Activation of the Endothelial Store-Operated I_{SOC} Ca^{2+} Channel Requires Interaction of Protein 4.1 With TRPC4

Donna L. Cioffi, Songwei Wu, Mikhail Alexeyev, Steven R. Goodman, Michael X. Zhu, Troy Stevens

Abstract—Store-operated calcium (SOC) entry represents the principal Ca^{2+} entry pathway into nonexcitable cells. Despite intensive investigation, mechanisms underlying activation of SOC entry have remained elusive. The endothelial I_{SOC} channel is a Ca^{2+}-selective SOC entry channel to which the transient receptor potential (TRP) proteins TRPC1 and TRPC4 contribute subunits. Activation of I_{SOC} is specifically regulated by the spectrin–actin membrane skeleton; however, the nature of coupling between the I_{SOC} channel and membrane skeleton is unknown. Here we demonstrate that protein 4.1 is an essential component of the I_{SOC} channel gating mechanism. Protein 4.1 interacts with TRPC4 and the membrane skeleton. Deletion of the protein 4.1 binding domain on TRPC4 or peptide competition to the protein 4.1 binding domain prevents I_{SOC} activation. These findings reveal that interaction of protein 4.1 with TRPC4 is required for activation of the endothelial I_{SOC} channel. **(*Circ Res*. 2005;97:1164-1172.)**

Key Words: cytoskeleton ■ I_{SOC} ■ spectrin ■ store-operated Ca^{2+} ■ entry ■ TRPC

Elevation in cytosolic calcium, $[Ca^{2+}]_i$, is an essential step in numerous physiological and pathophysiological signal transduction events. Ion channels allowing for the entry of extracellular Ca^{2+} into the cytosol are the principal source of increased $[Ca^{2+}]_i$. In nonexcitable cells, store-operated calcium (SOC), and to a lesser extent, receptor-operated calcium (ROC), entry channels represent the main source of calcium entry.[1,2] Although it is generally recognized that SOC entry occurs through Ca^{2+}-selective and nonselective channels, the molecular makeup, regulation, and downstream targets of specific channels remain to be determined.

In endothelial cells, activation of SOC entry contributes to the formation of intercellular gaps, leading to endothelial barrier disruption,[3–5] a common factor in the development and progression of systemic and pulmonary diseases, including hypertension, atherosclerosis, and acute respiratory distress syndrome. It is therefore therapeutically desirable to identify specific SOC entry channel(s) involved in endothelial barrier disruption. We recently reported that activation of the Ca^{2+}-selective SOC entry current I_{SOC} contributes to the increase in $[Ca^{2+}]_i$ necessary to disrupt the endothelial barrier.[6] However, the molecular makeup and regulation of the channel that provides I_{SOC} (ie, the I_{SOC} channel) are incompletely understood.

Transient receptor potential (TRP) proteins encode SOC and ROC entry channels. The TRPC (canonical) subfamily consists of seven known members.[7] It is not currently clear which TRPC homologues contribute to SOC versus ROC entry channels. Some studies have shown that TRPCs 1, 3, 4, and 5 can be activated by depletion of endoplasmic reticulum Ca^{2+}, suggesting they contribute to SOC entry channels, whereas other studies have proposed contribution of these homologues to ROC entry channels.[8] For the purpose of this study, we focused specifically on endogenous TRPC proteins that contribute to SOC entry in endothelial cells. Given this criterion, TRPC1 and TRPC4 contribute to the endothelial Ca^{2+}-selective I_{SOC} channel. Previously, our group used antisense technology to assess the contribution of endogenous TRPC1 to I_{SOC} in pulmonary artery endothelial cells (PAECs).[9] Inhibition of TRPC1 reduced but did not eliminate I_{SOC}. On the other hand, endothelial cells obtained from TRPC4-deficient mice completely lacked I_{SOC}.[10] These studies suggest that although TRPC1 and TRPC4 contribute subunits to the I_{SOC} channel molecular makeup, it is TRPC4 that is required for channel activation.

Activation of *Drosophila melanogaster* TRP and mammalian TRPC1 channels requires an intact C terminus.[11–13] However, to date, mechanisms regulating SOC entry channel activation are still unclear. Early studies in endothelial cells and platelets provided evidence supporting cytoskeletal regulation of SOC entry channel activation.[5,14,15] Actin and spectrin principally comprise cellular membrane and cytoskeletons, whereas protein 4.1 and ankyrin function to link transmembrane proteins to the underlying membrane skeleton.[16] Our group recently demonstrated that the specific interaction of protein 4.1 with spectrin was required for I_{SOC} activation.[17] Thus, we proposed that protein 4.1 functionally links the endothelial I_{SOC} channel to the membrane skeleton.

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From the Center for Lung Biology (D.L.C., S.W., M.A., T.S.), Department of Pharmacology (D.L.C., S.W., T.S.), and Department of Cell Biology and Neuroscience (M.A.), the University of South Alabama College of Medicine, Mobile; Department of Molecular and Cell Biology (S.R.G.), the University of Texas at Dallas; and Department of Neuroscience and Center for Molecular Neurobiology (M.X.Z.), the Ohio State University, Columbus.

Correspondence to Troy Stevens, PhD, Professor, Center for Lung Biology, Department of Pharmacology, University of South Alabama College of Medicine, Mobile, AL 36688. E-mail tstevens@jaguar1.usouthal.edu

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Although the spectrin–protein 4.1 interaction is necessary for I_{SOC} activation, the mode of coupling between the membrane skeleton and I_{SOC} channel is unknown. Because TRPC4 appears to be the subunit important for I_{SOC} channel activation, we sought to determine whether protein 4.1 interacts with TRPC4. We report that protein 4.1 binds to TRPC4, likely within a conserved protein 4.1 binding domain located on the C terminus of TRPC4. Deletion of the protein 4.1 binding domain on TRPC4, as well as peptide transfection targeted to disrupt endogenous TRPC4–protein 4.1 interaction, similarly prevents I_{SOC} activation. Collectively, these data support a regulatory role for protein 4.1 in I_{SOC} channel activation.

Materials and Methods

Reagents

All reagents were obtained from Sigma-Aldrich unless specified otherwise. Antibodies to TRPC4 were provided by M.Z. or purchased from Sigma-Aldrich; protein 4.1 antibody was received from S.R.G. Secondary antibodies and chemiluminescence reagents were purchased from Santa Cruz Biotechnology or Molecular Probes, Inc.

Isolation and Culture of Pulmonary Endothelial Cells

PAECs were isolated and cultured using a method described by Creighton et al.[18]

Immunocytochemistry

PAEC monolayers were methanol fixed and permeabilized. Primary antibody to TRPC4 (Sigma-Aldrich) was added at 1:200 dilution for 1 hour followed by secondary antibody (Alexa Fluor 488 goat anti-rabbit; Molecular Probes, Inc.) at 1:300 dilution for 40 minutes. Fluorescent and Normarski differential interference contrast images were obtained using a Leica TCS SP2 confocal laser scanning microscope fitted with a 63× oil immersion objective.

Preparation and Detergent Extraction of Membrane/Cytoskeleton Fractions[19]

For detailed protocol, refer to the online supplement, available at http://circres.ahajournals.org.

Thapsigargin Treatment

Cells were exposed to 1 μmol/L thapsigargin (Calbiochem) for 2.5 to 3 minutes at room temperature before beginning lysing procedure. In these experiments, the total time from addition of thapsigargin to end of lysing procedure (ie, samples at −80°C) was 12 to 14 minutes.

Immunoprecipitation Reactions and Western Blot Analysis

Immunoprecipitation Reactions

To 150 to 250 μL of supernatant was added TRPC4 antibody (1:100 dilution) or protein 4.1 antibody (1:130 dilution), and 2% protease inhibitor cocktail (10×). The sample was rocked at 4°C (typically overnight). This was followed by addition of 40 to 50 μL EZ View Red Protein A affinity gel (Sigma-Aldrich) and 2% protease inhibitor cocktail (10×). The sample was rocked for an additional 4 to 5 hours, pelleted, washed 3×750 μL ice-cold PBS/0.1% protease inhibitor cocktail (10×), and resuspended in 4× lithium dodecylsulfate (Invitrogen).

Western Blot Analysis

For SDS-PAGE, 7%, 8%, or 4% to 12% gradient (Invitrogen) gels were typically used. Samples were transferred to nitro-cellulose or polyvinylidene difluoride membranes at 30 V. Membranes were rocked with primary antibody for 2.5 hours at room temperature or overnight at 4°C and with secondary antibody for 1 hour. Proteins were visualized via enhanced chemiluminescence detection (Luminol Reagent; Santa Cruz Biotechnology).

Adenoviral Constructs

Recombinant adenoviruses were generated according to established protocols[20] using commercially available plasmids (AdMax; Microbix). For protocol description, refer to the online supplement.

Adenovirus Infection

Endothelial cells were seeded on 100- or 35-mm culture dishes±25-mm circle microscope glass coverslips (Fisher Scientific) and grown to ≈60% to 90% confluence. Cells were infected with adenovirus constructs at multiplicities of infection equal to 50:1, 25:1, or 10:1. Ca^{2+} and I_{SOC} measurements were performed at 36 to 50 hours after infection.

Peptide Transfection

Two peptides were synthesized by Alpha Diagnostic International, Inc. The prr/4.1 peptide corresponds to the region on TRPC4 encompassing the protein 4.1 binding domain and adjacent proline-rich region. A peptide with scrambled sequence was also prepared. Peptides were transfected into PAECs using Chariot reagent (Active Motif) generally following the suggested protocol of the manufacturer. For protocol description, refer to the online supplement.

Ca^{2+} Measurements

Endothelial cells were seeded onto 25-mm glass coverslips and grown to confluence. $[Ca^{2+}]_i$ was estimated with the fluorophore fura 2-acetoxymethylester (fura-2 AM; Molecular Probes) according to methods described previously.[21]

Patch-Clamp Electrophysiology

Conventional whole-cell voltage clamp configuration was performed to measure transmembrane currents in single rat PAECs using the protocol described by Wu et al.[17]

Results

TRPC4 Localizes to Sites of Cell–Cell Adhesion

TRPC4 is a subunit of the endothelial I_{SOC} channel that, when activated, promotes endothelial barrier disruption through the formation of interendothelial cell gaps. Immunocytochemistry was performed to determine cellular localization of endogenous TRPC4. Positive TRPC4 staining was identified in the plasma membrane of PAECs. Staining was particularly strong in areas of the membrane involved in cell–cell adhesion (Figure 1). Additional punctate staining was evident, likely because of the presence of TRPC4 in cholesterol-rich (eg, caveolae) areas of the membrane.[22] The localization of TRPC4 at sites of cell–cell adhesion supports a role for the endothelial I_{SOC} channel in intercellular gap formation.[6]

TRPC4 Binds to the PAEC Membrane Skeleton

Interaction between spectrin and protein 4.1 is required for activation of the I_{SOC} channel.[17] However, the nature of interaction between the membrane skeleton and proteins encoding the I_{SOC} channel is unknown. Because TRPC4 appears to be required for channel activation, we sought to determine whether TRPC4 physically interacts with the membrane skeleton. Membrane/cytoskeleton fractions of PAECs were detergent extracted using octyl-β-D-glucopyranoside to yield pellet and supernatant fractions. SDS-PAGE and immunoblotting of these fractions found

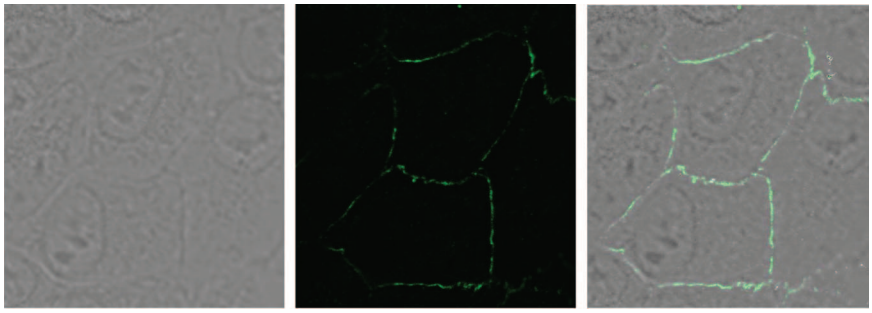

Figure 1. Endogenous TRPC4 localizes to PAEC plasma membrane. Immunocytochemistry of TRPC4 in PAECs. Left panel, Normarski image of several cells within PAEC monolayer. Center panel, Endogenous TRPC4 (green) resides in the plasma membrane, principally at sites of cell–cell adhesion. Punctate staining likely indicates TRPC4 presence in membrane-bound caveolae.[22] Right panel, Overlay of fluorescent and Normarski differential interference contrast images.

TRPC4 exclusively in the pellet (Figure 2a). Actin, a membrane skeleton marker, was also found in the pellet, whereas endothelial myosin light chain kinase 1 resided in the supernatant. Protein 4.1 was detected in pellet and supernatant fractions. Thus, we found that TRPC4 physically interacts with the membrane skeleton of PAECs because it was found in the pellet fraction extracted from membrane/cytoskeleton preparations. It is noteworthy that this physical association appears to be constitutive (ie, not requiring channel activation).

TRPC4 Interacts With the Membrane Skeleton in a Salt-Sensitive Manner

Having demonstrated that TRPC4 physically associates with the membrane skeleton, we next questioned the nature of this interaction. Salts are ionic compounds that weaken ionic and strengthen hydrophobic protein–protein interactions.[23] When PAEC membrane/cytoskeleton fractions were detergent extracted in the presence of 1 mol/L potassium iodide (KI), TRPC4 was found in the supernatant fraction instead of the pellet (data not shown). This suggested that TRPC4 associates with the membrane skeleton through an ionic interaction, which is sensitive to KI disruption. To more selectively probe the TRPC4–membrane skeleton interaction, we repeated the experiments using lower salt concentrations. Even at 150 mmol/L KI, TRPC4 resided in the supernatant (Figure 2b). We also probed for protein 4.1. Similar to TRPC4, the interaction of protein 4.1 with the membrane skeleton was sensitive to 150 mmol/L KI because the majority of protein 4.1 was now found in the supernatant. These data indicate that TRPC4 and protein 4.1 associate with the membrane skeleton through an ionic interaction sensitive to KI disruption.

To determine the minimal KI concentration necessary to dissociate TRPC4 from the membrane skeleton, we performed detergent/KI extractions over a low KI concentration range (0 to 100 mmol/L KI). Immunoblot analysis revealed that the interaction of TRPC4 with the membrane skeleton is

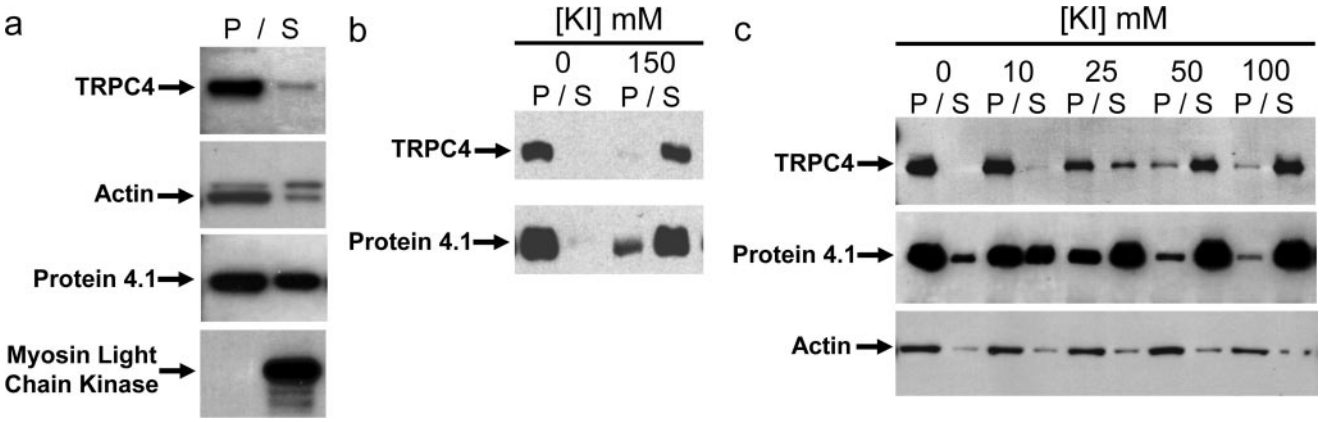

Figure 2. Association of TRPC4 with the PAEC membrane skeleton is KI sensitive. a, Immunoblot analysis of pellet (P) and supernatant (S) detergent extracted membrane/cytoskeleton fractions. TRPC4 (≈110 kDa) was detected in the pellet, indicating strong interaction with the membrane skeleton, represented by actin at 43 kDa. Protein 4.1 (≈110 kDa) was also found in the pellet. The endothelial isoform myosin light chain kinase 1 (210 kDa) resided exclusively in the supernatant. b, TRPC4 and protein 4.1 are dissociated from the membrane skeleton by 150 mmol/L KI. PAEC membrane/cytoskeleton fractions were detergent extracted in the presence of 150 mmol/L KI. At this ionic strength, the interaction of TRPC4 with the membrane skeleton was completely disrupted, and TRPC4 was detected exclusively in the supernatant. Likewise, protein 4.1 dissociated from the membrane skeleton because it was also mainly found in the supernatant. c, The sensitivity to ionic strength was evaluated for TRPC4 and protein 4.1 at the KI concentrations: 0, 10, 25, 50, and 100 mmol/L KI. Immunoblotting for TRPC4 and protein 4.1 revealed that both proteins similarly demonstrated salt-dependent dissociation from the pellet (at 0 mmol/L KI) to the supernatant (at 100 mmol/L KI). Actin was found predominantly in the pellet at all KI concentrations.

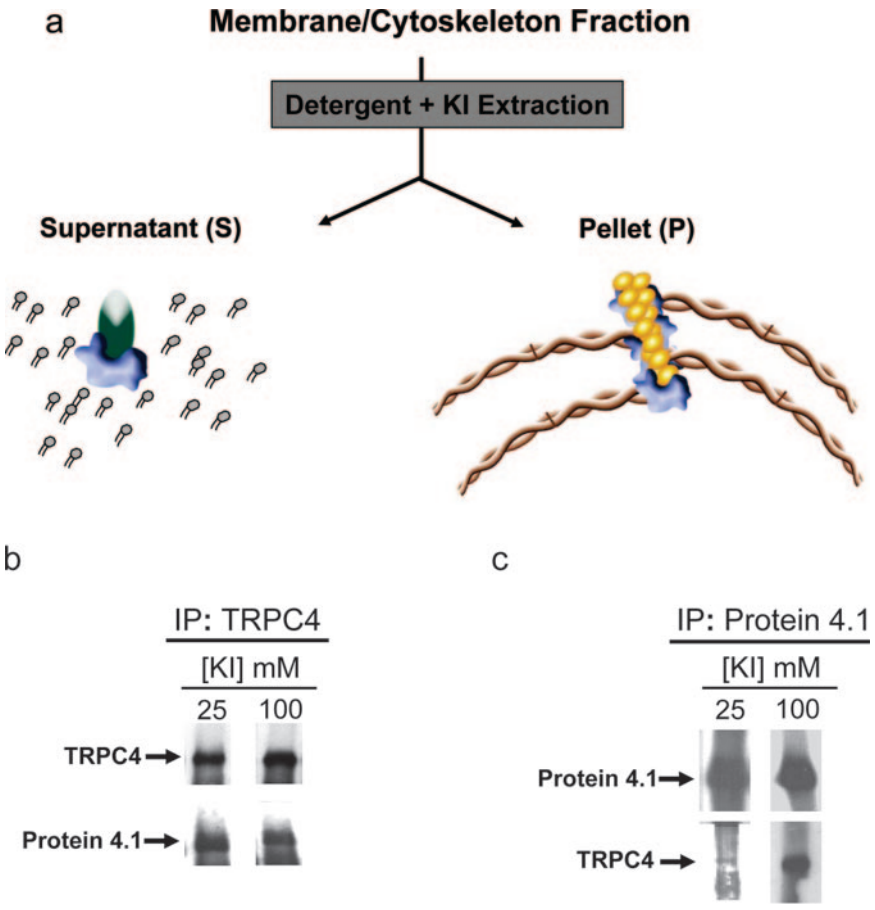

Figure 3. TRPC4 and protein 4.1 coimmunoprecipitate from salt-dissociated supernatants. a, Schematic representation of the pellet and supernatant fractions after KI/detergent extraction. As demonstrated in Figure 2, extraction of the membrane/cytoskeleton fractions in the presence of KI resulted in localization of TRPC4 and protein 4.1 to the supernatant, suggesting a TRPC4–protein 4.1 complex dissociated from the membrane skeleton. The model shows in the salt-dissociated supernatant, protein 4.1 (blue) bound to TRPC4 (green). In the pellet, actin (yellow), spectrin (brown), and protein 4.1 (blue) are shown interacting as the membrane skeleton. b, Immunoprecipitation of TRPC4 from salt-dissociated supernatants. At 25 and 100 mmol/L KI concentrations, protein 4.1 coimmunoprecipitated with TRPC4 in the supernatant. c, Immunoprecipitation of protein 4.1 from salt-dissociated supernatants. Probing for the coimmunoprecipitation of TRPC4 with protein 4.1 at 25 mmol/L KI resolved only a weak TRPC4 band. At 100 mmol/L KI, the coimmunoprecipitated TRPC4 band was strong.

exquisitely sensitive to this concentration range because it exhibited a dose-dependent shift from the pellet fraction to the supernatant (Figure 2c). Protein 4.1 was also sensitive to this KI concentration range (ie, at 0 mmol/L KI, the majority of protein 4.1 resided in the pellet fraction, whereas at 100 mmol/L KI, protein 4.1 was found predominantly in the supernatant). Actin, on the other hand, was found mainly in the pellet fraction irrespective of solution ionic strength. Collectively, these observations demonstrate that TRPC4 and protein 4.1 associate with the membrane skeleton through similar ionic interactions. These findings suggested to us that the KI-mediated dissociation of TRPC4 and protein 4.1 from the membrane skeleton occurred in the form of a TRPC4–protein 4.1 complex.

TRPC4 Interacts With Protein 4.1

If TRPC4 interacts with protein 4.1 and a TPRC4–protein 4.1 complex dissociates from the membrane skeleton in the presence of KI, then these proteins should coimmunoprecipitate from the salt-dissociated supernatant (Figure 3a). This is predicated on the idea that TRPC4–protein 4.1 binding is neither ionic in nature nor sensitive to disruption by KI. TRPC4 was immunoprecipitated from 25 and 100 mmol/L KI salt-dissociated supernatants and the coimmunoprecipitation of protein 4.1 assessed by immunoblot. Protein 4.1 coimmunoprecipitated with TRPC4 at both KI concentrations; these data indicate that indeed protein 4.1 binds to TRPC4 (Figure 3b). We next performed the reciprocal immunoprecipitation experiment and observed that TRPC4 coimmunoprecipitated with protein 4.1 (Figure 3c). Interestingly, in this reverse experiment, we observed that the TRPC4 coimmunoprecipitation was dependent on the salt concentration (ie, at 25 mmol/L KI, the coimmunoprecipitated TRPC4 appeared as a weak band, whereas at 100 mmol/L KI, the TRPC4 band was much stronger. Together, these data reveal that protein 4.1 binds to TRPC4, and further, increased KI concentrations promote interaction of TRPC4 with protein 4.1. Because salt strengthens hydrophobic interactions, we conclude that the interaction of TRPC4 with protein 4.1 is hydrophobic in nature.

TRPC4 Contains a Protein 4.1 Binding Domain

We screened TRPC4 for potential protein 4.1 binding domains and identified a sequence on the C terminus between amino acids 675 and 685 that resembled a consensus protein 4.1 binding sequence [(S/T)XXX(K/R)$_4$] found in membrane-associated guanylate kinase proteins.[24] Interestingly, the putative protein 4.1 binding sequence resides only 50 amino acids downstream from where the sixth transmembrane region exits the membrane, placing it in direct proximity to the pore region of the channel (Figure 4a and 4b). A proline-rich region (amino acids 654 to 663) lies immediately upstream of the protein 4.1 binding domain. Proline-rich regions are well recognized in signal transduction events, mediating dynamic

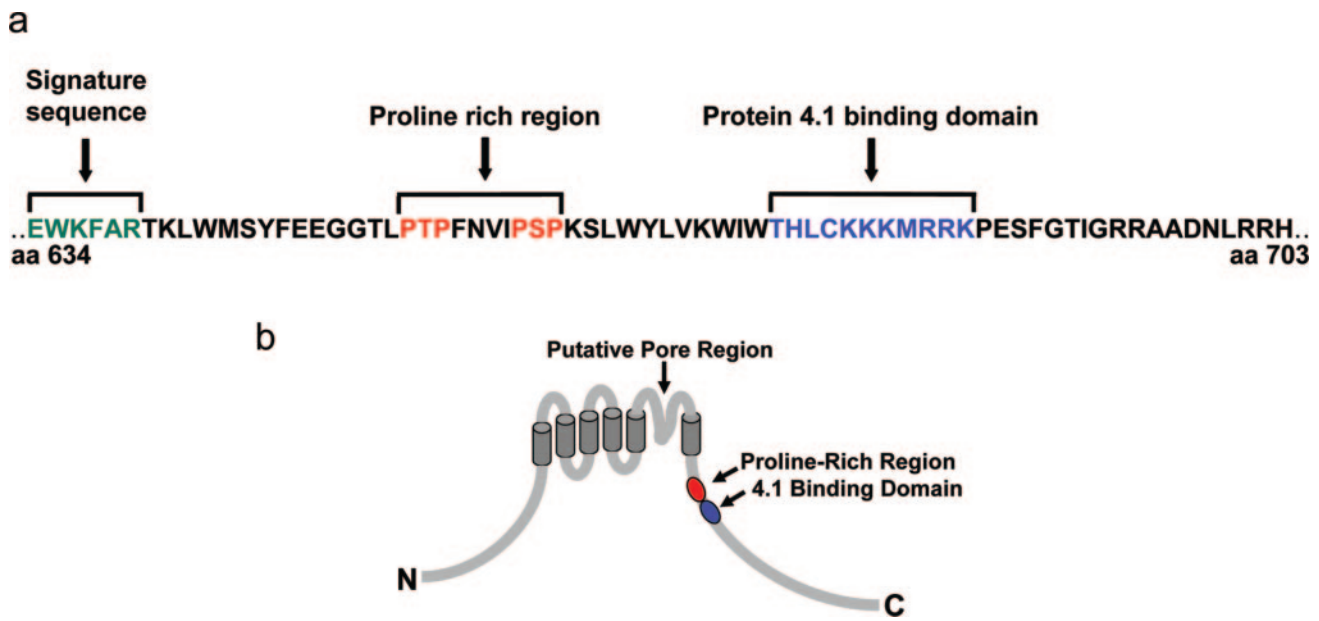

Figure 4. TRPC4 contains a conserved protein 4.1 binding sequence. a, A conserved protein 4.1 binding sequence was identified on TRPC4 between amino acids 675 and 685. Twelve amino acids upstream from the potential protein 4.1 binding domain resides a proline-rich region. The signature sequence is an invariant sequence that has been identified in all TRPC isoforms.[42] b, Schematic representation of TRPC4. The putative pore region lies between transmembrane regions five and six.[43] In immediate proximity to the pore region lay the potential protein 4.1 binding domain and an adjacent proline-rich region. N indicates amino terminus; C, carboxy terminus.

protein–protein interactions.[25,26] Typically, proline-rich regions act through hydrophobic attractive forces,[27] which, in some systems, can be modulated by phosphorylation.[28,29] The proline-rich region adjacent to the protein 4.1 binding domain contains threonine and serine as potential phosphorylation targets. Indeed, activation of SOC entry decreased gel mobility of TRPC4 isolated from the salt-dissociated supernatant, which was reversed by treatment with alkaline phospha-

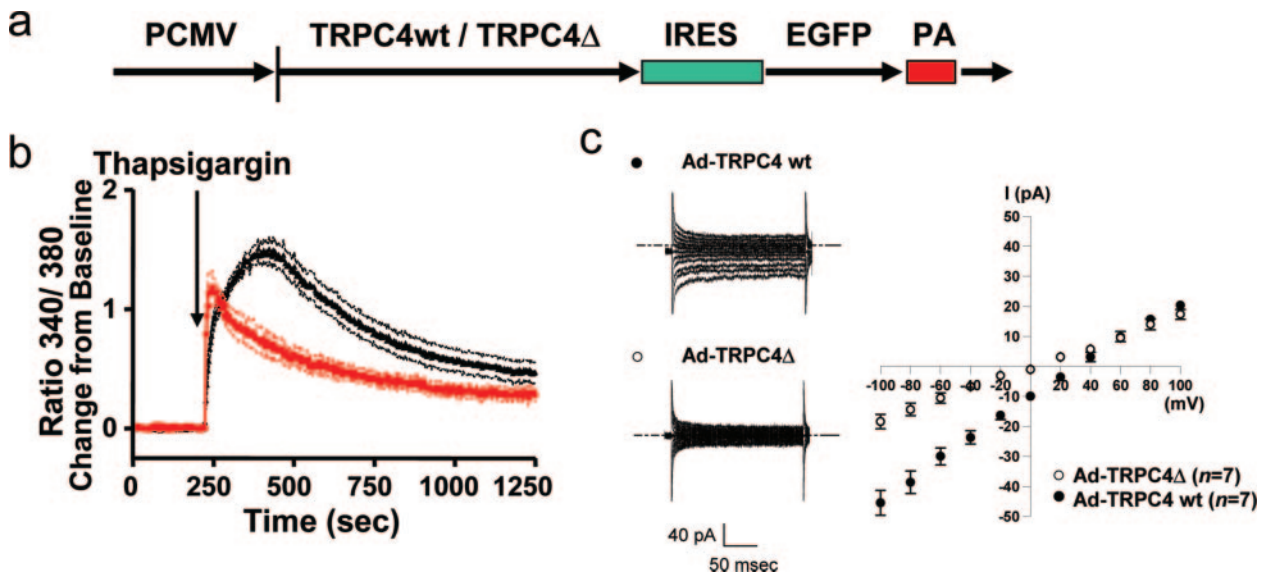

Figure 5. Deletion of the protein 4.1 binding domain and adjacent proline-rich region on TRPC4 decreases SOC entry and abolishes I_{SOC} activation. a, Schematic representation of the adenoviral vector used for expressing TRPC4wt and TRPC4Δ. PCMV indicates promoter cytomegalovirus; IRES, internal ribosomal entry site; EGFP, enhanced green fluorescent protein. b, Thapsigargin-induced SOC entry was decreased in PAECs expressing TRPC4Δ. PAECs were infected with Ad-TRPC4wt or Ad-TRPC4Δ at a multiplicity of infection of 25:1. Thirty-six to fifty hours after infection, cells were loaded with fura-2 AM and changes in $[Ca^{2+}]_i$ assessed by monitoring the 340 nm/380 nm ratio (*y* axis). The $[Ca^{2+}]_i$ response in PAECs expressing TRPC4Δ (red tracing) was 38% less than PAECs expressing TRPC4wt (black tracing). Change in $[Ca^{2+}]_i$ response was calculated at 10 minutes after thapsigargin application, reflective of Ca^{2+} entry only. Each tracing represents mean±SEM; n=6. c, I_{SOC} activation was abolished in PAECs expressing TRPC4Δ. PAECs were infected with Ad-TRPC4wt or Ad-TRPC4Δ at a multiplicity of infection of 25:1. Single cells were examined in a whole-cell voltage-clamp configuration. Cells were held at 0 mV and stepped from −100 mV to +100 mV for 200 ms, at 20-mV increments. Left panel, Infusion of thapsigargin (1 μmol/L) activated a small, inward Ca^{2+} current in Ad-TRPC4wt- but not in Ad-TRPC4Δ-infected PAECs. Right panel, Averaged current/voltage relationship of currents measured from the last 10 ms of each step in seven Ad-TRPC4wt- and seven Ad-TRPC4Δ-infected cells.

tase (data not shown), suggesting SOC entry activation induced TRPC4 phosphorylation. Thus, we propose that protein 4.1 binds to the protein 4.1 binding domain on TRPC4 to form an interaction dynamically mediated by the adjacent proline-rich region and that functions to regulate I_{SOC} channel activation.

I_{SOC} Activation Requires the Protein 4.1 Binding Domain/Proline-Rich Region on TRPC4

If the protein 4.1 binding domain and adjacent proline-rich region on the C terminus of TRPC4 are critical for the binding of protein 4.1 and regulation of I_{SOC} channel function, deletion of this region should disrupt thapsigargin-induced SOC entry. To test this hypothesis, adenoviral constructs were prepared to express either wild-type TRPC4 (TRPC4wt) or a mutant TRPC4 lacking the protein 4.1 binding domain and adjacent proline-rich region (TRPC4Δ; Figure 5a). PAECs were infected with adenovirus–TRPC4Δ or adenovirus–TRPC4wt at multiplicities of infection 50:1, 25:1, and 10:1. The global $[Ca^{2+}]_i$ response to thapsigargin was decreased in a dose-dependent manner in PAECs expressing TRPC4Δ compared with TRPC4wt (data not shown). At a multiplicity of infection of 25:1, the thapsigargin response was decreased 38% at the plateau phase (Figure 5b). Because the global $[Ca^{2+}]_i$ response reflects all SOC entry channels activated by thapsigargin, we conclude that the protein 4.1 binding domain and adjacent proline-rich region on TRPC4 is critical for activation of a subset of SOC entry channels. To determine the importance of the protein 4.1 binding domain and adjacent proline-rich region to I_{SOC} channel activation, whole-cell patch-clamp analysis was used. In PAECs expressing TRPC4wt, a typical thapsigargin-induced I_{SOC} current was observed (Figure 5c). Specifically, this current was small (50 pA at −100 mV), inwardly rectifying, and exhibited a reversal potential near +30 mV.[9,17] In PAECs expressing TRPC4Δ, the thapsigargin-induced I_{SOC} current was abolished (Figure 5c). The inward Ca^{2+} current was reduced to instrument noise level and the reversal potential left-shifted to 0 mV. Together, these data demonstrate a functional requirement for the protein 4.1 binding domain and adjacent proline-rich region on TRPC4 for I_{SOC} activation.

I_{SOC} Activation Is Disrupted by Peptide Competition With the Protein 4.1 Binding Domain/Proline-Rich Region on TRPC4

Heterologous expression of TRPC4Δ in PAECs, lacking the protein 4.1 binding domain and adjacent proline-rich region, reduced global SOC entry and blocked I_{SOC} activation. These studies clearly demonstrate functional significance of the protein 4.1 binding domain and adjacent proline-rich region on TRPC4. However, we could not determine from these data whether this effect was specifically attributable to the inability of protein 4.1 to bind to the TRPC4Δ subunit or whether the TRPC4Δ protein itself caused mistargeting of the I_{SOC} channel such that it was not able to insert properly into the plasma membrane. We therefore chose an alternate approach to determine whether the protein 4.1 binding domain and adjacent proline-rich region is critical for I_{SOC} channel function. Here, peptide competition experiments were performed.

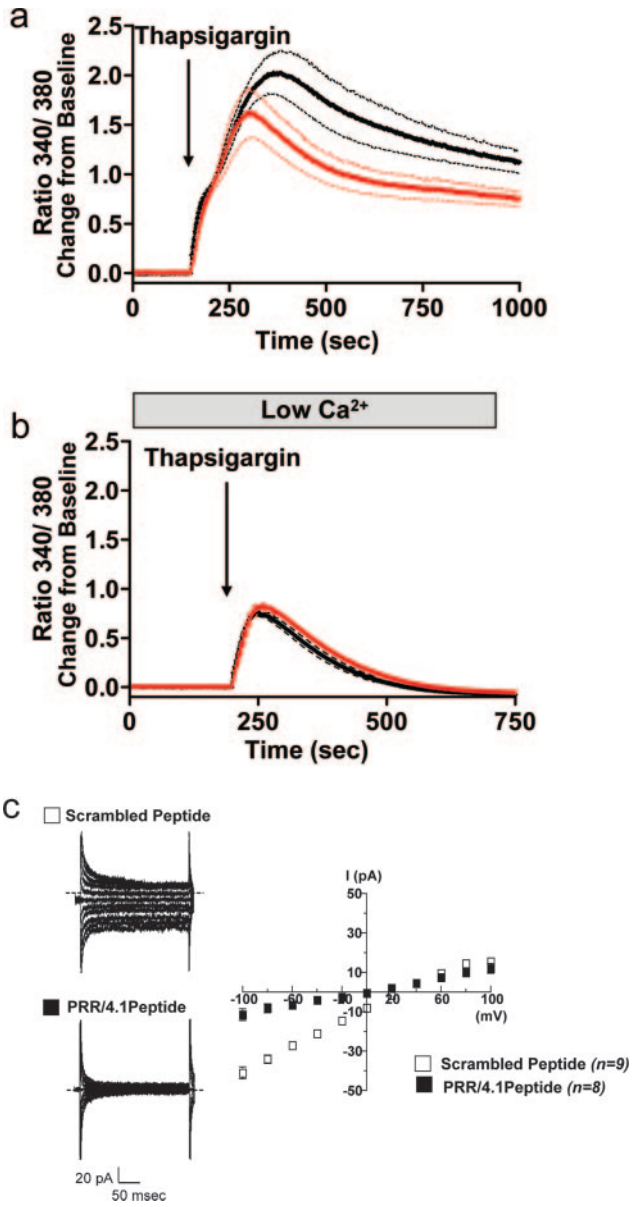

Figure 6. Peptide competition with the protein 4.1 binding domain/proline-rich region on TRPC4 decreases SOC entry and abolishes I_{SOC} activation. Peptides were transfected into PAECs using Chariot reagent. Cells were loaded with fura-2 AM and measured as described in Figure 5a. a, In PAECs transfected with prr/4.1 peptide (red tracing), thapsigargin-induced SOC entry was decreased 37% compared with cells transfected with scrambled peptide (black tracing). Each tracing represents mean±SEM; n=3. b, Transfection of prr/4.1 peptide does not affect Ca^{2+} release. There was no difference in Ca^{2+} release between PAECs transfected with prr/4.1 peptide (red tracing) compared with scrambled peptide (black tracing). Each tracing represents mean±SEM; n=4 to 5. c, I_{SOC} activation was completely blocked in PAECs transfected with prr/4.1 peptide. Whole-cell patch-clamp analysis was performed as described in Figure 5b. Left panel, PAECs transfected with scrambled peptide exhibited a typical I_{SOC} current, whereas no current was observed in PAECS transfected with prr/4.1 peptide, in eight of eleven experiments. Right panel, Averaged current/voltage relationship of currents measured from the last 10 ms of each step in eight prr/4.1 peptide and nine scrambled peptide experiments.

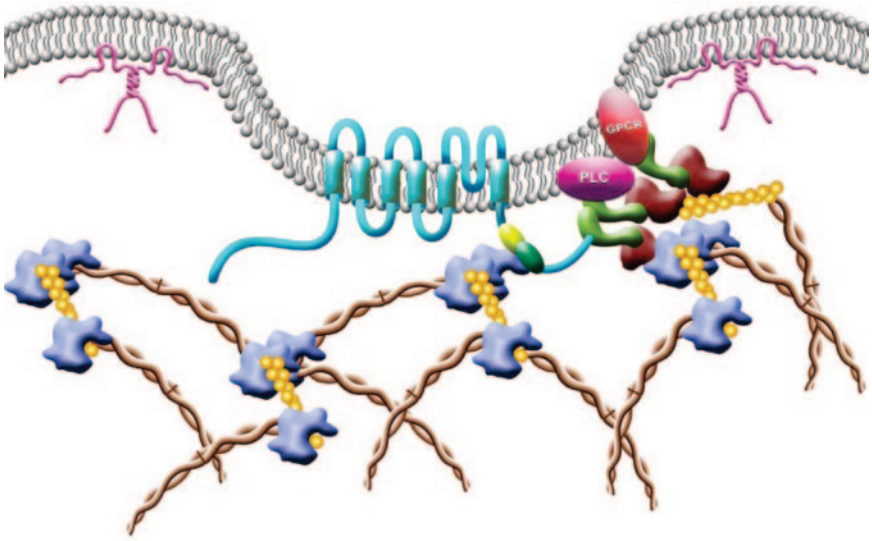

Figure 7. Model of TRPC4 in its signaling scaffold. TRPC4 (blue–green) contains six transmembrane domains. Its cytosolic C terminus contains several binding domains that tether TRPC4 to the membrane skeleton. TRPC4 likely interacts directly with protein 4.1 (blue) through its protein 4.1 binding domain (green). The distal C terminus contains a 5-aa sequence (VTTRL) that binds to a PDZ domain in the protein NHERF[36] (green), linked to actin (yellow) through ezrin-radixin-moesin proteins (red). NHERF is potentially a key scaffolding protein in that it can also bind directly to phospholipase C β (PLC)[36] and G-protein–coupled receptors (GPCR)[37] through its PDZ domains. The signaling scaffold is localized in cholesterol-rich caveolae containing caveolin-1 (pink).

A 41-aa residue peptide corresponding to the protein 4.1 binding domain and adjacent proline-rich region on TRPC4 (prr/4.1) and a scrambled peptide were synthesized. We reasoned that if the protein 4.1 binding domain (+/−adjacent proline-rich region) on TRPC4 is the site of direct binding between protein 4.1 and TRPC4 needed for I_{SOC} activation, then transfection of the prr/4.1 peptide into PAECs would disrupt endogenous TRPC4–protein 4.1 interaction and prevent I_{SOC} activation. Peptides were transfected into PAECs using the Chariot reagent. In cells transfected with prr/4.1 peptide, the global $[Ca^{2+}]_i$ response to thapsigargin was decreased 37% at the plateau phase compared with scrambled peptide (Figure 6a). Experiments performed in low Ca^{2+} buffer indicated no difference in Ca^{2+} release (Figure 6b). Patch-clamp experiments showed that although thapsigargin-induced I_{SOC} was normal in PAECs transfected with scrambled peptide, it was completely blocked by the prr/4.1 peptide in eight of eleven experiments (Figure 6c). Similar to our observations in PAECs expressing TRPC4Δ, the inward Ca^{2+} current was reduced to instrument noise level, and the reversal potential left-shifted to 0 mV. These results demonstrate a functional requirement for the protein 4.1 binding domain and adjacent proline-rich region located on the C terminus of TRPC4. We conclude that this region is essential for endothelial I_{SOC} activation, likely through direct interaction between protein 4.1 and TRPC4. Collectively, these data support a regulatory role for protein 4.1 in I_{SOC} channel activation.

Discussion

SOC entry was first described by Putney nearly two decades ago.[30] Since then, the structural and functional components underlying activation of SOC entry have been aggressively studied, hotly debated, and, to date, still remain elusive. Although most would agree that the TRPCs 1, 3, 4, and 5 proteins can form SOC entry channels, no one has yet described the oligomeric state of even one endogenous SOC entry channel. Equally puzzling is the regulatory mechanism(s) linking store depletion to activation of SOC entry channels. Although these questions remain to be answered, the physiological implications of SOC entry are beginning to be understood. In the pulmonary circulation, for example, activation of the endothelial I_{SOC} channel promotes endothelial barrier disruption,[6] and as such, the I_{SOC} channel may represent an attractive therapeutic target.

In this study, we identified protein 4.1 as a binding partner of TRPC4. This interaction likely occurs through a protein 4.1 binding domain +/−adjacent proline-rich region found on the C terminus of TRPC4. The TRPC4–protein 4.1 interaction appears to be a high affinity, constitutive interaction, such that an intact TRPC4–protein 4.1 complex can be dissociated from the endothelial membrane skeleton. It is important that this interaction is constitutive and does not require activation of SOC entry. Thus, physical association between the I_{SOC} channel and membrane skeleton is already established before Ca^{2+} store depletion. This observation supports our earlier evidence that disruption of the spectrin–protein 4.1 interaction abolishes I_{SOC}.[17]

A number of binding domains have been identified on the C terminus of TRPC4 as well as other TRPC homologues.[31] Although all the domains have not yet been studied, the majority of data to date show that discrete protein–protein interactions serve specific functions, including modulation of SOC entry channel activation states and proper assembly within the signaling scaffold. Rosado and Sage[32] demonstrated a requirement for interaction between the inositol-1,4,5-trisphosphate receptor (II) and TRPC1 for SOC entry in platelets; and Singh et al[33] showed that a TRPC1–calmodulin interaction was important for channel inactivation in human submandibular gland cells. The proline-rich region adjacent to the protein 4.1 binding domain represents an additional site of protein–protein interaction. Parts of this region have already been implicated in immunophilin FKBP52 binding to TRPCs 1, 4, and 5[34] and Homer binding to TRPC1.[35] The PSD-95/discs large protein/zonula occludens 1 (PDZ) binding domain located at the distal C terminus of TRPC4 and TRPC5 binds to the Na^+/H^+ exchange regulatory factor

(NHERF) scaffolding protein[36] (Figure 7). In addition to coupling TRPC4 to other members of the signaling cascade, such as G-protein–coupled receptors[37] and phospholipase Cβ,[36,38] NHERF may function to indirectly link TRPC4 to the membrane skeleton. NHERF binds to members of the ezrin-radixin-moesin protein family,[39] which can interact with transmembrane proteins and also bind directly to actin.[40] The TRPC4–NHERF interaction appears to be required for signalplex localization to organize signaling events into spatially[41] and temporally efficient cascades.

In this study, we demonstrated a functional requirement of the protein 4.1 binding domain and adjacent proline-rich region on TRPC4 for I_{SOC} channel activation. I_{SOC} was abolished by deletion of the protein 4.1 binding domain and adjacent proline-rich region. Transfection of a competitive peptide to this region also prevented I_{SOC} activation. Indeed, this is the first evidence for an I_{SOC} regulatory mechanism. Collectively, our data suggest that a protein 4.1 bridge between TRPC4 and the membrane skeleton is an absolute requirement for the regulation of channel activation and ion permeation.

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Activation of the Endothelial Store-Operated I_{SOC} Ca^{2+} Channel Requires Interaction of Protein 4.1 With TRPC4

Donna L. Cioffi, Songwei Wu, Mikhail Alexeyev, Steven R. Goodman, Michael X. Zhu and Troy Stevens

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Adenoviral Constructs. Recombinant adenoviruses were generated according to established protocols[1] using commercially available plasmids (AdMax, Microbix, Toronto, Canada). Briefly, the shuttle plasmid pDC512 was modified to incorporate a CMV promoter, a polylinker and an internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV). Two adenoviruses were generated in which the first cistron contained either a wild type mouse TRPC4 (Ad-TRPC4wt) or a mutant TRPC4 with deletion of amino acids 650-690 (Ad-TRPC4Δ). Both generated viruses contained an enhanced green fluorescent protein in their second cistron.

Preparation and Detergent Extraction of Membrane/Cytoskeleton Fractions Adapted from[2]. *Preparation of Endothelial Cell Lysates*: The lysing procedure was performed in the cold room (4°C) and all solutions used were pre-chilled. To prepare lysates, cell monolayers were rinsed with PBS and exposed to hypotonic lysis buffer [100 mmol/L Tris-HCl (pH 8.0), 1 mmol/L $MgCl_2$, 1% Protease Inhibitor Cocktail (10x)]. Cells were detached via scraping and frozen at -80°C.

Preparation and Extraction of Membrane/Cytoskeleton Fractions: Cell lysates were thawed in an ice-H_2O bath and centrifuged at 3000 x g (24 min., 4°C). The pellet was re-suspended in 500 μL ice-cold sucrose buffer [0.25 mol/L sucrose, 10 mmol/L Tris-HEPES (pH 7.4), 1 mmol/L dithiothreitol, 1% Protease Inhibitor Cocktail (10x)] and homogenized. The suspension was centrifuged at 3000 x g (17 min., 4°C) and protein content of the supernatant determined (modified Lowry Method; Sigma-Aldrich). The supernatant was divided into aliquots and centrifuged at 50,000 x g (30 minutes, 4°C).

The pellet was re-suspended in 300 μL ice-cold extraction buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, detergent (2% Triton X-100 (v/v), or 54 mmol/L octyl-β-D-glucopyranoside), 1% Protease Inhibitor Cocktail (10x)] +/- KI. Samples were incubated (40 minutes, 4°C) and centrifuged at 145,000 x g (50 minutes, 4°C). The pellet was washed 3 x 500 μL ice-cold H_2O-ethanol (1:1) and 300 μL SDS buffer [54 mmol/L Tris base, 5.2 mmol/L EDTA, 13% SDS (w\v)] was added. To solubilize the pellet, a combination of sonication and heating was used.

Peptide Transfection. Two peptides were synthesized by Alpha Diagnostic International, Inc. (San Antonio, TX). The prr/4.1 peptide corresponds to the region on TRPC4 encompassing the protein 4.1 binding domain and adjacent proline rich region. A peptide with scrambled sequence was also prepared. Peptides were transfected into PAECs using Chariot™ reagent (Active Motif, Carlsbad, CA) generally following the manufacturer's suggested protocol. Briefly, a peptide-Chariot™ complex was prepared to deliver 1200 ng peptide per 35 mm culture dish containing a PAEC monolayer at <60% confluence. To rinsed cells was added 200 μL prepared complex followed by 400 μL serum free media. Following a 1 hour incubation (37°C, 5% CO_2), 1 mL normal growth media (containing serum and antibiotic) was added. Cells were incubated for ≥ 3 hours or ≥ 1 hour before Ca^{2+} measurements in 2 mmol/L Ca^{2+} buffer or low Ca^{2+} buffer, respectively. For electrophysiology experiments, a single cell suspension of PAECs was distributed drop wise into 35 mm culture dishes. Peptide-Chariot™ complex (200 μL) was added directly over the drops followed by 200 μL serum free media. Cells were incubated for 1 hour; 1 mL normal growth media was then added to each dish and incubation resumed for a minimum of 3 hours.

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