RhoA Interaction with Inositol 1,4,5-Trisphosphate Receptor and Transient Receptor Potential Channel-1 Regulates Ca\(^{2+}\) Entry

ROLE IN SIGNALING INCREASED ENDOTHELIAL PERMEABILITY*

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We tested the hypothesis that RhoA, a monomeric GTP-binding protein, induces association of inositol trisphosphate receptor (IP\(_3\)R) with transient receptor potential channel (TRPC1), and thereby activates store depletion-induced Ca\(^{2+}\) entry in endothelial cells. We showed that RhoA upon activation with thrombin associated with both IP\(_3\)R and TRPC1. Thrombin also induced translocation of a complex consisting of Rho, IP\(_3\)R, and TRPC1 to the plasma membrane. IP\(_3\)R and TRPC1 translocation and association required Rho activation because the response was not seen in C3 transfection (C3)-treated cells. Rho function inhibition using Rho dominant-negative mutant or C3 dampened Ca\(^{2+}\) entry regardless of whether Ca\(^{2+}\) stores were emptied by thrombin, thapsigargin, or inositol trisphosphate. Rho-induced association of IP\(_3\)R with TRPC1 was dependent on actin filament polymerization because latrunculin (which inhibits actin polymerization) prevented both the association and Ca\(^{2+}\) entry. We also showed that thrombin produced a sustained Rho-dependent increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in endothelial cells overexpressing TRPC1. We further showed that Rho-activated Ca\(^{2+}\) entry via TRPC1 is important in the mechanism of the thrombin-induced increase in endothelial permeability. In summary, Rho activation signals interaction of IP\(_3\)R with TRPC1 at the plasma membrane of endothelial cells, and triggers Ca\(^{2+}\) entry following store depletion and the resultant increase in endothelial permeability.

The increase in cytosolic calcium concentration ([Ca\(^{2+}\)]\(_i\)) activated by depletion of Ca\(^{2+}\) stores is required for signaling multiple processes in non-excitable cells (1). The increase in [Ca\(^{2+}\)]\(_i\), regulates responses ranging from tension development through activation of actin and myosin motors to modulation of cell-cell and cell-extracellular matrix adhesive forces (2–4). For example in endothelial cells, increase in [Ca\(^{2+}\)]\(_i\), triggered by depletion of Ca\(^{2+}\) stores is essential for the increase in endothelial permeability induced by thrombin (5, 6). [Ca\(^{2+}\)]\(_i\), increases rapidly after the release of Ca\(^{2+}\) stores in the endoplasmic reticulum (ER). This is followed by a more sustained response secondary to Ca\(^{2+}\) entry through plasmalemmal channels (1, 7–11). The initial release of cytosolic Ca\(^{2+}\) occurs following the heterotrimeric G protein-coupled receptor-mediated generation of inositol 1,4,5-trisphosphate (IP\(_3\)), which in turn binds to inositol 1,4,5-trisphosphate receptor (IP\(_3\)R) on ER, and signals Ca\(^{2+}\) release from ER stores. Depletion of ER Ca\(^{2+}\) induces activation of store-operated channels (SOC), resulting in Ca\(^{2+}\) entry and replenishment of ER stores (8, 11–13). Molecular cloning and functional expression studies showed that the Drosophila transient receptor potential channel (TRPC) family of proteins are prominent plasmalemmal Ca\(^{2+}\) channels in non-excitable cells (10, 12–14). These channels are activated in response to stimulation of G protein-coupled receptors (1, 10, 12, 13, 15, 16). TRPC1, -2, -4, and -5 are likely candidates for endogenous SOCs because they are activated by Ca\(^{2+}\) store depletion (1, 12, 14, 17). TRPC1 activates Ca\(^{2+}\) entry upon store depletion in a variety of cell types including endothelial cells (12, 18–22). Despite their involvement in the mechanism of Ca\(^{2+}\) entry, the signals by which store depletion activate TRPC1-induced Ca\(^{2+}\) entry are unclear.

It is believed that interaction of IP\(_3\)R with TRPC is required for activation of store depletion-induced Ca\(^{2+}\) entry (1, 7, 8, 13, 16, 23–25). Interaction may be in the form of chemical or conformation coupling. Both models help to explain activation of Ca\(^{2+}\) entry through TRP channels (1, 7, 8, 13, 16). In the chemical-coupling model, Ca\(^{2+}\) store depletion induces release of diffusible messenger(s) from ER that activate SOC (26–29); however, identity of these mediator(s) is not known. In the conformational coupling model, store depletion causes a IP\(_3\)R conformational change that enables it to interact with TRPC, thereby resulting in channel opening (7, 8, 13, 16). If a change in IP\(_3\)R conformation is the sole requirement for its coupling to TRPC and its activation, Ca\(^{2+}\) entry through these channels should be essentially complete upon store depletion. However, patch clamp and whole cell fluorescence studies showed that complete activation of Ca\(^{2+}\) entry through SOC was achieved several tens to hundreds seconds after Ca\(^{2+}\) store depletion (1, 23, 30–34). Furthermore, this model fails to explain Ca\(^{2+}\) entry by TRPC1 as these channels have been shown to be localized.

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‡ The abbreviations used are: [Ca\(^{2+}\)]\(_i\), intracellular calcium concentration; IP\(_3\)R, inositol trisphosphate receptor; IP\(_3\), inositol trisphosphate; TRPC, transient receptor potential channel; ER, endoplasmic reticulum; SOC, store-operated Ca\(^{2+}\) channel; PM, plasma membrane; HPAEC, human pulmonary arterial endothelial cells; HIMEC, human microvessel endothelial cells; HBSS, Hanks’ balance salt solution; C3, C3 transferase; Abs, antibodies; RBD, Rho-kinase-Rho binding domain; GFP, green fluorescent protein; FBS, fetal bovine serum; GST, glutathione S-transferase; DAPI, 4,6-diamidino-2-phenylindole; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid.

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within intracellular membranes (35). These inconsistencies suggest that coupling involves other events such as translocation and docking of IP$_3$R and TRPC1 at the plasma membrane (1, 23, 24, 34). Thus, it is possible that proteins capable of translocating IP$_3$R and TRPC1 to the membrane induce association of components of the complex and trigger Ca$^{2+}$ entry. The monomeric GTP-binding proteins regulating SOC activation (24, 36–39) may be important in regulating the interaction of IP$_3$R and TRPC1, and thus in activation of Ca$^{2+}$ entry.

Activation of monomeric Rho family GTP-binding proteins, Rho, Rac, and Cdc42, depends on the GTP/GDP exchange cycle (40–42). These proteins can traffic from cytosol to plasma membrane on activation and they also regulate vesicle trafficking (40–42). We and others have shown that thrombin rapidly induces activation of RhoA (but not Rac or Cdc42) in endothelial cells (43–45). In the present study, we addressed the possibility that RhoA induces the interaction of IP$_3$R and TRPC1 required for activation of store depletion-induced Ca$^{2+}$ entry. We observed that RhoA associated with IP$_3$R and TRPC1 at the plasma membrane after thrombin stimulation of endothelial cells. Plasma membrane translocation of IP$_3$R and TRPC1 and store depletion-induced Ca$^{2+}$ entry were dependent on Rho because inhibition of Rho activation prevented these responses. We also showed that Rho-activated Ca$^{2+}$ entry has an important functional consequence in mediating the thornbin-induced increase in transendothelial permeability.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human e-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Human pulmonary arterial endothelial cells (HPAEC) and endothelial growth medium were obtained from Clonetics (San Diego, CA). Human microvascular endothelial cells (HMEC), a human dermal microvascular endothelial cell line, were obtained from Dr. Edwin W. Ades (National Center for Infectious Diseases, Center for Disease Control, Atlanta, GA). LipofectAMINE, Opti-MEM I, trypsin, Hanks’ balanced salt solution (HBSS), and pSRed1-N1 (dsRed) plasmid cDNAs were obtained from Invitrogen. MEM I, trypsin, Hanks’ balanced salt solution (HBSS), and pSRed1-N1 (dsRed) plasmid cDNAs were obtained from Invitrogen.

**Cell Transfection—**HMEC were grown to 50% confluence were incubated with LipofectAMINE-TRPC1 cDNA to determine the role of Rho in modulating TRPC1-induced Ca$^{2+}$ entry.

**Transendothelial Resistance Measurement—**The time course of endothelial cell retraction in real time, a measure of increased endothelial permeability, was determined as described (48). HPAEC cells (200,000 cells) grown to confluence on gelatin-coated small gold electrode (4.9 × 10$^{-4}$ cm$^2$) were left untreated or treated with C3, as reported previously (45). Cells were then stimulated with thrombin to measure changes in electrical resistance of endothelial monolayer in real time. The small electrode and larger counter electrode were connected to a phase-sensitive lock-in amplifier. A constant current of 1 μA was supplied by a lock-in amplifier with 0.1 Hz alternating current connected to a 100 kHz alternating current source. The voltage between the small electrode and large electrode was monitored by lock-in amplifier, stored, and processed on a computer. Data are presented as change in resistance (in-phase) portion of impedance normalized to its initial value at time.

**Ca$^{2+}$ Measurement—**Ca$^{2+}$ was measured using the fura-2-AM or fluo-3-AM fluorescent dyes fura-2-AM or fluo-3-AM. For loading cells with fura-2-AM, cells grown on 25-mm coverslips were incubated with 3 μM fura for 15 min at 37 °C. Cells were then washed 2 times with HBSS and imaged using an Attofluor Ratio Vision digital fluorescence microscopy system (Atto Instruments, Rockville, MD) equipped with a Zeiss Axioscop2 inverted microscope and a CoolSNAP ×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. The 334/380 excitation ratio that increased as a function of [Ca$^{2+}$], was captured at 5-s intervals.

For loading cells with fluo-3-AM, cells were grown on 4-well Lab-Tek chambers and incubated with 3 μM fluo-3-AM for 15 min at 37 °C. Cells were then washed 2× with HBSS and viewed using a LSM510 Zeiss confocal microscope with 63X 1.2 NA water immersion objective. A series of time lapse confocal images were acquired at 12.5-s intervals following thrombin stimulation using 488 and 568 nm excitation laser lines for fluo3 and dsRed fluorescence, respectively. We used the Zeiss AxioVision Imaging software to acquire these images independently of each other to avoid cross-talk between the two fluorescent indicators.

**Patch Clamping of Endothelial Cells—**Patch clamp in a whole cell configuration was performed on HMEC attached to a coverslip. Patch electrodes made from 1.5-mm borosilicate glass tubing without filament (Narishige, Japan) had a resistance typically between 3 and 6 MΩ when filled. Cell membrane and pipette capacitative transients were subtracted from the records by the amplifier circuitry before sampling. Voltages were not compensated for liquid junction potentials. Membrane currents measured were an EPC-7 amplifier in conjunction with pClamp 8.1 software and a Digidata 1322 A/D converter (Axon Instruments, Foster City, CA). The currents were filtered at 2 kHz (low-pass bessel filter) and 100 Hz (high-pass bessel filter) and sampled at an interval of 10 ms. The RGFP vector was a generous gift from Dr. M. Philips (New York University School of Medicine, NY), pCMV5 vector and vector containing dominant-negative (N19dn) Rho mutant were provided by Dr. T. Kozasa (University of Illinois at Chicago, IL). Transfection was performed on 50–70% confluent HPAEC grown on 4-well Lab-Tek chambers or 24-well plates using Superfect reagent following the supplier’s protocol. The efficiency of transfection in HPAEC ranged from 10 to 20%. We also determined whether Rho regulates Ca$^{2+}$ entry by modulating the activity of TRPC1.

We overexpressed TRPC1 by 3-fold in HMEC as in these cells a high efficiency of transfection can be obtained. Briefly, HMEC grown to 50% confluence were incubated with LipofectAMINE-TRPC1 cDNA or LipofectAMINE-pcDNA3.1 vector complexes for 4 h. LipofectAMINE DNA complexes were made by incubating 4 μg of LipofectAMINE with 0.5 μg of plasmid DNA in 0.2 ml of Opti-MEM I for 45 min at 32 °C. LipofectAMINE-DNA complexes were diluted with 0.8 μl of Opti-MEM before being added to HMEC, pre-washed 2 times with Opti-MEM I, for 4 h. To end the transfection procedure, 2 ml of MCDB 131-medium supplemented with 10% FBS was added to each well for up to 16 h after which they were fed again with fresh 10% FBS-MCDB medium and allowed to grow further (45). Cells expressing TRPC1 were then treated without or with C3 to determine the role of Rho in modulating TRPC1-induced Ca$^{2+}$ entry.

**Co-immunoprecipitation of Rho, IP$_3$R, and TRPC1—**HPAEC grown to 50-mm dishes were serum starved followed by quick washing in ice-cold phosphate-buffered saline. Cells were then lysed in buffer containing 50 μM Tris, pH 7.4, 150 mM NaCl, 0.25 mM EDTA, pH 8.0, 1% P. Koukis, unpublished observations.
deoxycholic acid, 0.5% Nonidet P-40, 0.1% SDS, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of leupeptin, aprotinin, and pepstatin A. The lysate was scraped and centrifuged at 4°C at 14,000 g for 10 min. Cell lysate containing an equal amount of protein was then incubated with rabbit IgG or incubated with anti-rabbit polyclonal Rho, IP3 receptor, or TRPC1 Abs for 3–4 h followed by addition of protein A-agarose beads overnight at 4°C. Beads were collected by centrifugation, washed 3 times with lysis buffer without detergents after which proteins were eluted from the beads by boiling the samples suspended in Laemmli sample buffer. Each sample was then electrophoresed on 10 or 4–20% linear gradient SDS-PAGE gels, transferred to nitrocellulose for Western blotting with IP3 receptor, TRPC1, or Rho Abs. Specificity of the TRPC1 Ab as described recently (22) was confirmed by using peptide immunogen as a negative control.

**Measurement of Rho Activity**—pGEX-2T containing Rhotekin-Rho binding domain was provided by Dr. M. A. Schwartz (Scripps Research Institute, La Jolla, CA). Bacterial expressed GST-Rhotekin Rho binding domain protein (GST-RBD) was purified from isopropyl-1-thio-D-galactopyranoside (1 mM)-induced DH5α cells previously transformed with the appropriate plasmid as described (49). Confluent HPAE cells grown in 100-mm dishes were stimulated for the indicated times with 50 nM thrombin. Cells were then quickly washed with ice-cold Tris-buffered saline and lysed in buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM TDAP). The lysates were centrifuged at 4°C at 14,000 g for 15 min. Cell lysate containing an equal amount of protein was then incubated with rabbit IgG or incubated with anti-rabbit polyclonal Rho, IP3 receptor, or TRPC1 Abs for 3–4 h followed by addition of protein A-agarose beads overnight at 4°C. Beads were collected by centrifugation, washed 3 times with lysis buffer without detergents after which proteins were eluted from the beads by boiling the samples suspended in Laemmli sample buffer. Each sample was then electrophoresed on 10 or 4–20% linear gradient SDS-PAGE gels, transferred to nitrocellulose for Western blotting with IP3 receptor, TRPC1, or Rho Abs. Specificity of the TRPC1 Ab as described recently (22) was confirmed by using peptide immunogen as a negative control.

**FIG. 1.** Rho regulates thrombin-induced Ca2+ transient in endothelial cells. A, HPAEC were stimulated with thrombin to assay Rho activity at the indicated times. Rho activity is evident by the amount of Rho A bound to Rho (Top) normalized to the amount of Rho in whole cell lysates (bottom). B, confocal microscopy of fluo3-loaded cells showing increase in [Ca2+]i, induced by thrombin at 25 and 120 s as indicated by the increase in fluo3 intensity after 0 s in the presence of 1.3 mM external Ca2+ in HPAEC co-transfected with dsRed (transfection marker) and either vector alone (left) or dnRho (right). A series of time lapse confocal images were acquired at 12.5-s intervals following thrombin stimulation using 488 and 568 nm excitation laser lines for fluo3 and dsRed fluorescence, respectively. Results are representative of at least two experiments. RBD, Rhotekin-Rho binding domain; α-T, α-thrombin; –, absence; +, presence. Outlined cells in the green channel represent the transfected cells.

**FIG. 2.** Rho inhibitor C3 transferase prevents thapsigargin-induced Ca2+ entry. HPAEC transfected without or with 3 μg/ml C3 transferase were loaded with fura2-AM, and stimulated with thapsigargin in the absence of extracellular Ca2+ to deplete ER Ca2+. This was followed by re-addition of 1.3 mM [Ca2+]o to determine Ca2+ entry. A, trace showing the average of real time data from 25 control or C3-treated cells from a single experiment. B, mean ± S.E. of the release of Ca2+ from stores and Ca2+ entry from multiple experiments calculated as the maximum increase over basal value in each condition (n = 3). *, values different from untreated cells (p < 0.001). Thap, thapsigargin.
X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, 10 µg/ml each of aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 14,000 × g for 2 min and equal volumes of cell lysates were incubated with GST-RBD beads (15 µg) at 4 °C for 1 h. The beads were washed three times with wash buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 10 µg/ml each of aprotinin and leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride), and bound Rho was eluted by boiling each sample in Laemmli sample buffer. Eluted samples from beads and total cell lysate were then electrophoresed on 14% gels, transferred to nitrocellulose, blocked with 5% nonfat milk, and analyzed by Western blotting using a polyclonal anti-RhoA, anti-IP₃R, or anti-TRPC1 Abs. Normalized band intensity was measured with SigmaPlot software. Differences in mean values were considered significant at p < 0.05.

**RESULTS**

**Activation of Rho Signals Inhibits Ca²⁺ Entry by Regulating TRPC1 Function**—We inhibited endogenous Rho function either by overexpressing the dominant-negative mutant of RhoA (19Nds-Rho) or by ADP ribosylating Rho with C3 transferase to determine the role of Rho in store depletion-mediated Ca²⁺ entry. In addition, we determined Rho activation and transendothelial electrical resistance changes (a measure of endothelial permeability in real-time) to address the role of Rho-regulated Ca²⁺ entry in mediating a thrombin-induced increase in endothelial permeability.

We showed using GST-Rhotekin fusion protein, which specifically binds to activated Rho, a 2–3-fold increase in Rho activity following thrombin stimulation of HPAEC (Fig. 1A). To determine the effects of N19Nds-Rho expression on thrombin-induced Ca²⁺ transients, dsRed plasmid cDNA (transfection marker) was co-expressed with empty vector or vector containing dn-Rho plasmid in HPAEC. These cells were then loaded with fluo3 to measure the Ca²⁺ transients in real time in the transfected cells (dsRed-positive) by confocal microscopy (Fig. 1B). In cells expressing the empty vector (outlined) or untransfected cells in the same field, we observed the characteristic Ca²⁺ transient following thrombin stimulation as indicated by increase in fluo3 fluorescence intensity after challenge (Fig. 1B). However, cells expressing dnRho (outlined) exhibited smaller and short-lived Ca²⁺ transients (Fig. 1B). These findings indicate that RhoA is important in regulating the increase in [Ca²⁺]ᵢ in response to thrombin exposure of HPAEC.

To determine whether Rho is responsible for activation of SOC, we used thapsigargin and IP₃ because these agents induce store depletion-mediated Ca²⁺ entry independent of ligand-receptor-G protein-coupled processes (12). Thapsigargin-induced Ca²⁺ entry was measured in fura 2-loaded control or C3-pretreated HPAEC incubated in Ca²⁺-free HBSS (Fig. 2). Inhibition of Rho markedly suppressed Ca²⁺ entry following the depletion of ER stores with thapsigargin without affecting the Ca²⁺ release response (Fig. 2, A and B). Fig. 3 shows the IP₃-activated store-operated current in control cells with a mean value
of 1.8 ± 0.05 pA, which was inactivated by perfusion of lanthanum, a blocker of the store-operated Ca^{2+} channels (Fig. 3A) (50). This value of mean current density is within the range reported in endothelial cells (12). However, inhibition of Rho by C3 transferase reduced the IP_{3}-induced Ca^{2+} current (Fig. 3, A and B). Thus, these observations show that Rho regulates Ca^{2+} entry by modulating the function of store-operated Ca^{2+} channels.

As TRPC1 is a primary store-operated Ca^{2+} channel in human endothelial cells (12, 20), we determined the role of Rho on endogenous TRPC1 function in HMEC in which TRPC1 expression was increased 3-fold. Cells expressing control vector or TRPC1 were pretreated without or with C3 transferase after which they were loaded with fura-2/AM to measure changes in cytosolic Ca^{2+} following thrombin stimulation (Fig. 4). We observed a sustained increase in [Ca^{2+}]_{i} in response to thrombin in TRPC1-overexpressing cells that was markedly reduced after inhibition of Rho by C3 transferase (Fig. 4). Furthermore, C3 transferase prevented a thrombin-induced decrease in transendothelial electrical resistance, indicating that Rho mediates the decrease in endothelial barrier function by regulating [Ca^{2+}]_{i} (Fig. 5).

**Rho Interacts with IP_{3}R and TRPC1**—Because the above results show Rho regulation of TRPC1-mediated Ca^{2+} entry, we addressed the possibility that Rho activates TRPC1 function by inducing its coupling with IP_{3}R. We first tested whether Rho interacts with IP_{3}R and TRPC1 by immunoprecipitating whole cell lysates with rabbit anti-Rho Ab. In addition, whole cell lysate immunoprecipitated with rabbit anti-Rho Ab or control IgG. We observed that IP_{3}R and TRPC1 were present in cell lysate immunoprecipitated using anti-Rho Ab, whereas these proteins were not detected in lysates immunoprecipitated using control IgG. Conversely, Rho was also present in lysates immunoprecipitated using IP_{3}R or TRPC1 antibody (Fig. 6B).

We then used the GST-Rhotekin pull-down assay in which active Rho is selectively bound by a GST fusion protein containing the Rho-binding region of Rhotekin to address whether IP_{3}R and TRPC1 interact with Rho following thrombin stimulation. Lysates from unstimulated or stimulated cells were incubated with GST-Rhotekin fusion protein after which they were subjected to Western blotting with anti-IP_{3}R, anti-TRPC1, or anti-Rho Abs. The resulting blot showed that thrombin induced the association of IP_{3}R and TRPC1 with activated Rho (Fig. 6C); however, as compared with TRPC1, the association of IP_{3}R with Rho was increased within 1 min after challenge (Fig. 6C). These findings show that thrombin activated the association of Rho with IP_{3}R and TRPC1 in a time-dependent manner consistent with its role in regulating Ca^{2+} signaling.

Studies show that Rho-GTPases are translocated to PM upon activation (51–53). Because the above results from the GST-pull down assay indicate that activated Rho associates with IP_{3}R and TRPC1, we used confocal imaging to address whether Rho mediates the recruitment of IP_{3}R and TRPC1 at the PM. Thrombin-induced co-localization of IP_{3}R and TRPC1 in cells expressing GFP-Rho is shown in Fig. 7. In an unstimulated cell (Fig. 7C), GFP-Rho was homogeneously distributed in the cytosol with little staining on the membrane. Furthermore, GFP-Rho was co-localized with IP_{3}R and TRPC1 near the perinuclear area (Fig. 7, C and D, yellow). Upon thrombin stimulation, we observed some of the Rho translocated to the PM. IP_{3}R was also present at the PM following thrombin stimulation. Marked increase in TRPC1 staining at the PM was observed following a 3-min thrombin stimulation. Interestingly, Rho was co-localized with IP_{3}R and TRPC1 at the membrane (Fig. 7, C and D). We also observed the appearance of condensed structures con-
Rho-dependent Coupling of IP₃R and TRPC1

containing GFP-Rho, IP₃R, and TRPC1 within the cytosol after thrombin challenge (Fig. 7, C and D). This pattern of IP₃R and TRPC1 staining and co-localization with Rho was specific because it was not observed in cells stained with control IgG or TRPC1 Ab pre-absorbed to its immunogenic peptide. However, in the absence of Rho activation in C3 transferase-treated cells,
thrombin failed to induce the translocation of IP₃R and TRPC1 to the membrane (Fig. 7, E and F). These imaging data along with biochemical results demonstrate that Rho signals recruitment of IP₃R and TRPC1 to the plasma membrane.

Rho Induced Association of IP₃R with TRPC1 Requires Actin Polymerization—Because our studies show that Rho regulates TRPC1 and IP₃R co-localization at the plasma membrane and Ca²⁺ entry through TRPC1 activation, we addressed the possibility that Rho induces association of IP₃R with TRPC1. We also determined whether actin polymerization (another Rho-dependent event) participates in the Ca²⁺ entry response. We treated HPAEC with C3 transferase and latrunculin-A (which inhibits actin polymerization by binding with actin monomers) to address the contribution of Rho and actin polymerization, respectively, in mediating association of TRPC1 and IP₃R. Lysates of control cells and cells treated with C3 transferase or latrunculin were immunoprecipitated using anti-IP₃R antibody, and Western blotted using anti-TRPC1 or IP₃R Abs. In addition, GFP-Rho expressing cells were left unstimulated or stimulated with thrombin after which they were fixed and stained for determining actin stress fiber formation as described above (C). This experiment was repeated at least 2 times; results are from a representative experiment. A, control HPAEC, HPAEC transfected with C3 transferase, or HPAEC pretreated with 250 nM latrunculin for 30 min were stimulated with thrombin for 2 min. Total cell lysate was immunoprecipitated with anti-IP₃R Ab, separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western blotted using anti-IP₃R or anti-TRPC1 Ab. Western blot shows the result from a representative experiment. B and C, HPAEC treated without or with 250 nM latrunculin for 15 min or C3 transferase pretreated cells were fixed following thrombin stimulation and then stained with Alexa-phallolidin (green) plus DAPI (blue) to label filamentous actin and nuclei (B). In addition, GFP-Rho expressing cells were left unstimulated or stimulated with thrombin after which they were fixed and stained for determining actin stress fiber formation as described above (C). This experiment was repeated at least 2 times; results are from a representative experiment. D and E, control cells or cells pretreated with 250 nM latrunculin-A for 15 min were loaded with fura2-AM in the presence or absence of latrunculin for 15 min to measure cytosolic Ca²⁺ in the presence of 1.3 mM external Ca²⁺ (D) or nominally Ca²⁺-free medium (E). As results from the experiments were similar, data from a representative experiment are shown (n = 3). α-T, α-thrombin; bar, 20 μm; −, absence; +, presence.

![Figure 8](image)

**Fig. 8.** Rho requires actin polymerization to couple IP₃R with TRPC1 and activate Ca²⁺ entry. A, control HPAEC, HPAEC transfected with C3 transferase, or HPAEC pretreated with 250 nM latrunculin for 30 min were stimulated with thrombin for 2 min. Total cell lysate was immunoprecipitated with anti-IP₃R Ab, separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western blotted using anti-IP₃R or anti-TRPC1 Ab. Western blot shows the result from a representative experiment. B and C, HPAEC treated without or with 250 nM latrunculin for 15 min or C3 transferase pretreated cells were fixed following thrombin stimulation and then stained with Alexa-phallolidin (green) plus DAPI (blue) to label filamentous actin and nuclei (B). In addition, GFP-Rho expressing cells were left unstimulated or stimulated with thrombin after which they were fixed and stained for determining actin stress fiber formation as described above (C). This experiment was repeated at least 2 times; results are from a representative experiment. D and E, control cells or cells pretreated with 250 nM latrunculin-A for 15 min were loaded with fura2-AM in the presence or absence of latrunculin for 15 min to measure cytosolic Ca²⁺ in the presence of 1.3 mM external Ca²⁺ (D) or nominally Ca²⁺-free medium (E). As results from the experiments were similar, data from a representative experiment are shown (n = 3). α-T, α-thrombin; bar, 20 μm; −, absence; +, presence.

![Figure 9](image)

**Fig. 9.** Rho-activated interaction of IP₃R with TRPC1 triggers Ca²⁺ entry. Upon activation Rho associates with IP₃R and TRPC1, and the complex is translocated to the plasma membrane. Rho couples IP₃R to TRPC1 in an actin filament polymerization-dependent manner, thereby linking Ca²⁺ store emptying to Ca²⁺ entry.

thrombin induced the formation of polymerized actin stress fibers in control and GFP-Rho-transfected cells (Fig. 8, B and C). In GFP-Rho-transfected cells, these fibers co-localized with Rho in the perinuclear area and plasma membrane (Fig. 8C). Lатrunculin pretreatment prevented the formation of actin stress fibers induced by thrombin; in some cells, actin was condensed in foci at the cell periphery (Fig. 8B). In addition,
inhibition of actin polymerization by latrunculin prevented Ca\textsuperscript{2+} entry while it had no effect on thrombin-induced Ca\textsuperscript{2+} release from ER (Fig. 8, D and E). Taken together, these results demonstrate the important role of Rho-dependent actin polymerization in the activating IP\textsubscript{3}R and TRPC1 coupling.

**DISCUSSION**

RhoA regulates discrete cellular events involving actin cytoskeletal dynamics, MLC phosphorylation, and vesicle trafficking (2, 40, 41). In the present study, we investigated the possible role of Rho in regulating Ca\textsuperscript{2+} entry in endothelial cells. We postulated a model in which Rho associates with IP\textsubscript{3}R and TRPC1, the predominant SOC in human endothelial cells (12, 20), and thus signals interaction of these two components of the complex required for Ca\textsuperscript{2+} entry. As RhoA activation has a central role in the mechanism of thrombin-induced increase in endothelial permeability subsequent to activation of MLC phosphorylation and actin filament re-arrangement (43–45), we also addressed the possible relevance of Rho-activated Ca\textsuperscript{2+} entry in signaling increased endothelial permeability.

Using pull-down assays, we showed that thrombin induced the association of IP\textsubscript{3}R and TRPC1 with activated Rho in a time frame corresponding to store depletion-induced Ca\textsuperscript{2+} entry. We also showed that thrombin stimulation of endothelial cells induced translocation of Rho, and subsequent co-localization of Rho with TRPC1 and IP\textsubscript{3}R at the plasmalemma. We showed that plasmalemmal distribution of TRPC1 and IP\textsubscript{3}R and co-localization of these components with Rho were specific in that these responses were not observed in cells stained with control IgG or with TRPC1 Ab preabsorbed to its immunizing peptide. Previous findings that IP\textsubscript{3}R can exist as an integral plasma membrane protein (54–56) lends credence to our observations. The present results also demonstrated that appearance of IP\textsubscript{3}R and TRPC1 at the membrane were required for the activated form of Rho because thrombin failed to induce PM translocation of these components in endothelial cells pretreated with C3. Inhibition of Rho function with either dnRho or C3 trans-ferase significantly reduced Ca\textsuperscript{2+} entry as well as SOC current in single cell recordings. This was the result of Rho activation of TRPC1 because overexpression of TRPC1 in endothelial cells produced a sustained C3 trans-ferase-sensitive increase in [Ca\textsuperscript{2+}], in response to thrombin exposure. We observed that thrombin-induced Ca\textsuperscript{2+} release from ER stores was reduced by C3 in TRPC1-overexpressing cells. The present studies showing that IP\textsubscript{3}R-induced Ca\textsuperscript{2+} release requires Rho activation are consistent with the findings that IP\textsubscript{3}R receptor activation occurs through a Rho-dependent pathway (57). Taken together, our results demonstrate an important role of Rho in inducing interaction of the key proteins required for activation of SOC-induced Ca\textsuperscript{2+} entry. In addition, we observed that inhibition of Rho activation prevented increased endothelial permeability occurring secondary to the rise in [Ca\textsuperscript{2+}], (5, 6). Thus, Ca\textsuperscript{2+} entry by this mechanism is important in increasing endothelial permeability.

Our results demonstrate that Rho activation is required for both thrombin and thapsigargin- or IP\textsubscript{3}R-induced Ca\textsuperscript{2+} entry. Studies have shown that thrombin binding to protease activated receptor-1 activates heterotrimeric G proteins, G\textsubscript{12}/G\textsubscript{13} and G\textsubscript{\alphaq} which in turn induce Rho activation (58–60). However, the mechanism of thapsigargin activation of Rho in the absence of the receptor-coupled pathway is not known. We observed only modest Rho activation induced by thapsigargin (data not shown); thus, it is possible that Rho can activate SOC independent of the mode of Ca\textsuperscript{2+} store emptying (i.e. an agonist in the case of thrombin versus ER calcium pump blockade in case of thapsigargin). Although evidence from several laboratories showed that IP\textsubscript{3}R coupling with TRPC1 can trigger Ca\textsuperscript{2+} entry, the mechanisms responsible for this critical association event are not clear (23, 25, 34, 61–64). Models involving chemical and conformational coupling have been suggested (1, 11, 14–16, 65). Although there is evidence in favor of chemical coupling, the identity of the messengers is unknown (26–29). There is also inconclusive evidence supporting the concept that coupling of IP\textsubscript{3}R with TRP channels is mediated through a IP\textsubscript{3}R conformational change. Additionally, as TRPC1 has been shown to be localized in intracellular membrane it is difficult to explain the activation of TRPC1-induced Ca\textsuperscript{2+} entry based on the above proposals (35). Our results are important in this regard because they help to explain Rho-activated interaction of IP\textsubscript{3}R with TRPC1 and subsequent activation of Ca\textsuperscript{2+} entry. RhoA may signal localization of IP\textsubscript{3}R with TRP channels by targeting these components of the Ca\textsuperscript{2+} entry machinery to the PM. Furthermore, Rho-dependent plasmalemmal recruitment of these channels provides an explanation for the observed delay in complete activation of SOC following Ca\textsuperscript{2+} store depletion (1, 23, 30–34). Our finding of Rho-dependent PM recruitment of IP\textsubscript{3}R and TRPC1, which promotes activation of Ca\textsuperscript{2+} entry following store emptying, is in accord with suggestions that SOC activation involves trafficking and docking of the channels in the plasmalemma (1, 24, 34, 36–39).

Spatial rearrangement of actin filaments promoted the association of IP\textsubscript{3}R with Ca\textsuperscript{2+} entry (62–64). Because RhoA regulates actin polymerization, we surmised that Rho-induced association of IP\textsubscript{3}R and TRPC1 is dependent on actin polymerization. We showed that thrombin failed to induce association of IP\textsubscript{3}R with TRPC1 in the absence of actin polymerization, and there was also no Ca\textsuperscript{2+} entry. Thus, these results point to an important role of actin polymerization in organizing the PM association of IP\textsubscript{3}R and TRPC1.

The present results are consistent with the Rho-activated coupling model in which Rho, by signaling trafficking of IP\textsubscript{3}R and TRPC1 to PM, promotes the interaction of these components of the complex (Fig. 9). Membrane insertion of this complex thereby triggers Ca\textsuperscript{2+} entry through TRPC1 after Ca\textsuperscript{2+} store depletion. Rho-activated SOC polymerization also participates in this process by enhanced signaling stabilized interaction of IP\textsubscript{3}R and TRPC1 channels at the PM. Rho activation of Ca\textsuperscript{2+} entry by this mechanism is functionally important in endothelial cells because it is a key determinant of increased endothelial permeability.

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