Impairment of Store-Operated Ca$^{2+}$ Entry in TRPC4$^{-/-}$ Mice Interferes With Increase in Lung Microvascular Permeability

Chinnaswamy Tiruppathi, Marc Freichel,* Stephen M. Vogel,* Biman C. Paria, Dolly Mehta, Veit Flockerzi, Asrar B. Malik

Abstract—We investigated the possibility that the TRPC gene family of putative store-operated Ca$^{2+}$ entry channels contributes to the increase in microvascular endothelial permeability by prolonging the rise in intracellular Ca$^{2+}$ signaling. Studies were made in wild-type (wt) and TRPC4 knockout (TRPC4$^{-/-}$) mice and lung vascular endothelial cells (LECs) isolated from these animals. RT-PCR showed expression of TRPC1, TRPC3, TRPC4, and TRPC6 mRNA in wt LECs, but TRPC4 mRNA expression was not detected in TRPC4$^{-/-}$ LECs. We studied the response to thrombin because it is known to increase endothelial permeability by the activation of G protein–coupled proteinase-activated receptor-1 (PAR-1). In wt LECs, thrombin or PAR-1 agonist peptide (TFLLRNPNDK-NH$_2$) resulted in a prolonged Ca$^{2+}$ transient secondary to influx of Ca$^{2+}$. Ca$^{2+}$ influx activated by thrombin was blocked by La$^{3+}$ (1 µmol/L). In TRPC4$^{-/-}$ LECs, thrombin or TFLLRNPNDK-NH$_2$ produced a similar initial increase of intracellular Ca$^{2+}$ secondary to Ca$^{2+}$ store depletion, but Ca$^{2+}$ influx induced by these agonists was drastically reduced. The defect in Ca$^{2+}$ influx in TRPC4$^{-/-}$ endothelial cells was associated with lack of thrombin-induced actin-stress fiber formation and a reduced endothelial cell retraction response. In isolated-perfused mouse lungs, the PAR-1 agonist peptide increased microvessel filtration coefficient ($K_{f,c}$), a measure of vascular permeability, by a factor of 2.8 in wt and 1.4 in TRPC4$^{-/-}$; La$^{3+}$ (1 µmol/L) addition to wt lung perfusate reduced the agonist effect to that observed in TRPC4$^{-/-}$. These results show that TRPC4-dependent Ca$^{2+}$ entry in mouse LECs is a key determinant of increased microvascular permeability. (Circ Res. 2002;91:70-76.)

Key Words: mouse lung endothelial cells ■ thrombin-induced Ca$^{2+}$ influx ■ TRPC4 knockout ■ lung microvascular permeability

Endothelial cell activation induced by thrombin plays an important role in the pathogenesis of vascular injury and tissue inflammation.$^{1,2}$ In lungs, thrombin increases vascular permeability and tissue water content.$^{1,2}$ Thrombin mediates these effects by activation of G protein–coupled proteinase-activated receptor-1 (PAR-1) expressed on the endothelial cell surface. We have shown thrombin fails to increase lung microvascular permeability in PAR-1 null mice.$^3$ Increase in intracellular Ca$^{2+}$ signaling is critical in the mechanism of increased endothelial permeability after activation of PAR-1.$^{1,4-7}$ Thrombin-induced increase in intracellular Ca$^{2+}$ concentration in endothelial cells is dependent on both inositol 1,4,5-trisphosphate (IP$_3$)–induced release of stored Ca$^{2+}$ and Ca$^{2+}$ store depletion-mediated Ca$^{2+}$ influx.$^5$ We have shown recently that the prevention of Ca$^{2+}$ influx in endothelial cells markedly reduced the thrombin-induced increase in permeability.$^5,6$ The Ca$^{2+}$ influx in this model required the activation of Src tyrosine kinase in endothelial cells.$^8$ Ca$^{2+}$ influx secondary to store depletion, the capacitative Ca$^{2+}$ entry, is mediated by store-operated cation channels (SOCs).$^9,10$ SOCs can be activated by IP$_3$–induced store depletion and by inhibition of Ca$^{2+}$-ATPase (SERCA) with agents such as thapsigargin, cyclopiazonic acid, or dihenzohydroquinone (BHQ).$^9,10$

Studies have identified the mammalian homologues of Drosophila transient receptor potential (TRP) gene family of channels expressed in the plasma membrane of cells that function as SOCs.$^9,10$ TRP genes encode a superfamily of proteins with 6 transmembrane helices, which can be divided into at least 3 subfamilies; ie, TRPC, TRPV, and TRPM.$^{11}$ Members of the TRPC protein subfamily contain 700 to 1000 amino acids and 7 isoforms (TRPC1 to 7) are expressed in mammalian cells.$^9$ Members of the TRPC family are divalent cation selective and nonselective cation channels.$^9,10$ They are...

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activated by Ca\(^{2+}\) store depletion induced by the G\(_{i}\)-protein–phospholipase C system.\(^{9,10,12-15}\) Primary endothelial cells in culture express TRPC1, TRPC2, TRPC3, TRPC4, TRPC5, and TRPC6.\(^{5,10,15,16}\) Studies have demonstrated the expression of TRPC1, TRPC2, TRPC3, TRPC4, and TRPC6 gene mRNA in mouse aortic endothelial cells.\(^{10,17}\) Freichel et al.\(^{17}\) investigated store-operated Ca\(^{2+}\) current and Ca\(^{2+}\) entry in aortic endothelial cells (AECs) derived from TRPC4 knockout (TRPC4\(^{-/-}\)) mice. They showed that IP3/BHQ-activated SOC current was absent in these AECs compared with wild-type (wt) AECs.\(^{17}\) Also, acetylcholine-induced endothelium-dependent smooth muscle relaxation was blunted in vessels lacking TRPC4 compared with wt. Because thrombin is a potent proinflammatory mediator and mediates increased endothelial permeability dependent on intracellular Ca\(^{2+}\) signaling, in the present study, we have investigated permeability response in the TRPC4\(^{-/-}\) mice. We show that Ca\(^{2+}\) entry in response to thrombin was drastically reduced in lung endothelial cells obtained from TRPC4\(^{-/-}\) mice. Further, the thrombin-induced increase in lung microvascular permeability was reduced 50% compared with controls. Thus, TRPC4-dependent Ca\(^{2+}\) entry is a critical determinant of the thrombin-induced increase in vascular endothelial permeability.

**Materials and Methods**

**Materials**

Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, Ind). PAR-1-specific agonist peptide (TFLRRNPNDK-NH\(_2\)) was synthesized as C-terminal amide at University of Illinois Protein Sciences Facility, Urbana, Ill. The peptide purity was greater than 95%. Endothelial Growth Medium (EGM-2) was obtained from Clonetics. Hanks' balanced salt solution (HBSS), L-glutamine, phosphate buffered saline (PBS), and trypsin were obtained from Life Technologies, Inc. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories, Inc. Fura 2-AM was purchased from Molecular Probes.

**Targeted Disruption of TRPC4 Gene in Mice**

TRPC4 gene was disrupted in mice as described by Freichel et al.\(^{17}\) using the homologous recombination method. TRPC4 transcript expression was absent in these TRPC4\(^{-/-}\) mice. In the present study, the loss of TRPC4 mRNA was confirmed by RT-PCR (shown in Figure 1). TRPC4 transcript expression was detected in wt mice lung endothelial cells, but TRPC4 transcript expression was not detectable in TRPC4\(^{-/-}\) mice. For all studies wild-type 129SvJ or TRPC4 mice. In the present study, we have investigated permeability response in the TRPC4\(^{-/-}\) mice. We show that Ca\(^{2+}\) entry in response to thrombin was drastically reduced in lung endothelial cells obtained from TRPC4\(^{-/-}\) mice. Further, the thrombin-induced increase in lung microvascular permeability was reduced 50% compared with controls. Thus, TRPC4-dependent Ca\(^{2+}\) entry is a critical determinant of the thrombin-induced increase in vascular endothelial permeability.

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**Lung Endothelial Cell Isolation and Culture**

According to an approved protocol of the University of Illinois Animal Care Committee, female 129SvJ (wild-type) or TRPC4\(^{-/-}\) mice weighing 30 to 35 g were anesthetized with 3% halothane, and heparin (50 U/mouse) was injected into the jugular vein. All animals used in this study were obtained from colonies located at the Institut fur Pharmakologie Universitat des Saarlandes, und Toxikologie, Homburg, Germany. The abdominal cavity was opened and the pulmonary artery was cannulated. Krebs-Henseleit solution supplemented with bovine serum albumin (5 g/100 mL) was infused to remove blood. Lungs were removed and placed inside a culture hood. Lung tissue slices from 3 mice were prepared, washed, and suspended in HBSS. Excess HBSS was aspirated, and the tissue slices were minced and transferred to a 15-mL sterile tube. The minced tissues were suspended in 10 mL of collagenase A (1.0 mg/mL in HBSS) and digested for 60 minutes at 37°C with gentle shaking. The released cells were centrifuged at 200g for 10 minutes. The pellet was suspended in 10 mL suspension buffer (Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS containing 0.5 g/100 mL bovine serum albumin, 2 mmol/L EDTA, and 4.5 mg/mL d-glucose), and filtered through 200-μm mesh filter. The filtered cell suspension was centrifuged at 200g for 10 minutes and suspended in 10 mL of suspension buffer. To this cell suspension, 1.5 μg/mL anti-mouse PECAM-1 antibody (BD Pharmingen) was added and incubated at 4°C for 30 minutes with gentle shaking. The cell suspension was centrifuged to remove unbound antibody and washed once with suspension buffer. The washed cells were incubated with Dynabeads M-450 (Sheep anti-rat IgG) for 30 minutes at 4°C. After this incubation period, the cell suspension was attached to a magnetic column and the unbound cells were aspirated. Cells bound to the magnetic beads were washed once with HBSS and digested with trypsin for 3 minutes at 22°C. The cells released from the magnetic beads were separated, washed, and suspended in growth medium (EGM-2 supplemented with 10% FBS). The cell suspension was plated on Matrigel (BD Bioscience)-coated 35-mm culture dish and allowed to grow to confluence for 10 days. Cells were then harvested from the Matrigel plates by disperse (BD Bioscience) for 60 to 90 minutes. Cells were washed after disperse treatment once with growth medium and plated on 0.1% gelatin coated culture dish. Cells passed between 3 and 4 times were used in experiments. Endothelial cells were characterized by their cobblestone morphology, platelet/endothelial cell adhesion molecule-1 (PECAM-1 or CD31) expression, and Dil-Ac-LDL uptake.

**Cytosolic Ca\(^{2+}\)**

Thrombin-induced increase in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) was measured using the Ca\(^{2+}\)-sensitive fluorescent dye fura 2.\(^{18}\) Cells were grown to confluence on 0.1% gelatin-coated 22-mm glass cover slips and then washed 2 times with HBSS and loaded with fura 2-AM (5 mg/mL in HBSS) and digested for 60 minutes at 37°C with gentle shaking. The released cells were centrifuged at 200g for 10 minutes. The pellet was suspended in 10 mL suspension buffer (Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS containing 0.5 g/100 mL bovine serum albumin, 2 mmol/L EDTA, and 4.5 mg/mL d-glucose), and filtered through 200-μm mesh filter. The filtered cell suspension was centrifuged at 200g for 10 minutes and suspended in 10 mL of suspension buffer. To this cell suspension, 1.5 μg/mL anti-mouse PECAM-1 antibody (BD Pharmingen) was added and incubated at 4°C for 30 minutes with gentle shaking. The cell suspension was centrifuged to remove unbound antibody and washed once with suspension buffer. The washed cells were incubated with Dynabeads M-450 (Sheep anti-rat IgG) for 30 minutes at 4°C. After this incubation period, the cell suspension was attached to a magnetic column and the unbound cells were aspirated. Cells bound to the magnetic beads were washed once with HBSS and digested with trypsin for 3 minutes at 22°C. The cells released from the magnetic beads were separated, washed, and suspended in growth medium (EGM-2 supplemented with 10% FBS). The cell suspension was plated on Matrigel (BD Bioscience)-coated 35-mm culture dish and allowed to grow to confluence for 10 days. Cells were then harvested from the Matrigel plates by disperse (BD Bioscience) for 60 to 90 minutes. Cells were washed after disperse treatment once with growth medium and plated on 0.1% gelatin coated culture dish. Cells passed between 3 and 4 times were used in experiments. Endothelial cells were characterized by their cobblestone morphology, platelet/endothelial cell adhesion molecule-1 (PECAM-1 or CD31) expression, and Dil-Ac-LDL uptake.
µmol/L) for 1 hour at 37°C. After the loading, cells were washed twice with HBSS. Cells were imaged using an Attoclor Ratio Vision digital fluorescence microscopy system (Atto Instruments) equipped with a Zeiss Axiovert S100 inverted microscope and F-Fluar 40×, 1.3 NA oil immersion objective. Regions of interest in individual cells were marked and excited at 334 and 380 nm with emission at 520 nm at 5-second intervals. At the end of each experiment, ionomycin (10 µmol/L) was added to obtain fluorescence of Ca²⁺ saturated fura 2 (2 high [Ca²⁺]) and EGTA (10 mmol/L) to obtain fluorescence of free fura 2 (2 low [Ca²⁺]), [Ca²⁺]i, was calculated based on a dissociation constant (Kd) of 225 mmol/L with a 2.5 point fit curve.

Transendothelial Cell Electrical Resistance
Thrombin-induced endothelial cell retraction response was measured as described. Briefly, endothelial cells were seeded on gelatin-coated gold electrodes (5.0 × 10⁻⁴ cm²) and grown to confluence. The small electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier. Constant current 1 µA was supplied by a 1 V, 4000 Hz AC signal connected serially to 1 MΩ resistor between the small electrode and larger counter electrode. Voltage between small electrode and large electrode was monitored by lock-in amplifier, stored, and processed by a PC. The computer controlled the output of the amplifier and switched the measurement to different electrodes in the course of each experiment. Before the experiment, confluent endothelial monolayer was kept in 1% FBS containing medium for 2 hours and then thrombin-induced change in resistance of endothelial monolayer was measured. Data are presented in resistance normalized to its value at time 0 as described.6,19

Distribution of Actin Stress Fibers
Endothelial cells were grown to confluence on gelatin-coated glass cover slips. Cells were incubated with 1% FBS supplemented growth medium for 2 hours before exposure with thrombin (5 µmol/L) for different time intervals. Cells were washed and fixed for 15 minutes with 2% paraformaldehyde in HBSS containing 10 mmol/L HEPES buffer (pH 7.4) at room temperature. Cells were thoroughly rinsed 3 times with HBSS, and then permeabilized with 0.1% Triton X-100 for 30 minutes. After rinsing, cells were incubated with 1% bovine serum albumin in HBSS followed by incubation with Alexa-488 labeled phalloidin plus DAPI to stain actin filaments and nuclei, respectively. Cells were then washed 3 times with HBSS, mounted with Prolong Antifade mounting medium and viewed with 60× objective in Zeiss LSM 510 confocal microscope.

Perfused Mouse Lung Preparation
According to approved protocol of University of Illinois Animal Care Committee, 129SvJ mice weighing 30 to 35 g were anesthetized using 2.5% halothane. Anesthesia was maintained with 1.5% halothane delivered through a nose cone. The trachea was cannulated with a stainless steel tube for constant positive pressure ventilation with the anesthetic gas mixture. Heparin (50 U) was injected into the right jugular vein to prevent blood clotting. A thoracotomy was performed to expose the heart and lungs. An incision was made in the right ventricle at the base of the pulmonary artery for introducing pulmonary arterial cannula. A polyethylene cannula (PE 60; Becton Dickinson) was advanced into the pulmonary artery and secured by a suture. For drainage of pulmonary venous effluent, a catheter (made of a 3-mm length of PE-50 tubing) was inserted through the left atrium and secured. The lung preparation was perfused in situ using a peristaltic pump. The anesthetic gas flow was terminated when perfusion was begun, and ventilation was continued with room air. The heart and the exanguinated lungs were rapidly excised and transferred en bloc to a perfusion apparatus, where lung preparations were suspended from an electronic beam balance. The isolated lung preparations were ventilated at 186 breaths/min and end-expiratory pressure of 2 cm H₂O, and perfused at constant flow (2 mL/min), temperature (37°C), and venous pressure (+1 cm H₂O) with a modified Krebs-Henseleit solution (composition in mmol/L: NaCl 118; KCl 4.7; CaCl₂ 1.0; MgCl₂ 0.5; HEPES sodium 4.43; HEPES free acid 5.57; NaHCO₃ 3; glucose 11; EDTA 0.025; pH 7.4) supplemented with 3.0 g/100 mL of bovine serum albumin (BSA, Fraction V, 99% pure and endotoxin-free; Sigma-Aldrich). Pulmonary arterial pressure was monitored using a Gould pressure transducer (Model P23ID; Gould Instruments Inc). Lung wet weight was electronically nulled when the tissue was mounted, and subsequent weight changes due to gain or loss of fluid from the lung were recorded. Lung weight and arterial pressure recordings were displayed on a computer video monitor with the aid of amplifiers (Model CP122; Astro-Med), an analog-to-digital converter (µCDAS-8PGA board; Keithley Metrabyte), and commercial software for acquisition and logging of data (Notebook Pro for Windows; Labtech). All lung preparations underwent a 20-minute equilibration perfusion. Lung preparations were discarded if they were not isogravimetric at the end of the equilibration period.

Capillary Filtration Coefficient Measurement
Pulmonary capillary filtration coefficient (Kf,c) was measured to determine microvascular permeability to liquid. Briefly, in the isogravimetric condition, outflow pressure was rapidly elevated by +5 cm H₂O for 4 minutes. The ensuing changes in lung wet weight reflect a rapid rise in vascular volume followed by a slower phase of net fluid extravasation. A double exponential function was fitted to the data (with the aid of a Lotus 1-2-3 macro); Kf,c was calculated from initial slope of slower exponential component normalized to the outflow pressure change and to lung wet weight. Lung wet weight was calculated from the dry weight of the lung (determined at the conclusion of each experiment) multiplied by the wet:dry weight ratio as described. This ratio in 6 freshly isolated (nonperfused) mouse lungs was 6.04±0.4. Preliminary experiments in normal control mouse lung preparations determined that, in the same lung preparation, 3 successive measurements of Kf,c separated by an interval of 20 minutes were identical.

Effects of PAR-1 Agonist Peptide on Kf,c
In experiments testing the effects of PAR-1 agonist peptide on Kf,c, the perfusion rate was reduced to 1.8 mL/min and perfusing liquid, containing either PAR 1 agonist peptide or no drug (baseline condition), was infused via a side-port at a rate of 0.2 mL/min. Kf,c measurements were made at baseline, and after a 20-minute exposure to PAR-1 agonist peptide (TFLRNPNDK-NH₂); in some preparations, a third Kf,c measurement was made after a 20-minute drug washout period. The values are expressed as the ratio of experimental-to-basal Kf,c values in the same lung preparation.

Statistical Analysis
Statistical comparisons were made using the 2-tailed Student’s t test. Values are reported as mean±SEM. Values were considered significant at P<0.05.

Results
Mouse Lung Endothelial Characterization and Validation of TRPC4⁻/⁻ Mice
We isolated LECs from wt and TRPC4⁻/⁻ mice. TRPC4 gene expression was absent in TRPC4⁻/⁻ mice (Figure 1). TRPC4 was markedly expressed in wt LECs compared with the other TRPC isoforms (Figure 1). TRPC2 and TRPC5 transcripts were not detectable, whereas TRPC1, TRPC3, and TRPC6 were expressed in wt and TRPC4⁻/⁻ mice.

Impaired Ca²⁺ Influx in TRPC4⁻/⁻ Endothelial Cells in Response to Thrombin
We measured the thrombin-induced rise in intracellular Ca²⁺ ([Ca²⁺]i) in mouse LECs isolated from wt or TRPC4⁻/⁻ mice. Fura 2–loaded wt LECs were challenged with either thrombin (5 U/mL) or the PAR-1–specific agonist peptide (5 µmol/L).
In the presence of extracellular Ca\textsuperscript{2+} (1.26 mmol/L), thrombin produced an increase in [Ca\textsuperscript{2+}], (peak value 750±55 mmol/L) followed by a gradual decline to baseline value (Figure 2A). PAR-1 agonist peptide produced a similar response in wt LECs (Table 1). In TRPC4\textsuperscript{−/−} LECs, thrombin-induced increase in initial peak [Ca\textsuperscript{2+}] was similar to wt LECs, but the sustained phase was considerably reduced in TRPC4\textsuperscript{−/−} (Figure 2A and Table 1). We compared the increase in [Ca\textsuperscript{2+}], in wt and TRPC4\textsuperscript{−/−} mice LECs by integrating the area under the Ca\textsuperscript{2+} transient for a 3-minute period after exposure to thrombin or PAR-1 agonist peptide. We observed a \(\approx\)55% reduction in the increase in [Ca\textsuperscript{2+}], in TRPC4\textsuperscript{−/−} LECs compared with wt LECs (Figure 2A and Table 1), regardless of the agonist used.

To further discriminate between increase of [Ca\textsuperscript{2+}], due to Ca\textsuperscript{2+} release from internal stores or Ca\textsuperscript{2+} entry, we first depleted the endoplasmic Ca\textsuperscript{2+} store with thrombin in the absence of extracellular Ca\textsuperscript{2+}, and then we reapplied Ca\textsuperscript{2+} to assess Ca\textsuperscript{2+} influx through the channels. In this experiment, thrombin produced a similar increase in peak [Ca\textsuperscript{2+}], in both wt and TRPC4\textsuperscript{−/−} cells (Figure 2B). In wt, replenishing Ca\textsuperscript{2+} (1.5 mmol/L) in the extracellular medium after store depletion caused a prolonged Ca\textsuperscript{2+} influx, which was completely blocked by the addition of La\textsuperscript{3+} (1 μmol/L) (Figure 2B). In contrast to wt cells, replenishing extracellular Ca\textsuperscript{2+} after store depletion with thrombin caused almost no significant Ca\textsuperscript{2+} influx in TRPC4\textsuperscript{−/−} LECs (Figure 2B). We compared the thrombin-induced Ca\textsuperscript{2+} influx between these two cell types by integrating the area over 4 minutes after Ca\textsuperscript{2+} reapplication. We observed a 75% to 80% reduction in Ca\textsuperscript{2+} influx in TRPC4\textsuperscript{−/−} LECs compared with wt LECs.

To address whether the reduced Ca\textsuperscript{2+} influx in TRPC4\textsuperscript{−/−} LECs was not secondary to an incomplete Ca\textsuperscript{2+} store depletion induced by thrombin, we measured the effects of ATP after challenging these cells with thrombin in the absence of extracellular Ca\textsuperscript{2+} (Figure 2C). In these experiments, cells were first challenged with thrombin, and after recovery to the baseline, the cells were challenged with ATP. Addition of ATP did not significantly increase [Ca\textsuperscript{2+}], in either wt or TRPC4\textsuperscript{−/−} mice LECs, suggesting that thrombin addition had depleted the stores. We also confirmed that ATP is as potent an agonist as thrombin in depleting the intracellular Ca\textsuperscript{2+} stores in LECs obtained from wt and TRPC4\textsuperscript{−/−} endothelial cells (data not shown).
measured actin-stress fiber formation both in wt and TRPC4−/− LECs (Figure 3A). Exposure of thrombin caused actin-stress fiber formation in wt but not in TRPC4−/− LECs (Figure 3A). We observed a characteristic pattern of peripheral actin staining in TRPC4−/− LECs (Figure 3A).

**Impaired Thrombin-Induced Endothelial Cell Retraction in TRPC4−/− Endothelial Cells**

Because store depletion–activated Ca2+ influx and actin-stress fiber formation were prevented in TRPC4−/− LECs, we measured thrombin-induced changes in transendothelial monolayer electrical resistance to assess endothelial cell retraction (ie, cell shape change), a prerequisite for the increase in endothelial permeability.20,21 Thrombin exposure to wt LEC monolayer produced a maximum decrease in transendothelial monolayer electrical resistance of ~35%, and the value returned to normal 1 hour after thrombin challenge (Figure 3B and Table 2). By contrast, exposure of thrombin to TRPC4−/− LECs produced only a ~16% decrease and the decrease in resistance returned to baseline within only 20 minutes after thrombin (Figure 3B and Table 2).

**Reduction in Thrombin-Induced Increase in Lung Vascular Permeability in TRPC4−/− Mice**

We measured microvessel liquid permeability in isolated lung preparations from wt and TRPC4−/− mice. Basal values of $K_{f,c}$ did not significantly differ between wt and TRPC4−/− lungs (mean±SD; n=5 to 6; 7×10−3±0.4×10−3 versus 6.3±0.6×10−3 mL/min per cm H2O/g). In wt mice, PAR-1 agonist peptide (5 μmol/L) produced a 3-fold increase in lung $K_{f,c}$ within 15 minutes (Figure 4). The factor of increase was only 1.5-fold in lungs from TRPC4−/− mice (Figure 4). After a 15-minute washout period to remove PAR-1 agonist, the changes in permeability were reversed both in wt and TRPC4−/− mice (Figure 4). Because we showed that La3+ (1 μmol/L) inhibited thrombin-induced Ca2+ influx, we predicted that La3+ should reduce PAR-1–mediated changes in vascular permeability in wt lung preparations to the same extent as deletion of TRPC4 gene. This was indeed the case because in wt mice the PAR-1 agonist peptide (5 μmol/L) produced only a 1.4-fold increase of $K_{f,c}$ in the presence of 1 μmol/L La3+ (see Figure 4).

**Discussion**

Activation of nonexcitable cells with $G_q$-linked receptor agonists results in release of Ca2+ from intracellular stores
followed by a transmembrane Ca\(^{2+}\) influx.\(^9,10,12–14\) The Ca\(^{2+}\) influx accounts for the second phase of the increased [Ca\(^{2+}\)]\(_i\).\(^9,10,12–14\) In endothelial cells, the sustained Ca\(^{2+}\) influx from the extracellular medium contributes to the increase of the cytosolic Ca\(^{2+}\) concentration, which is necessary for the release of vasoactive substances such as nitric oxide or prostaglandins,\(^9,10\) and may be involved in the mechanism of increased endothelial permeability.\(^4,5,7,14\) Several members of the mammalian TRPC gene family channels have been shown to function as store-operated cation channels.\(^9,10,12–14\) These channels are expressed in endothelial cells.\(^9,10,12–14\) Freichel et al.\(^17\) have recently developed TRPC4 knockout (TRPC4\(~/~\)) mice and investigated store-operated Ca\(^{2+}\) entry in aortic endothelial cells (AECs). In TRPC4\(~/~\)-AECs, IP\(_3\)/BHQ-activated SOC current was absent, and also the acetylcholine-induced endothelium-dependent vasorelaxation was reduced compared with wt.\(^17\)

In the present study, we showed that TRPC4 mRNA transcript was absent in TRPC4\(~/~\) mouse LECs, whereas marked expression was evident in wt LECs. Disruption of the TRPC4 gene in mice did not alter the expression profile of the other predominant TRPC isoforms in LECs (TRPC1, TRPC3, and TRPC6). We showed that thrombin produced a prolonged increase of the [Ca\(^{2+}\)]\(_i\) in wt LECs with the TRPC4\(~/~\) LECs. Further, Ca\(^{2+}\) entry after thrombin-induced Ca\(^{2+}\) store depletion caused a prolonged Ca\(^{2+}\) influx, which was inhibited by 1 \(\mu\)mol/L La\(^{3+}\) in wt LECs. In contrast, thrombin-induced Ca\(^{2+}\) influx was nearly absent in TRPC4\(~/~\) LECs. Previous studies showed that TRPC4 is highly specific in mediating Ca\(^{2+}\) entry in mouse endothelial cells.\(^17\) Our results demonstrate that TRPC4 is also essential for thrombin-induced Ca\(^{2+}\) entry.

We addressed whether the rise in [Ca\(^{2+}\)]\(_i\), secondary to activation of TRPC4 is involved in the mechanism of actin cytoskeletal reorganization in endothelial cells induced by thrombin.\(^20,21\) In wt LECs, we observed thrombin-induced actin stress fiber formation, and this was absent in TRPC4\(~/~\) LECs. These results show that TRPC4 activation-mediated Ca\(^{2+}\) influx is a critical determinant of the thrombin-induced cytoskeletal reorganization in mouse LECs. As a functional measure of cytoskeletal reorganization-dependent interendothelial gap formation, we quantified transendothelial electrical resistance in confluent monolayers of mouse endothelium. The results showed that cytoskeletal reorganization is necessary for gap formation (and hence increased endothelial permeability), because the cells from TRPC4\(~/~\) mice showed a smaller change in their transendothelial electrical resistance in response to thrombin.

We also investigated the possible role of TRPC4 channel activation in the thrombin-induced increase in the microvascular permeability in the intact lung (ie, by measurement of K\(_{f,c}\)). By comparing PAR-1 mediated increase in K\(_{f,c}\) in wt and TRPC4\(~/~\), we showed that TRPC4 expression is required for a substantial component of the increase in microvascular permeability in intact lungs. Thus, in the TRPC4\(~/~\) group, the agonist-induced permeability was reduced from wt value of \(\approx 3.0\) to 1.5. The involvement of TRPC4 channels in PAR-1-mediated permeability changes was supported by our finding of a marked inhibitory effect of La\(^{3+}\) (1 \(\mu\)mol/L) in wt lung preparations. The residual effect of agonist peptide seen in the TRPC4 knockout mice probably reflects the fact that agonists can still produce a rise in [Ca\(^{2+}\)]\(_i\) via other ion channels, albeit the response was attenuated and briefer owing to the lack of competent TRPC4 channels.

Our results suggest the following sequence of events mediating increase in lung vascular permeability after PAR-1 activation: (1) G\(_q\)-linked PLC activation and increase in IP\(_3\); (2) intracellular Ca\(^{2+}\) store depletion and Ca\(^{2+}\) influx determined by TRPC4; and (3) Ca\(^{2+}\)-dependent cytoskeletal reorganization and interendothelial gap formation.

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**References**

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