

## Thrombin Activates $\text{Ca}^{2+}$ -permeating Nonselective Cation Channels through Protein Kinase C in Human Umbilical Vein Endothelial Cells

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We analyzed  $\text{Ca}^{2+}$ -permeating nonselective cation channels (NSCs) mediating thrombin-induced contraction of human umbilical vein endothelial cells (HUVECs). A  $\text{Ca}^{2+}$  chelator, BAPTA-AM (10  $\mu\text{M}$ ), significantly inhibited the thrombin-induced contraction of HUVECs. Thrombin induced inward currents at -60 mV in the presence of intracellular MgATP. Removal of extracellular  $\text{Ca}^{2+}$  significantly decreased the currents. A selective phospholipase C inhibitor, U73122 (1  $\mu\text{M}$ ) but not its inactive analogue, U73343 (1  $\mu\text{M}$ ) almost completely inhibited the currents. Neither a selective inhibitor of  $\text{Ca}^{2+}$ -ATPase of endoplasmic reticulum, thapsigargin (1  $\mu\text{M}$ ) nor a diacylglycerol analogue, 1-oleoyl-2-acetyl-glycerol (30  $\mu\text{M}$ ) activated the currents. However, a selective protein kinase C inhibitor, bisindolylmaleimide I (500 nM) significantly inhibited the currents. The thrombin-induced currents were significantly inhibited by SKF96365 (50  $\mu\text{M}$ ) but not by  $\text{La}^{3+}$  (1 mM), ruthenium red (10  $\mu\text{M}$ ) or flufenamic acid (100  $\mu\text{M}$ ). As assessed with RT-PCR, HUVECs expressed transient receptor potential (TRP) M4, 7, TRPV1, 2, 4, TRPC1, 4 and 6 subunits of NSCs. These results indicate that thrombin activates  $\text{Ca}^{2+}$ -permeating NSCs containing TRPC4 through protein kinase C in HUVECs. Thus, drugs specifically inhibiting TRPC4-containing channels might be effective to control fatal diseases such as sepsis where thrombin mediates the vicious cycle between inflammation and coagulation. *Shinshu Med J* 59 : 13–26, 2011

(Received for publication June 21, 2010 : accepted in revised form September 8, 2010)

**Key words:** human umbilical vein endothelial cells, thrombin, nonselective cation channels, transient receptor potential C4, protein kinase C

### I Introduction

Endothelial cells (ECs) form a barrier between intra- and extra-vascular spaces and regulate the vessel wall permeability for fluid, electrolytes, proteins and blood cells<sup>1)</sup>. In the initial phase of inflammation, ligands for G protein-coupled receptors such as histamine induce contraction of ECs,

create gaps between ECs and increase vascular permeability<sup>2)</sup>. Plasma proteins leaking from the blood into tissues assemble into a provisional matrix that supports the attachment, survival and migration of invading neutrophils. Inflammation leads to activation of coagulation, whereas coagulation also markedly affects inflammation<sup>3)</sup>. In this crosstalk, thrombin plays a pivotal role. Thrombin potently induces the contraction of ECs through protease-activated receptors (PAR) 1 which activate  $\text{G}_{q/11}$  and  $\text{G}_{12/13}$  proteins<sup>4)</sup>.  $\text{G}_{q/11}$  induces phosphoinositide (PI) hydrolysis and thereby increases

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intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ )<sup>5)</sup>. This results in the phosphorylation of myosin light chain (MLC) by activating MLC kinase and induces the contraction of ECs.  $G_{12/13}$  activates Rho and Rho Kinase<sup>6)</sup>. Rho kinase suppresses a type I phosphatase, thereby increasing the phosphorylation of MLC and augmenting the contraction of ECs.

PI hydrolysis increases  $[\text{Ca}^{2+}]_i$  through two distinct mechanisms<sup>5)</sup>. Inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) induces rapid and transient  $\text{Ca}^{2+}$  release from the endoplasmic reticulum (ER). Subsequently, PI hydrolysis causes sustained  $\text{Ca}^{2+}$  influx through  $\text{Ca}^{2+}$  channels in the plasma membrane. For the contraction of ECs, the  $\text{Ca}^{2+}$  influx plays a critical role<sup>5)</sup>. These channels are conceptually classified into two types depending on the way in which the channels are activated<sup>7)</sup>. The “store-operated”  $\text{Ca}^{2+}$  channels (SOCs) are activated by the  $\text{IP}_3$ -mediated depletion of  $\text{Ca}^{2+}$  in ER. On the other hand, the “second messenger-operated”  $\text{Ca}^{2+}$  channels (SMOCs) are activated by second messengers/kinases recruited by PI hydrolysis such as diacylglycerol (DAG), arachidonic acid metabolites, protein kinase C (PKC), etc. Two major classes of molecules have been known to form these  $\text{Ca}^{2+}$  channels. “Transient receptor potential” (TRP) channels are the homo- or hetero-tetramers of putative six-transmembrane polypeptide subunits<sup>8)</sup>. The TRP superfamily comprises six related proteins: TRPC (canonical), TRPV (vallinoid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPA (ankyrin). Among them, TRPC, TRPV, TRPM, and TRPA channels can serve as SMOCs regulated by PI hydrolysis, whereas most of the TRPC channels can also operate as SOCs. Another class of  $\text{Ca}^{2+}$  channels regulated by PI hydrolysis is Orai/CRACM1 channels<sup>9)10)</sup>. Orai channels are oligomers of putative four-transmembrane polypeptide subunits and correspond to a special entity of SOCs termed  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channels. Orai channels sense a  $\text{Ca}^{2+}$  level in ER via single-transmembrane polypeptides termed stromal interaction molecules 1<sup>11)</sup>.

In ECs, TRPC channels have been reported to

form SOCs activated by thrombin. Freichel et al. found that TRPC4 knockout mice lacked SOCs in ECs<sup>12)</sup>, and Tirupathi et al. showed that in these mice, thrombin failed to induce the contraction of lung vascular ECs<sup>13)</sup>. On the other hand, it was shown that thrombin activates SOCs formed by TRPC1 through Rho-induced interaction of TRPC1 with  $\text{IP}_3$  receptors<sup>14)</sup> or through activation of PKC<sup>15)</sup>, thereby inducing contraction of human pulmonary ECs and human umbilical ECs (HUVECs), respectively. Thus, TRPC1 and/or TRPC4 may form SOCs mediating the thrombin-induced contraction of ECs. However, the whole-cell current density of SOCs in ECs is known to be extremely small in amplitude<sup>12)</sup>. In this study on HUVECs, we found that thrombin induces the contraction in a  $\text{Ca}^{2+}$ -dependent manner and activates  $\text{Ca}^{2+}$ -permeating nonselective cation channels that are likely to contain TRPC4 through PKC but not store depletion. These results suggest that selective blockers of TRPC4 channels may be utilized for the treatment of fatal diseases such as sepsis where thrombin mediates the vicious cycle between inflammation and coagulation.

## II Materials and Methods

### A Solutions and Chemicals

Modified Tyrode solution contained (in mM): NaCl 136.5; KCl 5.4;  $\text{CaCl}_2$  1.8;  $\text{MgCl}_2$  0.53; HEPES 10; and glucose 10 (pH adjusted to 7.4 with NaOH). Hypertonic extracellular solution contained (in mM): mannitol 220; Ca-gluconate 20; cesium methanesulfonate 5.4; HEPES 10; and glucose 10 (pH adjusted to 7.4 with CsOH). Pipette solution contained (in mM): cesium methanesulfonate 140; Ca-gluconate 1.74;  $\text{MgSO}_4$  3; HEPES 10; EGTA 5; BAPTA 2; ATP 3; and GTP 0.1 (pH adjusted to 7.2 with CsOH).

Primary human umbilical vein endothelial cells (HUVECs), endothelial growth medium (EBM-2), and a supplement for EBM-2 (EGM-2) were purchased from Cambrex Corporation (East Rutherford, NJ, USA); Penicillin/streptomycin, Trypsin-EDTA, BAPTA-AM, and SKF96365, from Sigma-

Aldrich, Inc. (St Louis, MO, USA); Cellmatrix type I-P, from Nitta Gertin Inc. (Osaka, Japan); FITC-phalloidin, from Invitrogen Corporation (Carlsbad, CA, USA); normal goat serum, from Dako A/S (Glostrup, Denmark); Isogen, from Nippon Gene (Tokyo, Japan); and GoTaq Master Mix, from Promega (Madison, WI, USA). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Stock solutions of BAPTA-AM, U73122, U73343, thapsigargin, bisindolylmaleimide I, 1-oleoyl-2-acetyl-glycerol, SKF96365, ruthenium red, and flufenamic acid were prepared in 100 % DMSO at 50, 2, 2, 1, 1, 100, 50, 10 and 100 mM, respectively. The maximum final concentration of DMSO (0.1 %) did not affect membrane currents.

### B Cell Culture

HUVECs were cultured in the EBM-2 medium supplemented with EGM-2 and 10 % fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO<sub>2</sub> and 95 % air until they formed subconfluent layers. Cells were then detached with trypsin-EDTA, resuspended in fresh culture medium and passaged. HUVECs between passages 1 and 7 were used for experiments.

### C Measurement of Contraction of Endothelial Cells

HUVECs were seeded at a density of 20,000 cell/cm<sup>2</sup> on glass coverslips coated with 0.01 % collagen, grown to confluence and incubated in serum-free medium for 2 h before experiments. HUVECs were then incubated with the indicated concentrations of thrombin in serum-free medium. In some experiments, HUVECs were pretreated with BAPTA-AM (10 μM) for 1 hr before addition of thrombin.

Contraction of HUVECs in response to thrombin was assessed by morphological and electrical methods. For the morphological assessment, the cells were treated with thrombin for the indicated intervals and then washed twice with PBS, fixed with 20 % formalin for 15 min and washed three times with PBS. The cells were then treated with blocking and permeation solution (1 % goat serum

and 0.02 % saponin in PBS) for 15 min, phalloidin solution (4 U/ml FITC-phalloidin and 0.02 % saponin in PBS) for 30 min and with propidium iodide solution (PI in PBS) for 15 min. After washing with PBS three times, the coverslips were mounted onto glass slides with the mounting medium. The morphology of HUVECs visualized with FITC fluorescence was pictured with a fluorescence microscopy (AX80, Olympus, Tokyo, Japan). The percentage of surface area of HUVECs per unit area was measured by Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). Images were acquired at x200 magnification, and 6 images on each sample were analyzed.

For the electrical assessment of the contraction of HUVECs, the impedance of a HUVEC monolayer was measured with a Culture Cell Analyzer (HIOKI E.E. Corp., Ueda, Nagano, Japan). This system comprises three components: an electronic sensor analyzer, a device station and 4×Indium Tin Oxide (ITO) electrode sensor wells. The ITO electrode was fabricated by sputtering ITO on a glass plate. HUVECs were grown on the surfaces of ITO electrodes. Sensor electrical impedance quantitatively reflects changes in the number, viability, morphology and adherence of cells.

To quantify cell status, a parameter termed reactance ratio was derived from the following equation:

$$\text{Reactance ratio} = X(t)/X(0), \quad \text{Eq. 1}$$

where X(0) and X(t) are the electrode reactances (i. e. a component of impedance) before or at time t after drug addition, respectively. When the number of cells in the well is constant, a change in the reactance ratio reflects a change in the cell status such as morphology. For instance, a decrease in cell adhesion or spreading reduces the cell-electrode contact area, leading to a decrease in X(t) and the reactance ratio. To monitor the thrombin-induced contraction of HUVECs, the cell-electrode impedance was continuously measured, and a corresponding time-dependent change in the reactance ratio was derived and recorded.

### D Electrophysiological Analyses

HUVECs were seeded at a density of 450 cell/cm<sup>2</sup> on glass coverslips coated with 0.01 % collagen and grown in the EBM-2 medium supplemented with EGM-2 and 10 % fetal bovine serum for 1–3 days until the days of experiments. The cells were treated with the serum-free medium for 2 hr before experiments.

The whole-cell membrane currents of single HUVECs were measured with the patch-clamp method<sup>16)</sup>. In all experiments, data acquisition was done with an in-house computer program. Patch pipettes were fabricated from borosilicate glass capillaries (Kimax-51, Kimble Glass Inc., Vineland, NJ, USA) and had a resistance of 2–4 M $\Omega$  when filled with the pipette solution. The pipette offset potential was adjusted with a patch clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA) when the tip of a pipette was immersed in the modified Tyrode solution (see below). The series resistance and cell capacitance compensated with the amplifier were  $8 \pm 1.0$  M $\Omega$  and  $73 \pm 6.9$  pF, respectively. All experiments were performed at room temperature (25–26 °C).

Before whole-cell recordings, cells were superfused with the modified Tyrode solution. After establishment of the whole cell configuration, cells were superfused with the hypertonic extracellular solution at a rate of 1.5 ml/min with a peristaltic pump (Dynamax, Rainin, Oakland, CA, USA). Both pipette and hypertonic extracellular solutions were devoid of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> to eliminate these currents<sup>17)</sup>. Drugs were dissolved in the hypertonic extracellular solution and applied to the cells.

An agar-bridge was used for a reference electrode to reduce the liquid junction potential. In this electrode, agar containing the hypertonic extracellular solution was connected to silver chloride via the pipette solution. The liquid junction potentials were directly measured with the patch-clamp amplifier and a 3 M KCl reference electrode<sup>18)</sup>. The potentials of the modified Tyrode solution in reference to the pipette and the hypertonic extracellular solution were +11 and +10 mV, respectively. Thus, the membrane potential differed by -1 mV from the

reading provided by the patch-clamp amplifier when the cells were perfused with the hypertonic extracellular solution and was thus disregarded in this study.

The holding potential was -60 mV. At the timings indicated in Figures, a ramp pulse from -100 to +60 mV for 1 sec was applied. In some experiments, the 500 msec rectangular pulses ranging from -100 to +60 mV with a 10 mV increment were applied every 3 sec.

Recorded cell currents were low-pass filtered at 10 kHz (-3 dB), digitized at 47.2 kHz with a PCM converter system (VR-10B, Instrutech Corp., New York, NY, USA) and recorded on videocassette tapes. For off-line analysis, data were reproduced, low-pass filtered at 2 kHz (-3 dB), digitized at 5 kHz with an AD converter (ITC16I, Instrutech Corp., New York, NY, USA) and analyzed with Patch Analyst Pro (MT Corp., Hyogo, Japan).

#### **E RT-PCR**

Total RNA of HUVECs was isolated with Isogen (Nippon Gene, Tokyo, Japan) according to the manufacture's instructions. For cDNA synthesis, 5  $\mu$ g of total RNA was reverse-transcribed with oligo(dT)<sub>20</sub> primer and SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). To assess the expression of the TRP channels of the HUVEC, PCR was performed with the above cDNA and GoTaq Master Mix (Promega, Madison, WI, USA). All primer sets used are listed in Table 1.

#### **F Data analysis**

Data values are shown as mean  $\pm$  SE. Statistical differences were evaluated with Student's paired or unpaired t-test. A value of  $P < 0.05$  was considered significant.

### **III Results**

#### **A Thrombin-induced contraction of HUVECs**

We first examined the effect of thrombin on the shape of cultured HUVECs (Fig. 1). In Fig. 1A, actin stress fibers and nuclei were visualized with FITC-phalloidin (green) and propidium iodide (red), respectively. Addition of thrombin (0.5 U/ml) to

Table 1 Primer sets used for RT-PCR

Gene	Sequence	Strand	Gene	Sequence	Strand
TRPM1	actatttctatcccctggaagaacc	Sense	TRPV4	aaccctgtgccaacatgaaggtgt	Sense
	cagacacaattactaagctgcttacactac	Antisense		ctcattcttgcccggtcctcgtt	Antisense
TRPM2	tcaccatggaccccatcctg	Sense	TRPV5	cttcgcatcattgccacact	Sense
	gtcacacgccacagccatt	Antisense		cccctactaagttcagtccaa	Antisense
TRPM3	ttctgtcggaatgatccttctg	Sense	TRPV6	tcatcgatggcccagccaact	Sense
	tgaccagcaagatggttggc	Antisense		tgttgattatcccacgcaggctctc	Antisense
TRPM4	tggaaccagtgcgacctagt	Sense	TRPC1	aaggattgggatgattccatcctac	Sense
	tgccctgtactttgccgaatg	Antisense		cgtacattgtcatcaagtagtaagcc	Antisense
TRPM5	agcgcgatgaaggagcttctt	Sense	TRPC3	tctgggttcttggatgatgtggt	Sense
	ggaagtctctcttgactgtctc	Antisense		agcattaacttagcccaaggtagt	Antisense
TRPM6	gcctccgtaaagctatgagagctgt	Sense	TRPC4	acgaggtccgctgtaactgtg	Sense
	gctctccccgagtgtactcatagg	Antisense		gaggctgtgtggcagataaac	Antisense
TRPM7	tctccattaaagccagctatggatac	Sense	TRPC5	acagccaactcccacttaggac	Sense
	acattctccacagcaaacct	Antisense		gcatgatcggcaataagctgatagg	Antisense
TRPM8	cgctggaggtggatattccgtt	Sense	TRPC6	gagttgtggaacatgcttga	Sense
	tgctctattctctgaggtgt	Antisense		tttgaccaatgtagtaggag	Antisense
TRPV1	tggcctatgtaattctcacctacatcc	Sense	TRPC7	gaccacaaattcatcgagaacattgg	Sense
	cttgaagacctcagcgtctct	Antisense		gagccgtttcatgattttctggtatc	Antisense
TRPV2	cgcttctctgatctacttagtcttc	Sense	TRPA1	cagctcatctgcagtggcaa	Sense
	aggacagggttctcgagagt	Antisense		aagttccacctgcatagctatcc	Antisense
TRPV3	agcagaactccaagtatcccatt	Sense			
	tttccgggaattcctcgactt	Antisense			

extracellular medium induced contraction of HUVECs. Fig. 1B shows quantification of the surface area of each HUVEC, indicating that thrombin induced the contraction of HUVECs in a time-dependent manner (open circles). Thrombin induced the contraction also in a concentration-dependent manner with a half-maximum effective concentration (EC<sub>50</sub>) of ~0.05 U/ml (Fig. 1C). We also assessed the shape change of HUVECs by electrically measuring the reactance ratio of their monolayer (Fig. 1D). Thrombin changed the ratio in a time- and concentration-dependent manner similar to that estimated morphologically (Fig. 1B, C). Preincubation of HUVECs with a cell-permeable Ca<sup>2+</sup> chelator, BAPTA-AM (10 μM) significantly attenuated the thrombin-induced contraction of

HUVECs (Fig. 1B, closed circles), indicating that thrombin induced the contraction at least partially by increasing intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) as previously reported<sup>4)19)20)</sup>.

### B Thrombin-induced Ca<sup>2+</sup>-permeable channels in HUVECs

It is known that thrombin causes the contraction of HUVECs mainly via protease-activated receptors (PAR) 1. G<sub>q/11</sub> proteins coupled to PAR1 activates phospholipase C (PLC), which produces inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate. This pathway causes an initial transient increase in [Ca<sup>2+</sup>]<sub>i</sub> by IP<sub>3</sub>-induced Ca<sup>2+</sup> release from the ER and a subsequent sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> by activating Ca<sup>2+</sup>-permeable channels in the plasma

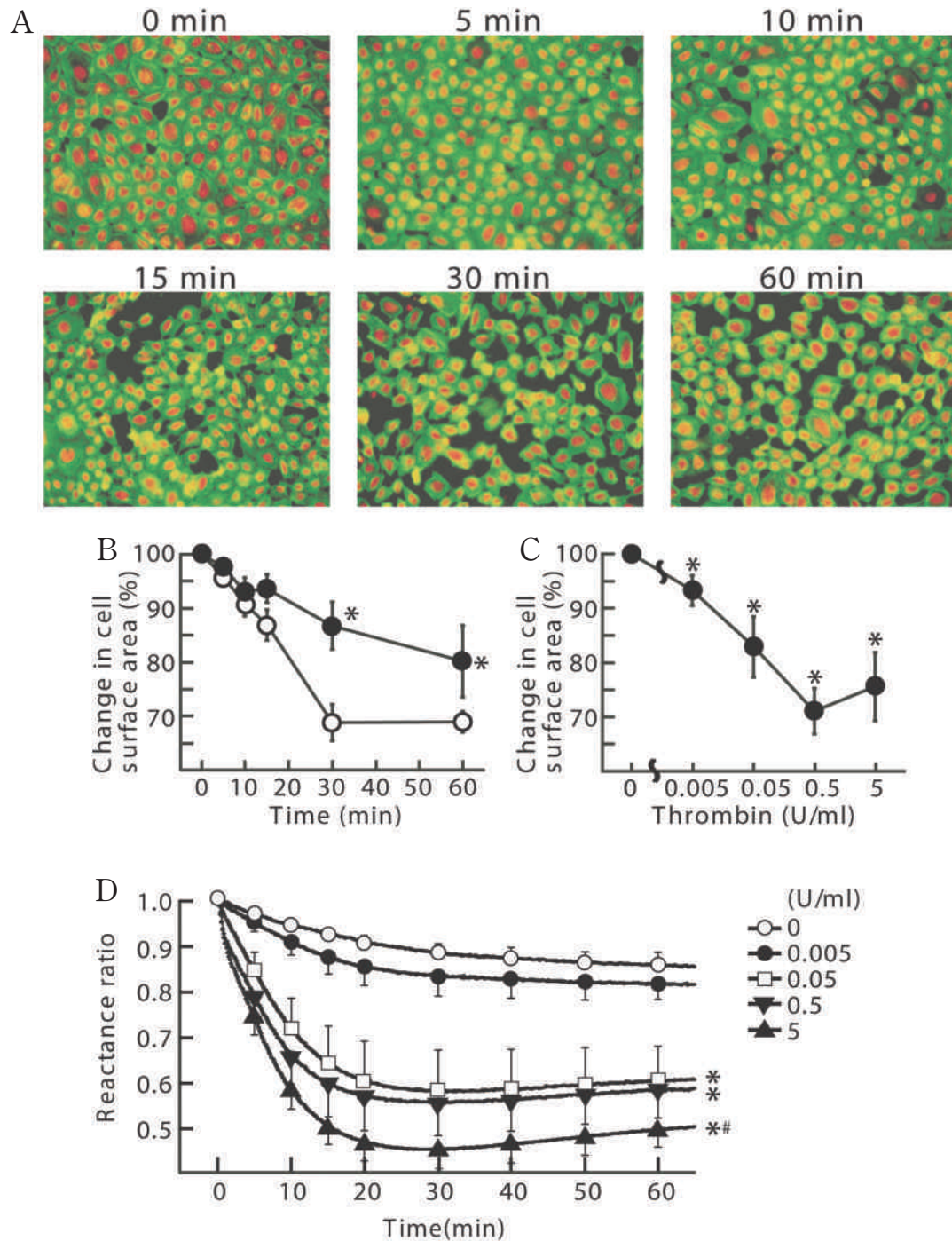


Fig. 1 Contraction of HUVECs induced by thrombin

A, Thrombin induced contraction of HUVEC in a time-dependent manner. Actin stress fibers and nuclei were visualized with FITC-phalloidin (green) and propidium iodide (red), respectively. Fluorescence images were taken at indicated timings after addition of thrombin (0.5 U/ml). B, Quantitative analysis of a change in cell surface area in response to thrombin (0.5 U/ml). Cell surface area at the indicated timings after application of thrombin is expressed as a percentage of that before application of thrombin. Data were obtained either under the control condition (open circles,  $n = 4$ ) or after pretreatment of HUVECs with BAPTA-AM ( $10 \mu\text{M}$ ) for 1 hr (filled circles,  $n = 4$ ).  $*p < 0.05$  versus control. C, Thrombin induced contraction of HUVECs in a concentration-dependent manner. Cell surface area was measured 60 min after application of different concentrations of thrombin.  $\text{EC}_{50}$  of thrombin was  $\sim 0.05$  U/ml ( $n = 4$ ).  $*p < 0.05$  versus the control. D, Thrombin-induced change in reactance ratio of HUVEC monolayers. HUVECs were grown to confluence on electrodes and treated with indicated concentrations of thrombin ( $n = 4-9$  for each concentration).  $*p < 0.05$  versus control,  $\#p < 0.05$  versus 0.5 U/ml.

membrane. In the sustained contraction of HUVECs induced by thrombin, the latter mechanism has been shown to play a more important role than the former<sup>5)20)</sup>. Thus, we measured the effect of thrombin on the whole-cell membrane currents with the patch-clamp technique. Under the present experimental condition where Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> currents were suppressed, virtually only Ca<sup>2+</sup> and Cs<sup>+</sup> carried membrane currents (see Materials and Methods). Under this condition, thrombin (2 U/ml) gradually increased inward currents at -60 mV (Fig. 2A). After reaching the maximum, thrombin-induced currents gradually decreased in the continuous presence of thrombin. The current-voltage (IV) relationship was linear or slightly outwardly rectified in the absence (a) and presence (b) of thrombin (2 U/ml) (Fig. 2B). The thrombin-induced currents reversed in direction at  $+56 \pm 10$  mV ( $n = 6$ ). Fig. 2C shows the membrane currents elicited by rectangular voltage pulses between -100 and +60 mV in a 10 mV increment (a) in the absence (b) and presence (c) of thrombin (2 U/ml). The thrombin-induced difference currents (d) were time- and voltage-independent and slightly outwardly rectified. Thrombin increased the amplitude of the inward currents at -60 mV in a concentration-dependent manner with EC<sub>50</sub> of 0.22 U/ml. Substitution of extracellular Ca<sup>2+</sup> with equimolar N-methyl-D(-)-glucamine significantly decreased the amplitude of the thrombin-induced inward currents (Fig. 2E, F). Thus, thrombin activated voltage-independent Ca<sup>2+</sup>-permeable channels in HUVECs.

### C Signal transduction pathways mediating the induction of Ca<sup>2+</sup> currents by thrombin

A selective PLC inhibitor, U73122 (1  $\mu$ M) but not its inactive analogue, U73343 (1  $\mu$ M) almost completely inhibited the thrombin-induced Ca<sup>2+</sup> currents (Fig. 3A-C), indicating that PLC mediated the thrombin-induced activation of Ca<sup>2+</sup> currents. Several mechanisms have been proposed for the PLC-mediated activation of Ca<sup>2+</sup> channels<sup>8)</sup>. First, IP<sub>3</sub>-mediated depletion of ER Ca<sup>2+</sup> results in activation of Ca<sup>2+</sup> currents (i.e. store-operated Ca<sup>2+</sup> channels (SOCs)). It has been shown that SOCs exist in en-

dothelial cells<sup>21)</sup>. Thus, we added 1  $\mu$ M thapsigargin, an inhibitor of Ca<sup>2+</sup>-ATPase in ER that effectively causes ER Ca<sup>2+</sup> depletion, to the pipette solution. However, it did not induce Ca<sup>2+</sup> currents in HUVECs where thrombin (0.2 or 2 U/ml) clearly did so (Fig. 3D, E). We also applied thapsigargin with 10  $\mu$ M IP<sub>3</sub> to the pipette solution or thapsigargin (1  $\mu$ M) to the extracellular solution and again failed to detect measurable Ca<sup>2+</sup> currents (data not shown). Second, DAG generated by PLC may mediate the activation of Ca<sup>2+</sup> channels. However, we could not activate Ca<sup>2+</sup> channels with a DAG analogue, 1-oleoyl-2-acetyl-glycerol (OAG) (30  $\mu$ M) as effectively as thrombin (0.2 U/ml) (Fig. 3F, G). Third, protein kinase C (PKC) activated by DAG may mediate the thrombin-induced activation of Ca<sup>2+</sup> channels. In fact, a selective PKC inhibitor, bisindolylmaleimide I (BIM) (500 nM) significantly attenuated the activation of inward currents by thrombin (2 U/ml) (Fig. 3H, I), indicating that thrombin induces Ca<sup>2+</sup> currents via PKC. Arachidonic acid metabolites can also mediate activation of Ca<sup>2+</sup> channels by PI hydrolysis<sup>8)</sup>. However, the addition of 10  $\mu$ M of arachidonic acids to HUVECs did not result in activation of Ca<sup>2+</sup>-permeable channels (data not shown).

### D Analyses of the molecules forming thrombin-activated Ca<sup>2+</sup> channels

The thrombin-activated Ca<sup>2+</sup> channels were voltage-independent. These channels were activated by PKC but not by store depletion. Thus, candidate genes responsible for these channels seem to be transient-receptor potential (TRP) C, TRPV, TRPM, and TRPA channels<sup>8)</sup>. As assessed with the RT-PCR technique, transcripts of TRPM4, 7, TRPV1, 2, 4, TRPC1, 4 and 6 genes existed in HUVECs (Fig. 4A). To analyze the molecules responsible for the thrombin-induced currents, we examined the effects of different channel blockers on thrombin-induced Ca<sup>2+</sup> currents (Fig. 4B-F). In Figs. 4B and C where blockers were applied after the addition of thrombin, experiments were conducted in the selected patches where the effect of thrombin was sustained rather than transient. A

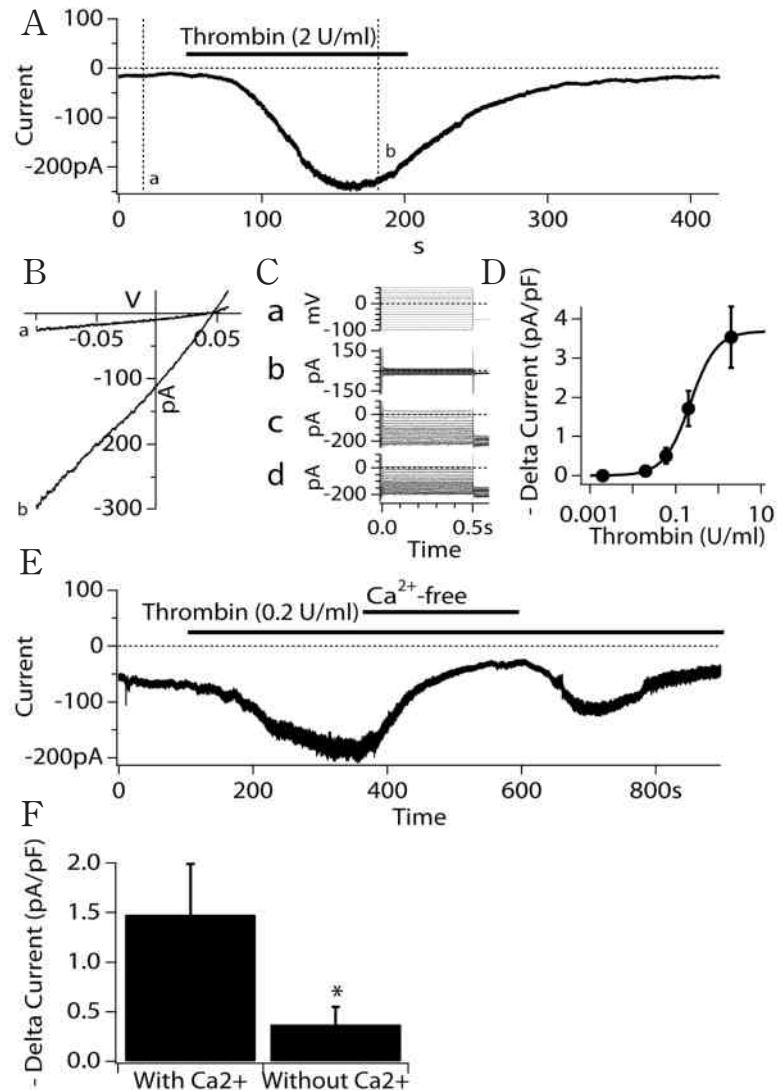


Fig. 2 Ca<sup>2+</sup> currents of HUVECs induced by thrombin

A, Whole-cell voltage-clamp recording of a HUVEC showed that thrombin (2 U/ml) transiently induced an inward current at a holding potential of -60 mV. A horizontal broken line indicates the zero current level. At timings indicated by vertical broken lines (a and b), a ramp pulse from -100 to +60 mV for 1 s was applied. B, The current-voltage relationships of the whole cell currents measured with the ramp pulse protocol in A. The current was measured before (a) and after (b) application of thrombin. The two curves crossed at +56 ± 10 mV (n = 6). C, The whole cell currents measured with rectangular voltage pulses. a. The voltage protocol. The membrane potential was changed from -100 mV to +60 mV for 500 ms with an increment of 10 mV every 3 s. The holding potential was -60 mV. The currents were measured before (b) and after (c) application of thrombin (2 U/ml). d shows the thrombin-induced difference currents. D, The relationship between concentrations of thrombin and the amplitude of currents induced by thrombin. The currents were measured at -60 mV. Symbols and bars indicate the mean and SE, respectively (n = 9-15 for each point). The curve indicates a fit of the data with the following Hill equation :

$$D = D_{\max} / (1 + (K_{0.5} / [\text{Thrombin}])^n),$$

where D is a thrombin-induced difference current density (- delta current density); D<sub>max</sub>, the maximum thrombin-induced difference current density; K<sub>0.5</sub>, the concentration of thrombin which gives the half-maximum activation; [Thrombin], the concentration of thrombin; and n, the Hill coefficient. The fitting yielded a D<sub>max</sub>, K<sub>0.5</sub> and n of 3.69 pA/pF, 0.22 U/ml and 1.42, respectively. E and F, The effect of removal of extracellular Ca<sup>2+</sup> on the membrane current induced by thrombin (0.2 U/ml). E shows a typical trace recorded at a holding potential at -60 mV. Substitution of extracellular Ca<sup>2+</sup> with equimolar N-methyl-D(-)-glucamine decreased the amplitude of thrombin-induced inward currents. F, The amplitude of thrombin (0.2 U/ml)-induced inward currents was significantly smaller in the absence than in the presence of extracellular Ca<sup>2+</sup>. Values are mean ± S.E. (n = 4). \*p < 0.05.



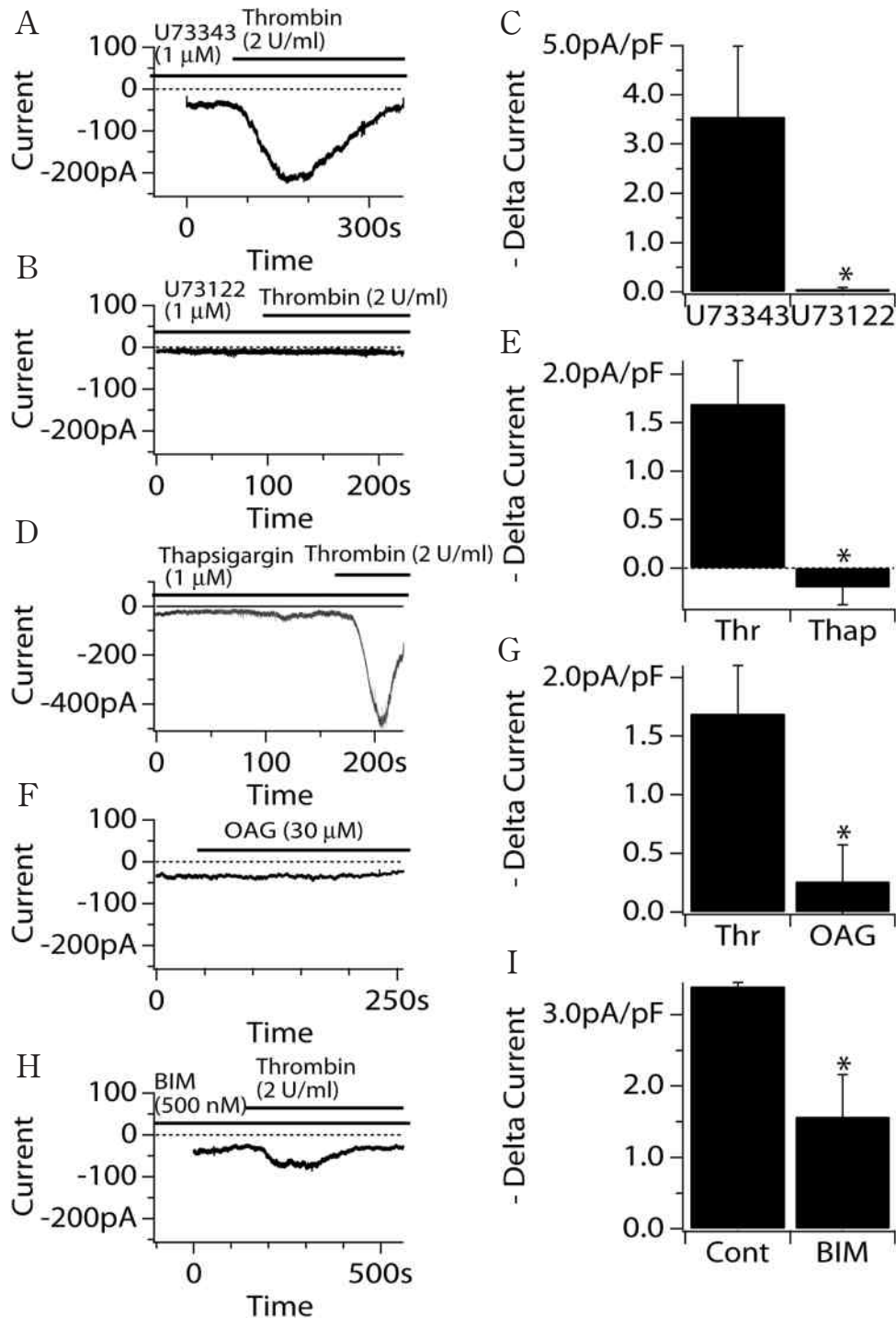


Fig. 3 The mechanism underlying the activation of inward currents by thrombin

A-B, Thrombin was applied to HUVECs pretreated with a selective inhibitor of phospholipase C, U73122 (1  $\mu$ M) (B) or its inactive analogue, U73343 (1  $\mu$ M) (A). U73343 or U73122 was applied to HUVECs  $\sim$ 3 min before application of thrombin. C. The summary of pooled data. U73122 inhibited thrombin-induced inward currents significantly more strongly than U73343. D, Application of an inhibitor of endoplasmic Ca<sup>2+</sup>-ATPase, thapsigargin (1  $\mu$ M) to the pipette solution failed to induce inward currents in HUVECs where thrombin (2 U/ml) did so. E, Thrombin (0.2 U/ml) induced inward currents, whereas intracellular thapsigargin (1  $\mu$ M) induced small outward rather than inward currents. F and G, An analogue of diacylglycerol, 1-oleoyl-2-acetyl-glycerol (OAG) (30  $\mu$ M) induced significantly smaller inward currents than thrombin (0.2 U/ml). H and I, A selective inhibitor of protein kinase C, bisindolylmaleimide I (BIM) (500 nM) significantly attenuated the activation of inward currents by thrombin (2 U/ml). BMI was applied to HUVECs  $\sim$ 1 hr before application of thrombin. Values are mean  $\pm$  S.E. (n = 4-6 for each experiment). \**p* < 0.05

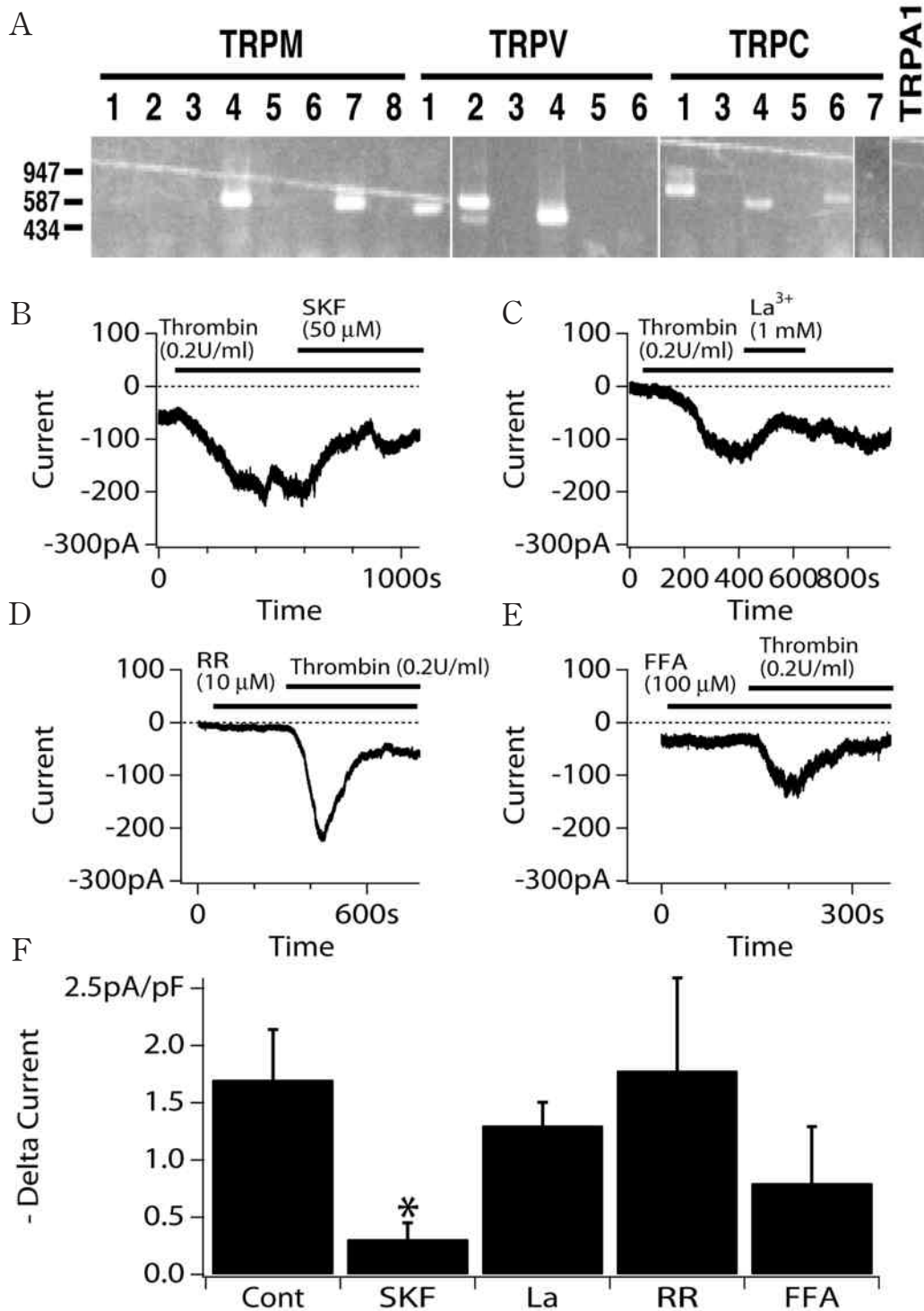


Fig. 4 Identification of the gene responsible for the thrombin-induced inward currents in HUVECs

A, As assessed with the RT-PCR technique, HUVECs expressed some members of transient receptor potential (TRP) families: TRPV1, 2, 4, TRPM4, 7, TRPC1, 4 and 6. B, SKF96365 (50  $\mu$ M) (a blocker of nonselective cation channels (NSCs)) applied to HUVECs in the presence of thrombin (0.2 U/ml) inhibited the thrombin-induced inward currents. C, La<sup>3+</sup> (1 mM) (a blocker of NSCs except for TRPC4 and TRPC5 channels) applied to HUVECs in the presence of thrombin (0.2 U/ml) slightly inhibited the thrombin-induced inward currents. D, ruthenium red (RR) (10  $\mu$ M) (a blocker of TRPV channels) applied  $\sim$ 3 min before application of thrombin did not prevent HUVECs from responding to thrombin (0.2 U/ml). E, Flufenamic acid (FFA) (100  $\mu$ M) (a blocker of TRPM2 and TRPM4 channels and an activator of TRPC6 channels) applied  $\sim$ 3 min before application of thrombin slightly inhibited thrombin (0.2 U/ml)-induced inward currents. F, The summary of pooled data. mean  $\pm$  S.E. (n = 4-6 for each experiment). \**p* < 0.05

blocker of non-selective cation channels (NSCs), SKF96365 (50  $\mu$ M) significantly inhibited the currents (Fig. 4B, F). However, La<sup>3+</sup> (1 mM), which also blocks NSCs except for TRPC4- or TRPC5-containing channels<sup>22)-24)</sup>, did not significantly inhibit the currents (Fig. 4C, F). SKF96365 and La<sup>3+</sup> exhibited a similar degree of blocking when applied before thrombin (data not shown), and these results are included in Fig. 4F. Pretreatment of HUVECs with a blocker of TRPV channels, ruthenium red (RR) (10  $\mu$ M) also failed to inhibit the currents (Fig. 4D, F). Flufenamic acid (FFA), a potent blocker of TRPM2 and 4 channels and an activator of TRPC6 channel tended to inhibit the currents (Fig. 4E), but this effect did not reach statistical significance (Fig. 4F). These results indicate that thrombin activated TRPC4-containing Ca<sup>2+</sup>-permeable channels in HUVECs (see Discussion).

#### IV Discussion

We have found that thrombin induced contraction of HUVECs in a [Ca<sup>2+</sup>]<sub>i</sub>-dependent manner, consistent with previous reports (Fig. 1B)<sup>4)19)20)</sup>. Because thrombin causes a Ca<sup>2+</sup> influx through the plasma membrane by activating PAR1/G<sub>q/11</sub>/PLC<sup>4)5)</sup>, we sought to characterize Ca<sup>2+</sup> currents induced by thrombin in detail.

Thrombin induced inward currents at -60 mV (Fig. 1). Under the present experimental condition, Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> currents were suppressed, and only Ca<sup>2+</sup> and Cs<sup>+</sup> carried membrane currents (see Materials and Methods). The calculated equilibration potentials for Ca<sup>2+</sup> and Cs<sup>+</sup> were +167 and -86 mV, respectively, whereas thrombin-induced currents reversed at +56  $\pm$  10 mV, indicating that thrombin activated Ca<sup>2+</sup>-permeable NSCs and that the thrombin-induced inward currents were mainly mediated by Ca<sup>2+</sup>. This is consistent with the finding that substitution of extracellular Ca<sup>2+</sup> with equimolar N-methyl-D(-)-glucamine suppressed the currents (Fig. 2E). Thrombin-induced Ca<sup>2+</sup> currents showed time- and voltage-independent kinetics and slightly outward rectification (Fig. 2). Groschner et al. found that histamine activated similar Ca<sup>2+</sup> currents in

human vascular ECs under the same experimental condition as ours<sup>17)</sup>. Thrombin induced the Ca<sup>2+</sup> currents less potently than the contraction in HUVECs (Fig. 1C, 2D). This is probably because thrombin induces contraction of ECs not only through a rise in [Ca<sup>2+</sup>]<sub>i</sub> but also through activation of Rho/Rho kinase<sup>5)6)</sup>.

A PLC inhibitor, U73122 but not its inactive analogue U73343 almost completely inhibited the thrombin-induced currents (Fig. 3A-C), indicating that thrombin activated the currents through PLC. PI hydrolysis activates Ca<sup>2+</sup> channels through IP<sub>3</sub>-induced depletion of Ca<sup>2+</sup> in ER (SOCs) and/or second messengers/kinases (SMOCs)<sup>5)7)</sup>. Thapsigargin, an inhibitor of Ca<sup>2+</sup>-ATPase in ER effectively causes depletion of Ca<sup>2+</sup> in ER and activates SOCs<sup>7)</sup>. Although Ahmed et al. showed  $\sim$ 1.5 pA/pF of thapsigargin-induced currents at -50 mV in HUVECs<sup>15)</sup>, we could not detect such a current irrespective of whether thapsigargin was applied to the pipette solution alone or in combination with IP<sub>3</sub> or whether thapsigargin was applied to the intra- or extra-cellular solution (Fig. 3D, E, data not shown). This discrepancy might have occurred because Ahmed et al. (2004) buffered intracellular Ca<sup>2+</sup> more strongly than we did: their pipette solution contained 10 mM of BAPTA without additional Ca<sup>2+</sup> (i.e. virtual absence of free Ca<sup>2+</sup>) whereas ours had 5 mM of EGTA, 2 mM of BAPTA and 1.74 mM of Ca<sup>2+</sup> (i.e. free Ca<sup>2+</sup> concentration of 44 nM). Whatever the reason is, thrombin clearly induced Ca<sup>2+</sup> currents in HUVECs in which thapsigargin failed to do so (Fig. 3D, E), indicating that thrombin did not activate the Ca<sup>2+</sup> channels through store depletion.

Although we examined whether second messengers produced by PI hydrolysis other than IP<sub>3</sub> activated the currents<sup>7)8)</sup>, a DAG analogue, OAG (30  $\mu$ M) or arachidonic acid (10  $\mu$ M) failed to activate Ca<sup>2+</sup> currents in HUVECs (Fig. 3F, G, and data not shown). On the other hand, a selective PKC inhibitor, BIM (500 nM) significantly inhibited thrombin-induced Ca<sup>2+</sup> currents (Fig. 3H, I). Thus, it is likely that thrombin activated the Ca<sup>2+</sup> channels

through PKC in HUVECs.

From these results, candidate genes for the thrombin-activated  $\text{Ca}^{2+}$  channels in HUVECs are TRPC, TRPV, TRPM, and TRPA<sup>8)</sup>. RT-PCR analysis indicated that TRPM4, 7, TRPV1, 2, 4, TRPC1, 4 and 6 were expressed in HUVECs (Fig. 4A). TRPM4 is unlikely to mediate thrombin-induced currents because TRPM4 channels are voltage-dependent,  $\text{Ca}^{2+}$ -impermeable and potentially blocked by FFA (Fig. 4E, F)<sup>25)</sup>. TRPM7 also cannot be the candidate gene for thrombin-activated  $\text{Ca}^{2+}$ -permeable channels because TRPM7 channels are strongly inhibited by intracellular MgATP that amply existed in the pipette solution used in this study<sup>26)</sup>. TRPV channels differ from thrombin-activated channels because a TRPV blocker, RR did not inhibit the currents (Fig. 4D, F)<sup>27)–29)</sup>. It is also unlikely that TRPC6 forms thrombin-activated  $\text{Ca}^{2+}$ -permeable channels because FFA activates rather than inhibits TRPC6 channels (Fig. 4E, F)<sup>30)</sup>. Thus, the remaining candidate genes for thrombin-activated  $\text{Ca}^{2+}$ -permeable channels are TRPC1 and/or TRPC4. It is known that TRPC1 and TRPC4 form either homomeric channels or heteromeric TRPC1/TRPC4 channels<sup>23)31)32)</sup>.

The thrombin-activated channels in HUVECs were similar to TRPC4-containing channels in their relative insensitivity to  $\text{La}^{3+}$  (Fig. 4C, F), the transient response to agonists (Fig. 2A), the insensitivity to OAG (Fig. 3F, G), and the permeability to  $\text{Ca}^{2+}$

(Fig. 2B, E)<sup>22)24)</sup>. However, it has been reported that TRPC4 forms SOCs in mouse aortic ECs<sup>12)</sup>, and that thrombin failed to induce a  $\text{Ca}^{2+}$  influx in lung vascular ECs of TRPC4 knockout mice<sup>13)</sup>. The discrepancy between these and the present studies might have arisen from species difference or again from different  $[\text{Ca}^{2+}]_i$ . Indeed, the activation of TRPC4-containing channels depends on  $[\text{Ca}^{2+}]_i$ <sup>22)24)</sup>. Thus, thrombin might activate TRPC4-containing channels through store depletion in the virtual absence of intracellular  $\text{Ca}^{2+}$  but directly through PKC in the presence of a certain amount of intracellular  $\text{Ca}^{2+}$  in ECs. However, further studies are clearly needed to determine the subunit composition of the thrombin-activated  $\text{Ca}^{2+}$  channels in HUVECs by using antisense oligonucleotides or small interference RNA.

To summarize, thrombin induced the contraction of HUVECs in a  $[\text{Ca}^{2+}]_i$ -dependent manner. It was likely that thrombin induced this response by activating  $\text{Ca}^{2+}$ -permeable SMOCs containing TRPC4 through PKC. In future, drugs specifically inhibiting TRPC4-containing channels might be developed and used to control fatal diseases such as sepsis where thrombin mediates the vicious cycle between inflammation and coagulation.

#### Acknowledgement

We are grateful to Ms. Reiko Sakai for secretarial assistance.

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(2010. 6. 21 received ; 2010. 9. 8 accepted)

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