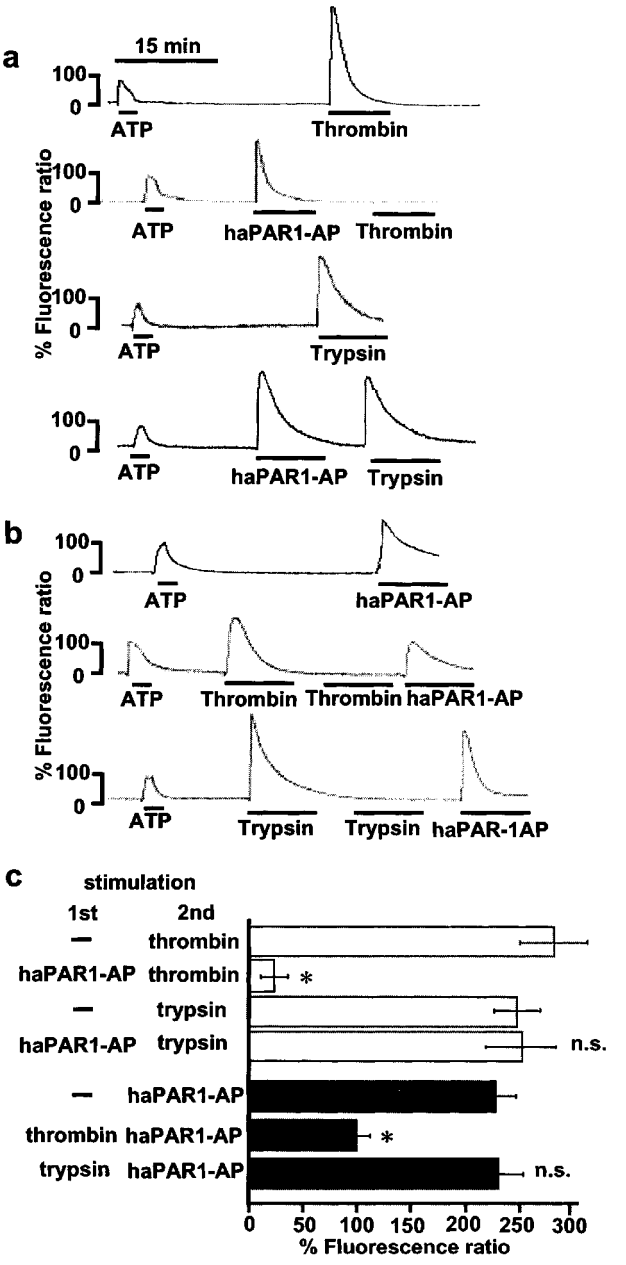

Figure 6 Effects of high affinity PAR1-AP on the response to thrombin and trypsin in HUVEC. (a,b) Representative traces showing the responses to 6 units ml^{-1} thrombin and 100 nM trypsin and after preceding stimulations with 10 μM haPAR1-AP (a), and 10 μM haPAR1-AP 15 min after preceding stimulations with 6 units ml^{-1} thrombin or 100 nM trypsin (b). The levels of $[Ca^{2+}]_i$ at rest and at peak response to 10 μM ATP were assigned values of 0% and 100%, respectively. (c) Summary of five independent measurements. The data are the mean±s.e.mean. *$P<0.05$, n.s., not significant ($P>0.05$).

trypsin induced a relaxation during the sustained contraction induced by 100 nM U46619, a thromboxane A2 analogue. However, thrombin did not induce any effect on the U46619-induced contraction after the preceding stimulation with trypsin (Figure 7b), while bradykinin did induce a relaxation similar to that seen without any preceding stimulation (data not shown).

Discussion

It is well known that the activity of thrombin receptor is regulated by both proteolysis and phosphorylation of the receptor, which cause the internalization of the receptor and the inhibition of intracellular signal transduction (Dery *et al*., 1998; Macfarlane *et al*., 2001; Mizuno *et al*., 2000). We herein propose a new mode for the regulation of the thrombin receptor activity by proteolytic cleavage in the vascular endothelial cells. We suggest that cleavage at the site(s) C-terminal to the thrombin cleavage site makes thrombin receptor unresponsive to thrombin, while it leaves the responsiveness to the activating peptides intact. The most critical observation which supports this conclusion is that the preceding stimulation with trypsin abolished the subsequent response to thrombin, however, the response to PAR1-AP remained intact. This inhibition of the response to thrombin required the proteolytic activity of trypsin but not the preceding elevation of $[Ca^{2+}]_i$ induced by the PAR2 activation. This notion is supported by the observation that the response to thrombin was inhibited by trypsin at the concentrations slightly lower than those required to induce elevation of $[Ca^{2+}]_i$ through activation of PAR2. The functional significance of the proteolytic inactivation of PAR1 in endothelial cells was supported by the observation that the preceding stimulation with trypsin inhibited the endothelium-dependent relaxation induced by the subsequent application of thrombin.

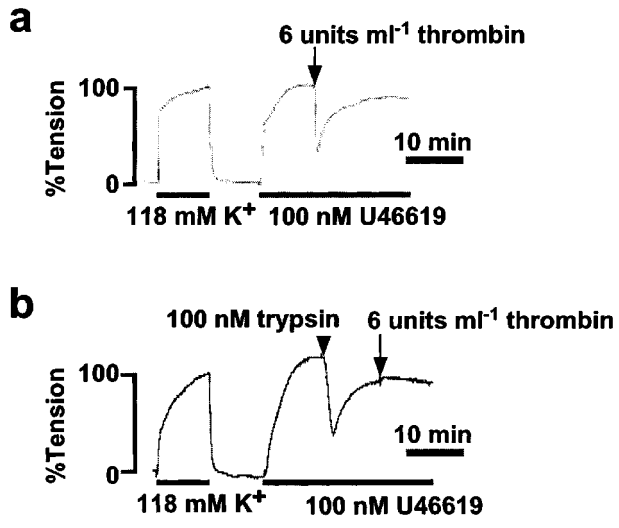

Figure 7 Effect of the preceding stimulation with trypsin on the thrombin-induced relaxation in the strips of the porcine coronary artery. Representative traces of change in tension induced by 6 units ml^{-1} thrombin without (a) and with (b) the preceding stimulation with 100 nM trypsin during the sustained contraction induced by 100 nM U46619. The tension obtained at rest and during the sustained contraction induced by 118 mM K^+ was assigned to be 0% and 100%, respectively. Shown are the representative traces of three independent experiments.

It is also known that the regulation of thrombin receptor activity varies depending on the types of cells (Dery *et al*., 1998; Macfarlane *et al*., 2001). For example, thrombin receptor is rapidly cleared from the cell surface after stimulation with thrombin, and an intact receptor reappeared within 30 min and recovered to 90% of the prestimulation level within 5 h in the HUVEC (Woolkalis *et al*., 1995). On the other hand, no intact receptor recovers by 90 min after the stimulation in the *in situ* endothelial cells of the porcine aortic valve and human megakaryoblast HEL cells (Hoxie *et al*., 1993; Mizuno *et al*., 2000; Woolkalis *et al*., 1995). Regarding the trypsin-induced inactivation of PAR1, this phenomenon does not seem to be specific to the *in situ* endothelial cells of the porcine aortic valve, as we observed the similar inactivation of PAR1 by trypsin in HUVEC (the present study) and the pregnant rat myometrium (Shintani *et al*., 2001). We thus suggest that the proteolytic inactivation of PAR1 by trypsin is a general mechanism for the regulation of the thrombin receptor activity.

PAR1, PAR3 and PAR4 were reported to serve as thrombin receptor, and PAR1, PAR2 and PAR4 were reported to serve as trypsin receptor (Dery *et al*., 1998; Macfarlane *et al*., 2001). In the present study, the preceding stimulation with thrombin did not have any effect on the subsequent response to trypsin, but it did completely abolish the response to thrombin itself. These observations exclude the involvement of PAR1 and PAR4 in the response to trypsin in the endothelial cells of porcine aortic valve. Moreover, the response to trypsin showed a similar sensitivity toward the preceding stimulation with thrombin, trypsin, PAR1-AP and PAR2-AP to that observed with the response to PAR2-AP. It is thus conceivable that the response to trypsin is mostly mediated by PAR2 in the endothelial cells of porcine aortic valves. On the other hand, PAR1 is considered to be the major receptor for thrombin, because the preceding stimulation with PAR1-AP substantially inhibited the subsequent response to thrombin as well as the response to PAR1-AP. However, the possible involvement of PAR3 and/or PAR4, in addition to PAR1, in the trypsin-induced inhibition of the response to thrombin remains to be elucidated.

Trypsin has been reported to cleave PAR1 at the thrombin site, and it is thus considered to activate PAR1 (Dery *et al*., 1998; Macfarlane *et al*., 2001). In fact, trypsin was shown to cleave the recombinant extracellular domain of PAR1 at the thrombin site within 5 min in the *in vitro* experiment (Loew *et al*., 2000). However, evidence for the cleavage of PAR1 at the thrombin site and the resultant activation of PAR1 by trypsin *in vivo* still appears to be insufficient. In the present study, we proposed that trypsin cleaves PAR1 but does not activate it, based on the following observations: (1) The preceding stimulation with trypsin completely abolished the response to thrombin thus suggesting that PAR1 was cleaved by trypsin. (2) However, the response to trypsin after the preceding stimulation with thrombin did not differ from that obtained without the preceding stimulation. If the activation of PAR1 were involved in the response to trypsin, the response to trypsin after stimulation with thrombin should be

smaller than the control response. It is thus suggested that trypsin cleaved thrombin receptor, but its cleavage was unproductive and did not lead to any activation of PAR1.

Regarding the desensitization of the response to thrombin after the preceding stimulation with trypsin, there is a possibility that the desensitization resulted from the receptor internalization. PAR1 activated by either thrombin or activating peptides was shown to be rapidly phosphorylated and internalized (Hammes *et al*., 1999). In fact, 60% of the receptors are internalized within 10 min after stimulation with thrombin in the HUVEC (Woolkalis *et al*., 1995). Therefore, we examined the responsiveness to the activating peptides, non-proteolytic stimulation, after the preceding stimulation with trypsin. The most important observation is that the preceding stimulation with trypsin had no effect on the response to PAR1-AP. In the same situation, thrombin did not induce any response. This observation suggests that the receptor responsive to PAR1-AP but not to thrombin fully remained on the cell surface. Namely, the desensitization of PAR1 by trypsin is not considered to be due to the receptor internalization but due to the cleavage of the receptor. Furthermore, it is suggested that trypsin does not disintegrate PAR1 into pieces while, in addition, the trypsin cleavage of PAR1 is not considered to be due to non-specific digestion of the membrane proteins, because the responsiveness to bradykinin and substance P remained intact. Trypsin is rather suggested to cleave PAR1 at limited site(s). Since it was suggested that PAR1 was not activated during trypsin stimulation, the thrombin site is not the major site for trypsin. We thus propose that trypsin cleaved PAR1 somewhere closer to the C-terminus than the thrombin site.

A mass-spectrosocopic analysis of the *in vitro* trypsin-cleavage products of the extracellular domain of PAR1 has recently shown that the residues 41–42 (thrombin site), 70–71 and 82–83 were cleaved within 5 min, while the residues 47–48 were cleaved between 5 min to 3 h (Loew *et al*., 2000). The early cleavage sites other than residues 41–42 are thus the candidate sites for the trypsin-induced unproductive cleavage and inactivation of PAR1 in the endothelial cells. However, the precise location of the sites responsible for the unproductive cleavage of PAR1 *in vivo* remains to be determined. It has been further suggested that the cleavage of PAR1 at any sites closer to the C-terminus than the thrombin site can make PAR1 unresponsive to thrombin. Loew *et al*. (2000) reported that other proteases cleaved the extracellular domain of PAR1 at such sites within 5 min. These proteases and their cleavage sites include plasmin (residues 70–71 and 76–77), cathepsin G (residues 55–56 and 69–70), elastase (residues 72–73 and 86–87), proteinase 3 (residues 48–49, 72–73 and 92–93) and calpain I (residues 76–77). It is thus possible that these proteases can also cause unproductive cleavage of PAR1, and make PAR1 unresponsive to thrombin as trypsin did. However, this possibility still remains to be proven.

The preceding stimulation with PAR1-AP attenuated the subsequent responses to trypsin and PAR2-AP similarly to those seen with the preceding stimulation with PAR2-AP. These observations suggest that PAR1-AP also desensitizes PAR2 as PAR2-AP does. This desensitization is not due to the heterologous desensitization caused by the activation of PAR1, because haPAR1-AP had no effect on the subsequent response to trypsin and PAR2-AP in HUVEC. Since the porcine aortic valve responded poorly to haPAR1-AP, we used HUVEC. However, we confirmed that PAR1-AP attenuated the subsequent response to trypsin and PAR2-AP in the HUVEC as well as in porcine aortic valve. Therefore, the desensitization of PAR2 induced by PAR1-AP was due to the activation of PAR2 by PAR1-AP. Namely, PAR1-AP is suggested to activate and subsequently desensitize both PAR1 and PAR2. PAR1-AP was indeed reported to serve as agonist for both PAR1 and PAR2 (Blackhart *et al*., 1996; Lerner *et al*., 1996). In the present study, the maximal $[Ca^{2+}]_i$ elevation obtained with PAR1-AP was approximately 3 fold as high as that obtained with thrombin. This observation is also consistent with double activation of PAR1 and PAR2 by PAR1-AP. On the other hand, the preceding stimulation with trypsin and PAR2-AP had no effect on the subsequent response to PAR1-AP, thus suggesting that the activation of PAR2 does not induce heterologous desensitization of PAR1. However, the activation of PAR2 by either trypsin or PAR2-AP is suggested to induce the desensitization of PAR2 itself.

Although the precise site of cleavage by trypsin remains to be determined, residues 70–71 and 82–83 are the candidate sites for the trypsin-induced unproductive cleavage of PAR1. These residues are suggested to be cleaved by trypsin much faster than the residues 41–42, thus the inactivation of PAR1 due to the cleavage at the residues 70–71 and/or 82–83 dominated the activation of PAR1 due to the cleavage at the residues 41–42. Therefore, PAR1 is thought to have a negligible contribution to the trypsin-induced $[Ca^{2+}]_i$ elevation. Since trypsin is not activated in vascular tissue, the trypsin-induced cleavage of PAR1 may not play a physiological or pathophysiological role in the regulation of thrombin receptor activity. However, any proteases which cleave PAR1 at the sites C-terminal to the thrombin site can inactivate PAR1. Plasmin is one of the proteases which were shown to cleave PAR1 at such sites. Plasmin is activated in the fibrin clot, an end product of the activation of thrombin, and lyses the clot (Loew *et al*., 2000). It is thus an intriguing possibility that plasmin may also antagonize thrombin at the receptor level by inactivating the PAR1 responsiveness to thrombin. However, such a possibility remains to be examined.

In conclusion, we herein propose a new mode for regulating the PAR1 activity by cleavage at the sites closer to C-terminus than the thrombin site. In the present study, we demonstrated trypsin to be a representative protease which induces the unproductive cleavage and inhibition of the PAR1 activity.

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