Various cellular signals initiate calcium entry into cells, and there is evidence that lipid rafts and caveolae may concentrate proteins that regulate transmembrane calcium fluxes. Here, using mice deficient in caveolin-1 (Cav-1) and Cav-1 knock-out reconstituted with endothelium-specific Cav-1, we show that Cav-1 is essential for calcium entry in endothelial cells and governs the localization and protein-protein interactions between transient receptor channels C4 and C1. Thus, Cav-1 is required for calcium entry in vascular endothelial cells and perhaps other specialized cell types containing caveolae.

The generation of receptor-induced cytosolic calcium signals involves the rapid, transient release of calcium from stores, mainly located in the endoplasmic reticulum (ER) followed by sustained entry of extracellular calcium (1). Ligand stimulation of G protein- and tyrosine kinase-coupled receptors activates phospholipase C generating the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The former functions as a calcium messenger that interacts with IP3 receptors (IP3Rs) to rapidly release calcium from the ER lumen (1). The initial release of calcium is followed by a more sustained entry of calcium into cells via several potential mechanisms, including calcium release-activated calcium channels, store-operated calcium channels, and receptor-operated calcium channels (2). The tonic phase of calcium entry mediates long term calcium signaling and provides calcium to replenish intracellular stores. The relative role of calcium release from ER stores versus other second messenger systems (such as DAG and Gαq/11 (3, 4)) activating calcium influx pathways is complex and controversial and may depend on the channel composition and cell type being studied.

There is growing evidence that members of the transient receptor potential (TRP) cation channel family can assemble in the plasma membrane to participate in cation influx pathways. TRPs are members of a large superfamily of mammalian TRP channels (TRPCs), and to date, seven TRPCs (TRPC1–7) have been identified (5). Heterologous expressions of the highly homologous TRPC3, -6, and -7 channels are clearly activated by phospholipase C-derived DAG (6, 7), whereas activations of TRPC1, -4, and -5 are less clear. Although TRPC1 was characterized as a component of SOC in nerve cells (8), subsequent studies indicated that TRPC3, TRPC4, and TRPC5 might also contribute to calcium entry mechanisms after agonist stimulation (9–11). In vascular endothelial cells, sustained calcium entry into the cytosol is necessary to release vasoactive agents such as nitric oxide or prostaglandins (12, 13), and mice deficient in TRPC4 (10) show markedly reduced acetylcholine (ACH) agonist-induced calcium entry and endothelium-dependent, nitric oxide-mediated vasorelaxation. The compositions of the native TRPC channels in endothelial cells are not known and are likely complex and species-specific. However, there is recent evidence that TRPC3/C4 heteromers are necessary for cation influx activated by redox signaling in porcine endothelial cells (14).

Caveolae are 50–100 nm cholesterol-rich invaginations of the plasma membrane that have been implicated in a variety of cellular functions, including endocytosis and signal transduction events (15). There are three family members of caveolins (Cav-1, -2, and -3) (16–18). Cav-1 is predominantly expressed in endothelial cells, smooth muscle cells, fibroblasts, and adipocytes, and Cav-3, which is specifically expressed in striated muscle, is sufficient for formation of caveolae in these cell types or tissues. Recent studies have demonstrated that isolated caveolae contain complex sets of functionally organized signaling molecules, including Ca2+ signaling molecules (19–21), and have been suggested to regulate Ca2+ entry in living cells (22).

Recently, we have developed a mouse model to study the effects Cav-1 on endothelial function in vivo by breeding the Cav-1 knock-out mice to endothelium-specific Cav-1 transgenic mice, thus rescuing caveolae in the endothelium only (23). Here we show using genetic criterion in intact blood vessels and isolated cells that Cav-1 is necessary for the second phase of calcium entry in endothelial cells. Mechanistically, Cav-1 is associated with a dynamic protein complex consisting of...
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of TRPC4, TRPC1, and IP₃Rs, and the loss of Cav-1 impairs the localization of TRPC4 and agonist-simulated complex formation. These data favor the idea that Cav-1 in caveolae of specialized cells, such as endothelia, may serve as a scaffold to integrate TRPC regulation of calcium influx pathways.

EXPERIMENTAL PROCEDURES

Generation of Cav-1-deficient and Endothelium-specific Cav-1 Transgenic Reconstituted (Cav-1 RC) Animals

Endothelium-specific Cav-1 transgenic mice (Cav-1 TG, F4) carrying the canine CAV-1 transgene under the prepro-endothelin-1 promoter (24) were crossed with F4 generation Cav-1-deficient mice (18) to generate endothelial-specific Cav-1 reconstituted mice as described (Cav-1 RC (23)). Male mice (8–10 weeks old) were used for the experiments.

Isolation of Murine Lung Endothelial Cells

Lungs were dissected from the mice, minced in 0.1% collagenase, and homogenized by passing through a needle (14-gauge). After centrifugation (1000 rpm, 5 min), cells were plated into flasks and were cultured for 2 days. Cells were then immortalized by infection with a retrovirus expressing the polyoma middle T antigen, and cultured again for 2 days. Magnetic beads (Dynal) coated with anti-ICAM-2 antibodies (Pharmingen) were added to cells and incubated at 4 °C for 1 h, and cells were detached by trypsin. Then the endothelial cells were collected using a magnetic holder. After washing, cells were plated and passaged for use. After 48 h serum-starved murine lung endothelial cells (MLEC) (passage 7–15) were used for experiments.

Immunostaining

Whole-mount Staining—The mouse intrapulmonary arteries were fixed with 4% paraformaldehyde (4 °C; 10 min), permeabilized with 0.3% Triton X-100/PBS (room temperature, 30 min), and blocked by 3% normal goat serum containing PBS (room temperature, 30 min). Then the arteries were probed with rabbit anti-Cav-1 polyclonal antibody (BD Transduction Laboratories) and rat anti-PECAM (BD Biosciences) polyclonal antibody (1:200 dilution; 4 °C; 12 h). Alexa 488 anti-rabbit IgG and 488 anti-rat IgG (Molecular Probe) were used as secondary antibodies. Alexa 488 anti-rabbit IgG and 568 anti-mouse IgG (Molecular Probe) were used as secondary antibody (1:500 dilution, room temperature, 1 h). For actin staining, we applied Alexa Fluor 488 phalloidin (Molecular Probe) to samples (1:200 dilution, room temperature, 10 min) after the paraformaldehyde fixation.

After mounting of 4,6-diamidino-2-phenylindole, all of the images were captured using a Carl Zeiss scanning microscope Axiovert 200M imaging system (×400–630), and images were digitized under constant exposure time, gain, and offset. After capturing images, protein co-localization and cell area were measured by the OpenLab program (Improvision).

Electron Microscopy

Cav-1 WT, KO, and RC mice were perfused for 10 min at room temperature under anesthesia with oxygenated Dulbecco’s modified Eagle’s medium through the left ventricle, followed by fixation by perfusion (10 min at room temperature) with 2.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M sodium cacodylate buffer. Specimens were taken from lung and trimmed into small blocks. The blocks were immersed into fresh fixative (1 h at room temperature), washed twice (15 min at room temperature) in 0.1 M cacodylate, postfixed in Palade’s OsO₄ (1 h on ice), stained en bloc in Kellemberger’s uranyl acetate (overnight at room temperature), dehydrated in graded ethanol, and embedded in LX112 resin (Ladd Research Industries). Thin sections (50 nm) were cut, stained with lead citrate, and examined and photographed under an electron microscope (JEOL 1200EX or CM10, Phillips Electronic Instruments).

Prostacyclin Release

After 12 h of incubation, pulmonary arterial segments (4 mm) were placed in sterile 48-well tissue culture plates with 300 μl of HEPES solution at 37 °C and kept in the incubator for 30 min. Aliquots of 100 μl were then taken to measure the basal release of prostacyclin. ACh (3 μM) and ionomycin (3 μM) were added, and the plate was incubated for an additional 30 min, after which the aliquots were again taken to measure the ACh-stimulated release of prostacyclin. This protocol was carried out both in the absence and in the presence of ibuprofen (10 μM) and La³⁺ (300 μM). The aliquots were frozen, and prostacyclin release was measured as the stable breakdown product, 6-keto-PGF₁α, by immunoassay (Amersham Biosciences).

IP₃ Accumulation

Serum-starved MLECs were stimulated by ACh (3 μM) for 3 min, homogenized in 7.5% trichloroacetic acid solution, and centrifuged at 2,000 × g for 15 min at 4 °C, as described previously. The cell suspensions were applied to an IP₃ (25) Biotrak Assay System (GE Healthcare), and pellets were used to deter-
mine protein content. IP$_3$ contents are expressed as pmol/mg protein content.

**Tissue and Cell [Ca$^{2+}$]$_i$ Measurements**—Measurement of the [Ca$^{2+}$]$_i$ in endothelial cells *in situ* was performed using fluo-4 AM (Molecular Probe) according to the method described previously (26). The artery (0.6 mm wide) and confluent MLEC were incubated in physiological salt solution (136.9 mM NaCl, 5.4 mM KCl, 2.0 mM CaCl$_2$, 1.0 mM MgCl$_2$, 23.8 mM NaHCO$_3$, and 5.5 mM glucose) with 5-$\mu$M fluo-4 AM and 0.2% cromophor EL for 30 min at room temperature. The opened strip of pulmonary artery or MLEC was mounted on the stage of a microscope, and the images were captured by a Carl Zeiss scanning microscope Axiovert 200M imaging system (×100–400) using 488-nm (excitation) and 540-nm (emission) filters. Images of vascular endothelial cells stained with fluo-4 were taken every 1.8–10 s at 37 °C, and data were expressed as the change in fluorescence after agonist challenge relative to base line ($F/F_0$) as a function of time.

**Western Blotting**

Confluent MLEC were washed twice with phosphate-buffered saline and lysed on ice in 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40 (v/v), sodium orthovanadate, 20 mM NaF, 1 mM Na$_4$P$_2$O$_7$, 1 mM Pefabloc SC, and protease inhibitor mixture (Roche Diagnostics) and rotated for 1 h at 4 °C. Lysates were Dounce-homogenized (30 strokes), and insoluble material was removed by centrifugation at 13,000 $\times$ g for 10 min at 4 °C, and 20–50 µg of each protein was subjected to regular Western blot analysis.

**Knockdown of TRPC1 and 4 by siRNA**

Small inhibitory RNA (siRNA) duplexes for mouse TRPC1 (GenBank™ accession number NM_011643) and TRPC4 (GenBank™ accession number NM_016984) were designed by Qiagen. Individual TRPC1 siRNA duplexes (sense sequences) were as follows: TRPC1, r(CGAGGACUGAAGAAAG)dTdT and r(GCGUGUAGCCACCCUGUAG)dTdT, and TRPC4, r(CGUUAUUGCAGACUCUAC)dTdT and r(GGAGCAGUCUGGCGAAU)dTdT. MLEC maintained in serum-free EB-M2 were transfected with siRNAs (40 nm) using Lipofectamine 2000 following the manufacturer’s protocol. Fresh medium was added 6 h post-transfection, and experiments were conducted 72 h after transfection to assess mRNA levels using each specific primers against TRPC1 and -4 and protein using Western blotting.

**Cellular Fractionation**

Confluent MLEC (3 × 150-mm dish) were serum-starved for 48 h and were washed twice with phosphate-buffered saline, scraped in 2 ml of ice-cold hypotonic buffer (500 mM Na$_2$CO$_3$, pH 11), and sonicated (3 times, 20-s bursts). The homogenate was then adjusted to 42.5% sucrose by the addition of 2 ml of 85% sucrose prepared in MBS (25 mM MES, 150 mM NaCl, pH 6.5) and placed at the bottom of an ultracentrifuge tube. A 5–30% discontinuous sucrose gradient was formed and centrifuged at 35,000 rpm for 17 h in an SW40 rotor (Beckman Instruments). Gradient fractions (1 ml) were collected from the top of the tube to yield a total of 12 fractions, and each fraction (fractions 1–12) was used for Western blot analysis. Blots were probed with the anti-Cav-1 monoclonal (1:500), anti-TRPC1 polyclonal (1:200), and anti-TRPC4 polyclonal (1:200) antibodies (as described above), rabbit anti-IP$_3$R1 polyclonal antibody (1:500; Calbiochem), anti-IP$_3$R3 monoclonal antibody (1:1000; BD Transduction Laboratories), anti-flotillin-2 monoclonal (1:5000; BD Transduction Laboratories) and anti-α-actin monoclonal anti-bodies (1:5000; Sigma).

**Immunoprecipitation Studies**

Tissues and MLECs for Western blot analysis were resuspended in lysis buffer (for TRPC1 and -4, and IP$_3$Rs immunoprecipitation: 50 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.1 mM EGTA, 0.1 mM EDTA, 0.75% Nonidet P-40, 1 mM sodium orthovanadate, 20 mM NaF, 1 mM Na$_4$P$_2$O$_7$, 1 mM Pefabloc SC, and protease inhibitor mixture (mentioned above); for Cav-1 immunoprecipitation: 10 mM Tris, pH 8.0, 60 mM n-octyl-β-D-glucopyranoside, 150 mM NaCl, 1 mM sodium orthovanadate, 20 mM NaF, 1 mM Na$_4$P$_2$O$_7$, 1 mM Pefabloc, and protease inhibitor mixture (Roche Diagnostics)). Insoluble material was removed by centrifugation at 13,000 $\times$ g for 10 min at 4 °C. One thousand (for TRPC1 and -4, and IP$_3$Rs) or 500 µg (Cav-1) of protein from lysates was subjected to immunoprecipitation. Anti-TRPC1 and -4, IP$_3$Rs, and 50 µl of protein A-agarose (Sigma) were added to the supernatant, which was then rotated at 4 °C for 3 h. After washing 3 times with lysis buffer, binding protein was eluted with SDS sample buffer and analyzed by Western blot.

**Purification of GST-Caveolin-1 Fusion Proteins and in Vitro Interactions**

Construction of the Cav fusion proteins was performed as follows. Full-length Cav-1 (residues 1–178, Cav-FL) and Cav subdomains (the N-terminal domains, residues 1–61 and 61–101) and the C-terminal domain (residues 135–178) were separately amplified by PCR and subcloned into the vector pGEX-4T-1 (XmaI-XhoI sites) as described previously (27). All fusions in *Escherichia coli* strain BL-21, were induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside and lysed by sonication (two 20-s bursts) after addition of purification buffer (7.5 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1.5% N-lauryl Sarkosyl (w/v), 2% Triton X-100, 100 µM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 3 mM EDTA). Debris was removed by centrifugation. Purified GST-Cavs were immobilized on glutathione-agarose beads (GE Healthcare). Cells were lysed in a modified RIPA buffer (50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% SDS, 0.1% deoxycholic acid, 1 mM Pefabloc, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 2 µg/ml pepstatin) and centrifuged for 10 min at 13,000 $\times$ g at 4 °C. Lysates (500 µg of proteins) were incubated for 2 h at 4 °C with beads containing either GST alone or GST-Cav. After binding, the beads were washed (3 times) with wash buffer containing 50 mM Tris-Cl, pH 7.4, 125 mM NaCl, 1 mM EDTA, and 1 mM EGTA. Binding protein was eluted with SDS sample buffer, and analyzed by Western blot.
FIGURE 1. Impaired calcium fluxes in Cav-1 KO mice; rescued by endothelium-specific Cav-1 reconstitution. A, reverse transcription-PCR analysis for genotyping of Cav-1 WT, KO, and RC mice. B and C, Cav-1 (green in B, red in C), smooth muscle α-actin (red in B), and specific endothelial cell marker, PECAM-1 (green in C), expression in Cav-WT, KO, and RC intrapulmonary arteries in cross-sections and in situ whole-mount immunostaining (C). These figures are typical traces of four experiments. Bar = 20 μm in B and 10 μm in C. D, representative transmission electron micrographs of lung endothelial cells from WT, Cav-1 KO, and Cav-1 RC mice. Bar = 100 nm. E, increases in [Ca^{2+}]_{i} induced by ACh (3 μM, left) in endothelial cells of Cav-1 WT, KO, and RC mouse pulmonary arteries in situ. F, prostacyclin release (quantified by the stable breakdown product 6-keto-PGF1α) from Cav-1 WT, KO, and RC pulmonary arteries. Results are expressed as means ± S.E., n = 5 individual experiments. Significantly different from WT in each treatment: *, p < 0.05, or **, p < 0.01; significantly different from the amount elicited by 3 μM ACh in WT with ##, p < 0.01.
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Statistical Analysis

The results of the experiments were expressed as the means ± S.E. Statistical evaluation of the data was performed by analysis of variance, followed by the Tukey post-test for comparison between groups using Prism 3.0. A value of $p < 0.05$ was regarded as statistically significant.

RESULTS

Cav-1 Is Necessary for Acetylcholine-induced Calcium Fluxes and Prostacyclin Production—Previously, we have established mice deficient in Cav-1 that were bred to transgenic mice expressing Cav-1 in endothelial cells only (23). As seen in Fig. 1A, PCR genotyping of WT mice expressing endogenous murine Cav-1, Cav-1−/− mice (labeled Cav-1 KO) was determined by the presence of the neomycin cassette and lack of the endogenous gene, whereas the Cav-1−/− mice bred to the EC specific transgenic Cav-1 mice (Cav-1−/−, TG EC-Cav-1; labeled Cav-1 RC) contains the neomycin cassette and the transgenic canine Cav-1 gene but lacks the endogenous gene. Fig. 1B documents Cav-1 protein expression in intrapulmonary arteries isolated from these mice. WT mice demonstrate the presence of Cav-1 (Fig. 1B left column) in both endothelial and smooth muscle layers ($n = 6$, top panel shows Cav-1 alone, middle panel shows labeling of smooth muscle $\alpha$-actin, and bottom panel show the merged images), whereas Cav-1 KO (middle column) lacks Cav-1 immunostaining in both layers ($n = 9$). Reconstitution of Cav-1 into the endothelium generated by crossing Cav-1 KO to EC-specific Cav-1 TG mice reintroduces Cav-1 into the endothelium but not into smooth muscle layers marked by smooth muscle $\alpha$-actin staining ($n = 22$; Fig. 1B, right columns). Next we used whole-mount staining of the endothelium to examine the extent of Cav-1 reconstitution in these strains. As seen in Fig. 1C, WT and Cav-1 RC have relatively equal levels of immunoreactive Cav-1 in endothelial cells ($n = 6$ and 10, respectively; top row, left and right columns) defined by the endothelial cell marker, PECAM-1 ($n = 6$ each; bottom row). Cav-1 KO vessels lack endothelial cell Cav-1 immunoreactivity without changing the levels of PECAM-1 ($n = 10$ and 6, respectively). In multiple experiments, we estimate ~85–90% of the endothelial cells from intrapulmonary arteries are Cav-1-positive. Cav-1 KO mice lack caveolae organelles in several tissues examined, including blood vessels, adipocytes, and fibroblasts (18, 28). As shown in Fig. 1D, transmission electron micrographs document the presence of ample caveolae in EC lining pulmonary vessels, the loss of EC caveolae in Cav-1 KO vessels, and the presence of the organelle in the Cav-1 RC vessels. Thus, we have generated a model of Cav-1 re-expression in the endothelium only, resulting in formation of endothelial caveolae.

Next, we examined the changes in endothelial [Ca$^{2+}$], induced by the calcium-mobilizing agonist ACh (3 $\mu$M) by imaging fields of endothelial cells lining the intrapulmonary arteries loaded with the calcium indicator fluo-4AM. As shown in Fig. 1E, ACh induced a transient increase in [Ca$^{2+}$], followed by a sustained increase in all of the arteries ($n = 5$ each). However, the sustained phases of [Ca$^{2+}$], stimulated by ACh was reduced in the endothelium of Cav-1 KO pulmonary arteries compared with that seen in WT and Cav-1 RC mice. Thus, Cav-1 reconstitution in the endothelium completely restored the impairment in [Ca$^{2+}$], fluxes during the tonic phase of the response. Next we sought to examine if the reduction in stimulated changes in calcium entry may impair endothelial function. To this end, we examined ACh-stimulated increases in prostacyclin production (an index of endothelial function; quantified by the stable breakdown product 6-keto-PGF$_{1\alpha}$), which is known to be calcium-dependent (13). The loss of Cav-1 reduced resting and ACh but not ionomycin-induced 6-keto-PGF$_{1\alpha}$ production in vessels compared with vessels from WT and Cav-1 RC mice ($n = 5$ each, Fig. 1F). The cyclooxygenase inhibitor ibuprofen completely inhibited 6-keto-PGF$_{1\alpha}$ production in all of the arteries ($n = 5$ each). To confirm the dependence of 6-keto-PGF$_{1\alpha}$ release on extracellular calcium, vessels were treated with the nonspecific blocker lanthanum (La$^{3+}$; 300 $\mu$M). La$^{3+}$ inhibited ACh-induced prostacyclin release in the pulmonary arteries from WT mice ($n = 4$). These results demonstrate that the impairment in endothelial cell calcium handling in Cav-1 KO mice results in reduced endothelial function quantified by the release of prostacyclin, and both calcium fluxes and prostacyclin production could be genetically rescued by the endothelium-specific transgenic Cav-1.

Ca$^{2+}$ Fluxes and Entry, but Not IP$_{3}$, Production, Are Impaired in Isolated Endothelial Cells of Cav-1 KO Mice and Rescued by Reintroduction of Endothelial Cav-1—To examine potential mechanisms of how the loss of Cav-1 and caveolae impaired the tonic phase of agonist-stimulated changes in calcium, MLEC were isolated from WT, Cav-1 KO, and Cav-1 RC mice. ACh-, but not ionomycin, stimulated increases in [Ca$^{2+}$], were decreased in Cav-1 KO MLEC compared with cells isolated from WT and Cav-1 RC mice ($n = 5$ individual experiments; five individual cells each; see Fig. 2A). These data are consistent with the data collected monitoring intracellular calcium in the endothelium of intact blood vessels.

It is well accepted that Ca$^{2+}$ enters the cytosol from two general sources, namely Ca$^{2+}$ release from mainly the ER and Ca$^{2+}$ entry across the plasma membrane. Next, we investigated whether Cav-1 deficiency would affect Ca$^{2+}$ release from the intracellular calcium stores. Fig. 2B shows typical traces of calcium signal intensities, and C and D show the summary from individual experiments quantifying phasic (C) and tonic (D) calcium levels after ACh ($n = 5$ experiments per cell type; three individual cells shown). In the absence of external Ca$^{2+}$ (plus 0.5 mM EGTA), stimulation with ACh (Fig. 2B, top, left panel) induced a transient increase in [Ca$^{2+}$], due to primarily Ca$^{2+}$ release from intracellular stores. Cav-1 deficiency slightly decreased the amount of ACh-induced peak Ca$^{2+}$ release, an effect rescued by Cav-1 (Fig. 2C).

Next, we investigated the effects of Cav-1 on Ca$^{2+}$ entry. Re-addition of external Ca$^{2+}$ after ACh stimulation sustained Ca$^{2+}$ entry (Fig. 2B), and the loss of Cav-1 markedly attenuated the entry of Ca$^{2+}$ by at least 50% (see top left panel after re-addition of 2 mM Ca$^{2+}$ after stimulation by ACh and summary data in Fig. 2D). We further investigated calcium entry by examining the effects of three nonspecific inhibitors of cation entry, gadolinium (Gd$^{3+}$), 2-aminoethoxydiphenyl borate (2-APB), and lanthanum (La$^{3+}$; $n = 4$ experiments) in
FIGURE 2. Cav-1 deficiency slightly impairs calcium release and markedly inhibits calcium entry but not IP₃ production in cultured lung endothelial cells. A, effect of Cav-1 KO and RC on intracellular Ca²⁺ ([Ca²⁺]ᵢ) increase by ACh (3 μM; left panel) and ionomycin (3 μM; right panel) in MLEC. B and C, effects of Cav-1 on Ca²⁺ release and entry induced by ACh. B, typical recordings of the increase in [Ca²⁺]ᵢ, in fluo-4AM-loaded MLECs. The external solution was replaced with Ca²⁺-free solution for the last 45 s of preincubation. Cells were then stimulated with ACh (3 μM) for 5 min, followed by the readdition of external Ca²⁺ (2 mM). In other experiments, cells were treated without (control) or with Gd³⁺ (100–300 μM, at the same time with Ca²⁺ readdition), 2-APB (100 μM) 15 min before, during, and after the ACh treatment or La³⁺ (100–300 μM). Areas under the curve were calculated for Ca²⁺ 5 min after the treatment with ACh (C) or the readdition of external Ca²⁺ (D). The results are expressed as the mean ± S.E. of 30–40 cells in five to six independent experiments. * and ** show significant differences from WT in each treatment with p < 0.05 and 0.01, respectively. ## reflects significant differences from the amount elicited by ACh in WT mice with p < 0.01. E, basal and ACh-stimulated IP₃ production in MLEC from WT, Cav-1 KO, and Cav-1 RC. Data are mean ± S.E., n = 4 in duplicate experiments.
WT endothelial cells. Gd3+ (100–300 μM), 2-APB (100 μM), and La3+ (100–300 μM) all decreased ACh-induced Ca2+ entry (see Fig. 2B and summary of data in Fig. 2D).

ACh binding to muscarinic receptors leads to phospholipase Cγ activation and the generation of DAG and IP3. To examine if Cav-1 is important for IP3 generation, we measured basal and stimulated IP3 accumulation in WT, Cav-1 KO, and Cav-1 RC MLEC. As seen in Fig. 2E, basal and ACh-stimulated IP3 accumulations were identical from these cells suggesting that defective calcium influx was not attributable to defective IP3 production.

**Caveolin-1 Is Crucial for the Subcellular Distribution of TRPC1 and -4 in Endothelial Cells**—Recently, both the N and C termini of TRPC1 have been shown to bind to Cav-1, and perhaps binding to Cav-1 may regulate the trafficking of TRPC and calcium influx pathways (29). Initially the protein levels of TRPCs (1 and 4) and Cav-1 were determined in MLEC lysates using isoform-specific antisera. To examine Ab specificity, siRNA and peptide blocking experiments were performed. Transfection of specific siRNAs (but not control siRNAs) for TRPC1 and TRPC4 into MLEC reduced the mRNA expression (by PCR; Fig. 3A) and protein levels (Fig. 3B) of TRPC1 (93 kDa) and TRPC4 (95 kDa). Commercially available Abs to TRPC3 detected a 160-kDa band, which was inconsistent with the predicted molecular weight of TRPC3; therefore, we could not study this TRPC further. In addition, preincubation of the peptide immunogen reduced the immunoreactivity of the TRPC1 and TRPC4 Abs (data not shown). These data are consistent with recent publications using these Abs for the respective TRPCs (30–32) and are in contrast with other studies using these Abs (33, 34).

In the three lines of MLEC used (WT, Cav-1 KO, and Cav-1 RC), there were no significant changes in the levels of TRPC1 (93 kDa) and TRPC4 (95 kDa; n = 4–7; Fig. 3C, far right panel, the levels of β-actin as a loading control were identical, and Cav-1 was present at equal levels in WT and Cav-1 RC cells). Because TRPCs are known to have a tissue-specific variation of subtype expression, we compared the protein expression of TRPCs (Fig. 3D, left panel) in lysates prepared from MLEC or the cerebral cortex (from 3-week-old mice). MLEC expressed primarily TRPC1 and -4, whereas TRPC1 and -4 were equally expressed in cerebral cortex; thus for further analysis of TRPCs in MLEC we focused on TRPC1 and -4.

Because caveolae have been implicated as microdomains that segregate signal transduction mechanisms, we next determined if the presence or absence of Cav-1 (and caveolae) could influence the subcellular distributions of TRPC1 and -4. Thus, cholesterol- and caveolin-1-enriched microdomains from WT, Cav-1 KO, and Cav-1 RC MLEC were subfractionated using a sucrose density gradient that separates the lipid rafts/caveolae from other intracellular cellular membranes and cytosolic proteins. As demonstrated in Fig. 3E, Cav-1 (left panel, rows 1–3) and lipid raft marker flotillin-2 (Flot-2, left panel, rows 4–6) were correctly targeted to low density, cholesterol-enriched membranes (fractions 1–4), and the loss of Cav-1 did not impair the distribution of the raft marker Flot-2 (n = 4 experiments). Conversely, β-actin (Fig. 3E, left panel, rows 7–9) was localized in heavy membrane fractions (fraction 8–12). Using this methodology, TRPC1 and TRPC4 (Fig. 3E, left panel, rows 1–6) were found enriched with Cav-1/Flot-2 representing caveolae/lipid rafts (n = 4 each). In Cav-1 KO MLEC, TRPC1 and TRPC4 were mislocalized away from fractions that co-sediment with Cav-1/Flot-2 and were relocated into heavy fractions (n = 4). Interestingly, the reintroduction of Cav-1 (Cav-1 RC) rescued the mislocalization of TRPCs (n = 4 each). These data suggest that in MLEC, Cav-1 and presumably caveolae were required for TRPC1 and -4 sedimentation into cholesterol-rich caveolae/rafts.

To examine this possibility at the cellular level, we determined the localization of TRPC1 and -4 by immunofluorescence microscopy. As shown in Fig. 3F, immunofluorescent staining of MLECs with anti-TRPC1 and anti-Cav-1 antibodies (left panels) revealed a diffuse cytoplasmic pattern for TRPC1 in WT MLEC with a punctate, dot-like, monocillia pattern near the nucleus (top panel, n = 6 experiments). Cytosolic localization of TRPC1 was confirmed with the use of two other antibodies, and the staining was eliminated by preincubating with the immunogen peptide (data not shown). Cav-1 had a diffuse staining pattern throughout the membranes and co-localized with TRPC1 in WT and Cav-1 RC cells (see merged images); however, the loss of Cav-1 did not affect TRPC1 localization (n = 6 each). TRPC4 had a distinct junctional, plasmalemmal pattern (Fig. 3G, see arrowheads) that partially co-localized with Cav-1 in WT or Cav-1 RC MLEC (n = 6 each); however, the loss of Cav-1 reduced the plasmalemmal staining pattern of TRPC4 (n = 6).

**Caveolin-1 Is Necessary for a Regulated Channel Complex Consisting of TRPC1, TRPC4, and IP3R in Endothelial Cells**—There is evidence that IP3R may bind directly or indirectly via the adaptor protein junctate (35) to the TRPC family of channels; however, the functional role of this interaction is not completely understood. As shown Fig. 4A, upon immunoprecipitation of TRPC4, a protein complex consisting of IP3R3, TRPC1, and Cav-1 was detected in WT and Cav-1 RC cell lines (n = 5 each). Stimulation of WT or Cav-1 RC MLEC with ACh increased the association of TRPC4 with IP3R1, IP3R3, and TRPC1 (n = 5 each). Interestingly, ACh-stimulated complex formation and recruitment of IP3R1 and IP3R3 were markedly reduced in cells lacking Cav-1 and rescued by reintroducing Cav-1 (n = 5 each). In all lines of MLEC under conditions where we could detect a TRPC4-TRPC1-IP3R3-Cav-1 complex, we could not detect any interaction of IP3Rs with TRPC1 and -3 when these TRPCs were immunoprecipitated (n = 5 each, data not shown). Using a pan-IP3R antibody for immunoprecipitation, a complex of IP3R3, TRPC4, and Cav-1 existed (Fig. 4B; n = 5 experiments). ACh increased the interaction of IP3R3 with TRPC4 (n = 5), and the levels of IP3R3 and Cav-1 were constant. TRPC1 was undetectable in the IP3R immunoprecipitate under basal conditions. Again, the loss of Cav-1 reduced the ACh-stimulated assembly of the Cav-1-IP3R3-TRPC4 complex (n = 5 each). In contrast to results immunoprecipitating TRPC4 where TRPC1 is associated with the complex in IP3R3 immunoprecipitates, TRPC1 did not co-precipitate (n = 5). Finally, immunoprecipitation of Cav-1 recovered a basal complex of IP3R3 and TRPC4 (Fig. 4C). Cav-1 is required for...
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A

TRPC1 Control 356 bp  
TRPC4 Control 304 bp

B

TRPC1 Control  
TRPC4 Control

100 kDa  
75 kDa  
95 kDa

TRPC1  
TRPC4

95 kDa  
22 kDa  
22 kDa

C

WT Cav-1 KO Cav-1 RC

TRPC1 150 kDa  
75 kDa  
50 kDa

TRPC4 150 kDa  
95 kDa

WT Cav-1 KO Cav-1 RC

β-actin 40 kDa

TRPC1 95 kDa

TRPC4 95 kDa

WT Cav-1 KO Cav-1 RC

D

WT MLEC Cerebral cortex  
WT MLEC Cerebral cortex

5%  
25%  
45%

5%  
25%  
45%

E

Sucrose concentration

1 2 3 4 5 6 7 8 9 10 11 12

Sucrose concentration

1 2 3 4 5 6 7 8 9 10 11 12

WT  
Cav-1 KO  
Cav-1 RC

Cav-1 (22 kDa)

Cav-1 (22 kDa)

β-actin (40 kDa)

β-actin (40 kDa)

F

WT Cav-1 KO Cav-1 RC

TRPC1  
Cav-1

TRPC1 & Cav-1

G

WT Cav-1 KO Cav-1 RC

TRPC4  
Cav-1

TRPC1 & Cav-1

TRPC4  
Cav-1
the interaction because the loss of Cav-1 obviates any interaction, and the subsequent reconstitution of Cav-1 permits recovery of the complex.

There is growing evidence that TRPCs can assemble into homo- and heteromers between themselves and can create a large variety of different channels. Especially in brain, various combinations of TRPCs have been reported (3, 36, 37), as well as in porcine endothelial cells. As shown in Fig. 4A, TRPC1 co-immunoprecipitates with TRPC4 and, vice versa, TRPC4 co-immunoprecipitates with TRPC1 in WT and Cav-1 RC MLEC (Fig. 4D). Therefore, immunoprecipitation of either TRPC4 or TRPC1 permitted the detection of heteromers of these TRPCs. Cav-1 KO reduced the interaction of TRPC1 with TRPC4 suggesting that regulated communication of IP₃Rs with TRPCs requires Cav-1 and presumably caveolae for this complex to assemble properly.

FIGURE 3. Characterization of TRPC1 and -4 expression in endothelial cells. Loss of Cav-1 changes the flotation of TRPCs into cholesterol-rich microdomains and localization in endothelial cells. A and B, characterization of TRPC1 and TRPC4 mRNA (A) and protein (B) expression in MLEC using siRNA. C, TRPC1 and -4 protein expression in lysates from WT, Cav-1 KO, and Cav-1 RC MLEC. Lysates (50 μg for TRPCs or 20 μg for Cav-1 and β-actin) from each MLEC were loaded onto SDS-PAGE and blotted with respective antibodies. D, relative expression of TRPCs in MLEC versus mouse cerebral cortical lysates. Similar amounts of protein were loaded as in A. E, subcellular fractionation of caveolin-enriched membranes on a sucrose gradient. WT, Cav-1 KO, and Cav-1 RC MLECs were lysed, homogenized, and subjected to sucrose density gradient centrifugation as described under “Experimental Procedures.” Twelve fractions were collected, and an aliquot of each fraction (50 μl) was subjected to Western blot analysis with anti-Cav-1, Flot-2, β-actin, TRPC1, and TRPC4 antibodies. Localization of TRPC1 (F), TRPC4 (G), and Cav-1 in WT, Cav-1 KO, and Cav-1 RC endothelial cells by immunofluorescent microscopy. Cav-1 is shown in red, and TRPC1 and -4 are labeled in green. Arrowheads indicate TRPC4 localized at edges of the plasma membrane. These results are representative of 6–9 separate experiments.

FIGURE 4. Cav-1 regulation of TRPC/IP₃R protein complexes. Immunoprecipitation (IP) with TRPC4 (A), IP₃Rs (B), Cav-1 (C), and TRPC1 (D). Control or treated (ACh for 5 min) MLECs were immediately lysed, homogenized, and subjected to immunoprecipitation with the respective Abs. E, TRPC1, TRPC4, or IP₃Rs were immunoprecipitated from mouse cerebral cortical extracts, and the protein complex was detected by Western blotting (IB). F and G, expression and interaction of recombinant GST-Cav-1 with TRPC4 and IP₃Rs in lysates from Cav-1 KO MLEC. Lysates were incubated with GST alone or GST-Cav fusion proteins (Cav-FL), Cav-(1–61), Cav-(61–101), and Cav-(135–178), and the binding of TRPC4 and IP₃Rs was assessed by Western blotting. Equivalent amounts of cell lysates, GST, and GST fusions (F) were used in these experiments. These data are representative of five independent experiments.
The Presence of Cav-1 in Endothelial Cells Specializes the Interaction between IP$_3$Rs and TRPCs—Cav-1 is expressed in vasculature and adipose tissue but not in neural cells, whereas non-neural and neural cells both exhibited calcium influx pathways that have been linked to TRPC expression. To specify the role of Cav-1 in regulating calcium entry in endothelial cells and to examine the assembly of TRPCs in a native tissue lacking Cav-1, we examined IP$_3$R and TRPC interactions in lysates prepared from the cerebral cortex of 3–4-week-old mice (see Fig. 3D and Fig. 4E). First, we confirmed that the cerebral cortex did not express Cav-1 protein (Fig. 3D, n = 4 experiments). Compared with MLEC, the murine cerebral cortex expressed less TRPC1 and TRPC4. In addition, IP$_3$R1, not IP$_3$R3, was the primary subtype of the IP$_3$R family in the cerebral cortex as mentioned before (n = 4 experiments). In co-immunoprecipitation experiments from cortical extracts, precipitation of both TRPC1 and TRPC4 did not result in the significant recovery of IP$_3$R or additional TRPCs in the complex. However, precipitation of IP$_3$R with pan-IP$_3$R antibody did not result in co-precipitation of TRPC1 or TRPC4 in cortical extracts (n = 4 each), suggesting that Cav-1 may uniquely regulate the assembly of IP$_3$R-TRPC interactions in endothelial cells.

Binding of Recombinant Cav-1 to TRPC4 and IP$_3$R Isolated from Cav-1 KO MLEC—Because both IP$_3$R and TRPC4 co-immunoprecipitate with Cav-1 and vice versa in cellular extracts, we sought to examine if either protein could co-purify and interact with recombinant Cav-1 using GST-Cav-1 as bait and Cav-1 KO lysates (Fig. 4, F and G). Fig. 4F depicts the expression of several distinct modules of the Cav-1 GST proteins as follows: GST alone, GST-Cav-(1–61), GST-Cav-(61–101), GST-Cav-(135–178), as well as full-length Cav-1, GST-Cav-(1–178). Incubation of GST alone (Fig. 4F, lane 1) or Cav-(1–61, lane 2) with Cav-1 KO lysates did not permit recovery of immunoreactive IP$_3$Rs or TRPCs from the lysates. On the other hand, incubation of equal amounts of GST-Cav-(61–101, see lane 3), GST-Cav-(135–178, lane 4), or full-length Cav-(1–178, lane 5) with Cav-1 KO lysates resulted in the recovery of an IP$_3$R1-IP$_3$R3-TRPC4 complex (Fig. 4G; n = 4 each). No detectable interactions between the GST-Cav constructs and TRPC1 were found (n = 4 experiments), although TRPC1 was present in the starting lysate (Fig. 4G, last lane labeled WT total).

DISCUSSION

Caveolins/caveolae have been proposed to influence calcium homeostasis in mammalian cells for over 3 decades. Initial experiments in the 1970s demonstrated close juxtapositioning between the endoplasmic reticulum and caveolae in muscle cells (38, 39), suggesting communication between the two organelles. Later studies using fluorescent techniques, co-precipitation experiments, subcellular fractionation, and disruption of lipidic microdomains by using cholesterol-modifying agents to disassemble caveolae and lipid rafts in cells have shown that caveolins/caveolae may influence calcium regulatory pathways (20, 29, 40–42). Recent studies using Cav-1-deficient mice have demonstrated that the loss of Cav-1 and caveolae reduces spontaneous transient outward currents and Ca$^{2+}$ sparks (local increases in subsarcolemmal Ca$^{2+}$ activity) in vascular smooth muscle cells (18); however, the mechanistic basis of this phenotype was unknown. In this study, we used genetic and biochemical criterion to define the role of Cav-1 on calcium influx pathways and TRPC assembly in the vascular endothelium. We show the following: 1) Cav-1 deficiency impairs endothelial Ca$^{2+}$ entry but not IP$_3$ production, which results in a decrease in ACh-induced prostacyclin release; 2) Cav-1 is essential for TRPC4 localization to the plasma membrane; and 3) Cav-1 interacts with a TRPC4-IP$_3$R complex especially after agonist stimulation. Most importantly, the abnormal phenotypes in calcium handling in Cav-1 KO mice can be restored to physiological levels by genetic reconstitution of Cav-1 back into endothelial cells. These results propose a firm genetic basis for Cav-1 regulating the assembly of TRPCs at the cell membrane and regulating calcium influx pathways (see Fig. 5 for proposed model).

The lack of embryonic lethality in Cav-1-specific knock-out mice has allowed investigators to test the importance of this protein and corresponding organelle in several post-natal functions. By breeding this KO strain to a newly generated strain of mice expressing Cav-1 specifically in the endothelium, we were able to reconstitute (Cav-1 RC) caveolae in endothelia confirming the idea that Cav-1 is responsible for caveolae biogenesis in the endothelium in vivo. Recently, using this model, we have shown that Cav-1 is necessary for flow-dependent remodeling and mechanotransduction in endothelial cells (23). In intact endothelia lining intrapulmonary arteries, Cav-1 deficiency impairs endothelial Ca$^{2+}$ metabolism, primarily agonist-mediated calcium entry, which is required for production of the vasodilator, anti-thrombotic lipid prostacyclin (Fig. 2B). Whether the defects in mechanotransduction in carotid arteries as described relate to impaired calcium influx in intrapulmonary arteries in this paper are not clear. Furthermore, qualitatively similar results were seen in endothelial cells isolated and cultured from Cav-1 WT, Cav-1 KO, and Cav-1 RC mice thus demonstrating the necessary role of Cav-1 in regulating the tonic increase in [Ca$^{2+}$], after ACh challenge.

Cav-1 deficiency did not influence basal or ACh-stimulated IP$_3$ production (Fig. 2E), but significantly reduced endothelial cell calcium influx (Fig. 2B), prostacyclin release (Fig. 1F), and TRPC1/4-IP$_3$R interactions (Fig. 5, A and B) primarily after stimulation with ACh. Interestingly, ACh-induced Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores was slightly less in Cav-1 KO MLEC and rescued by the re-addition of Cav-1 (Fig. 2C), suggesting partial Cav-1 regulation of calcium loading perhaps by its effect in reducing influx mechanisms because Ca$^{2+}$ entry is required for replenishment of Ca$^{2+}$ into intracellular Ca$^{2+}$ stores. As a result, the changes in Ca$^{2+}$ metabolism may reduce both basal and agonist-stimulated increases in prostacyclin release.

Subsequently, quenching followed by the re-addition of extracellular Ca$^{2+}$ to endothelial cells (Fig. 2) demonstrates that Cav-1 deficiency mainly impairs Ca$^{2+}$ entry across the plasma membrane and, to a lesser degree, the above-mentioned Ca$^{2+}$ release from internal stores. This phenotype is most evident in the cultured MLEC when ACh responses were examined in the absence of extracellular calcium, followed by the re-addition of calcium. Previous work has shown that endothelial cells express the mRNAs for several TRPC subtypes 1, 3, 4, 6,
and 7 (43), and in porcine endothelial cells, TRPC3 and TRPC4 form a redox-sensitive cation channel (14). The idea that TRPC4 is necessary for calcium influx in murine endothelial cells is supported by data showing that loss of TRPC4 in knock-out mice reduces calcium influx into endothelial cells in vivo (10, 44).

There is evidence that certain TRPCs (such as TRPC1 and TRPC3) and IP3R may be regulated by Cav-1 (29, 45, 46). However, these studies are largely based on overexpression studies, peptide antagonism, or cholesterol-modifying drugs (which disrupt caveolae and raft domains but also extract membranes and change cellular integrity) (47). In our experiments, we used a variety of techniques, including flotation of protein complexes, cellular localization, and protein complex formation by co-precipitation in cells containing or lacking endogenous Cav-1. In WT endothelial cells, TRPC1 and TRPC4 co-fractionate with Cav-1 and the raft marker, flotillin-2. Cav-1 deficiency causes anomalous sedimentation of the channel complex but does not change the fractionation of the raft marker flotillin-2. This suggests that the loss of Cav-1 does not nonspecifically impair membrane raft assembly (delineated by flotillin-2) but only the assembly of proteins requiring Cav-1. Importantly, the impairments in TRPC1 and TRPC4 flotation were corrected by re-expression of Cav-1 in the knock-out background. By confocal microscopy, Cav-1 can co-localize with intracellular TRPC1 and TRPC4, and the loss of Cav-1 results in no conspicuous changes in the apparent subcellular localization of TRPC1 but markedly disrupts TRPC4 localization to the plasma membrane (Fig. 3G), again effects corrected by reintroduction of Cav-1. Taken together these data suggest that in resting endothelial cells, TRPC1 and TRPC4 can be isolated in similar buoyant microdomains that are dependent on Cav-1 expression and heteromerization of TRPC or IP3R subtypes, the interactions of TRPC complexes with IP3Rs, and the presence or absence of specific scaffolds/adaptors may determine the regulation of TRPC-mediated Ca2+ entry in specialized cells such as endothelial cells and neurons.

Using co-precipitation techniques (detergent-soluble, native complexes) in resting endothelial cells, TRPC4 can interact with TRPC1, IP3R3, and Cav-1. The loss of Cav-1 does not

![Figure 5. Proposed model of endothelial calcium entry by caveolin-1.](image)

A. Endothelial cells

**WT**

**Basal state**

![Cell diagram](image)

**Cav-1 KO**

**Basal state**

![Cell diagram](image)

**Activated**

![Cell diagram](image)

**Caveolin-1 Regulates Endothelial Calcium**

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markedly influence the basal interaction of the TRCs with the IP₃Rs. However, upon challenge with ACh, the interactions of TRPC4 with IP₃Rs and Cav-1 are enhanced with no changes in the levels of co-associated TRPC1. In contrast, following immunoprecipitation of either IP₃R3 or Cav-1, there is an increase in TRPC4 recovered in the complex suggesting that TRPC4 and IP₃R3 are regulated in the Cav-1 complex. The lack of TRPC1 in these complexes appears to be at odds with fractionation studies that demonstrate the loss of both TRPC1 and TRPC4 from buoyant membranes isolated from Cav-1 KO cells (shown by fractionation studies). However, the co-association of TRPC1 with TRPC4 under these differing biochemical conditions and the interactions of TRPC1 with other components may stabilize/destabilize TRPC1 in these complexes. Alternatively, during immunoprecipitation of soluble, native complexes, perhaps only a small fraction of the total TRPC1/4 interactions was recovered. Regardless of these technical differences, there is evidence supporting TRPC1 and TRPC4 interactions in regulating TRPC function because co-expression of TRPC4 with TRPC1 results in heteromultimer assembly and proper trafficking of TRPC1 to the cell membrane (48). Also, Cav-1, via its scaffolding domain, can interact with TRPC1 (46); however, we did see a direct interaction of TRPC1 with GST-Cav-1 in our experiments (Fig. 4, F and G). In this study, the loss of Cav-1 reduces ACh-mediated calcium entry and the co-precipitation of TRPCs with IP₃Rs, phenotypes rescued in Cav-1 RC cells, suggesting that Cav-1 is responsible for the assembly of an activated calcium-influx protein complex consisting of TRPCs and IP₃Rs in endothelial cells.

Three subtypes of IP₃Rs (IP₃R1, -2, and -3) are expressed in a tissue-specific manner and form heterotetrameric channels (49, 50), and endothelial cells express mainly IP₃R3, whereas the brain mainly expresses IP₃R1 (Figs. 3B and 4E). In endothelial cells, an endogenous TRPC4/IP₃R3 interaction occurs, and while in cerebral cortical extracts lacking caveolins, a TRPC3/IP₃R3 interaction occurs (Fig. 4E) suggesting that the expression of Cav-1 regulates specialized calcium-dependent processes in endothelial cells. Previous studies also reported TRPC1- and TRPC3/IP₃R interactions when overexpressed in HEK293 cells, and the N-terminal fragment of the IP₃R directly gates TRPC3 in patch clamp studies (9); however, a definitive link between the ER or ER-derived calcium and activation of native TRPCs has not been established unequivocally. Thus, interactions between TRPCs and Cav-1 may regulate TRPC localization, or the proximity of TRPCs in invaginated caveolae juxtaposed to the IP₃Rs in the ER may explain our data. Clearly, when Cav-1 and caveolae are lacking, ACh-stimulated calcium entry and TRPC4/IP₃R complex formation are reduced. Our data with Cav-1 in endothelial cells are similar to recent study showing that juncate, IP₃R-associated protein, serves as a scaffold for TRPC4 and TRPC5 in sperm (35). In neurons, other molecules may subserve a similar adaptor function for TRPCs such as Homer, NHERF-2, and the immunophillin FKBP59 (36, 51, 52). Collectively, specialized cells may have different combinations of TRPC subtype expression, heterologous channel multimerization, TRPC/IP₃R interaction, and adaptors to control the multitude of cellular functions in a tissue-dependent manner.

Control of calcium entry via Cav-1 defines a new, clear in vivo function for this protein and caveolae in endothelial cells. The expression of Cav-1 in other specialized cells such as adipocytes, epithelial cells, and vascular smooth muscle cells suggests Cav-1 may influence additional pathways of calcium regulation and calcium-mediated signal transduction in these cell types. The role of caveolins and lipid rafts as plasma membrane microdomains in signal transduction is a attractive hypothesis to integrate disparate pathways into concentrated lipid domains; however, this concept has been met with skepticism despite clear genetic evidence showing impaired mechanosignaling, nitric oxide synthesis, transcytosis, pulmonary and cardiac abnormalities, and abnormal adipogenesis in mice deficient in Cav-1 (18, 23, 53, 54). The cell-specific reconstitution of Cav-1 or perhaps Cav-3, in the respective deficient backgrounds, provides another level of support for the vital role of this protein and organelle in physiological processes.

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