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Proteolysis and phosphorylation-mediated regulation of thrombin receptor activity in in situ endothelial cells

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Abstract

The regulatory mechanism of thrombin receptor responsiveness in in situ endothelial cells was investigated by evaluating elevations of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura-2-loaded porcine aortic valvular strips. Once stimulated with thrombin, endothelial cells did not respond to the second thrombin stimulation within 90 min. However, applying thrombin receptor activating peptide (TRAP7) at 15 min after the thrombin stimulation caused $[Ca^{2+}]_i$ elevation, which was smaller than that seen without preceding stimulation. After 90 min, response to TRAP7 recovered to the control level. When stimulated with TRAP7, the subsequent responses to thrombin and TRAP7 were attenuated at 15 min, and fully recovered after 90 min. Staurosporine partially prevented the TRAP7-induced desensitization. The recovery of responsiveness was inhibited completely by calyculin-A and partially by okadaic acid. Proteolysis and phosphorylation thus play an important role in thrombin receptor desensitization in in situ endothelial cells. Both cleaved and uncleaved receptors were desensitized through phosphorylation in part by staurosporine-sensitive kinase, and restored the responsiveness through dephosphorylation by type 1 phosphatase. The mechanism of regulation of thrombin receptor activity in in situ endothelial cells differed from those reported in cultured endothelial cells. We suggest that the cell-specific regulatory mechanism may be altered by culture conditions.

Keywords: Endothelial cell; Thrombin receptor; Protein phosphatase

1. Introduction

Thrombin, a serine protease, induces diverse biological responses through its actions on specific receptors in a wide variety of cells, including smooth muscle cells and endothelial cells (Shuman, 1986). Molecular cloning of a functional thrombin receptor revealed activation of thrombin receptor to be unique in that thrombin cleaves its receptor's amino-terminal exodomain, and unmasks a new amino terminus, which then acts as a tethered ligand and activates subsequent signal transduction (Vu et al., 1991). Synthetic peptides corresponding to the new amino-terminal region of thrombin-cleaved receptor mimic many, if not all, biological action of thrombin (Tiruppathi et al., 1992; Vouret-Craviari et al., 1992; Derian et al., 1995; Marsen et al., 1995). These peptides are thus referred to as thrombin receptor-activating peptides (TRAPs). Since the activation of thrombin receptor by TRAPs is independent of proteolysis, TRAPs are a unique tool for investigating the regulation of thrombin receptor activity.

Thrombin receptor has been shown to be rapidly desensitized following the stimulation (Grand et al., 1996). Since not only thrombin but also TRAPs induce desensitization, both cleavage-dependent and independent mechanisms are considered to contribute to the desensitization (Brass, 1992). Thrombin receptor has been shown to be rapidly internalized and disappear from the cell surface (Hoxie et al., 1993; Shapiro et al., 1996), and also to be rapidly phosphorylated (Vouret-Craviari et al., 1995). Phosphorylation inhibits interaction of thrombin receptor with G protein and also promotes receptor internalization, both of which can cause desensitization (Grand et al., 1996; Shapiro et al., 1996). The kinase(s) responsible for desensitization remain to be identified. On the other hand, when cells are kept unstimulated for a certain time after the first stimulation, they recover their responsiveness to

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thrombin (Brass, 1992; Grand et al., 1996). It was suggested that synthesis of a new receptor molecule or supply of an intact receptor from the intracellular pool contributes to the recovery of such intact receptors (Brass, 1992). The recycling of the internalized receptor was shown to contribute to the reappearance of the cleaved receptor on cell surface (Hoxie et al., 1993). Dephosphorylation may also restore the ability of receptor to interact with G protein. However, the precise mechanisms of dephosphorylation and responsible phosphatase(s) remain to be clarified.

One important aspect regarding thrombin receptor desensitization is that the relative contribution of mechanisms of desensitization and its recovery tends to vary with the type of cell (Brass, 1992; Woolkalis et al., 1995). In human umbilical vein endothelial cells, an intact receptor became detectable on cell surface within 30 min (Woolkalis et al., 1995). On the other hand, human magakaryoblastic erythroleukemia (HEL) and human magakaryoblastic tumor cell line (CHRF-288) recovered their responsiveness to TRAPs but not to thrombin within several hours after desensitization by thrombin (Brass, 1992). We hypothesize that the cell-specific mechanism may be involved in such cell-specific manner of desensitization and recovery. The mechanism of regulation regarding the thrombin receptor activity in in situ endothelial cells thus remains to be elucidated.

In the present study, we investigated the mechanisms of regulation of thrombin receptor activity in in situ endothelial cells, by measuring the changes in $[Ca^{2+}]_i$ in response to thrombin and TRAPs in fura-2-loaded strips of porcine aortic valve (Kanaide, 1998). We found that in situ endothelial cells never recovered their responsiveness to thrombin within 90 min, in contrast to the findings on cultured endothelial cells (Woolkalis et al., 1995). Furthermore, the effects of several kinase inhibitors on the desensitization process were examined to characterize kinase(s) responsible for the desensitization, and the effects of phosphatase inhibitors were also examined to identify the phosphatase responsible for the recovery.

2. Materials and methods

2.1. Materials

Thrombin (bovine plasma, specific activity 1880 NIH units/mg), probenecid, U46619 (a thromboxane A_2 analogue; 9,11-Dideoxy-11α, 9α-epoxymethano-prostaglandin F2α), calyculin-A, okadaic acid, wortmannin, H-7 (1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine), ML-9 (1-(5-Chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine) and staurosporine were purchased from Sigma (St. Louis, MO, USA). The concentration of thrombin was basically expressed as NIH units/ml in this study. When necessary, the molar concentration of thrombin was estimated from units according to the conversion rate given by manufacturer (10 nM ≈ 1 U/ml). Sodium salt of ATP was obtained from Boehringer Mannheim (Germany). Y27632 ((*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride, monohydrate) was kindly donated by Yoshitomi Pharmaceutical (Osaka, Japan). Fura-2-acetoxymethyl ester (fura-2/AM) was obtained from Dojindo (Kumamoto, Japan). TRAPs [TRAP7 (SFLLRNP) and TRAP14 (SFLLRNPNDKYEPF)] were purchased from Bachem (Bubendorf, Switzerland). Fetal bovine serum was obtained from Sanko Junyaku (Tokyo, Japan). Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). The normal physiological salt solution (PSS) was composed of 123 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.25 $CaCl_2$ and 11.5 D-glucose (in mM) and aerated with a mixture of 5% CO_2 and 95% O_2, with resulting pH to be 7.4.

2.2. Tissue preparations

For the measurement of $[Ca^{2+}]_i$ of in situ endothelial cells, strips of porcine aortic valve were prepared as previously described (Kuroiwa et al., 1995; Mizuno et al., 1998). Valvular strips for fura-2 fluorometry were approximately 3 mm × 5 mm. For the simultaneous measurement of $[Ca^{2+}]_i$ and tension of smooth muscle, vascular strips with an endothelium were prepared from the proximal part of the left circumflex coronary arteries of porcine heart as previously described (Kuroiwa et al., 1995; Mizuno et al., 1998). Vascular strips were approximately 1 mm × 5 mm × 0.1 mm (thickness).

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

Strips of aortic valve were loaded with fura-2 by incubating in DMEM containing 50 μM fura-2/AM, 5% fetal bovine serum and 1 mM probenecid for 90 min at 37°C (Kuroiwa et al., 1995; Mizuno et al., 1998). Probenecid was added to prevent any leakage of fluorescent dye (Di Virgilio et al., 1989). After loading with fura-2, the strips were washed at least five times and equilibrated in normal PSS for 1 h at room temperature. The strips were mounted vertically in a quartz organ bath filled with normal PSS. Changes in $[Ca^{2+}]_i$ in endothelial cells of the valvular strips were monitored, using a front-surface fluorometer CAM-OF-3 designed in collaboration with the Japan Spectroscopic (Tokyo, Japan), as described (Kuroiwa et al., 1995; Mizuno et al., 1998). Fluorometry was performed at 25°C to prevent the leakage of fluorescent dye (Kuroiwa et al., 1995; Mizuno et al., 1998). The fluorescence intensities (500 nm) at 340 and 380 nm excitation and their ratio were continuously monitored. The control response was obtained by exposing the strip to 10 μM ATP for 1 min at the beginning of the experimental protocol (Kuroiwa et al., 1995; Mizuno et al., 1998). The fluorescence ratio was

expressed as a percentage, assigning the values obtained in normal PSS and with 10 μM ATP to be 0% and 100%, respectively. The absolute values of $[Ca^{2+}]_i$ were estimated in separate measurements as previously described (Kanaide, 1998), in which the $[Ca^{2+}]_i$ levels of valvular endothelial cells at 0% and 100% were determined to be 70.1 ± 10 nM and 190 ± 30 nM, respectively ($n = 5$).

2.4. Simultaneous measurement of $[Ca^{2+}]_i$ and tension of smooth muscle in coronary arterial strips with endothelial cells

The vascular strips with an endothelium were loaded with fura-2 by incubating in DMEM containing 25 μM fura-2/AM and 5% fetal bovine serum for 3 h at 37°C (Kuroiwa et al., 1995; Mizuno et al., 1998). The fura-2 loaded strips were equilibrated in normal PSS for 1 h at room temperature. 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. Strips were placed with the adventitial side of the strip facing to the fiber optics for fluorometry. The resting tension was adjusted to 200 mg in normal PSS. The changes in $[Ca^{2+}]_i$ in the vascular strips were monitored, using a front-surface fluorometer CAM-OF-3. Fluorescence ratio was continuously monitored as an indicator of $[Ca^{2+}]_i$. The response of each strip to 118 mM K^+ depolarization was recorded as a control response before starting the experimental protocols. The fluorescence ratio and tension development were expressed as a percentage, assigning the values in normal PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively. The $[Ca^{2+}]_i$ levels at rest (0%) and during 118 mM K^+ depolarization (100%) were 110 ± 5.0 nM and 710 ± 5.0 nM, respectively ($n = 10$) (Hirano et al., 1990). The fura-2 fluorescence of the vascular strip with an endothelium originated exclusively from the smooth muscle cells during measurement at 37°C, while the fluorescence of endothelial cells was, if any, only negligible (Kuroiwa et al., 1995).

2.5. Data analysis

The data are the mean ± S.E.M. of the number of experiments as indicated. All values were statistically analyzed by the unpaired Student's *t*-test and an analysis of variance (ANOVA). A P value of less than 0.05 was considered to be statistically significant. The concentrations for a half-maximal response (EC_{50} or IC_{50}) were determined by fitting the concentration–response curves fitted to a four-parameter logistic model (De Lean et al., 1978). 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, USA).

3. Results

3.1. Effects of thrombin, TRAP7 and TRAP14 on $[Ca^{2+}]_i$ in in situ endothelial cells

In aortic valvular strips, thrombin induced a rapid and transient increase in $[Ca^{2+}]_i$ (Fig. 1A), in a concentration-dependent manner within the range between 0.1 and

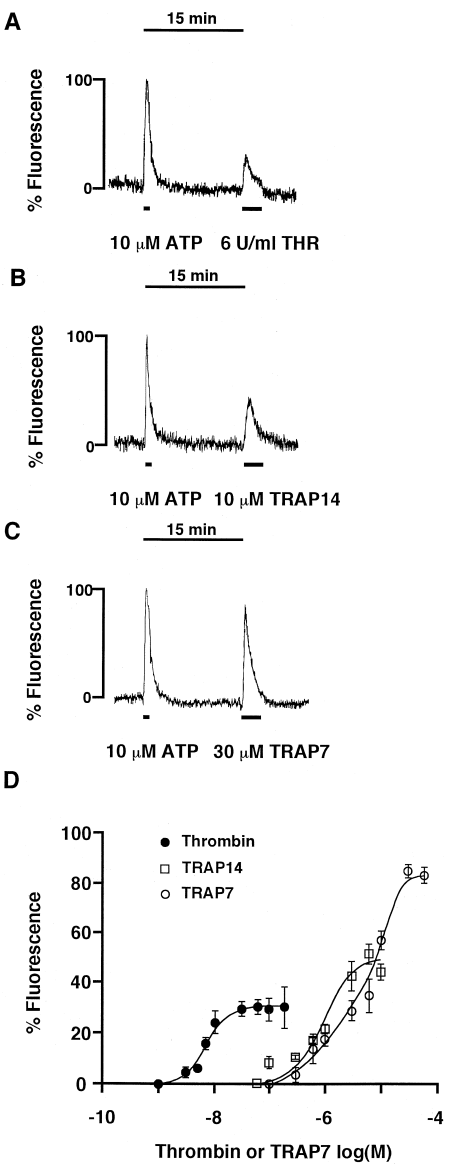


Fig. 1. The changes in cytosolic Ca^{2+} concentrations induced by thrombin, TRAP14 and TRAP7 in in situ endothelial cells of the porcine aortic valvular strips. (A–C) Representative recording of changes in $[Ca^{2+}]_i$ induced by 10 μM ATP and 6 U/ml thrombin (A), 10 μM TRAP14 (B) and 30 μM TRAP7 (C). (D) The concentration–response curve for the $[Ca^{2+}]_i$ transients induced by thrombin (●), TRAP14 (□) and TRAP7 (○) in in situ endothelial cells. The data are the mean ± S.E.M. ($n = 3$–6). The fluorescence ratio was expressed as a percentage, assigning the levels obtained in normal PSS and at the maximum elevation induced by 10 μM ATP to be 0% and 100%, respectively.

10 U/ml (Fig. 1D). The maximum response (30.1 ± 2.9%, $n = 6$) was obtained with 6 U/ml thrombin in normal PSS (Fig. 1D). EC_{50} of thrombin (a concentration required to induce half-maximal response) was 0.70 ± 0.04 U/ml ($n = 4$–9). Similarly, TRAP14 (Fig. 1B) and TRAP7 (Fig. 1C) induced a rapid and transient increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 1D). The maximum response was obtained with 6 μM TRAP14 (52.0 ± 3.9%, $n = 5$) and 30 μM TRAP7 (85.1 ± 2.6%, $n = 4$). EC_{50} of TRAP14 and TRAP7 were 1.1 ± 0.4 μM ($n = 3$–8) and 9.4 ± 0.5 μM ($n = 3$–6), respectively. The order of potency (the reciprocal of EC_{50}) to induce $[Ca^{2+}]_i$ elevation was thrombin ≫ TRAP14 > TRAP7. The order of maximal $[Ca^{2+}]_i$ elevation was TRAP7 > TRAP14 > thrombin.

3.2. Thrombin-, TRAP7- and TRAP14-induced endothelium-dependent relaxation in the porcine coronary arterial strips

U46619 induced sustained contractions in the porcine coronary arterial strips with an endothelium (Fig. 2A). Upon exposure to 100 nM U46619, the $[Ca^{2+}]_i$ and ten-

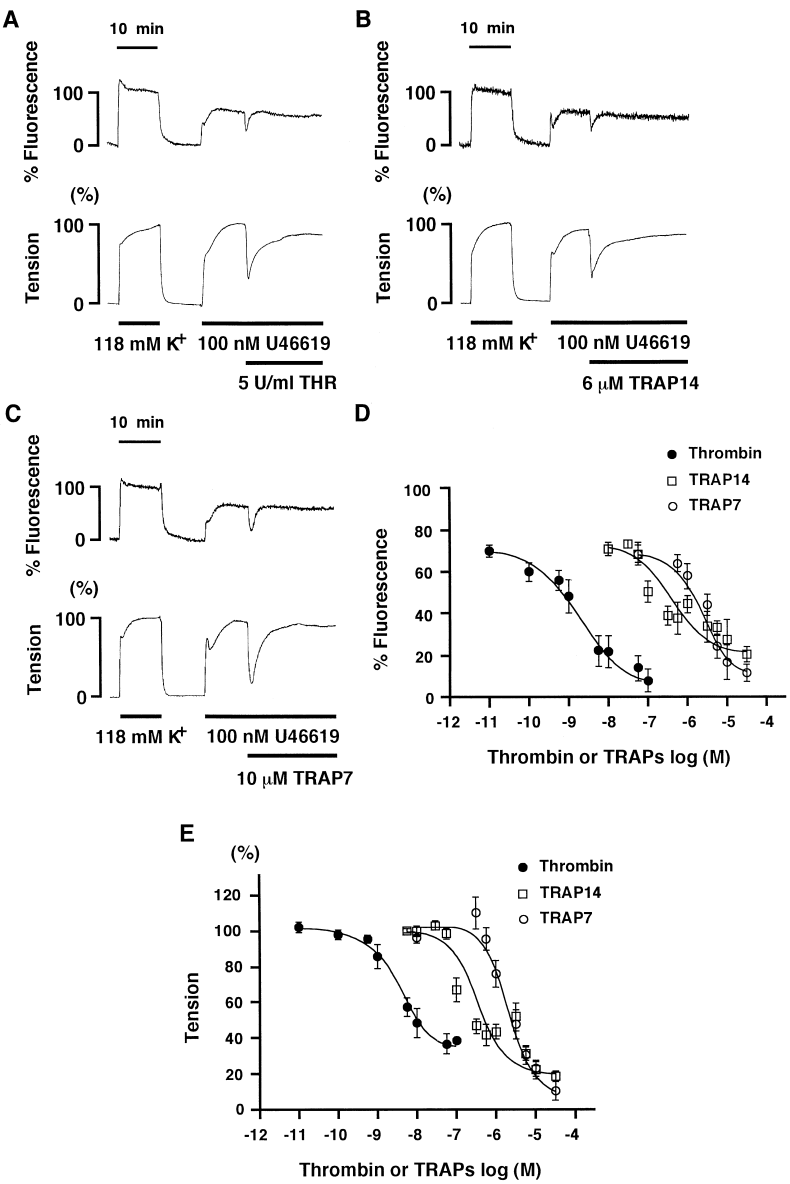


Fig. 2. The endothelium-dependent relaxations induced by thrombin, TRAP14 and TRAP7 in porcine coronary artery. (A–C) Representative recordings showing the effect of thrombin (THR; A), TRAP14 (B) and TRAP7 (C) on $[Ca^{2+}]_i$ and tension of smooth muscle in the coronary arterial strips with an endothelium, precontracted with 100 nM U46619. After recording the control response to 118 mM K^+ depolarization, the strip was precontracted with 100 nM U46619. THR (5 U/ml), TRAP14 (6 μM) and TRAP7 (10 μM) were applied 10 min after the application of U46619. (D, E) The concentration–response curves for the maximal decrease in $[Ca^{2+}]_i$ (D) and tension (E) induced by thrombin (●), TRAP14 (□) and TRAP7 (○) during U46619-induced contraction. The data are the mean ± S.E.M. ($n = 4$–8). The fluorescence ratio and tension development were expressed as a percentage, assigning the values in normal PSS (5.9 mM K^+) and 118 mM K^+ depolarization to be 0% and 100%, respectively.

sion of smooth muscle increased rapidly and reached steady state within 10 min. The sustained levels of $[Ca^{2+}]_i$ and tension at 10 min after the application of U46619 were 70.9 ± 1.7% and 102.0 ± 3.3% ($n = 4$) of those seen with 118 mM K^+-depolarization (Fig. 2D and E), respectively. When 5 U/ml thrombin was applied during the steady state of contraction, an early transient relaxation and a subsequent small sustained relaxation were both observed (Fig. 2A). The early transient relaxation was associated with a transient decrease in $[Ca^{2+}]_i$, while the sustained relaxation was not associated with any apparent $[Ca^{2+}]_i$ decrease. The decreases in $[Ca^{2+}]_i$ and tension were concentration-dependent, with IC_{50} of thrombin for the peak decrease in $[Ca^{2+}]_i$ and tension being 0.2 ± 0.1 and 0.3 ± 0.9 U/ml ($n \geq 4$) (Fig. 2D and E), respectively. These values were similar to the EC_{50} values obtained for the $[Ca^{2+}]_i$ elevation in in situ endothelial cells. The maximal decreases in $[Ca^{2+}]_i$ (13.9 ± 5.9%) and tension (36.8 ± 5.5%) was obtained with 10 and 5 U/ml thrombin, respectively. These concentration values were also similar to those required to induce a maximum $[Ca^{2+}]_i$ elevation in in situ endothelial cells.

Similarly, TRAP14 (6 μM) and TRAP7 (10 μM) caused an endothelium-dependent decrease in $[Ca^{2+}]_i$ and tension (Fig. 2B and C). The maximal decreases in $[Ca^{2+}]_i$ induced by TRAP14 (20.6 ± 3.4%; $n = 3$) and TRAP7 (11.7 ± 0.8%, $n = 3$) were both obtained at 30 μM. The maximal relaxations induced by TRAP14 (19.0 ± 2.3%; $n = 3$) and TRAP7 (12.0 ± 2.0%, $n = 3$) were both observed at 30 μM. IC_{50} of TRAP14 for inhibition of $[Ca^{2+}]_i$ and tension were 1.7 ± 1.0 and 0.6 ± 0.4 μM ($n = 3$–11), respectively, and those of TRAP7 were 4.1 ± 0.3 and 3.0 ± 0.6 μM ($n = 3$–8), respectively. The concentration ranges for TRAPs to induce endothelium-dependent relaxations were thus similar to those required to induce the $[Ca^{2+}]_i$ elevations in in situ endothelial cells. The orders of potency of the relaxing effects (thrombin ≫ TRAP14 > TRAP7) correlated closely with those obtained for the effect on $[Ca^{2+}]_i$ elevations in in situ endothelial cells. As a result, the concentration of thrombin and TRAPs required to induce $[Ca^{2+}]_i$ elevation in in situ endothelial cells were functionally relevant to endothelium-dependent relaxation.

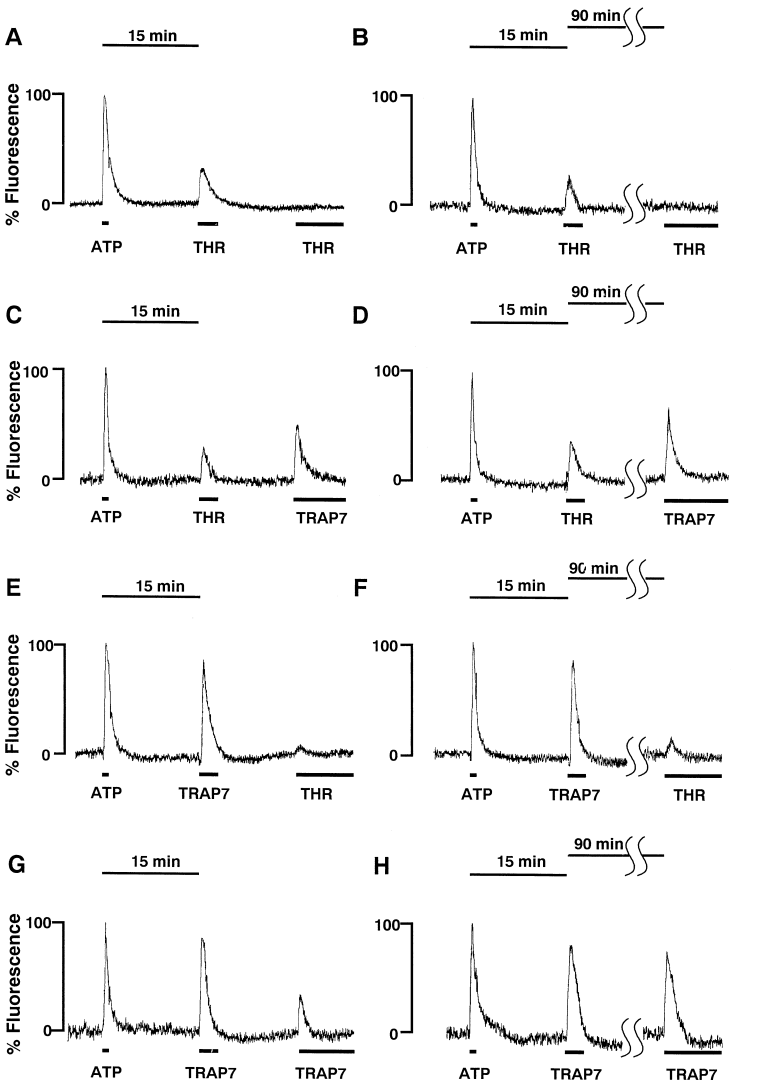
3.3. Desensitization and recovery of responsiveness to thrombin and TRAP7

Using thrombin and TRAP7 as two different stimulations to activate thrombin receptor, the mechanism of desensitization of thrombin receptor was investigated in in situ endthelial cells. To stimulate receptors, the concentration required to induce the maximal response was used; 6 U/ml thrombin and 30 μM TRAP7 (see Fig. 1D). Valvular strips were sequentially stimulated in various combinations of thrombin and TRAP7 at two different intervals, namely, 15 and 90 min (Fig. 3). The desensitization of response to thrombin or TRAP7 was evaluated by comparing the extent of $[Ca^{2+}]_i$ elevation induced by the second stimulation to that obtained by the same stimulation without the preceding stimulation. The levels of $[Ca^{2+}]_i$ elevation induced by thrombin (30.1 ± 2.9%, $n = 9$) or TRAP7 (83.1 ± 2.8%, $n = 18$) 15 min after the stimulation with 10 μM ATP were the same as those (36.0 ± 8.9% for thrombin, $n = 4$; 87.4 ± 19.3% for TRAP7, $n = 4$) obtained 90 min after the stimulation with ATP (Fig. 3I, columns 1–4). As a result, the first stimulation by thrombin and TRAP7 induced the same $[Ca^{2+}]_i$ elevation as that observed during 90 min observation.

Once endothelial cells were stimulated with thrombin for 3 min, the subsequent application of thrombin after 15 or 90 min produced no $[Ca^{2+}]_i$ elevations (Fig. 3A and B). On the other hand, TRAP7 induced a $[Ca^{2+}]_i$ elevation 15 min after the first stimulation by thrombin (Fig. 3C). However, the level of the $[Ca^{2+}]_i$ elevation (46.3 ± 3.5%, $n = 3$) was smaller than that observed without pretreatment by thrombin (Fig. 3I, column 7). Earlier stimulation at 5 and 10 min with TRAP7 induced more attenuated $[Ca^{2+}]_i$ elevation than that seen with 15 min interval. However, we previously observed that the $[Ca^{2+}]_i$ elevations in response to the repetitive stimulations with 10 μM ATP became lower when repetitions were performed at an interval shorter than 15 min (Aoki et al., 1991). An interval of 15 min or longer was thus allowed to observe the consistent full responses to the repetitive stimulations with ATP. Thus, we used 15-min intervals between two sequential stimulation with thrombin and TRAP7 as the shortest interval in the present study. When TRAP7 was applied 90 min after the first stimulation by thrombin, the response recovered to 69.4 ± 13.9% ($n = 5$). This level did not significantly differ from that seen without pretreatment by thrombin (Fig. 3D and I, column 8). On the other hand, once endothelial cells were stimulated with TRAP7 for 3 min, the subsequent application of thrombin (Fig. 3E) or TRAP7 (Fig. 3G) after 15 min induced smaller increases in $[Ca^{2+}]_i$ than those observed without pretreatment with TRAP7 (Fig. 3I, columns 9, 11). The levels of $[Ca^{2+}]_i$ elevation obtained with the second stimulations by thrombin and TRAP7 were 7.8 ± 3.0% ($n = 5$) and 30.4 ± 5.3% ($n = 4$), respectively. When applied 90 min after the first stimulation with TRAP7, the response to thrombin and TRAP7 recovered to 21.4 ± 9.9% ($n = 4$) and 68.7 ± 9.9% ($n = 4$), respectively. In addition, these levels did not significantly ($P > 0.05$) differ from those seen without pretreatment (Fig. 3I, columns 10, 12).

3.4. Effects of kinase inhibitors on the desensitization of thrombin receptor

Since protein phosphorylation plays an important role in the desensitization of thrombin receptor (Sibley et al., 1987; Ishii et al., 1994), the effects of various kinase inhibitors on the desensitization of thrombin receptor were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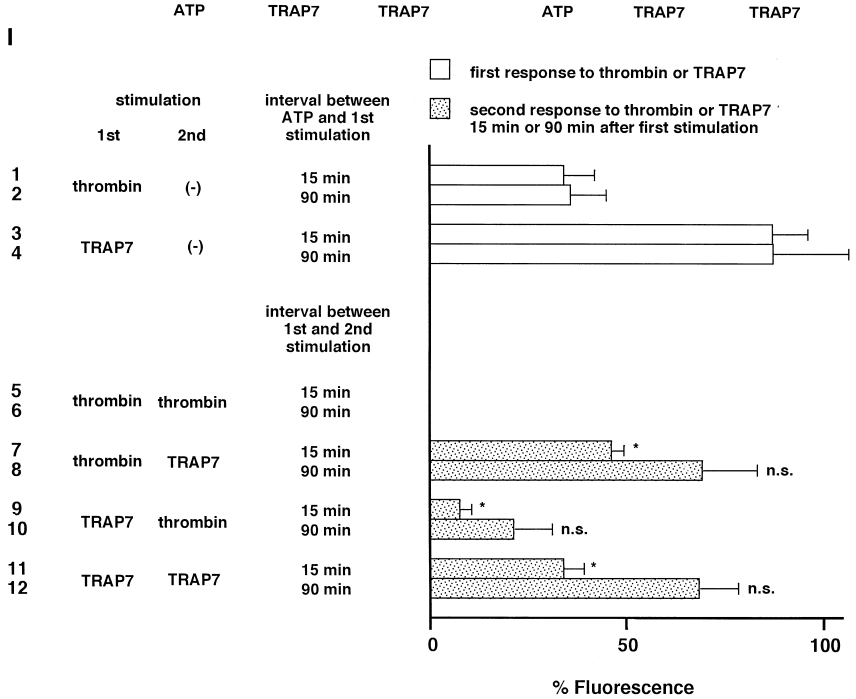
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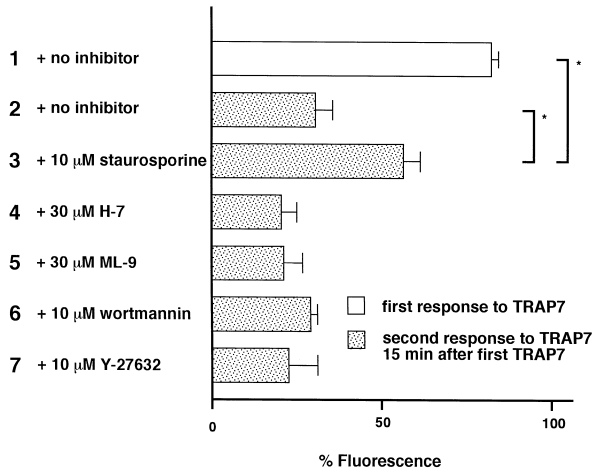

Fig. 4. Effects of kinase inhibitors on the TRAP7-induced desensitization of thrombin receptor in in situ endothelial cells of the porcine aortic valve. Columns indicate the $[Ca^{2+}]_i$ level obtained with the first stimulation by 30 μM TRAP7 in the absence of kinase inhibitors and with the second stimulation by TRAP7 with a 15 min interval after the first stimulation by TRAP7, in the absence and presence of 10 μM staurosporine, 30 μM H-7, 30 μM ML-9, 10 μM wortmannin and 10 μM Y27632. Kinase inhibitors were applied just after the stimulation with 10 μM ATP and 14 min before the first TRAP7 stimulation. The fluorescence ratio was expressed as a percentage, assigning the levels obtained in normal PSS and with 10 μM ATP to be 0% and 100%, respectively. All data are the mean ± S.E.M. ($n = 3$–5). * Significantly ($P < 0.05$) different.

investigated in in situ endothelial cells (Fig. 4). The desensitization of the receptor was induced by 30 μM TRAP7, and evaluated by the response to the second stimulation by 30 μM TRAP7 15 min after the first stimulation. The inhibitors used in the present study were staurosporine, H-7, ML-9, wortmannin, and Y27632 (Rho-kinase inhibitor). These kinase inhibitors were applied just after the 1 min stimulation with 10 μM ATP and 14 min before the first stimulation by TRAP7. Treatment with these inhibitors had no effect on the response to the first TRAP7 stimulation (data not shown). The response to the second stimulation with TRAP7 at 15 min after the first stimulation decreased to 30.4 ± 5.3% ($n = 4$) in the absence of kinase inhibitors (Fig. 4, column 2). Among the kinase inhibitors examined, only staurosporine significantly inhibited the receptor desensitization. The response to the second TRAP7 in the presence of 10 μM staurosporine was 56.5 ± 5.0% ($n = 5$; $P < 0.01$). However, this level was significantly lower than that obtained with the first stimulation (Fig. 4, column 1), thus, indicating the desensitization not to be completely inhibited by staurosporine. On the other hand, other inhibitors had no effects on the receptor desensitization. The response to the second TRAP7 obtained in the presence of 30 μM H-7 (20.5 ± 2.5%), 30 μM ML-9 (21.2 ± 5.6%), 10 μM wortmannin (28.9 ± 2.1%) and 10 μM Y27632 (22.6 ± 8.4%) did not significantly differ from that obtained without any inhibitors (Fig. 4, columns 4–7, respectively).

3.5. Effect of phosphatase inhibitors on the recovery of the receptor responsiveness

Involvement of protein phosphatases in the recovery of the desensitized receptor in in situ endothelial cells was investigated by using phosphatase inhibitors calyculin-A and okadaic acid (Fig. 5A and B). In this protocol, thrombin receptor was desensitized by 30 μM TRAP7 and the recovery of the responsiveness was evaluated by the response to the second TRAP7 stimulation 90 min after the first TRAP7 stimulation. Phosphatase inhibitors were applied 15 min after the first TRAP7 stimulation. In the absence of phosphatase inhibitors, the extent of $[Ca^{2+}]_i$ elevation induced by the second stimulation with TRAP7 was 69.5 ± 6.9% ($n = 4$) (Fig. 5C, column 2). In the presence of 100 nM calyculin-A, the $[Ca^{2+}]_i$ elevation induced by the second stimulation was significantly inhibited to 21.6 ± 4.5% ($n = 3$) (Fig. 5A and C, column 3). This level did not significantly differ from that obtained by the second stimulation 15 min after the first stimulation (Fig. 5C, column 1), thus indicating that treatment with calyculin-A completely inhibited the recovery of responsiveness. Similarly, okadaic acid inhibited the recovery of the response to TRAP7 (Fig. 5B). The $[Ca^{2+}]_i$ elevation induced by the second stimulation in the presence of 1 μM okadaic acid was inhibited to 40.2 ± 2.6% ($P < 0.01$) ($n = 6$) (Fig. 5C, column 4). However, the level was significantly ($P < 0.05$) higher than that obtained by the second stimulation 15 min after the first stimulation (Fig. 5C). In contrast, the treatment with calyculin-A or okadaic

Fig. 3. Desensitization of thrombin receptor induced by thrombin and TRAP7 in endothelial cells in the in situ porcine aortic valve. (A–H) After recording reference response to 10 μM ATP, in in situ endothelial cells were sequentially stimulated by 6 U/ml thrombin and 30 μM TRAP7 in various combinations. Two stimulations were separated by 15 min (A, C, E, G) or 90 min (B, D, F, H) interval of incubation in the normal PSS. A–D, cells were first stimulated with thrombin for 3 min and secondarily by thrombin (A, B) or TRAP7 (C, D). E–H, cells were first stimulated by TRAP7 and secondarily by thrombin (E, F) or TRAP7 (G, H). (I) Summary of 4–6 independent measurements. Columns 1–4, the level of $[Ca^{2+}]_i$ elevation induced by the first stimulation by thrombin and TRAP7 with 15 min and 90 min interval after recording the reference response to 10 μM ATP. Columns 5–12, the level of $[Ca^{2+}]_i$ elevations induced by the second stimulation with thrombin or TRAP7 after the first stimulation by thrombin or TRAP7 with 15 min and 90 min interval between two stimulations. The fluorescence ratio was expressed as a percentage, assigning the level obtained in normal PSS and that at the maximum elevation induced by 10 μM ATP to be 0% and 100%, respectively. All data are the mean ± S.E.M. * Significantly ($P < 0.05$) different from the $[Ca^{2+}]_i$ elevation obtained with the same stimulation by the time-matched first application. (n.s.) no significant difference.

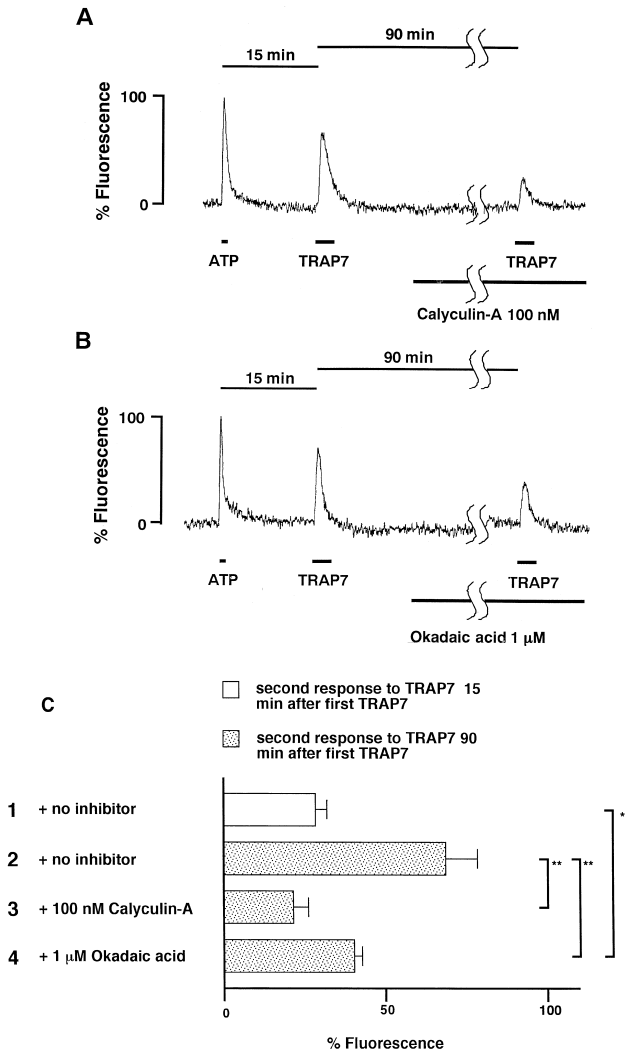

Fig. 5. Effects of phosphatase inhibitors on the recovery of the thrombin receptor responsiveness from the TRAP7-induced desensitization. (A, B) Representative recordings of $[Ca^{2+}]_i$ elevation sequentially induced by 10 μM ATP, 30 μM TRAP7 and 30 μM TRAP7 90 min after the first stimulation of TRAP7, in the presence of 100 nM calyculin-A (A) and 1 μM okadaic acid (B). Calyculin-A and okadaic acid were added 15 min after the first stimulation and 75 min before the second stimulation. (C) Summary of 3–6 independent measurements. Columns 1 and 2, $[Ca^{2+}]_i$ elevation induced by the second stimulation with TRAP7 with 15 min and 90 min interval after the first stimulation with TRAP7 in the absence of phosphatase inhibitors. Columns 3 and 4, $[Ca^{2+}]_i$ elevation induced by the second stimulation with TRAP7 with 90 min interval in the presence of 100 nM calyculin-A and 1 μM okadaic acid. The fluorescence ratio was expressed as a percentage, assigning the levels obtained in normal PSS and with 10 μM ATP to be 0% and 100%, respectively. All data are the mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$.

acid had no effects on the $[Ca^{2+}]_i$ elevation induced by the first stimulation by TRAP7 (data not shown).

4. Discussion

In the present study, we investigated the mechanism of regulation of thrombin receptor activity in in situ endothelial cells, by examining the $[Ca^{2+}]_i$ elevation in response to thrombin and TRAP. The major findings in this study are as follows: (1) Thrombin receptor was rapidly desensitized after both stimulations with thrombin and TRAP7; (2) The response to thrombin did not recover at all within 90 min after the first stimulation by thrombin, while the response to TRAP7 fully recovered after 90 min; (3) The thrombin receptor desensitized by TRAP7 fully recovered its responsiveness to both thrombin and TRAP7 by 90 min; (4) Staurosporine partially inhibited the desensitization of thrombin receptor; (5) Calyculin A completely inhibited the recovery of thrombin receptor activity, while okadaic acid partially inhibited the recovery process. These findings suggest that thrombin receptor was thus desensitized by two different mechanisms in in situ endothelial cells; one is proteolytic cleavage-dependent mechanism while the other is a phosphorylation-dependent mechanism. Thrombin induced both mechanisms, while TRAP7 induced the latter mechanism. This is consistent with previous reports in which thrombin receptor desensitization was due to a combination of proteolysis and phosphorylation, while the recovery of response involves protein synthesis and dephosphorylation (Brass, 1992; Woolkalis et al., 1995).

Thrombin receptor activation was unique as a G protein coupled receptor (Vu et al., 1991). Thrombin cleaves an amino-terminus of its receptor and the resulting new amino-terminus acts as a tethered ligand to activate thrombin receptor. As a result, the peptides corresponding to the tethered ligand (TRAPs) mimicked the effect of thrombin and activated the undigested receptor. In the present study, the digested receptor underwent rapid desensitization and cells never recovered responsiveness to thrombin. There is a possibility that thrombin receptors were internalized and disappeared from cell surface after stimulation by thrombin. However, since the response to TRAP7 fully recovered after 90 min, it was suggested that no intact receptor recovered but rather digested receptors that are no longer desensitized to stimulation by TRAPs fully recovered. On the other hand, the receptor activated by TRAP7 rapidly lost its responsiveness to both thrombin and TRAP7. The desensitized receptor also fully recovered its responsiveness to both thrombin and TRAP7 after 90 min, thus indicating that the number of intact receptors fully recovered by 90 min. These findings suggest that both digested and undigested receptors were desensitized by the similar mechanism. The inhibition of TRAP7-induced desensitization by staurosporine and the complete inhibition of its recovery by calyculin A thus suggested that phosphorylation and dephosphorylation play a major role in this desensitization and its recovery.

In general, two different mechanisms underlies the rapid desensitization of G protein-coupled receptor. The first mechanism is the phosphorylation of receptor. Following activation, the receptor is phosphorylated by kinases such as G protein receptor kinases, cAMP-dependent kinase or protein kinase C (Lohse et al., 1992). The phosphorylated

receptors are subsequently recognized by an arrestin-like protein which inhibit interaction of receptor with G proteins (Lohse et al., 1992). The second mechanism is internalization of receptor into intracellular compartments, and thereby the receptor becomes inaccessible to its ligand. The phosphorylation of receptors may trigger the internalization of receptor (Shapiro et al., 1996). In the present study, the activated thrombin receptor either digested or undigested can be desensitized by these two mechanisms in in situ endothelial cells. The phosphorylated receptors may be retained on cell surface but incapable to induce subsequent signal transduction. Since we did not directly determine the number of thrombin receptor remained after the stimulation, the involvement of receptor internalization in in situ endothelial cells remains to be elucidated.

The involvement of internalization in receptor desensitization was clearly shown in cultured human umbilical vein endothelial cells or human megakaryoblastic HEL cell line. In these studies, the receptor molecules remaining on cell surface after stimulation were directly determined by immunofluorescence staining with monoclonal anti-thrombin receptor antibodies (Hoxie et al., 1993; Woolkalis et al., 1995). In human umbilical vein endothelial cells, receptors were rapidly cleared from cell surface by stimulation with thrombin and intact receptors reappeared within 30 min and recovered to 90% of the prestimulation level by 5 h (Woolkalis et al., 1995). Since this rapid recovery of intact receptor is not dependent on protein synthesis, the intact receptors were suggested to be supplied from intracellular receptor reservoir (Woolkalis et al., 1995). These findings with cultured endothelial cells are distinct from our results obtained with in situ endothelial cells. In in situ endothelial cells, no intact receptor reappeared by 90 min. This is rather similar to the findings observed with HEL cells. In HEL cells, 90% of digested receptors were rapidly internalized and up to 40% of the cleaved receptors reappeared, none of which were reactivated by thrombin (Hoxie et al., 1993). Since the primary structure of the thrombin receptor and activation mechanism of the receptor is apparently the same in all cells, the thrombin receptor desensitization and its recovery both appear to depend on the cell-specific mechanisms. The present study therefore suggests that such cell-specific mechanisms may alter during culture.

Controversy remains regarding kinase which phosphorylates and desensitizes the thrombin receptor. At least two distinct types of protein kinases may be involved: the second messenger-activated kinases and the second messenger-independnt G protein-coupled receptor kinas (Inglese et al., 1993). The kinases that were shown to be activated by thrombin receptor are protein kinase C (Grand et al., 1996), myosin light chain kinase (Moy et al., 1996; Shasby et al., 1997; Majumdar et al., 1998) or a $p21^{cdc42/rac1}$-activated serine/threonine kinase (Teo et al., 1995). Among kinase inhibitors we tested, only staurosporine inhibited the receptor desensitization induced by TRAP7. Myosin light chain kinase inhibitors, ML-9 and wortmannin (Yano et al., 1993) had no effect. Protein kinase C thus is considered to be one of the candidate kinases. Yan et al. (1998) proposed that the protein kinase C_β isoform is involved in the heterologous desensitization of thrombin receptor. However, H-7, another inhibitor of protein kinase C (Hidaka et al., 1984), had no effect in the present study. Moreover, in line with the finding of a previous report (Brass, 1992), staurosporine did not completely inhibit the desensitization induced by TRAP7, thus suggesting that either other kinases are involved in the desensitization or the responsible kinases are relatively resistant to staurosporine. Recently, thrombin receptor has been shown to couple with a novel family of trimeric G proteins, G_{12} (Aragay et al., 1995; Post et al., 1996) which activates small GTP-binding proteins such as Rho and Rac (Buhl et al., 1995; Collins et al., 1996). RhoA was shown to activate protein kinase and Rho-associated kinase (Leung et al., 1995; Teo et al., 1995; Amano et al., 1996; Ishizaki et al., 1996; Matsui et al., 1996; Watanabe et al., 1996). However, Y-27632, an inhibitor of Rho-associated kinase (Uehata et al., 1997) did not inhibit the receptor desensitization. On the other hand, G protein-coupled receptor kinase, especially G protein-coupled receptor kinase 3 has been reported to phosphorylate thrombin receptor co-expressed in *Xenopus oocyte* and inhibited the receptor signaling (Ishii et al., 1994). The involvement of these kinases, however, remains to be determined in in situ endothelial cells.

There have been few reports on the phosphatases responsible for dephosphorylation and the reactivation of the phosphorylated and desensitized thrombin receptor. Brass (1992) showed that calyculin-A and okadaic acid inhibited the recovery of the thrombin receptor desensitization in HEL cells, with calyculin-A being more effective. This observation is consistent with our observation of in situ endothelial cells. An important finding was the fact that calyculin-A completely inhibited the recovery of desensitized receptor, thus suggesting that serine/threonine but not tyrosine phosphorylation can account for the desensitization. Furthermore, 1 μM okadaic acid only partially inhibited the desensitization, suggesting that type 1 protein phosphatase is the major phosphatase responsible for dephosphorylation and the recovery of the desensitized receptors.

In conclusion, thrombin receptor is rapidly desensitized in in situ endothelial cells, following the activation by both thrombin and TRAP in the functionally relevant concentration ranges. Proteolysis and phosphorylation are the major mechanisms of such desensitization. Both thrombin-cleaved and TRAP-activated (uncleaved) receptors underwent the phosphorylation-dependent desensitization. The phosphorylated receptors were dephosphorylated mainly by type 1 phosphatase and fully recovered its ability to induce $[Ca^{2+}]_i$ elevation upon activation. The activation and desensitization of thrombin receptor observed in in situ endothelial cells are basically similar to those observed in HEL cell

but apparently different from those seen with cultured endothelial cells. Intact receptor recovered rapidly in cultured endothelial cells, while it was not detected by 90 min after thrombin stimulation in in situ endothelial cells. Since the primary structure and activation mechanism of thrombin receptor is fundamentally the same in all cells, some other cell-specific mechanism may be involved in such cell-specific manner of desensitization and recovery, and this cell-specific mechanism may be altered by culture conditions.

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References

Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., Kaibuchi, K., 1996. Identification of a putative target for Rho as the serine–threonine kinase protein kinase N. Science 271, 648–650.

Aoki, H., Kobayashi, S., Nishimura, J., Yamamoto, H., Kanaide, H., 1991. Endothelin induces the Ca^{2+}-transient in endothelial cells in situ. Biochem. Biophys. Res. Commun. 181, 1352–1357.

Aragay, A.M., Collins, L.R., Post, G.R., Watson, A.J., Feramisco, J.R., Brown, J.H., Simon, M.I., 1995. G_{12} requirement for thrombin-stimulated gene expression and DNA synthesis in 1321N1 astrocytoma cells. J. Biol. Chem. 270, 20073–20077.

Brass, L.F., 1992. Homologous desensitization of HEL cell thrombin receptors. Distinguishable roles for proteolysis and phosphorylation. J. Biol. Chem. 267, 6044–6050.

Buhl, A.M., Johnson, N.L., Dhanasekaran, N., Johnson, G.L., 1995. $G_{\alpha 12}$ and $G_{\alpha 13}$ stimulate Rho-dependent stress fiber formation and focal adhesion assembly. J. Biol. Chem. 270, 24631–24634.

Collins, L.R., Minden, A., Karin, M., Brown, J.H., 1996. $G_{\alpha 12}$ stimulates c-Jun NH_2-terminal kinase through the small G proteins Ras and Rac. J. Biol. Chem. 71, 17349–17353.

De Lean, A., Munson, P.J., Rodbard, D., 1978. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose–response curves. Am. J. Physiol. 235, E97–E102.

Derian, C.K., Santulli, R.J., Tomko, K.A., Haertlein, B.J., Andrade-Gordon, P., 1995. Species differences in platelet responses to thrombin and SFLLRN. Receptor-mediated calcium mobilization and aggregation, and regulation by protein kinases. Thromb. Res. 78, 505–519.

Di Virgilio, F., Steinberg, T.H., Silverstein, S.C., 1989. Organic-anion transport inhibitors to facilitate measurement of cytosolic free Ca^{2+} with fura-2. In: Tartakoff, A.M. (Ed.), Methods in Cell Biology. Academic Press, San Diego, CA, pp. 453–462.

Grand, R.J., Turnell, A.S., Grabham, P.W., 1996. Cellular consequences of thrombin-receptor activation. Biochem. J. 313, 353–368.

Hidaka, H., Inagaki, M., Kawamoto, S., Sasaki, Y., 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23, 5036–5041.

Hirano, K., Kanaide, H., Abe, S., Nakamura, M., 1990. Effects of diltiazem on calcium concentrations in the cytosol and on force of contractions in porcine coronary arterial strips. Br. J. Pharmacol. 101, 273–280.

Hoxie, J.A., Ahuja, M., Belmonte, E., Pizarro, S., Parton, R., Brass, L.F., 1993. Internalization and recycling of activated thrombin receptors. J. Biol. Chem. 268, 13756–13763.

Inglese, J., Freedman, N.J., Koch, W.J., Lefkowitz, R.J., 1993. Structure and mechanism of the G protein-coupled receptor kinases. J. Biol. Chem. 268, 23735–23738.

Ishii, K., Chen, J., Ishii, M., Koch, W.J., Freedman, N.J., Lefkowitz, R.J., Coughlin, S.R., 1994. Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase. Functional specificity among G-protein coupled receptor kinases. J. Biol. Chem. 269, 1125–1130.

Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., Narumiya, S., 1996. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J. 15, 1885–1893.

Kanaide, H., 1998. Measurement of $[Ca^{2+}]_i$ in smooth muscle strips using front-surface fluorometry. In: Lambert, D.G. (Ed.), Methods in Molecular Biology. Humane Press, Totowa, NJ, pp. 269–277.

Kuroiwa, M., Aoki, H., Kobayashi, S., Nishimura, J., Kanaide, H., 1995. Mechanism of endothelium-dependent relaxation induced by substance P in the coronary artery of the pig. Br. J. Pharmacol. 116, 2040–2047.

Leung, T., Manser, E., Tan, L., Lim, L., 1995. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J. Biol. Chem. 270, 29051–29054.

Lohse, M.J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J.P., Caron, M.G., Lefkowitz, R.J., 1992. Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the β2-adrenergic receptor and rhodopsin systems. J. Biol. Chem. 267, 8558–8564.

Majumdar, M., Seasholtz, T.M., Goldstein, D., de Lanerolle, P., Brown, J.H., 1998. Requirement for Rho-mediated myosin light chain phosphorylation in thrombin-stimulated cell rounding and its dissociation from mitogenesis. J. Biol. Chem. 273, 10099–10106.

Marsen, T.A., Simonson, M.S., Dunn, M.J., 1995. Thrombin induces the preproendothelin-1 gene in endothelial cells by a protein tyrosine kinase-linked mechanism. Circ. Res. 76, 987–995.

Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, T., Iwamatsu, A., Kaibuchi, K., 1996. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. EMBO J. 15, 2208–2216.

Mizuno, O., Hirano, K., Nishimura, J., Kubo, C., Kanaide, H., 1998. Mechanism of relaxation induced by thrombin in pig coronary arterial strips with endothelium. Eur. J. Pharmacol. 351, 67–77.

Moy, A.B., Van Engelenhoven, J., Bodmer, J., Kamath, J., Keese, C., Giaever, I., Shasby, S., Shasby, D.M., 1996. Histamine and thrombin modulate endothelial focal adhesion through centripetal and centrifugal forces. J. Clin. Invest. 97, 1020–1027.

Post, G.R., Collins, L.R., Kennedy, E.D., Moskowitz, S.A., Aragay, A.M., Goldstein, D., Brown, J.H., 1996. Coupling of the thrombin receptor to G_{12} may account for selective effects of thrombin on gene expression and DNA synthesis in 1321N1 astrocytoma cells. Mol. Biol. Cell. 7, 1679–1690.

Shapiro, M.J., Trejo, J., Zeng, D., Coughlin, S.R., 1996. Role of thrombin receptor's cytoplasmic tail in intracellular. Distinct determinants for agonist-triggered vs. tonic internalization and intracellular localization. J. Biol. Chem. 271, 32874–32880.

Shasby, D.M., Stevens, T., Ries, D., Moy, A.B., Kamath, J.M., Kamath, A.M., Shasby, S.S., 1997. Thrombin inhibits myosin light chain

dephosphorylation in endothelial cells. Am. J. Physiol. 272, L311–L319.

Shuman, M.A., 1986. Thrombin–cellular interactions. Ann. N.Y. Acad. Sci. 485, 228–239.

Sibley, D.R., Benovic, J.L., Caron, M.G., Lefkowitz, R.J., 1987. Regulation of transmembrane signaling by receptor phosphorylation. Cell 48, 913–922.

Teo, M., Manser, E., Lim, L., 1995. Identification and molecular cloning of a $p21^{cdc42/rac1}$-activated serine/threonine kinase that is rapidly activated by thrombin in platelets. J. Biol. Chem. 270, 26690–26697.

Tiruppathi, C., Lum, H., Andersen, T.T., Fenton, J.W. II, Malik, A.B., 1992. Thrombin receptor 14-amino acid peptide binds to endothelial cells and stimulates calcium transients. Am. J. Physiol. 263, L595–L601.

Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., Narumiya, S., 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature 389, 990–994.

Vouret-Craviari, V., Auberger, P., Pouyssegur, J., Van Obberghen-Schilling, E., 1995. Distinct mechanisms regulate 5-HT_2 and thrombin receptor desensitization. J. Biol. Chem. 270, 4813–4821.

Vouret-Craviari, V., Van Obberghen-Schilling, E., Rasmussen, U.B., Pavirani, A., Lecocq, J.P., Pouyssegur, J., 1992. Synthetic alpha-thrombin receptor peptides activate G protein-coupled signaling pathways but are unable to induce mitogenesis. Mol. Biol. Cell 3, 95–102.

Vu, T.K.H., Hung, D.T., Weheaton, V.I., Coughin, S.R., 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64, 1057–1068.

Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A., Narumiya, S., 1996. Protein kinase N (PKN) and PKN-related protein rhophilin as targets of small GTPase Rho. Science 271, 645–648.

Woolkalis, M.J., DeMelfi, T.M. Jr., Blanchard, N., Hoxie, J.A., Brass, L.F., 1995. Regulation of thrombin receptors on human umbilical vein endothelial cells. J. Biol. Chem. 270, 9868–9875.

Yan, W., Tiruppathi, C., Lum, H., Qlao, R., Malik, A.B., 1998. Protein kinase Cβ regulates heterologous desensitization of thrombin receptor (PAR-1) in endothelial cells. Am. J. Physiol. 274, C387–C395.

Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y., Matsuda, Y., 1993. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J. Biol. Chem. 268, 25846–25856.