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Involvement of STIM1 in the Proteinase-Activated Receptor 1-Mediated Ca^{2+} Influx in Vascular Endothelial Cells

Katsuya Hirano,* Mayumi Hirano, and Akiko Hanada

Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

ABSTRACT

Thrombin increases the cytosolic Ca^{2+} concentrations and induces NO production by activating proteinase-activated receptor 1 (PAR_1) in vascular endothelial cells. The store-operated Ca^{2+} influx is a major Ca^{2+} influx pathway in non-excitable cells including endothelial cells and it has been reported to play a role in the thrombin-induced Ca^{2+} signaling in endothelial cells. Recent studies have identified stromal interaction molecule 1 (STIM1) to function as a sensor of the store site Ca^{2+} content, thereby regulating the store-operated Ca^{2+} influx. However, the functional role of STIM1 in the thrombin-induced Ca^{2+} influx and NO production in endothelial cells still remains to be elucidated. Fura-2 and diaminorhodamine-4M fluorometry was utilized to evaluate the thrombin-induced changes in cytosolic Ca^{2+} concentrations and NO production, respectively, in porcine aortic endothelial cells transfected with small interfering RNA (siRNA) targeted to STIM1. STIM1-targeted siRNA suppressed the STIM1 expression and the thapsigargin-induced Ca^{2+} influx. The degree of suppression of the STIM1 expression correlated well to the degree of suppression of the Ca^{2+} influx. The knockdown of STIM1 was associated with a substantial inhibition of the Ca^{2+} influx and a partial reduction of the NO production induced by thrombin. The thrombin-induced Ca^{2+} influx exhibited the similar sensitivity toward the Ca^{2+} influx inhibitors to that seen with the thapsigargin-induced Ca^{2+} influx. The present study provides the first evidence that STIM1 plays a critical role in the PAR_1-mediated Ca^{2+} influx and Ca^{2+}-dependent component of the NO production in endothelial cells. J. Cell. Biochem. 108: 499–507, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: CA^{2+} INFLUX; ENDOTHELIUM; NITRIC OXIDE; PROTEINASE-ACTIVATED RECEPTOR; STROMAL INTERACTION MOLECULE 1; THROMBIN

Store-operated Ca^{2+} influx, which is activated following the depletion of the intracellular Ca^{2+} stores, is a major Ca^{2+} influx pathway in non-excitable cells including vascular endothelial cells [Parekh and Putney, 2005]. Recently, the molecular mechanism underlying this type of Ca^{2+} influx has been emerging [Putney, 2007b]. Stromal interaction molecule 1 (STIM1) was identified to function as a sensor of the stored Ca^{2+} content [Liou et al., 2005; Roos et al., 2005]. STIM1 is a single-pass transmembrane protein, which exits on the endoplasmic reticulum membrane as well as on the plasma membrane [Putney, 2007b; Stathopulos et al., 2008]. The N-terminal luminal region contains a Ca^{2+}-binding motif and coordinates a single Ca^{2+} ion [Stathopulos et al., 2008]. The depletion of the stored Ca^{2+} causes a loss of Ca^{2+} from STIM1, which induces oligomerization and redistribution of STIM1 at the proximity to the plasma membrane and thereby activates the Ca^{2+} influx channels [Wu et al., 2006; Yuan et al., 2007; Luik et al., 2008; Stathopulos et al., 2008]. The Orai proteins have been shown to serve as Ca^{2+} influx channels regulated by STIM1, and they mediate the Ca^{2+} selective type of store-operated Ca^{2+} influx such as that seen in T lymphocytes [Feske et al., 2006]. On the other hand, a canonical type of transient receptor potential channel (TRPC) has been suggested to mediate the non-selective type of store-operated Ca^{2+} influx such as that seen in endothelial cells [Mehta and Malik, 2006; Putney, 2007b]. The recent studies with exogenously expressed TRPC and STIM1 have shown that TRPC was regulated by STIM1 [Huang et al., 2006; Yuan

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*Correspondence to: Dr. Katsuya Hirano, MD, PhD, Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: khirano@molcar.med.kyushu-u.c.jp

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et al., 2007]. Recently, it was demonstrated that the knockdown of STIM1 was associated with the inhibition of the store-operated Ca^{2+} influx and proliferation of endothelial cells [Abdullaev et al., 2008]. However, the production of nitric oxide (NO) and barrier function in endothelial cells are also known to be regulated by Ca^{2+} signal [Sessa, 2004; Mehta and Malik, 2006]. Therefore, the functional role of STIM1 in these endothelial functions still remains to be investigated.

Thrombin is known to induce NO production and impairment of barrier function by activating proteinase-activated receptors (PARs) in endothelial cells [Coughlin, 2000; Hirano, 2007]. Among the four members of PARs, PAR_1 and PAR_4 serve as major signaling receptors for thrombin in endothelial cells [Macfarlane et al., 2001]. PAR_1 activation has been shown to induce NO production in a manner partly dependent on and partly independent of Ca^{2+} in porcine aortic and human umbilical vein endothelial cells, while PAR_4 activation induced NO production in a Ca^{2+}-independent manner [Momota et al., 2006; Hirano et al., 2007a]. The store-operated as well as receptor-operated Ca^{2+} influx have been reported to mediate the thrombin-induced Ca^{2+} signal in endothelial cells [Ahmmed et al., 2004]. However, the role of STIM1 in the thrombin-induced Ca^{2+} influx still remains to be determined.

The present study thus investigated the involvement of STIM1 in the thrombin-induced, PAR_1-mediated Ca^{2+} influx and NO production in endothelial cells. Because human umbilical vein endothelial cells exhibited a low viability after the transfection, porcine aortic endothelial cells (PAEC) was utilized in the present study. As a result, the present study provides the first evidence that STIM1 plays an important role in the PAR_1-mediated store-operated Ca^{2+} influx and NO production in endothelial cells.

MATERIALS AND METHODS

DESIGN OF SMALL INTERFERING RNA (siRNA) TARGETED TO PORCINE STIM1

A partial nucleotide sequence of the porcine STIM1 was determined using a PCR product derived from a cDNA library of PAEC [Hirano et al., 2001a]. The sense and anti-sense PCR primers were designed according to the sequence of human STIM1 (accession # NM003156) and they were 5′-ggT gAT gTg gAT gTg gAA gA-3′ and 5′-CAA TgC TgC TgT CAC CTC gC-3′, respectively. A PCR was conducted with a protocol consisting of an initial 2-min denaturation at 94°C and a following 30-cycle amplification step with 10-s denaturation at 98°C, 30-s annealing at 55°C and 1-min extension at 68°C. The nucleotide sequence data of the porcine STIM1, which corresponds to the nucleotide region 830–1812 of human STIM1, have been deposited to the DDBJ/EMBL/GenBank under accession number AB374252 (Fig. S1). Based on this sequence data, siRNA targeted to the region corresponding to nucleotide 1558–1576 of human STIM1 was synthesized with a 3′-UU overhang by Dharmacon (Tokyo, Japan), as siRNA targeted to this region has been reported to effectively down-regulate the STIM1 expression in HEK293 [Wedel et al., 2007]. The sequence of the sense strand of the siRNA for the porcine STIM1 was 5′-AGA AGG AGC UGG AGU CUC A-3′ (Fig. S1). The control siRNA (siControl Non-targeting siRNA pool #2) and the pre-designed and pooled siRNA targeted to the four regions of the human STIM1 (siGenome smart pool M-011785) were purchased from Dharmacon. Among four siRNA targeted to the human STIM1, three had 2 mismatches with the porcine STIM1 and one had 3 mismatches (Fig. S1). All siRNA were dissolved at the stock concentration of 20 μmol/L in 6 mmol/L HEPES, pH 7.5, 20 mmol/L KCl, 0.2 mmol/L $MgCl_2$ and kept at −20°C.

CELL CULTURE AND TRANSFECTION OF siRNA

PAEC were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum, as described previously [Hirano et al., 2007a,b], but no antibiotics. PAEC of passages 9–16 were used for the experiments. HeLa cells and HEK293 cells were also cultured in DMEM containing 10% serum. The cells were transfected with siRNA 24 h after the plating at 7.8×10^3 cells/cm^2 for fura-2 fluorometry and 1.5×10^4 cells/cm^2 for DAR-4M fluorometry. On the day of transfection, the growth medium was renewed and then the mixture of siRNA and lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) prepared in Opti-MEM I (Invitrogen) were added to the growth medium. The different concentrations of siRNA (0.01–20 nmol/L) were used in the studies as indicated, while the amount of lipofectamine RNAiMAX was fixed at 1 μl/ml medium. The cells were then used for the evaluation 3 days after transfection.

FURA-2 FLUOROMETRY

The cells on 35-mm dish were loaded with fura-2 by incubating them in DMEM containing 5 μmol/L fura-2 acetoxymethyl ester (Dojin, Kumamoto, Japan) and 1 mmol/L probenecid at 37°C for 1 h, as described previously [Hirano et al., 2007a]. After fura-2 loading, the cells were equilibrated in HEPES-buffered saline (HBS; 10 mmol/L HEPES, pH 7.4, 135 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 5.5 mmol/L D-glucose) at room temperature for 30 min before starting fluorometry. The changes in fura-2 fluorescence of cell population (excitation at 340 ± 10 and 380 ± 10 nm; emission at 500 ± 10 nm) were monitored using a front-surface fluorometer CAM230-OF2, as described previously [Hirano et al., 2007a]. The response to 50 μmol/L ionomycin was recorded at the end of each recording as a reference response to normalize the fluorescence data.

DAR-4M FLUOROMETRY OF NO PRODUCTION

PAEC was plated on Cell Desk LF1 (Sumitomo Bakelite, Tokyo, Japan) in 24-well culture dish and then subjected to DAR-4M fluorometry, as described previously [Hirano et al., 2007a]. In brief, Cell Desk LF1 with cells was inserted into a quartz cuvette containing 10 μmol/L DAR-4M (Daiichi Pure Chemicals, Tokyo, Japan) in 1 ml HBS. The cells were then stimulated by changing the bathing solution to that containing 10 μmol/L DAR-4M with agonist or vehicle. The changes in DAR-4M fluorescence (excitation at 540 ± 5 nm; emission at 580 ± 10 nm) were continuously measured at 25°C with a fluorescence spectrophotometer 650–40 (Hitachi, Tokyo, Japan). The NO production was evaluated 3 min after the stimulation. The fluorescence data were expressed in arbitrary units, as described previously [Hirano et al., 2007a]. A separate in vitro experiment demonstrated the linearity of the DAR-4M fluorometry within the range of measurement in the cells [Hirano et al., 2007a].

WESTERN BLOT ANALYSIS

The cells on 60-mm culture dish were washed in ice-cold PBS and then immediately lysed in 100 μl of the buffer consisting of 50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Nonidet P-40, 0.5 mmol/L Na_3VO_4, 10 μmol/L 4-amidinophenyl-methanesulfonyl fluoride hydrochloride, 10 μg/ml leupeptin and 10 μg/ml aprotinin, followed by a snap freeze in liquid N_2, as described previously [Hirano et al., 2001b]. After thawing on ice, the cell lysate was clarified by centrifugation at 12,000 rpm, 15 min, on a microcentrifuge in the cold room. The protein concentration of the lysate was determined with a Coomassie protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. Thirty micrograms protein samples were subjected to immunoblot analyses. After incubation with the primary and secondary antibodies in an immunoreaction enhancer solution named Can-Get-Signal (Toyobo, Osaka, Japan), the immune complex was detected with an enhanced chemiluminescence technique (ECL plus kit, GE Healthcare Bioscience, Buckinghamshire, UK). The light emission was detected and analyzed with ChemiDoc XRS-J and Quantity One software (BioRad, Tokyo, Japan). After immunoblot detection, the polyvinylidene difluoride membranes were stained with naphthol blue black to detect the band of actin. The density of actin was used to normalize the sample loading.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS OF STIM1 EXPRESSION

Total RNA was isolated from PAEC as described previously [Hirano et al., 2001b], and subjected to a RT-PCR analysis with a High Fidelity RT-PCR kit ReverTra-Plus™, according to the manufacturer's instructions (Toyobo). One microgram RNA was used in the RT reaction with a random primer and then 2 and 1 μl of RT product were used in PCR for STIM1 and β-actin, respectively. The sense and anti-sense PCR primers for the porcine STIM1 were 5′-CTT ACA CCG AGC CGA GCA GA-3′ and 5′-CCT CCT CCG CAT ACT TCT GG-3′, respectively. The primers for porcine β-actin were 5′-GTG CGG GAC ATC AAG GAG AA-3′ and 5′-TGT CCA CGT CGC ACT TCA T-3′. STIM1 cDNA (209 bp) was amplified with KOD plus DNA polymerase (Toyobo) and a protocol consisting of a 2-min initial denaturation at 96°C and a following 30-cycle amplification step with 10-s denaturation at 98°C, 30-s annealing at 55°C and 1-min extension at 68°C. β-actin cDNA (241 bp) was amplified with Taq DNA polymerase (New England Biolab, Ipswich, MA) and a protocol consisting of 2-min initial denaturation at 94°C and a following 25-cycle amplification step with 1-min denaturation at 94°C, 1-min annealing at 55°C and 1-min extension at 72°C.

OTHER MATERIALS

Anti-STIM1 antibody (#610954), anti-STIM2 antibody (#54681), and anti-PAR_1 antibody (sc-13503) were obtained from BD Bioscience Japan (Tokyo, Japan), Anaspec (San Jose, CA), and Santa Cruz (Santa Cruz, CA), respectively. The secondary antibodies conjugated with horseradish peroxidase, α-thrombin (bovine plasma, 2,539 U/mg protein), ionomycin, SKF96365 and thapsigargin were purchased from Sigma. TFLLR-NH_2 (PAR_1-activating peptide; PAR_1-AP) and SFLLRNPNDKYEPF (thrombin receptor-activating peptide 14; TRAP14) were obtained from Bachem (Bubendorf, Switzerland). TRAP14 was used as an antigen peptide to pre-absorb the anti-PAR_1 antibody.

STATISTICAL ANALYSIS

An analysis of variance (ANOVA) followed by Bonferroni/Dunn's post hoc test and Student's *t*-test was used to evaluate any statistical significance. $P < 0.05$ was considered to be significantly different.

RESULTS

EFFICACY AND SPECIFICITY OF STIM1-TARGETED siRNA

The expression level of STIM1 protein was concentration-dependently suppressed 3 days after transfection of STIM1-targeted but not control siRNA in PAEC (Fig. 1a). The maximal suppression was obtained with 1 nmol/L siRNA (Fig. 1a). A substantial suppression of the STIM1 expression was obtained 2–3 days after transfection (data not shown). The suppression of the STIM1 protein expression was associated with the suppression of the mRNA expression (Fig. 1b). On the other hand, STIM1-targeted siRNA had no effect on the level of STIM2, a second isoform in mammals, which has been shown to function as a store site Ca^{2+} sensor under resting conditions and regulate the resting level of the cytosolic and endoplasmic Ca^{2+} concentrations [Brandman et al., 2007] (Fig. 1a).

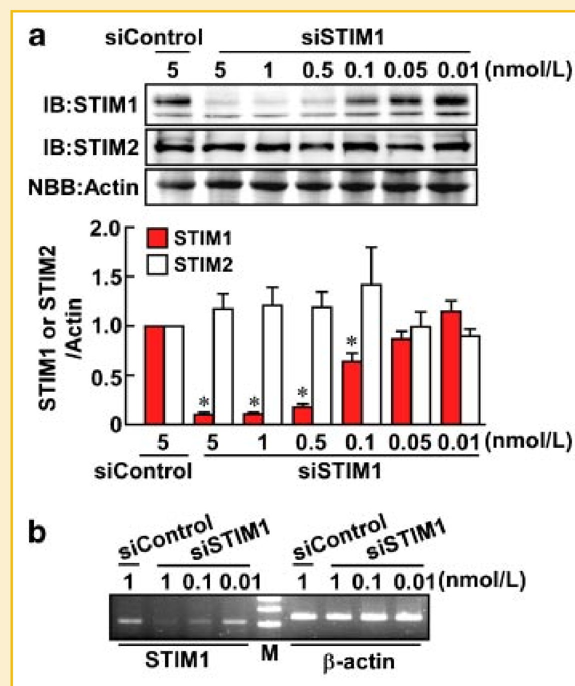

Fig. 1. The specific knockdown of STIM1 by the transfection of STIM1-targeted siRNA in PAEC. Immunoblot (a) and RT-PCR analyses (b) of the expression of STIM1, STIM2 and β-actin in PAEC, 3 days after transfection of control (siControl) or STIM1-targeted siRNA (siSTIM1) at the indicated concentrations. Since the upper band in panel a was apparently down-regulated by the STIM1-targeted siRNA, the level of this band was therefore evaluated to represent the level of STIM1. The data are the mean ± SEM (n = 6 for STIM1, n = 3 for STIM2). $^*P < 0.05$ versus siControl. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

These observations thus support the efficacy and specificity of the siRNA designed for porcine STIM1. Accordingly, the 3-day transfection of 1 nmol/L siRNA was used as a standard protocol.

EFFECT OF STIM1 KNOCKDOWN ON THE THAPSIGARGIN-INDUCED CA^{2+} INFLUX IN PAEC

Thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase, is widely used as a standard stimulation to activate the store-operated Ca^{2+} influx [Liou et al., 2005; Roos et al., 2005; Putney, 2007b]. A Ca^{2+} add-back protocol was used to examine the thapsigargin-induced Ca^{2+} influx (Fig. 2a). Preliminary experiments determined 100 nmol/L thapsigargin to induce the maximal elevation of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (data not shown). In the Ca^{2+} add-back protocol, cells were first exposed to the Ca^{2+}-free media containing 2 mmol/L EGTA and then they were stimulated with 100 nmol/L thapsigargin, which induced a transient elevation of $[Ca^{2+}]_i$ (release component; Fig. 2a). After $[Ca^{2+}]_i$ returned to the pre-stimulation level, the extracellular Ca^{2+} was replenished, which induced a transient and subsequent sustained elevation of $[Ca^{2+}]_i$ (influx component; Fig. 2a). The Ca^{2+} influx was evaluated at the peak $[Ca^{2+}]_i$ elevation seen after the Ca^{2+} replenishment. The extent of the Ca^{2+} influx increased as the concentrations of Ca^{2+} in the replenishment were increased, while the maximal Ca^{2+} influx was observed with the replenishment of 2 mmol/L Ca^{2+} (data not shown). The Ca^{2+} replenishment without the stimulation with thapsigargin induced only a slight elevation of $[Ca^{2+}]_i$, thus returning $[Ca^{2+}]_i$ to the level similar to the resting level seen in the presence of 1 mmol/L Ca^{2+} (Fig. 2a,b).

STIM1-targeted siRNA concentration-dependently inhibited the thapsigargin-induced Ca^{2+} influx, but not the Ca^{2+} release component (Fig. 2b). The inhibition of the thapsigargin-induced Ca^{2+} influx was observed with the concentrations of

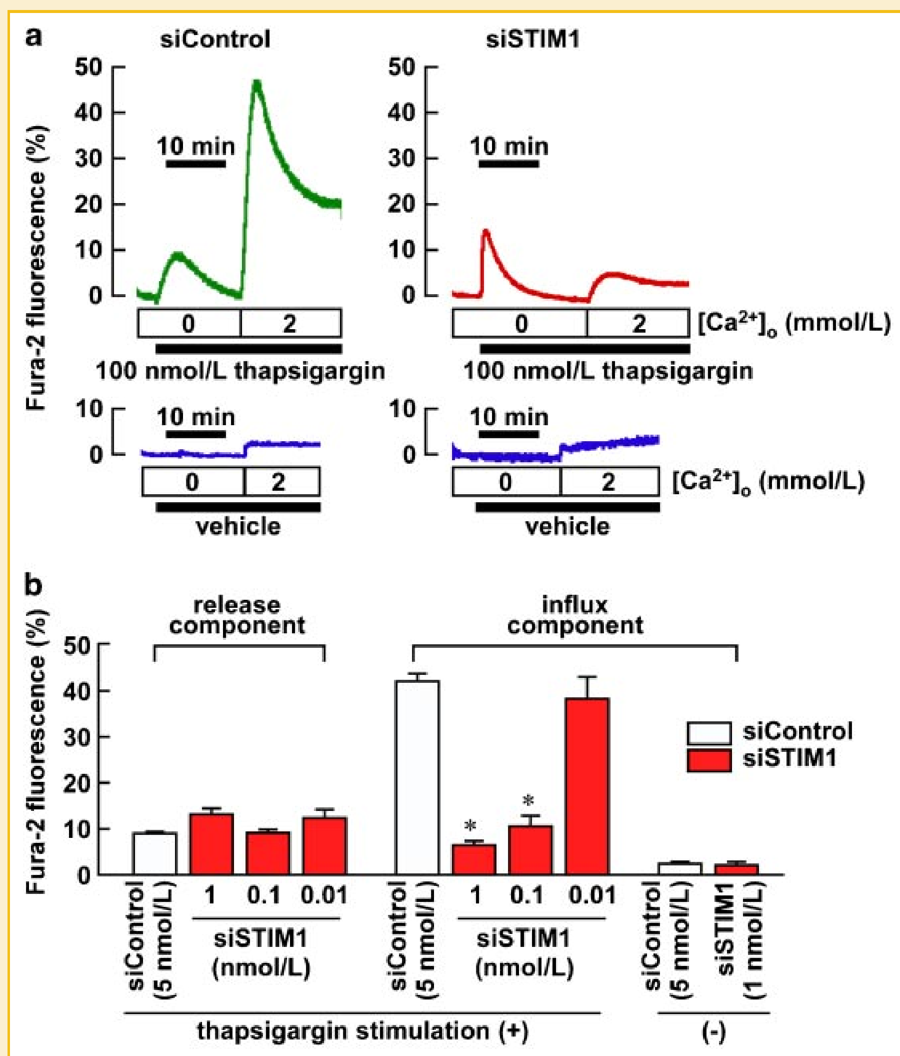

Fig. 2. Effect of STIM1 knockdown on the thapsigargin-induced Ca^{2+} influx in PAEC. a: Representative recordings of fura-2 fluorometry during the Ca^{2+} add-back experiments in the presence (upper traces) and absence (lower traces) of 100 nmol/L thapsigargin in PAEC, 3 days after transfection of 5 nmol/L control (siControl) or 1 nmol/L STIM1-targeted siRNA (siSTIM1). b: Summary of the effect of STIM1 knockdown on the Ca^{2+} release and Ca^{2+} influx components. The data are the mean ± SEM (n = 4–6). *$P < 0.05$ versus siControl. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

STIM1-targeted siRNA similar to those required to suppress the STIM1 expression. Next, the pre-designed and pooled siRNA targeted to human STIM1, which contained 2–3 mismatches to porcine STIM1 (Fig. S1), was used as a second siRNA to knockdown the expression of STIM1. As shown in Figure 3a,b, the pooled siRNA was less effective, even at 20 nmol/L (5 nmol/L for each siRNA duplex in the pool), in suppressing the STIM1 expression and inhibiting the thapsigargin-induced Ca^{2+} influx in PAEC. However, in HeLa cells, a human cell line, the pooled siRNA substantially suppressed the STIM1 expression and inhibited the thapsigargin-induced Ca^{2+} influx at much lower concentrations (Fig. 3c,d). The maximal suppression was obtained with 1 nmol/L pooled siRNA (Fig. 3c). The pooled siRNA had no effect on the expression of STIM2 in both PAEC and HeLa cells (Fig. 3a,c). As a result, the degree of the suppression of STIM1 expression correlated to the degree of inhibition of the thapsigargin-induced Ca^{2+} influx.

EFFECT OF STIM1 KNOCKDOWN ON THE PAR_1-MEDIATED CA^{2+} INFLUX AND NO PRODUCTION IN PAEC

In the Ca^{2+} add-back protocol, both thrombin and PAR_1-AP (TFLLR-NH_2) induced a transient $[Ca^{2+}]_i$ elevation in the absence of the extracellular Ca^{2+} and the subsequent replenishment of the extracellular Ca^{2+} induced a sustained $[Ca^{2+}]_i$ elevation (Fig. 4a). In case of the PAR_1 stimulation, 5 mmol/L Ca^{2+} was required to obtained the maximal Ca^{2+} influx (data not shown). However, even with 5 mmol/L Ca^{2+} replenishment, the level of $[Ca^{2+}]_i$ elevation observed with the PAR_1 stimulation was lower than that seen with thapsigargin and 2 mmol/L Ca^{2+} replenishment, thus indicating that the PAR_1 stimulation activated the Ca^{2+} influx to the extent much lower than to that seen with thapsigargin. The lower level of the Ca^{2+} influx seen with thrombin was associated with the lower level of the Ca^{2+} release. Namely, once the Ca^{2+} release was induced by thapsigargin, the subsequent stimulation with thrombin induced no Ca^{2+} release. In contrast, thapsigargin induced a significant

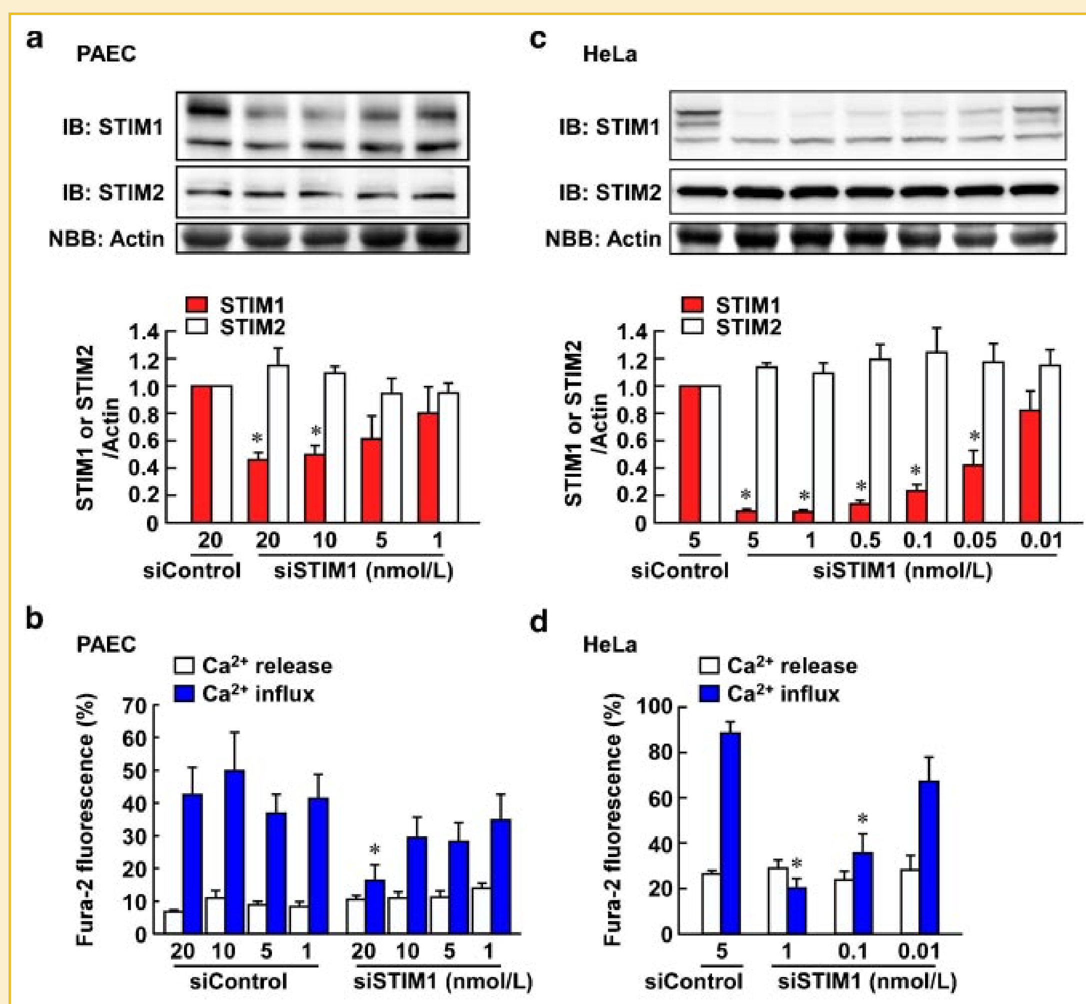

Fig. 3. Effects of pooled siRNA targeted to human STIM1 on the expression of STIM1 and the thapsigargin-induced Ca^{2+} influx in PAEC (a,b) and HeLa cells (c,d). a,c: An immunoblot analysis of the expression of STIM1 and STIM2 in PACE (a) and HeLa cells (c), 3 days after transfection with pooled siRNA targeted to the human STIM1 (siSTIM1) or control siRNA (siControl) at the indicated concentrations. The expression level of STIM1 in PAEC was evaluated as in Figure 1. The expression level of STIM1 in HeLa cells was evaluated by the sum of the level of the upper two bands, which were apparently down-regulated by the siRNA in HeLa cells. b,d: The effects of STIM1 knockdown on the Ca^{2+} release and Ca^{2+} influx induced by 100 nmol/L thapsigargin in PAEC (b) and HeLa cells (d), according to the Ca^{2+} add-back protocol as in Figure 2. Data are the mean ± SEM (n = 5 in a; n = 6–8 in b; n = 6 in c,d). $^{*}P < 0.05$ versus siControl. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

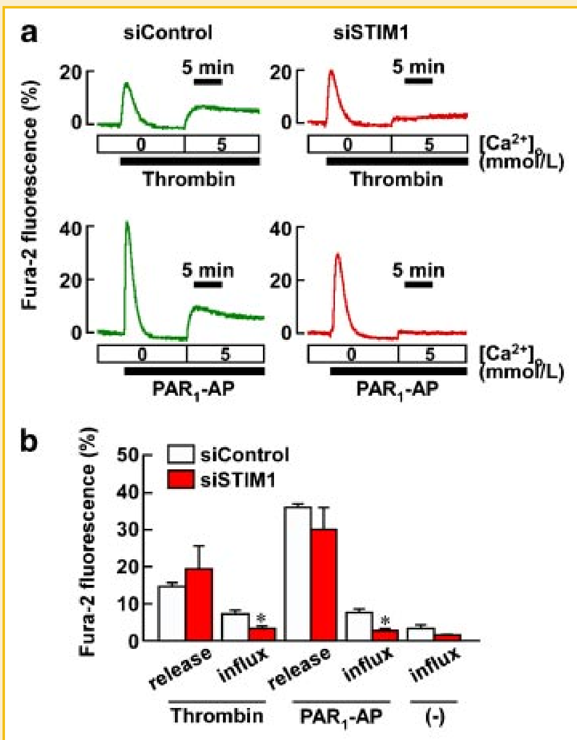

Fig. 4. Effect of STIM1 knockdown on the PAR_1-mediated Ca^{2+} influx in PAEC. a: Representative recordings of fura-2 fluorometry during the Ca^{2+} add-back experiments with 1 U/ml thrombin or 30 μmol/L PAR_1-AP, 3 days after transfection of control (siControl) or STIM1-targeted siRNA (siSTIM1) at 1 nmol/L. b: Summary of the effect of STIM1 knockdown on the Ca^{2+} release and influx. The data are the mean ± SEM (n = 3–5). *$P < 0.05$ versus siControl. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

degree of Ca^{2+} release after the preceding activation of the Ca^{2+} release by thrombin (data not shown). Transfection of 1 nmol/L STIM1-targeted siRNA significantly inhibited the Ca^{2+} influx induced by thrombin and PAR_1-AP, while having no significant effect on the release components (Fig. 4a,b). The level of Ca^{2+} influx obtained in the cells transfected with 1 nmol/L STIM1-targeted siRNA was similar to the level obtained in the absence of stimulation, thus indicating the complete inhibition of the PAR_1-mediated Ca^{2+} influx by the STIM1 knockdown (Fig. 4b).

The physiological significance of the STIM1-mediated Ca^{2+} influx induced by thrombin was then evaluated under physiological conditions. In the presence of 1 mmol/L extracellular Ca^{2+}, thrombin induced a transient elevation of $[Ca^{2+}]_i$ followed by a small sustained elevation. This transient $[Ca^{2+}]_i$ elevation was significantly inhibited by the transfection of STIM1-targeted siRNA (Fig. 5a). In contrast, the thrombin-induced transient $[Ca^{2+}]_i$ elevation in the absence of extracellular Ca^{2+} was resistant to STIM1 knockdown (Fig. 5b). This observation is consistent with that shown in Figure 4. The thrombin-induced NO production in PAEC is partly dependent on $[Ca^{2+}]_i$ elevation [Hirano et al., 2007a]. The NO production was thus evaluated using DAR-4M fluorometry to investigate the functional role of the STIM1-mediated Ca^{2+} influx in endothelial cells. The knockdown of the STIM1 expression significantly, but only partly, inhibited the thrombin-induced NO production (Fig. 5c).

The off-target effect of siRNA on the expression of PAR_1 and its contribution to the observed inhibition of the Ca^{2+} influx were ruled out by an immunoblot analysis (Fig. 5d). The anti-human PAR_1 antibody used in the study detected two bands with an apparent molecular size of 60 and 71 kDa in both PAEC (Fig. 5d) and HEK293 (data not shown). The detection of these bands was abolished by pre-absorbing the primary antibody with the antigen peptide in both cell types (data not shown). The two bands may thus represent PAR_1 with different post-translational modification, such as glycosylation or ubiquitination [Compton, 2003; Wolfe et al., 2007]. Nevertheless, transfection of STIM1-targeted siRNA had no significant effect on the levels of both bands (Fig. 5d).

The Ca^{2+} influx components seen with thapsigargin, thrombin, and PAR_1-AP exhibited a similar sensitivity toward the STIM1 knockdown, thus suggesting the involvement of the similar mechanism of Ca^{2+} influx. The properties of Ca^{2+} influx were further characterized by examining the effects of several Ca^{2+} influx inhibitors on the Ca^{2+} influx components (Fig. 6). SKF96365, $NiCl_2$, and $LaCl_3$ were used in the present study. SKF96365 has been used as an inhibitor of the store-operated Ca^{2+} influx [Liou et al., 2005; Roos et al., 2005], although it can inhibit other channels over similar concentration ranges [Parekh and Putney, 2005]. Divalent and trivalent cations act as a rather non-specific Ca^{2+} influx inhibitor, while lanthanides can block the store-operated Ca^{2+} influx at the micro molar concentrations [Parekh and Putney, 2005]. The Ca^{2+} influx induced by thapsigargin, thrombin, and PAR_1-AP exhibited a similar sensitivity toward SKF96365, $NiCl_2$, and $LaCl_3$ (Fig. 6).

DISCUSSION

The store-operated Ca^{2+} influx is a major Ca^{2+} influx pathway in endothelial cells, thereby contributing to the regulation of various endothelial functions including the NO production [Parekh and Putney, 2005; Putney, 2007b]. Recent studies have identified STIM1 to function as a sensor of the content of the stored Ca^{2+} and activate the store-operated Ca^{2+} influx [Liou et al., 2005; Roos et al., 2005]. The observation of the present study that the degree of suppression of the STIM1 expression correlated with the degree of inhibition of the thapsigargin-induced Ca^{2+} influx suggests STIM1 to play an essential role in the store-operated Ca^{2+} influx in vascular endothelial cells. This was consistent with the observations in other cell types [Putney, 2007a,b]. However, the functional significance of STIM1 in endothelial cells still remains to be established, especially in a native setting. The present study thus provides the first evidence that STIM1 is involved in the PAR_1-mediated Ca^{2+} influx and Ca^{2+}-dependent component of the NO production in endothelial cells.

The efficacy and specificity of siRNA in suppressing the expression of STIM1 is a prerequisite for concluding the involvement of STIM1. This was supported by the several lines of evidence: (1) When the species-matched STIM1-targeted siRNA was used, the expression of STIM1 was significantly suppressed at a level as low as 0.1 nmol/L, while the maximal and substantial (>90% suppression)

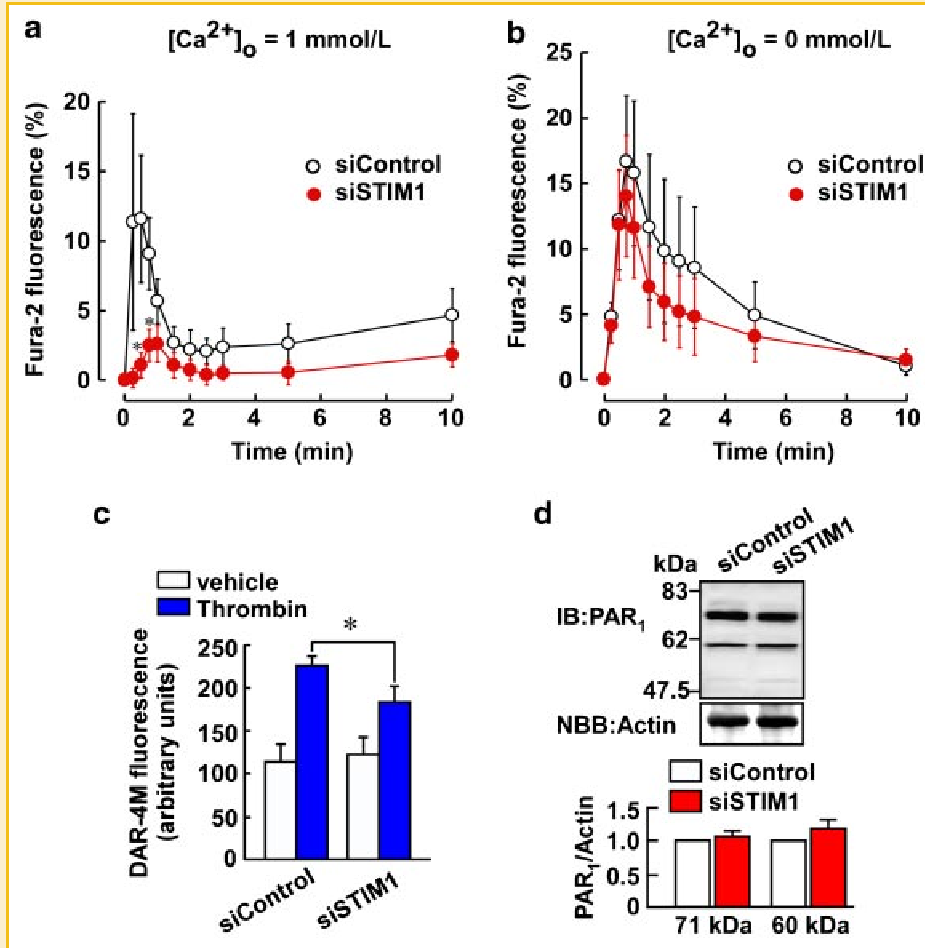

Fig. 5. Role of STIM1 in the thrombin-mediated Ca^{2+} mobilization and NO production in PAEC. a,b: Summary of the effect of STIM1 knockdown on the thrombin-induced $[Ca^{2+}]_i$ elevation in the presence (a) and absence (b) of 1 mmol/L extracellular Ca^{2+}. c: The NO production 3 min after the stimulation with 1 U/ml thrombin in PAEC, 3 days after transfection of siControl or siSTIM1 at 1 nmol/L. d: Immunoblot analysis of the PAR_1 expression. The data are the mean ± SEM (n = 4 in a,b,d; n = 5 in c). $^*P < 0.05$ versus siControl. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

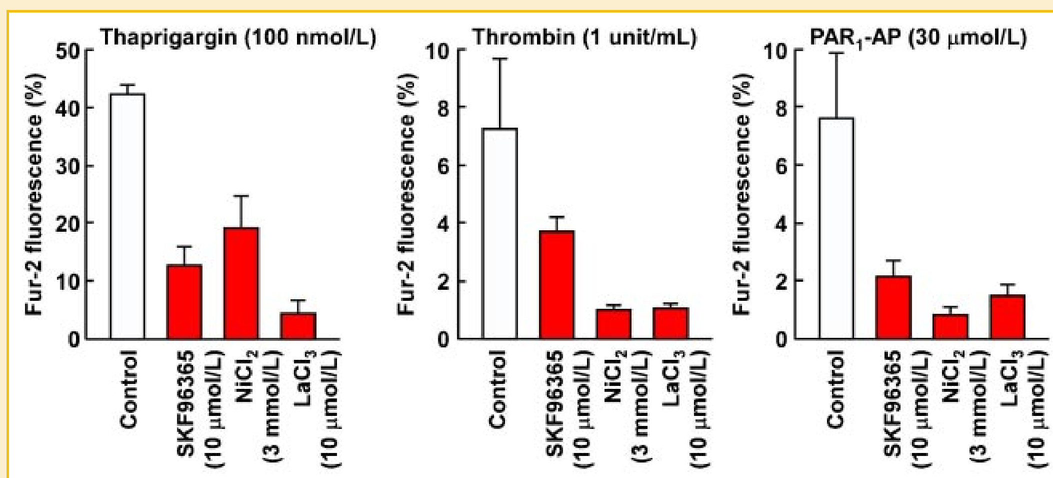

Fig. 6. Effects of Ca^{2+} channel blockers on the Ca^{2+} influx induced by thapsigargin, thrombin and PAR_1-AP in PAEC. The Ca^{2+} influx components obtained with 100 nmol/L thapsigargin, 1 U/ml thrombin, and 30 μmol/L PAR_1-AP the during the Ca^{2+} add-back protocol with and without SKF96365, $NiCl_2$, and $LaCl_3$ are shown. The data are the mean ± SEM (n = 3–7). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

suppression was obtained at 1 nmol/L; (2) siRNA with 2–3 mismatches caused a less effective suppression of the STIM1 expression; (3) It has no effect on the expression of actin, STIM2 and PAR_1. Accordingly, the off-target effect of STIM1-targeted siRNA was less likely.

Thrombin has been shown to activate store-operated as well as receptor-operated Ca^{2+} influxes in human umbilical vein endothelial cells [Ahmmed et al., 2004; Singh et al., 2007]. TRPC1 is suggested to mediate the store-operated Ca^{2+} influx, while TRPC6 is suggested to mediate the receptor-operated Ca^{2+} influx [Ahmmed et al., 2004; Singh et al., 2007]. In the present study, (1) the PAR_1-mediated Ca^{2+} influx exhibited the similar sensitivity toward three Ca^{2+} influx inhibitors to that seen with the thapsigargin-induced Ca^{2+} influx; (2) the knockdown of STIM1 caused the complete abolishment of the Ca^{2+} influx induced by thapsigargin and PAR_1 agonist. The thapsigargin-induced Ca^{2+} influx represents the store-operated Ca^{2+} influx [Parekh and Putney, 2005]. These observations thus suggested that the PAR_1-mediated Ca^{2+} influx was mainly attributable to the store-operated Ca^{2+} influx and the STIM1-dependent mechanism. The relative contribution of the store-operated Ca^{2+} influx in the agonist-induced Ca^{2+} influx could vary depending on the type of agonist [Nilius and Droogmans, 2001]. Therefore, the dependence of the agonist-induced Ca^{2+} influx on STIM1 could vary with the type of agonist in accordance with the relative contribution of the store-operated Ca^{2+} influx.

The identity of the channels involved in the store-operated Ca^{2+} influx in endothelial cells is still controversial [Beech, 2009]. TRPC1 has been suggested to be involved in the store-operated Ca^{2+} influx in human pulmonary artery endothelial cells as well as in other cell types [Brough et al., 2001; Parekh and Putney, 2005], and it was also shown to be involved in the thrombin-induced Ca^{2+} influx [Ahmmed et al., 2004; Singh et al., 2007] in human umbilical vein endothelial cells. On the other hand, Orai1, but not TRPC1 or TRPC4, has recently been shown to mediate the store-operated Ca^{2+} influx in human umbilical vein endothelial cells [Abdullaev et al., 2008]. The identity of the channel involved in the thrombin-induced, STIM1-mediated Ca^{2+} influx thus remains to be investigated.

Among four subtypes of PARs, PAR_1 and PAR_4 serve as major signaling receptors for thrombin [Coughlin, 2000; Hirano, 2007]. PAR_4 has been shown to induce NO production in a Ca^{2+}-independent manner in PAEC as well as in bovine aortic endothelial cells and human umbilical vein endothelial cells [Momota et al., 2006; Hirano et al., 2007a]. However, PAR_1 activation induced NO production in a manner partly dependent on and partly independent of Ca^{2+} in PAEC [Mizuno et al., 2000; Hirano et al., 2007a]. The substantial inhibition of the thrombin-induced $[Ca^{2+}]_i$ elevation by loading BAPTA was associated with a partial inhibition of the NO production [Hirano et al., 2007a]. In the present study, the knockdown of STIM1 significantly inhibited the thrombin-induced $[Ca^{2+}]_i$ elevation seen in the presence of extracellular Ca^{2+}, while it was associated with a partial inhibition of the NO production. These observations are thus consistent with the previous observations [Hirano et al., 2007a]. The present study also noted that the STIM1-dependent mechanism, presumably the store-operated Ca^{2+} influx, contributed differently to the thrombin-induced Ca^{2+} mobilization according to the presence or absence of the extracellular Ca^{2+}. The significant part of the transient $[Ca^{2+}]_i$ elevation seen in the presence of extracellular Ca^{2+} was thus suggested to be attributable to the STIM1-dependent mechanism, while that seen in the absence of extracellular Ca^{2+} was largely independent of STIM1.

The anti-STIM1 antibody used in the present study detected doublet in PAEC (Figs. 1 and 3a) and triplet in HeLa cells (Fig. 3c). The upper band in PAEC and the upper two bands in HeLa cells were down-regulated by STIM1-targeted siRNA, thus suggesting these bands to represent STIM1. The side-by-side immunoblot analysis aligned the upper band in PAEC to the first band in HeLa cells, and the lower band in PAEC to the third band in HeLa cells (data not shown). The second band seen in HeLa cells could thus be some additional isoform of STIM1 or a degradation production of the first band. The lowest bands in both cell types, which were resistant to the siRNA-mediated knockdown, are suggested to be non-specific bands in immunoblot detection.

In conclusion, the present study demonstrated, for the first time, the STIM1-mediated store operated Ca^{2+} influx to play an important role in the PAR_1-mediated Ca^{2+} influx, thus contributing to a Ca^{2+}-dependent component of the NO production in vascular endothelial cells.

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REFERENCES

Abdullaev IF, Bisaillon JM, Potier M, Gonzalez JC, Motiani RK, Trebak M. 2008. Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation. Circ Res 103:1289–1299.

Ahmmed GU, Mehta D, Vogel S, Holinstat M, Paria BC, Tiruppathi C, Malik AB. 2004. Protein kinase Ca phosphorylates the TRPC1 channel and regulates store-operated Ca^{2+} entry in endothelial cells. J Biol Chem 279:20941–20949.

Beech DJ. 2009. Harmony and discord in endothelial calcium entry. Circ Res 104:e22–e23.

Brandman O, Liou J, Park WS, Meyer T. 2007. STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca^{2+} levels. Cell 131:1327–1339.

Brough GH, Wu S, Cioffi D, Moore TM, Li M, Dean N, Stevens T. 2001. Contribution of endogenously expressed Trp1 to a Ca^{2+}-selective, store-operated Ca^{2+} entry pathway. FASEB J 15:1727–1738.

Compton SJ. 2003. Glycosylation and proteinase-activated receptor function. Drug Dev Res 59:350–354.

Coughlin SR. 2000. Thrombin signalling and protease-activated receptors. Nature 407:258–264.

Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. 2006. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 441:179–185.

Hirano K. 2007. The Roles of proteinase-activated receptors in the vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 27:27–36.

Hirano K, Hirano M, Zeng Y, Nishimura J, Hara K, Muta K, Nawata H, Kanaide H. 2001a. Cloning and functional expression of a degradation-resistant novel isoform of $p27^{Kip1}$. Biochem J 353:51–57.

Hirano M, Hirano K, Nishimura J, Kanaide H. 2001b. Transcriptional upregulation of $p27^{Kip1}$ during the contact-induced growth arrest in the vascular endothelial cells. Exp Cell Res 271:356–367.

Hirano K, Nomoto N, Hirano M, Momota F, Hanada A, Kanaide H. 2007a. Distinct Ca^{2+} requirement for NO production between proteinase-activated receptor 1 and 4 (PAR_1 and PAR_4) in vascular endothelial cells. J Pharmacol Exp Ther 322:668–677.

Hirano M, Kanaide H, Hirano K. 2007b. Rac1-dependent transcriptional upregulation of $p27^{Kip1}$ by homophilic cell-cell contact in vascular endothelial cells. Biochim Biophys Acta-Mol Cell Res 1773:1500–1510.

Huang GN, Zeng W, Kim JY, Yuan JP, Han L, Muallem S, Worley PF. 2006. STIM1 carboxyl-terminus activates native SOC, I_{crac} and TRPC1 channels. Nat Cell Biol 8:1003–1010.

Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE, Jr., Meyer T. 2005. STIM is a Ca^{2+} sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+} influx. Curr Biol 15:1235–1241.

Luik RM, Wang B, Prakriya M, Wu MM, Lewis RS. 2008. Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. Nature 454:538–542.

Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. 2001. Proteinase-activated receptors. Pharmacol Rev 53:245–282.

Mehta D, Malik AB. 2006. Signaling mechanisms regulating endothelial permeability. Physiol Rev 86:279–367.

Mizuno O, Kobayashi S, Hirano K, Nishimura J, Kubo C, Kanaide H. 2000. Stimulus-specific alteration of the relationship between cytosolic Ca^{2+} transients and nitric oxide production in endothelial cells ex vivo. Br J Pharmacol 130:1140–1146.

Momota F, Hirano K, Hirano M, Nishimura J, Kanaide H. 2006. Involvement of $G_{i/o}$ in the PAR-4-induced NO production in endothelial cells. Biochem Biophys Res Commun 342:365–371.

Nilius B, Droogmans G. 2001. Ion channels and their functional role in vascular endothelium. Physiol Rev 81:1415–1459.

Parekh AB, Putney JW, Jr. 2005. Store-operated calcium channels. Physiol Rev 85:757–810.

Putney JW, Jr. 2007a. Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). Cell Calcium 42:103–110.

Putney JW, Jr. 2007b. New molecular players in capacitative Ca^{2+} entry. J Cell Sci 120:1959–1965.

Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Velicelebi G, Stauderman KA. 2005. STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol 169:435–445.

Sessa WC. 2004. eNOS at a glance. J Cell Sci 117:2427–2429.

Singh I, Knezevic N, Ahmmed GU, Kini V, Malik AB, Mehta D. 2007. $G_{\alpha q}$-TRPC6-mediated Ca^{2+} entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin. J Biol Chem 282:7833–7843.

Stathopulos PB, Zheng L, Li GY, Plevin MJ, Ikura M. 2008. Structural and mechanistic insights into STIM1-mediated initiation of store-operated calcium entry. Cell 135:110–122.

Wedel B, Boyles RR, Putney JW, Bird GS. 2007. Role of the store-operated calcium entry proteins Stim1 and Orai1 in muscarinic cholinergic receptor-stimulated calcium oscillations in human embryonic kidney cells. J Physiol 579:679–689.

Wolfe BL, Marchese A, Trejo J. 2007. Ubiquitination differentially regulates clathrin-dependent internalization of protease-activated receptor-1. J Cell Biol 177:905–916.

Wu MM, Buchanan J, Luik RM, Lewis RS. 2006. Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. J Cell Biol 174:803–813.

Yuan JP, Zeng W, Huang GN, Worley PF, Muallem S. 2007. STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. Nat Cell Biol 9:636–645.