Orai1 Determines Calcium Selectivity of an Endogenous TRPC Heterotetramer Channel

Donna L. Cioffi, Songwei Wu, Hairu Chen, Mikhail Alexeyev, Claudette M. St. Croix, Bruce R. Pitt, Stefan Uhlig, Troy Stevens

Rationale: Canonical transient receptor potential 4 (TRPC4) contributes to the molecular composition of a channel encoding for a calcium selective store-operated current, $I_{\text{SOC}}$, whereas Orai1 critically comprises a channel encoding for the highly selective calcium release activated calcium current, $I_{\text{CRAC}}$. However, Orai1 may interact with TRPC proteins and influence their activation and permeation characteristics. Endothelium expresses both TRPC4 and Orai1, and it remains unclear as to whether Orai1 interacts with TRPC4 and contributes to calcium permeation through the TRPC4 channel.

Objective: We tested the hypothesis that Orai1 interacts with TRPC4 and contributes to the channel’s selective calcium permeation important for endothelial barrier function.

Methods and Results: A novel method to purify the endogenous TRPC4 channel and probe for functional interactions was developed, using TRPC4 binding to protein 4.1 as bait. Isolated channel complexes were conjugated to anti-TRPC protein antibodies labeled with Cy3-cy5 pairs. Förster Resonance Energy Transfer among labeled subunits revealed the endogenous protein alignment. One TRPC1 and at least 2 TRPC4 subunits constituted the endogenous channel (TRPC1/4). Orai1 interacted with TRPC4. Conditional Orai1 knockdown reduced the probability for TRPC1/4 channel activation and converted it from a calcium-selective to a nonselective channel, an effect that was rescued on Orai1 reexpression. Loss of Orai1 improved endothelial cell barrier function.

Conclusion: Orai interacts with TRPC4 in the endogenous channel complex, where it controls TRPC1/4 activation and channel permeation characteristics, including calcium selectivity, important for control of endothelial cell barrier function. (Circ Res. 2012;111:00-00.)

Key Words: store operated calcium entry □ $I_{\text{SOC}}$ calcium selective store operated calcium entry current □ $I_{\text{CRAC}}$ calcium release activated calcium current □ protein 4.1 □ endothelium

Diffuse environmental signals provoke transient cytosolic calcium elevations that initiate physiologically relevant cellular adaptations. Such calcium transitions occur in all eukaryotic cells, in response to chemical, mechanical, and physical first messengers. In endothelium, neurohumoral inflammatory mediators induce a transient rise in cytosolic calcium that serves as a proximal signal to reorganize the cytoskeleton, disrupt cell-cell junctions, and induce intercellular gaps. These gaps form a paracellular pathway for plasma-rich exudation, and delivery of immunoglobulins and anti-inflammatorins to sites of injury or infection.

Calcium entry across the cell membrane, and not calcium reticulum, is necessary to induce endothelial cell gaps and increase permeability. Transient receptor proteins 1, 3, 4, and 6 within the canonical subfamily (TRPC) have all been incriminated in the calcium influx that is responsible for increased permeability, yet functional TRPC channels are thought to form by some combination of 4 subunits, and the subunit stoichiometry of an endogenously expressed TRPC channel has yet to be resolved. Recently, the TRPC4 carboxy-terminus was found to directly interact with protein 4.1, near the channel’s projected pore. Disrupting the TRPC4–protein 4.1 interaction abolished calcium entry through the TRPC4 channel and prevented interendothelial cell gap formation, indicating that TRPC4 is directly tethered to the...
membrane skeleton as a means to localize the calcium signal to physiologically important effectors. Both TRPC1 and TRPC4 contribute subunits to the protein 4.1-bound channel.\textsuperscript{12,15,16} This channel encodes for a calcium selective store-operated current, \( I_{\text{SOC}} \), in endothelia, which is small (~1.5 pA/pF), inwardly rectifying, and possesses a reversal potential near +40 mV.\textsuperscript{11,12,14,17} \( I_{\text{SOC}} \) is activated by thapsigargin, inositol 1,4,5 trisphosphate, and TPEN, all of which deplete endoplasmic reticulum calcium. Questions remain as to whether \( I_{\text{SOC}} \) is the same as, or different from, the calcium release activated calcium current, \( I_{\text{CRAC}} \), which displays similar biophysical properties to \( I_{\text{SOC}} \), although it is thought to be more highly calcium selective.\textsuperscript{18–20} \( I_{\text{SOC}} \) is the same as, or different from, the calcium release activated calcium current, \( I_{\text{CRAC}} \), which displays similar biophysical properties to \( I_{\text{SOC}} \), although it is thought to be more highly calcium selective.\textsuperscript{18–20} Orai1 is a 4 transmembrane spanning domain protein that forms a pore through the coordination of 4 subunits and encodes for \( I_{\text{CRAC}} \).\textsuperscript{21–25} Endothelial cells express Orai1.\textsuperscript{18,26–27} Trebak and colleagues\textsuperscript{18} suggest that thapsigargin activates both \( I_{\text{SOC}} \) and \( I_{\text{CRAC}} \) in endothelial cells, and further work indicated that \( I_{\text{SOC}} \) controls permeability whereas \( I_{\text{CRAC}} \) regulates proliferation, migration, and angiogenesis.

Despite the evidence that Orai1 can form a channel, it remains unclear as to whether Orai1 is sufficient to comprise the pore-forming channel accounting for \( I_{\text{CRAC}} \) in vivo. For example, Orai1 appears to be nearly ubiquitously expressed, and not all cells that express Orai1 possess \( I_{\text{CRAC}} \).\textsuperscript{28–30} Moreover, the Birnbaumer,\textsuperscript{31} Ambudkar,\textsuperscript{32} and Flockerzi\textsuperscript{33} groups have independently noted that Orai1’s anatomy is more reminiscent of channel ancillary proteins, such as the T channel’s \( \gamma \) subunit.\textsuperscript{27} Orai1 may be an essential subunit of TRPC channels that is required for TRPC proteins to sense calcium store depletion, and hence to be store-operated.\textsuperscript{32} At present this central issue remains unresolved. To address this issue, we developed a means to enrich for the endogenous TRPC4 channel from endothelial cell membrane fractions, using protein 4.1 as bait. Using purified fractions, we probed for intermolecular interactions by Förster resonance energy transfer (FRET). Cells were engineered for the conditional expression of Orai1 short hairpin RNA (shRNA), so that in the same cells, Orai1 expression could be suppressed and then rescued. Our results indicate that Orai1 constitutively interacts with TRPC4 in endothelial cells, and with TRPC1 after calcium store depletion. Furthermore, Orai1 increases the probability that TRPC4 will be activated following calcium store depletion, promotes calcium selectivity of the TRPC4 channel, and critically controls the transmembrane calcium flux that serves as a proximal signal for endothelial cell barrier disruption.

**Methods**

An expanded Methods section is provided in the online-only Data Supplement.

**Ethical Approval**

All animal use studies were approved by the University of South Alabama’s Institutional Animal Care and Use Committee.

**Cell Cultures**

Rat pulmonary artery and microvascular endothelial cells were isolated and cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin using previously described methods.\textsuperscript{33,34}

**Reagents**

See the online-only Data Supplement.

**Membrane/Cytoskeleton Fractions and Immunoprecipitation**

Performed as described in Ref. 12.

**Preparation of Isolated Immunocomplex**

See the online-only Data Supplement.

**SDS-PAGE and Western Blot**

As described in Ref. 12.

**Fluorescently Tagged Antibodies**

Antibodies were tagged with the cyanine reagents Cy3 and Cy5 following the manufacturer’s directions (Cy3 mAb Labeling Kit #PA33001 and Cy5 mAb Labeling Kit #PA35001; GE Healthcare Bio-Sciences; Piscataway, NJ).

**FRET Experiments**

The FRET-based approach to determine subunit stoichiometry was adapted from Refs. 35 and 36. Procedure details are presented in the online-only Data Supplement.

** Conditional Expression of Orai1 shRNA**

Four pairs of shRNA-encoding oligonucleotides were designed using BLOCK-iT RNAi Designer program (Invitrogen), synthesized by Integrated DNA Technologies (Coralville, IA), reconstituted in water, annealed, and cloned into BsuRI-digested pMAM267; this approach was described previously.\textsuperscript{37} The identity of the constructs was verified by sequencing. Lentiviruses containing supernatants were produced using standard procedures, as shown previously.\textsuperscript{37} Supernatants were used to infect tetracycline-regulated (Tet-On) pulmonary artery endothelial cells, which were then selected to homogeneity using puromycin.

**Whole Cell Patch Clamp Electrophysiology**

Patch clamp experiments were performed as described extensively elsewhere.\textsuperscript{11,12,14,15,17} Details regarding intra- and extracellular solutions are available in the online-only Data Supplement.

**Statistical Analysis**

Results were analyzed using 1-way ANOVA, where appropriate, using GraphPad Prism 4.0 software. All data represent mean±SEM. \( P<0.05 \) is considered statistically significant for the comparisons.

**Results**

**Protein 4.1 Functions as Bait for Isolation of an Endogenous TRPC4 Channel**

We used the physical interaction between TRPC4, protein 4.1, and the membrane skeleton as a way to enrich for the endogenous channel. Pulmonary artery endothelial cells were lysed and detergent extracted using octyl-\( \beta \)-D-glucopyranoside. The resulting pellet fraction contained elements of the cytoskeleton and membrane skeleton, including spectrin bound to protein 4.1. In the presence of salt, protein 4.1 was displaced from the pellet.
fraction into a supernatant fraction with TRPC1, TRPC4, and Orai1 (Figure 1A), whereas actin and other cytoskeletal proteins remained in the pellet fraction (data not shown). Immunoprecipitation of protein 4.1 and immunoblotting of TRPC proteins revealed that TRPC1, TRPC4, and Orai1 coimmunoprecipitate with protein 4.1.

FRET Resolves Intermolecular Interactions Between TRPC4 and Orai1 in Endothelium

We next developed a method to resolve the nature of the protein 4.1-bound TRPC4 channel purified within the salt-dissociated supernatant, based on the use of FRET as a molecular ruler to define intermolecular interactions within a channel (Online Figure I). Protein 4.1 antibody was affixed to Protein A/agarose beads and incubated with the salt-dissociated supernatant fraction, which tethered associated TRPC subunits to the beads as part of the channel complex. Our FRET approach was derived from studies resolving the GABA$_A$ and cyclic nucleotide gated cation channel stoichiometries, with the notable exception that in our system TRPC subunits were not overexpressed. Rather, cy3 and cy5 FRET pairs were conjugated to TRPC protein antibodies and then incubated with Protein A/agarose beads containing the protein 4.1 bound TRPC channel over a range of concentrations. Fluorescence saturation binding curves were determined, and the half maximal concentration of each antibody was determined to allow for efficient FRET (Online Figure II). FRET detection occurs only when the distance between the fluorophores is $\frac{R_0}{2}$, where $R_0$ is the Förster radius. Using an $R_0$ of 50 Å for the cy3-cy5 pair, positive FRET reflects a distance of $\frac{R_0}{2}$ between proteins.

Because TRPC1 and TRPC4 coimmunoprecipitate and because TRPC4 appears to be required for TRPC1 membrane targeting, we began by testing whether TRPC1 and TRPC4 directly interact. TRPC1 and TRPC4 were labeled with cy3 and cy5 conjugated antibodies, respectively, and FRET signals examined. As anticipated, strong FRET signals were observed between TRPC1 and TRPC4, indicating they directly interact within the native channel (Figure 1B).

Several control approaches were developed to ensure FRET signals are due to intrachannel subunit interactions. Dilution experiments were performed where one-fifth the amount of salt-dissociated supernatant was immunoprecipitated for protein 4.1 (Online Figure III). FRET detection occurs only when the distance between the fluorophores is $<1.5 R_0$, where $R_0$ is the Förster radius. Using an $R_0$ of 50 Å for the cy3-cy5 pair, positive FRET reflects a distance of $<75$ Å between proteins.
would be retained. To do this, FRET between cy3-labeled TRPC1 and cy5-labeled TRPC4 was tested. In undiluted samples, FRET was readily apparent between TRPC1 and TRPC4. The overall fluorescence intensity was decreased in all of the diluted samples compared to the undiluted samples. However, strong TRPC1-TRPC4 FRET signals were retained in the diluted samples (Online Figure III), demonstrating FRET detects intrachannel subunit interactions.

To confirm efficient energy transfer between cy3 and cy5 was occurring as predicted, photobleaching studies were undertaken. In this case, the cy5-labeled TRPC4 channel was irradiated to photobleach fluorescence. Such cy5 inactivation prevented energy transfer from cy3-labeled TRPC1 to cy5-labeled TRPC4. Consequently, increased cy3 fluorescence was measured, indicating the signal measured in the FRET channel is specific (Online Figure IV). Collectively, these data confirm that our FRET approach detects intrachannel subunit interactions.

To determine whether the channel possesses 1 TRPC1 subunit, TRPC1 antibody was labeled with cy3 and cy5 and FRET responses examined. FRET could not be detected under these conditions (Figure 1B). However, labeling the TRPC4 antibody with cy3 and cy5 revealed strong FRET responses, indicating 1 TRPC4 protein exists within the channel (Figure 1B).

These initial studies were conducted using pulmonary artery endothelial cells, yet considerable heterogeneity among endothelial cell phenotypes has been reported, particularly in regard to calcium signaling. To determine whether stoichiometry of the protein 4.1-bound TRPC channel is the same among endothelial cell phenotypes, FRET experiments were repeated using salt-dissociated supernatants isolated from pulmonary microvascular endothelial cells (Figure 1C). The results of these experiments revealed an identical TRPC protein alignment.

Because TRPC1 and 4 proteins reside in caveolin-rich fractions,41,42 and because caveolin-rich fractions can be isolated from pulmonary endothelium in the intact circulation,43 we used caveolin fractions obtained from an isolated perfused lung preparation to examine TRPC channel subunit alignment. These caveolin-rich fractions retain elements of the membrane skeleton, including spectrin, actin, and protein 4.1. Detergent extracted caveolin fractions were prepared for FRET studies and protein–protein interactions probed. Once again, identical FRET responses were recorded (Figure 1C), indicating the channel’s subunit alignment is the same in cultured cells and in vivo.

**Figure 2. Orai1 interacts with transient receptor potential canonical (TRPC)4 in the protein 4.1-bound TRPC channel.** Protein 4.1-bound immunocomplex was isolated from pulmonary artery endothelial cells either before (A) or after (B) thapsigargin treatment (1 μmol/L for 15 min). The TRPC channel was treated with cy3- (donor channel) and cy5- (acceptor channel) labeled antibodies at their EC50 concentrations. Förster Resonance Energy Transfer (FRET) analyses were performed using the sensitized emission approach (FRET channel). FRET pairs refer to the cy3- and cy5-labeled proteins, respectively. Whereas Orai1 only interacted with TRPC4 in the unstimulated state, it interacted with both TRPC1 and TRPC4 following thapsigargin treatment. FRET efficiency is derived from the summary of a minimum of 15 separate measurements. ND indicates to not detected.

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**Orai1 Interacts With TRPC4 in the Protein 4.1-Enriched Channel Complex**

TRPC proteins are generally believed to form nonselective cation channels. However, the protein 4.1-bound TRPC1/4 channel described presently is calcium selective,11,12,14,15 albeit less so than the calcium release-activated calcium current, I_{CRAC}, that is thought to be encoded by Orai1.21–25 Controversy remains as to whether TRPC channels interact with Orai1, and as to whether TRPC channels are activated by calcium store depletion. Orai1 has been reported to interact with TRPC1 following calcium store depletion, and this interaction may be necessary for TRPC1 to sense calcium store depletion.31

FRET was used to examine whether Orai1 interacts with the protein 4.1-bound TRPC1/4 channel. Whereas Orai1 was found to constitutively interact with TRPC4, it did not interact with TRPC1 (Figure 2A). We next examined whether the nature of this Orai1–TRPC4 interaction changed following channel activation. Thapsigargin was used to activate store-operated calcium entry in pulmonary artery endothelial cells, and salt-dissociated supernatants were prepared for FRET studies. Under these conditions, Orai1 interacted with both TRPC1 and TRPC4, generally consistent with previous observations (Figure 2B).

**Orai1 Impacts TRPC4 Channel Activation and Calcium Permeation**

The functional significance of an Orai1–TRPC1/4 interaction is not known. We developed an approach to test whether
Within 72 hours of doxycycline treatment, Western blot analysis revealed that Orai1 expression was nearly abolished, and within 48 hours after withdrawing doxycycline Orai1 expression had returned to near normal levels. Doxycycline concentrations as low as 0.1 μg/mL effectively reduced Orai1, with maximal protein inhibition ranging from 1 to 3 μg/mL. Salt-dissociated supernatant fractions were generated from these engineered cells, and FRET studies were performed. As shown previously in control cells, constitutive FRET was seen between Orai1 and TRPC4 (Figure 3B). In doxycycline-treated cells, shRNA expression abolished Orai1 fluorescence on the agarose beads; TRPC protein expression was not decreased by Orai1 knockdown. As anticipated, in the absence of Orai1 protein, an interaction between Orai1 and TRPC4 could not be detected by FRET; yet on doxycycline withdrawal, Orai1 fluorescence was rescued, as was the Orai1–TRPC4 interaction.

Whole cell electrophysiology experiments were performed using doxycycline naïve cells and following doxycycline treatment and withdrawal. Thapsigargin was delivered in the patch pipette using internal solutions designed to clamp intracellular calcium at 100 nmol/L. Under these conditions, thapsigargin induced a small, inward calcium current that displayed a reversal potential near +40 mV (Figure 4A). Eighty-six percent of the control cells tested displayed the current. Four important differences were noted in cells treated with doxycycline for 72 hours to reduce Orai1 expression. First, thapsigargin activated a current in only 47% of the cells lacking Orai1, suggesting that Orai1 plays a critical role in coupling calcium store depletion to channel activation (Odds ratio = 5.25; P < 0.05 χ²). Second, of the cells that responded to thapsigargin, the current density was decreased by approximately 25% at all of the negative test potentials, although the current was not abolished indicating Orai1 is not necessary for cation permeation through the I_{SOC} (Figure 4B). Third, the reversal potential was left shifted to +20 mV, indicating a loss of calcium selectivity (Figure 4B). Fourth, calcium-dependent inactivation was reduced (Figure 4C). The TRPC1/4 channel displays a calcium-dependent inactivation, where the macroscopic current is decreased approximately 40% in 60 seconds. In cells lacking Orai1, TRPC1/4 inactivation was reduced by approximately 20%, suggesting less calcium was permeating through the channel. When doxycycline was withdrawn and Orai1 expression returned to normal levels, the thapsigargin-induced current, including the percent of cells activated, current density, and reversal potential, were normalized.

We next tested the anomalous mole fraction behavior of I_{SOC} in the presence and absence of Orai1. In cells possessing Orai1, typical anomalous mole fraction behavior was observed, where the I_{SOC} current was greatest in 100 nmol/L extracellular calcium, reflecting sodium permeation through the TRPC1/4 channel (Figure 5A). Increasing extracellular calcium concentrations reduced this sodium current, revealing smaller calcium currents, especially at 10 nmol/L extracellular calcium. In the absence of Orai1, the sodium current was preserved whereas the calcium-dependent inhibition of the sodium current was nearly abolished. Orai1 did not influence the reversal potential when extracellular calcium concentrations were below 1 mmol/L, but when 10 mmol/L calcium concentrations were studied to reveal...
a calcium current, cells possessing Orai1 displayed a reversal potential of approximately \(-40\) mV whereas cells lacking Orai1 had a reversal potential near \(-20\) mV (Figure 5B). These findings suggest that Orai1 is necessary for the TRPC4-dependent \(I_{\text{SOC}}\) to display calcium selectivity. Using this anomalous mole fraction protocol, \(I_{\text{SOC}}\) inactivation was again tested (Figure 5C). The decay fraction was greatest in cells expressing Orai1, indicating the presence of Orai1 is an important determinant of the amount of calcium that permeates the TRPC1/4 channel.
Orai1 Contributes to the Calcium Entry That Disrupts the Endothelial Cell Barrier

Inflammatory agonists that activate store-operated current entry induce gaps between adjacent endothelial cells as a paracellular pathway for exudation; TRPC1/4 localizes to junctional complexes. Here, we tested whether the presence of Orai1 contributes to endothelial cell barrier disruption. Time-lapse videomicroscopy was performed to investigate whether thapsigargin induces gaps in the presence and absence of Orai1 (Figure 6 and Online Figure VII). As previously reported, thapsigargin induced multiple small gaps ranging in size from 20 to 200 μm², and fewer gaps larger than 200 μm². Interendothelial cell gap formation was transient, as most gaps resealed within 30 minutes. This effect was dependent on the presence of Orai1, as Orai1 knockdown prevented thapsigargin from inducing gaps.

Discussion

In this study, we illustrate the privileged intermolecular interaction within caveolin-rich membrane domains between the protein 4.1-bound TRPC1/4 channel and Orai1. This interaction is functionally important, as Orai1 increases the probability that TRPC1/4 will be activated by calcium store depletion, and it critically influences the calcium selectivity of the TRPC1/4 channel and, hence, the calcium concentration that permeates through the channel. These results prompt further consideration of the molecular structure and function of the endothelial cell TRPC1/4 channel.

Few channels are known to bind to protein 4.1. Precedence for a role of protein 4.1 in both the scaffolding and regulation of ion channels was first vetted in neurons, where protein 4.1R was shown to coassociate with NaV1.5 channels in the Nodes of Ranvier. Protein 4.1R knockout mice were later revealed to display a cardiac phenotype, with bradycardia accompanied by prolongation of the QT interval. In this case, a delay in NaV1.5 channel inactivation was reported. Similar findings were reported in TRPC4-channels, as a protein 4.1–TPRC4 interaction was shown to be essential for channel gating. There is an absolute dependence on protein 4.1-actin binding for thapsigargin to activate I_{SOC}, and protein 4.1 in turn directly links the spectrin membrane skeleton to the TRPC4 channel. Both the introduction of a peptide targeting the conserved protein 4.1 binding domain on the TRPC4 carboxy-terminus and heterologous expression of a chimeric TRPC4 protein lacking the protein 4.1 binding domain prevent I_{SOC} activation. We recently recognized that Orai1 also possesses a conserved protein 4.1 binding domain (residues 24–33; TSGSRRSRRR). Unlike TRPC4, however, the Orai1 protein 4.1 binding domain is located in the amino-terminus; structural analysis revealed no such protein 4.1 binding domain in either Orai2 or Orai3. At this time, the function of the Orai1 protein 4.1 binding domain is unknown.

Recognition that relatively few ion channels interact with protein 4.1 prompted us to use protein 4.1 as bait to enrich for the endogenous TRPC4 channel and probe for its molecular binding partners. As in earlier studies, we found that TRPC4 and TRPC1 coimmunoprecipitate with one another. However, by using FRET as a molecular ruler we were able to identify that the TRPC4 and TRPC1 interaction is within 75 Å, indicative of direct protein–protein binding. Using this
FRET approach, only 1 TRPC1 subunit was resolved within the complex, while at least 2 TRPC4 subunits were identified. Along with these 6 transmembrane domain proteins, Orai1 was found to interact with TRPC4. There are at least 3 explanations for resolving Orai1 within the TRPC4 complex. First, because Orai1 possesses a protein 4.1 binding domain it could have been selected by our use of protein 4.1 as bait, and hence, represent an independent channel that is not associated directly with TRPC4. This possibility is unlikely, as in dilution experiments—studies designed to dilute channels that are not a part of the same molecular complex—strong TRPC4–Orai1 interaction was retained. Second, Orai1 constitutes an independent channel that directly binds with the TRPC1/TRPC4 channel. This possibility has not been entirely ruled out by our studies, although as discussed below, if Orai1 is an independent channel, calcium permeation through the channel is not required to activate TRPC1/4. Third, Orai1 directly interacts with the TRPC4 protein complex; this possibility is most likely. Data to this point do not permit us to conclude whether Orai1 directly interacts with TRPC4 through membrane-spanning domains, or whether it interacts with TRPC4 through protein 4.1 or another scaffolding protein. FRET studies would suggest the interaction is sufficiently close to constitute direct binding.

Orai1 is recognized as the protein responsible for the I_{CRAC} of endothelium using our FRET approach as a molecular ruler, and in cells devoid of the I_{CRAC}, the current can be rescued by Orai1 expression. Nevertheless, I_{CRAC} has not been measured in all of the cells that express Orai1, and the predicted channel membrane topology is atypical for known calcium channels, prompting the hypothesis that Orai1 may function as an ancillary subunit for TRPC channels. In some cell types, Orai1 interacts with TRPC1, or interacts with TRPC1 following calcium store depletion, suggesting it may confer sensitivity of TRPC channels to calcium store depletion. We did not detect a constitutive interaction between Orai1 and TRPC1 in endothelium using our FRET approach as a molecular ruler, however, we did detect an Orai1–TRPC1 interaction following calcium store depletion. Our findings are consistent with the previous studies concluding that Orai1 confers calcium store depletion sensitivity to TRPC channels.

There remains some skepticism as to whether TRPC channels are indeed activated by calcium store depletion in endothelium. Although thapsigargin activates a TRPC4-dependent I_{SOC}, questions persist as to whether calcium release from the endoplasmic reticulum or calcium entry through another store-operated channel, such as Orai1, is sufficient to activate the channel. In our studies, intracellular calcium was buffered to 100 nmol/L, making it unlikely that calcium release from the endoplasmic reticulum activates TRPC4 channels. Moreover, Orai1 silencing did not prevent the I_{SOC} activation, suggesting that calcium permeation through an Orai1-dependent pore was not required for TRPC1/4 activation, at least not in an absolute sense, as with the protein 4.1–actin and protein 4.1–TRPC4 interactions.

Our data reveal that the Orai1–TRPC4 interaction is functionally important, however, as Orai1 silencing decreased the probability for I_{SOC} activation. Loss of Orai1 likely decreased the coupling efficiency between stromal interaction molecule 1 and store-operated calcium entry channels, most notably between stromal interaction molecule 1 and Orai1 and TRPC channels. The independent or combined roles of stromal interaction molecule 1 and protein 4.1 in channel activation are not presently known, although their binding sites on Orai1 and TRPC proteins are distinct.

Loss of Orai1 coincided with a left shift in the reversal potential. We interpreted these data to mean that Orai1 influences the calcium selectivity of the TRPC4 channel directly, or alternatively, by contributing a calcium current that is embedded within the macroscopic current. To better understand this issue, we studied the anomalous mole fraction behavior of I_{SOC} in the presence and absence of Orai1.

Thapsigargin-induced ion permeability was measured in the absence of extracellular calcium, and then in the presence of increasing calcium concentrations. With calcium-selective channels, sodium permeates through the channel in the absence of extracellular calcium. At relatively low extracellular calcium concentrations, this sodium permeability is inhibited, and then at higher extracellular calcium concentrations a smaller, calcium current is revealed. In our studies, Orai1 right shifted the calcium current reversal potential, consistent with the idea that Orai1 contributes to calcium selectivity of the TRPC4 macroscopic current.

It was of interest to determine how Orai1 organizes into the larger TRPC1/4 complex, and how it controls TRPC1/4 channel calcium selectivity, as our data suggest the nature of interaction between Orai1 and TRPC1/4 changes as a function of channel activation status. TRPC1/4 and Orai1 are resolved within caveolin rich membrane fractions, along with junctional complex proteins such as cadherins. These membrane regions are highly dynamic. It is presently unknown whether a larger TRPC1/4–Orai complex is preassembled and stably inserted into the membrane, or whether, as with K^+ channels, channels or subunits of channels dynamically enter and exit the membrane.

The junctional locale of the TRPC1/4–Orai complex suggests its calcium signal controls endothelial cell barrier integrity. Calcium permeation through the TRPC1/4 channel has previously been incriminated in endothelial cell barrier disruption. Here, however, we identify that Orai1’s regulation of the TRPC1/4 calcium selectivity is critically important. Recent work by Abdullaev and coworkers indicates Orai1 also controls endothelial cell proliferation. Together, these results suggest that Orai1 regulation of calcium influx disrupts the endothelial cell barrier while activating a mechanism that promotes vascular repair. Future studies will be needed to determine how the calcium signal is coordinated to regulate diverse intracellular effector mechanisms.

In summary, our studies reveal a previously unappreciated interaction between an endogenous TRPC1/4 channel and Orai1, where Orai1 constitutively interacts with TRPC4. This interaction is important for TRPC1/4 channel activation, and for the biophysical properties of the TRPC1/4 channel once it is activated, as Orai1 determines calcium selectivity of the TRPC1/4 macroscopic current and the calcium concentration permeating the channel. This tight control of calcium influx is
physiologically relevant, as calcium that permeates through the TRPC1/4 channel disrupts the endothelial cell barrier.

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Disclosures
None.

References


What New Information Does This Article Contribute?

- Orai1 interacts with TRPC4 and is a component of the TRPC1- and TRPC4-containing channel in endothelium, both in vitro and in vivo.
- Orai1 increases calcium selectivity of, and calcium permeation through, the TRPC channel.
- Orai1 is required for calcium influx through store-operated calcium channels to induce interendothelial cell gaps.

In acute inflammatory conditions, inflammatory mediators increase cytosolic calcium in the endothelium, which serves as a signal for reorganization and contraction of the actin cytoskeleton, loss of cell-cell apposition, and interendothelial cell gap formation, which increases tissue edema. This increase in cytosolic calcium is due to calcium influx across the cell membrane. Whereas store-operated calcium channels mediate calcium entry, the molecular composition and function of endothelial cell store-operated calcium channels remain incompletely understood. Presently, we found that pulmonary artery endothelial cells express TRPC1, TRPC4, and Orai1 proteins, which coimmunoprecipitate with protein 4.1 within caveolin-rich membrane fractions. To ascertain the molecular composition of the endogenous endothelial cell store-operated calcium channel complex, we developed a novel Förster Resonance Energy Transfer approach using protein 4.1 as bait. Isolated channel complexes were conjugated to anti-TRPC protein or anti-Orai1 antibodies labeled with Cy3-cy5 pairs. The protein 4.1-bound channel complex contains 1 TRPC1 and at least 2 TRPC4 subunits. Orai1 constitutively interacts with TRPC4, and with TRPC1 after the channel has been activated. The presence of Orai1 in this channel complex is necessary to optimize channel activation, and to control the amount of calcium that permeates through the TRPC channel. Moreover, the presence of Orai1 within the complex is necessary for calcium entry through the store-operated calcium channel to induce interendothelial cell gaps. These findings define Orai1 as an essential constituent of the endogenous endothelial cell store-operated calcium channel that controls endothelial cell permeability in the acute inflammatory response.
Orai1 Determines Calcium Selectivity of an Endogenous TRPC Heterotetramer Channel
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*Orai1 determines calcium selectivity of an endogenous TRPC heterotetramer channel*

Donna L. Cioffi¹, Songwei Wu¹, Hairu Chen², Mikhail Alexeyev³, Claudette M. St. Croix⁶, Bruce R. Pitt⁶, Stefan Uhlig⁷, and Troy Stevens²,⁴,⁵

Departments of Biochemistry and Molecular Biology¹, Pharmacology², Cell Biology and Neuroscience³, and Medicine⁴, Center for Lung Biology⁵, University of South Alabama, Mobile AL 36688; Department of Environmental and Occupational Health⁶, University of Pittsburgh, Pittsburgh PA 15219; Institute of Pharmacology and Toxicology⁷, University Hospital Aachen, RWTH Aachen, Wendlingweg 2, 52074 Aachen
SUPPORTING ONLINE MATERIAL

Materials and Methods

Reagents. Reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise noted. TRPC antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Sigma-Aldrich Co. (St. Louis, MO), Millipore Corp. (Billerca, MA) or received as gifts. Additionally, TRPC4 antibody was received from Dr. M. Zhu (The Ohio State University), TRPC1 antibody was received from Dr. I. Ambudkar (National Institutes of Health, Secretory Division) and protein 4.1 antibody was received from Dr. S.R. Goodman (Syracuse University). ImmunoPure® Immobilized Protein A was purchased from Thermo Scientific, Inc. (Rockford, IL).

Isolation and Culture of Pulmonary Artery and Microvascular Endothelial Cells. Extralobar pulmonary arteries are isolated, inverted, collagenase treated, scraped into primary cultures, and grown in standard cell culture media supported with 10-20% fetal or calf bovine serum. Cultures are intensely selected in the first weeks after isolation to eliminate contaminating cells, and enrich for and expand pure populations of pulmonary artery endothelial cells. Once expanded, these cells exhibit a cobblestone morphology that is typical of endothelium. At confluence, pulmonary artery endothelial cells reach a baseline resistance near 1200-1400 Ω, measured using the electrical cell-substrate impedance sensing approach. Upon trypsinization and reseeding, these cells grow to confluence with a doubling time of approximately 57 hours. They express typical endothelial cell markers, including endothelial cell nitric oxide synthase, vascular endothelial cell adhesion molecule (CD144), and platelet-endothelial cell adhesion molecule (CD31), and they display typical endothelial cell behaviors, such as the uptake of low density lipoprotein and formation of vascular networks on Matrigel in vitro and in vivo. Pulmonary artery endothelial cells interact with Helix pomatia, but not with Griffonia simplicifolia.

Pulmonary microvascular endothelial cells are isolated by a modification of the pleural cut technique, in which lungs are dissected approximately 0.5 mm deep along the pleural surface, minced into small pieces, exposed to collagenase and strained. Cells collected following straining are seeded onto culture plates, and grown into primary cultures in standard cell culture media supported with 10-20% fetal or calf bovine serum. Cultures are intensely selected in the first weeks after isolation to eliminate contaminating cells, and enrich for and expand pure populations of pulmonary microvascular endothelial cells. Once expanded, these cells exhibit a cobblestone morphology that is typical of endothelium. At confluence, pulmonary microvascular endothelial cells reach a baseline resistance near 1600-2000 Ω, using the electrical cell-substrate impedance sensing system. Upon trypsinization and reseeding, they grow to confluence with a doubling time of approximately 39 hours. They express typical endothelial cell markers, including endothelial cell nitric oxide synthase, vascular endothelial cell adhesion molecule (CD144), and platelet-endothelial cell adhesion molecule (CD31), and they display typical endothelial cell behaviors, such as the uptake of low density lipoprotein and formation of vascular networks on Matrigel in vitro and in vivo. Pulmonary microvascular endothelial cells interact with Griffonia simplicifolia, but not with Helix pomatia.

Isolation of Caveolin-Rich Fractions from the Intact Pulmonary Circulation. Rat lungs were perfused through the pulmonary artery at a constant hydrostatic pressure (12 cm H2O) with Krebs-Henseleit-buffer (containing 2% albumin, 0.1% glucose, 0.3% HEPES) and ventilated with 2 mL tidal volume at 60 breaths/min.
Membrane fractions from endothelial cells were isolated by use of colloidal silica beads essentially as described. Briefly, after 40 min of lung perfusion, the flow rate was reduced to 2-3 mL/min and perfusion with 1% cationic colloidal silica beads was started. Lung tissue was homogenized and the homogenate was mixed with an equal volume of 1.02 g/ml Nycodenz (containing 20 mM KCl) and layered over 0.5-0.7g/ml Nycodenz containing 60 mM sucrose in a centrifuge tube. After centrifugation (Beckman SW 28 rotor, 20000 rpm) for 30 min at 4°C the floating tissue debris was removed and the pellet containing the silica-coated endothelial membrane fragments was resuspended with 1ml MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl).

Triton-X-100 (final concentration 1%, 4°C) was added to the membranes for 60 min. After incubation the suspension was homogenized and the homogenate mixed with 80% sucrose to achieve a 40% membrane-sucrose-solution. A 30-5% sucrose gradient was layered on top. Samples were centrifuged in a Beckman·centrifuge (SW55Ti rotor, 30000 rpm) at 4°C overnight for 16-18h. Fractions were sampled from the top to the bottom. The fraction used for this study represents caveolae; it was rich in caveolin-1 and flotillin (caveolae marker proteins), but was lacking angiotensin converting enzyme (a non-caveolar plasma membrane protein).

Membrane/Cytoskeleton Fractions and Immunoprecipitation. Endothelial cell monolayers were lysed and scraped in a lysis buffer. The lysate was then centrifuged at 3000 x g, the pellet was resuspended in a sucrose buffer, homogenized and centrifuged at 3000 x g. The supernatant was then divided into aliquots and centrifuged at 50,000 x g. The resulting pellet was incubated in extraction buffer in the presence of KI for 40 minutes at 4°C, and then was centrifuged at 145,000 x g to resolve a pellet fraction and a salt-dissociated supernatant fraction.

Preparation of Isolated Immunocomplex. Protein 4.1 antibody was incubated with ImmunoPure® Immobilized Protein A beads for 4-5 hours. The antibody affixed beads were washed once in extraction buffer plus 100 mM KI. Salt-dissociated supernatant (40 μL or 8 μL) and protease inhibitor cocktail was added to the washed beads. The mixture was incubated at 4°C overnight. Beads were washed five times with extraction buffer/100 mM KI and stored under extraction buffer/100 mM KI at -80°C.

SDS-PAGE and Western Blot. Samples were run on 4-12% Bis-Tris Gels (Invitrogen Corp.) and transferred to nitrocellulose overnight at 30V and 4°C. Membranes were rocked in blocking buffer [phosphate-buffered saline with 5% non-fat dry milk/0.1% Tween 20 or Protein-free (TBS) blocking buffer (Thermo Scientific, Inc.) with 0.05% tween] for 1 hour at room temperature, primary antibody for either 2 hours at room temperature or overnight at 4°C and then secondary antibody (horseradish peroxidase conjugated) for one hour at room temperature. Proteins were visualized using SuperSignal West Pico or West Femto Chemiluminescent Substrates (Thermo Scientific, Inc.).

Fluorescently Tagged Antibodies. Antibodies were tagged with the cyanine reagents cy3 and cy5 following the manufacturer’s directions (Cy3 mAb Labeling Kit #PA33001 and Cy5 mAb Labeling Kit #PA35001; GE Healthcare Bio-Sciences Corp.; Piscataway, NJ).

Förster resonance energy transfer (FRET) Experiments. To construct an antibody dose response curve, isolated immunocomplex (prepared above) was divided into eight aliquots. IgG (0.5 to 1.0 mg) from rabbit serum in 0.05% Triton X-100/PBS was added to bind free protein A sites on the beads. Samples were rotated overnight at 4°C, then washed once with 0.05% Triton X-100/PBS. Fluorescently tagged antibody was added to each sample over a range of
concentrations. Samples were rotated at 4 °C for 5 – 6 hours, then washed three times with 0.05% Triton X-100/PBS. Samples were re-suspended in 0.05% Triton X-100/PBS, transferred to a 96 well plate, and fluorescence counts read on a Spectra Max M5 plate reader (Molecular Devices; Sunnyvale, CA). Fluorescence counts were plotted as a function of antibody concentration.

For FRET experiments, isolated immunocomplex was divided into eight aliquots, rotated overnight in the presence of 0.5 to 1.0 mg IgG from rabbit serum in 0.05% Triton X-100/PBS and washed once with 0.05% Triton X-100/PBS. Cy3- and Cy5-tagged antibodies to the TRPC subunits were added to the samples at their EC50 concentrations. Samples were rotated at 4 °C for 5 – 6 hours, washed three times with 0.05% Triton X-100/PBS and resuspended in 0.05% Triton X-100/PBS. Samples were transferred to glass slides containing mounting medium and a cover slip placed on top. FRET experiments were performed on a Leica TCS SP2 Confocal Microscope (63x oil objective) using the sensitized emission approach. Spectral bleed-through was corrected for by measuring donor only and acceptor only samples. Regions of interest (roi’s) were selected and FRET efficiency calculated by the Leica software using the formula EA(i) = [B-Axb-Cx(c-axb)]/C. EA is the apparent FRET efficiency; A,B,C are the donor, FRET and acceptor channel intensities, respectively; a,b,c are correction factors. FRET signals, as determined via the sensitized emission approach, were confirmed using acceptor photobleaching.

**Engineering Conditional Expression of Orai1 shRNA.** Doxycycline-regulated knockdown in the rat pulmonary artery endothelial cells (PAECs) was performed using a hybrid retro-lentiviral system. Low passage PAECs first infected with retrovirus 2641, which encodes the Long Terminal Repeat (LTR)-promoter driven reverse tetracycline transactivator (rtTA) gene component of the Tet-On Advanced system (Clontech, Mountain View, CA). Infection was achieved by incubating target cells in 35 mm dishes at 20% confluence with retrovirus-containing supernatants overnight 8 µg/ml polybrene (Sigma–Aldrich Corp., St. Louis, MO). The next day supernatant was removed and cells were allowed to recover for 24 hours in DMEM, after which time cells were trypsinized, transferred into 140 mm dishes and blasticidin selection (30 µg/ml) was applied for 5 days. shRNA-encoding oligonucleotides were designed against coding sequence of Orai1 using BLOCK-iT RNAi Designer program (Invitrogen, Carlsbad, CA), and cloned into BsuAI-digested lentiviral vector pMA2867. The identity of cloned oligonucleotides was verified by sequencing, and lentivirus-containing supernatants were produced from the resulting plasmids by CaPO4-mediated transfection of the HEK293FT cell line using established protocols. Gag, Pol and Env functions for lentiviral constructs were provided in trans by cotransfection of the vector plasmid with two helper plasmids, psPAX2 and pMD2.G (Addgene, Cambridge, MA). Lentivirus infection of rtTA expressing cells was conducted similarly to retroviral infection described above, except puromycin selection (1 µg/ml) was applied for at least 3 days.

**Patch Clamp Electrophysiology.** Transmembrane current recordings were performed using the conventional whole-cell voltage-clamp configuration in single pulmonary artery endothelial cells by the standard giga seal patch-clamp technique. Confluent cells were trypsin dispersed, seeded onto 35-mm plastic culture dishes, and allowed to reattach at least 4 hours before patch-clamp experiments were performed. Patch-clamp recordings were obtained from single electrically isolated pulmonary artery endothelial cells, exhibiting a flat, polyhedral morphology consistent with the cells from a confluent monolayer. Recording pipettes were heat polished to produce a tip resistance in the range of 3 to 5 megohms in the pipette (internal) solution. Currents were recorded with a computer-controlled EPC9 patch-clamp amplifier (HEKA; Lambrecht, Germany). The step–wise voltage pulses were applied from -100 to +60 mV in 20-mV increments after the whole-cell configuration was achieved, with a 200-ms duration during each voltage step and a 2-s interval between steps. The holding potential between each step was 0 mV. For the time-dependent current decay examination, this protocol was applied
approximately every 30 s. Data acquisition and analysis were performed with Pulse/PulseFit software (HEKA), current was measured as the mean value of the current amplitude during the last 20 ms of each step. Current decay was evaluated at a time corresponding to the peak of current (fraction of peak) at the -80 mV voltage pulse. All experiments were performed at room temperature (20-22 °C).

The standard pipette solution contained (in mmol/liter) 130 N-methyl-D-glucamine, 10 Hepes, 2 Mg$^{2+}$-ATP, 1 N-phenylanthranilic acid, 0.1 5-nitro-2-(3-phenylpropylamino)benzoic acid, 2 EGTA, 1 Ca(OH)$_2$ (pH 7.2, adjusted with methane sulfonic acid; the free [Ca$^{2+}$] was estimated as 100 nmol/liter, as calculated by the CaBuf program <G. Droogmans, ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip>). To examine calcium currents, the bath (external) solution contained (in mmol/liter) 120 aspartic acid, 5 Ca(OH)$_2$, 5 CaCl$_2$, 10 Hepes, 0.5 3,4-diaminopyridine (pH 7.4 adjusted with tetrathyamine hydroxide). To examine the anomalous mole fraction effect, the initial Ca$^{2+}$-free external solution contained (in mmol/liter) 130 sodium methanesulfonate, 10 Hepes, 0.5 3,4-diaminopyridine, 10 BAPTA (pH 7.4 adjusted with tetrathyamine hydroxide; the free [Ca$^{2+}$] was estimated as below 1 nmol/liter). To obtain various free [Ca$^{2+}$] in the bath solution, the appropriate amount of Ca$^{2+}$, in the form of CaCl$_2$ or CaCl$_2$/Ca(OH)$_2$, was added in as calculated with the CaBuf program. For instance, in the presence of 10 mmol/liter BAPTA, 6.928, 9.966, 11.000, or 20.000 mmol/liter Ca$^{2+}$ was added to obtain 100 nmol/liter, 10 µmol/liter, 1 mmol/liter, or 10 mmol/liter free Ca$^{2+}$, respectively. All the solutions were adjusted to 290–300 mosmol/liter with sucrose.
Enriching for the TRPC Channel. By incubating the salt dissociated supernatant with antibody bound to protein 4.1 conjugated to Protein A/agarose beads, a population of channels uniquely tethered to protein 4.1 was selected. Prior studies have established that this channel possesses TRPC4, as TRPC4 binds directly to protein 4.1. Functional studies reveal the protein 4.1-bound TRPC4 channel is calcium selective and, further, that other calcium non-selective channels are not inhibited by disrupting the protein 4.1-actin or protein 4.1-spectrin interaction. Hence, utilizing protein 4.1 as bait to enrich for the TRPC4-containing channel purifies a selective population of calcium channels (Online Figure I).

Establishing Antibody Half-Maximal Saturation. Prior to all FRET experiments full binding saturation curves were generated to establish the EC$_{50}$ antibody concentration, which optimizes cy3-cy5 energy transfer. The TRPC1-cy5 binding curve is shown in Online Figure II. From these data a 70 µg/mL TRPC1-cy5 antibody concentration was calculated to represent the EC$_{50}$.

Determining Specificity of Intra-Molecular Interactions using FRET. Several control approaches were developed to ensure FRET signals are due to intra-channel subunit interactions. Dilution experiments were performed where one-fifth the amount of salt-dissociated supernatant was immunoprecipitated for protein 4.1. If the FRET measurements are non-specific, then the distance between adjacent TRPC1/4 channel complexes should increase as the sample becomes more dilute. In this instance, the FRET signal would be abolished. If the FRET measurements are specific, then the distance between individual proteins in the TRPC1/4 complex should not increase as the sample becomes more dilute. In this case, the FRET signal would be retained. To do this, FRET between cy3-labeled TRPC1 and cy5-labeled TRPC4 was tested. In undiluted samples, FRET was readily apparent between TRPC1 and TRPC4. The overall fluorescence intensity was decreased in all of the diluted samples compared to the undiluted samples. However, strong TRPC1-TRPC4 FRET signals were retained in the diluted samples (Online Figure III), demonstrating FRET detects intra-channel subunit interactions.

To confirm efficient energy transfer between cy3 and cy5 was occurring as predicted, photobleaching studies were undertaken. In this case, the TRPC4-cy5 channel was irradiated to photobleach fluorescence. Such inactivation of the cy5 channel prevented energy transfer from cy3-labeled TRPC1 to cy5-labeled TRPC4. Consequently, increased fluorescence was measured in cy3-labeled TRPC1, indicating the signal measured in the FRET channel is specific (Online Figure IV). Collectively, these data confirm that our FRET approach detects intra-channel subunit interactions.

Generating shRNA to Conditionally Knockdown Orai1. A series of shRNA targeting rat Orai1 mRNA were initially transiently transfected and screened for their ability to downregulate Orai1 protein (Online Figure V), allowing for development of a system in which the Orai1 shRNA could be introduced into cells, and then withdrawn, to test the role of Orai1 on TRPC1/4 channel function in wild type cells. To do this, pulmonary artery endothelial cells were engineered to express the Tet-On reverse transactivator protein (rtTA). The construct was stably introduced using a retro-lentiviral system (Online Figure VI). rtTA was followed by an internal ribosome entry site and a fusion between enhanced green fluorescent protein and blasticidin resistance gene. Once these cells were selected to homogeneity, they were re-infected with a second virus containing the shRNA sequence driven by doxycycline stabilized Tet (Online Figure VI). Cells expressing shRNA were selected using puromycin, and confirmed by measuring mCherry fluorescence.
**Time-lapse movies.** PAECs were grown to confluence on 35 mm plates in standard growth media, washed with physiological salt solution and placed on the microscope stage. Thapsigargin (1 µmol/L) was added to the media, and pictures were taken at 15-second intervals for a period of 1-hour. Pictures were then compressed to generate the movie. Doxycycline treatment was initiated daily prior to the application of thapsigargin. **Online Figure VII** shows the typical monolayer response to thapsigargin in cells with and without Orai1 (representative of 3 experiments/treatment). Orai1 silencing prevents gap formation. Studies using wild type endothelial cells revealed no effect of doxycycline treatment; doxycycline treatment did not prevent thapsigargin from inducing gaps (data not shown).
**Figure Legends**

**Online Figure I.** Schematic representing the protein 4.1-bound TRPC channel complex interacting with agarose beads. Protein 4.1 antibodies were bound onto agarose beads and used to isolate the endogenous TRPC1/4 channel. Channel proteins were then labeled with cy3 or cy5 to enable FRET measurements using the sensitized emission approach.

**Online Figure II.** Binding saturation curves reveal the half maximal antibody concentration needed to optimize cy3-cy5 energy transfer. TRPC1 antibody cy3 fluorescence was tested over a range of concentrations. Using this approach, the half maximal antibody concentration is determined for subsequent experiments.

**Online Figure III.** Dilution experiments reveal high FRET efficiency is retained even with lower total fluorescence. Labeled agarose beads were diluted 1:5, and FRET measurements repeated. Despite a decrease in total fluorescence (data not shown) under these experimental conditions, the FRET efficiency was retained, supporting the idea that FRET detected interactions between proteins within the channel complex.

**Online Figure IV.** Energy transfer from TRPC1-cy3 to TRPC4-cy5 is illustrated by photobleach of the cy5 channel. A region of interest (shown in the hatched black box) was identified and irradiated in the cy5 channel. Photobleach of the cy5 fluorescence prevented energy transfer from cy3 to cy5 channels within this region, resulting in increased cy3 fluorescence.

**Online Figure V.** shRNA sequences utilized to decrease Orai1 expression. The coding region for the rat Orai1 gene is shown, with shRNA target regions highlighted in red and green. The most effective shRNA sequence is shown red. The targets were selected and prioritized (based on the probability of successful knockdown) using Invitrogen’s BLOCK-iT™ RNAi Designer program. Note that targets for shRNAs #1 and #2 overlap.

**Online Figure VI.** Map for the conditional expression of Orai1 shRNA in endothelium. Pulmonary artery endothelial cells were first infected with pMA2641 and selected to homogeneity using blasticidin. These purified cells were then infected with pMA2879 and selected to homogeneity using puromycin. Abbreviations: 5’ and 3’ mir, 5’ and 3’ flanking sequences derived from the murine mir-155 micro-RNA gene; Bsr, blasticidin resistance gene; EGFP, enhanced green fluorescent protein; HIV RRE, human immunodeficiency virus rev response element; IRES EMV, encephalomyocarditis virus internal ribosome entry site; LTR, retro/lentiviral long terminal repeat; PAC, puromycin resistance gene; PSV40, SV40 promoter; P_TET, doxycycline-regulated promoter; tTA, tetracycline controlled transactivator protein; wPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

**Online Figure VII.** Orai1 silencing prevents thapsigargin-induced inter-endothelial cell gap formation. Pulmonary artery endothelial cells engineered for conditional expression of Orai1 shRNA were grown in the presence (+) or absence (-) of doxycycline to confluence on 25 mm glass coverslips and imaged. Images were captured every 15-seconds for 1-hour, and the images compressed into a time-lapse movie. Thapsigargin (1 µM) was applied to the monolayer to activate store operated calcium entry. Whereas thapsigargin induced transient gap formation in the presence of Orai1, gap formation was not observed in cells lacking Orai1.
Protein A/Agarose Bead

Excite Cy3

FRET

Emit Cy5

Online Figure I
## FRET Efficiency

<table>
<thead>
<tr>
<th>FRET Pair</th>
<th>PAEC ½ Dilution</th>
<th>PAEC ½/10 dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC 1/4</td>
<td>0.27 ± 0.09</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>TRPC 1/1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TRPC 4/4</td>
<td>0.30 ± 0.13</td>
<td>0.22 ± 0.06</td>
</tr>
</tbody>
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Online Figure IV
Online Figure V
pMA2641 (Tet-On)

- LTR
- rtTA advanced
- IRES EMV
- EGFP
- Bsr
- wPRE
- LTR

pMA2879

- LTR
- HIV RRE
- P_{TET}
- 5' mir
- 3' mir
- P_{SV40}
- PAC
- LTR

- mCherry
- Orai1 shRNA
- BfuAI
- BfuAI

Online Figure VI
See accompanying time-lapse movie