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Store-operated calcium entry and increased endothelial cell permeability

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Norwood, Natalie, Timothy M. Moore, David A. Dean, Rakesh Bhattacharjee, Ming Li, and Troy Stevens. Store-operated calcium entry and increased endothelial cell permeability. *Am J Physiol Lung Cell Mol Physiol* 279: L815–L824, 2000.—We hypothesized that myosin light chain kinase (MLCK) links calcium release to activation of store-operated calcium entry, which is important for control of the endothelial cell barrier. Acute inhibition of MLCK caused calcium release from inositol trisphosphate-sensitive calcium stores and prevented subsequent activation of store-operated calcium entry by thapsigargin, suggesting that MLCK serves as an important mechanism linking store depletion to activation of membrane calcium channels. Moreover, in voltage-clamped single rat pulmonary artery endothelial cells, thapsigargin activated an inward calcium current that was abolished by MLCK inhibition. F-actin disruption activated a calcium current, and F-actin stabilization eliminated the thapsigargin-induced current. Thapsigargin increased endothelial cell permeability in the presence, but not in the absence, of extracellular calcium, indicating the importance of calcium entry in decreasing barrier function. Although MLCK inhibition prevented thapsigargin from stimulating calcium entry, it did not prevent thapsigargin from increasing permeability. Rather, inhibition of MLCK activity increased permeability that was especially prominent in low extracellular calcium. In conclusion, MLCK links store depletion to activation of a store-operated calcium entry channel. However, inhibition of calcium entry by MLCK is not sufficient to prevent thapsigargin from increasing endothelial cell permeability.

lung; myosin light chain kinase; signal transduction; inositol trisphosphate; capacitative calcium entry

MAJNO AND PALADE (16) originally suggested that inflammatory mediators stimulate endothelial cell retraction necessary to increase permeability. General support for this hypothesis continues today as the molecular events that control barrier function are examined (15, 17, 18). Recent measurements indicate that endothelial cells possess a constitutive inward tension resulting from the interaction of F-actin with nonmuscle myosin that forms an actomyosin complex (10, 26, 27). Actomyosin interaction is stimulated by reversible phosphorylation of 20-kDa myosin light chain (MLC_{20}). An endothelial cell-specific MLC kinase (MLCK) is the primary isoform that regulates phosphorylation of MLC_{20} (34, 35). G_q-coupled agonists like histamine and thrombin activate MLCK, which increases MLC_{20} phosphorylation from its constitutive level of $\cong$0.4 to $\cong$1.2 mol phosphate/mol MCL_{20} and further promotes centripetally directed tension (10, 19, 20, 27, 38, 39).

Although a central role for MLCK in endothelial cell barrier function has been demonstrated, the precise relationship between MLCK-induced retraction and generation of intercellular gaps is not fully established. MLCK activation is clearly linked to increased permeability, and inhibition of MLCK reduces permeability evoked by G_q-coupled agonists (9, 19, 27). However, direct inhibition of cell-cell and cell-matrix tethering under conditions of constitutive MLC_{20} phosphorylation is sufficient to increase permeability. Furthermore, MLCK may play a secondary or alternate role in regulating the endothelial barrier response by inhibiting cytosolic calcium concentration ($[Ca^{2+}]_i$) responses to neurohumoral inflammatory agonists (11, 36, 37). Thus the specific function of MLCK in linking cell activation to increased permeability is not completely understood.

An elevation in $[Ca^{2+}]_i$ associated with activation of store-operated calcium entry is sufficient to increase endothelial cell permeability (4, 13, 18, 28, 29). Activation of store-operated calcium entry occurs after depletion of intracellular calcium stores either by stimulation of calcium release (e.g., histamine or thrombin) or by inhibition of calcium reuptake (e.g., thapsigargin) into storage sites. Although the mechanism linking store depletion to activation of calcium entry is unknown, a conformational or physical coupling model has previously been proposed (1, 24). The original hypothesis suggested that a decrease in stored calcium alters the inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] receptor conformation that directly opens a membrane calcium channel. The possibility that cytoskeletal elements tether intracellular organelles or the Ins(1,4,5)P_3 receptor to membrane channel function

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has also been considered (3, 12, 23). An extension of this latter possibility is that intracellular organelles possessing calcium stores (e.g., endoplasmic reticulum) or the Ins(1,4,5)P_3 receptor are coupled to store-operated calcium entry channels through the cytoskeleton that is held under tension. Thus changes in MLCK-dependent tension may directly regulate activation of store-operated calcium entry, suggesting that MLCK may influence endothelial cell barrier function by controlling calcium responses to G_q agonists. Our present studies tested the hypothesis that MLCK activation by inflammatory calcium agonists regulates calcium entry that is important for control of endothelial cell barrier function.

METHODS

Measurement of $[Ca^{2+}]_i$. Rat pulmonary artery endothelial cells (RPAECs) were isolated and cultured for study at *passages 9–17*. For calcium measurements, the cells were seeded at ~1.5 × 10^5 cells/ml on two-chambered glass coverslips (Nalge Nunc International) and grown to confluence in serum-containing medium continuously for 4–7 days without a change in medium. Cells were loaded with a fura 2-AM loading buffer (2 ml of Krebs buffer with 25 mM HEPES plus 2 mM or 100 nM calcium, 3 mM fura 2-AM, and 6 μl of Pluronic acid) for 20 min in a CO_2 incubator at 37°C. The cells were then washed with 2 ml of Krebs buffer and treated with deesterification medium (2 ml of Krebs buffer with 25 mM HEPES plus 2 mM or 100 nM calcium) for an additional 20 min. After deesterification, $[Ca^{2+}]_i$ was assessed with an Olympus IX70 inverted microscope at ×400 with a xenon arc lamp photomultiplier system (Photon Technologies, Monmouth Junction, NJ), and data were acquired and analyzed with PTI Felix software. Epifluorescence was measured from three to four endothelial cells in a confluent monolayer, and the changes in $[Ca^{2+}]_i$ are expressed as the fluorescence ratio of Ca^{2+}-bound (340-nm) to Ca^{2+}-unbound (380-nm) excitation wavelengths (ratio 340/380) emitted at 510 nm. In vitro calibrations were then performed with the fura 2 calcium imaging calibration kit (Molecular Probes).

Microinjections. RPAECs were trypsin dispersed on etched glass coverslips placed in 60-mm plastic culture dishes. The cells were allowed to reattach for at least 24 h in serum-containing growth medium. Approximately 75–100 cells in small confluent patches were then microinjected with glass capillary pipettes pulled with a pipette puller (World Precision Instruments, Sarasota, FL) and a Narishige micromanipulator. Heparin (5 U/ml) in phosphate-buffered saline was microinjected. Approximately 3 × 10^{-10} ml was injected into each cell (5).

Electrophysiology. Whole cell patch clamp was utilized to measure transmembrane ion flux in thapsigargin-stimulated RPAECs according to previously described methods (18). Confluent RPAECs were enzyme dispersed, seeded onto 35-mm plastic culture dishes, and then allowed to reattach for at least 24 h before the patch-clamp experiments. Single RPAECs exhibiting flat polyhedral morphology were studied. These cells were chosen for study because their morphology was consistent with RPAECs from a confluent monolayer. These single cells have previously been shown to possess electrophysiological recordings generally similar to those observed in confluent monolayers (28). The extracellular solution was composed of (in mM) 110 tetraethylammonium aspartate, 10 calcium aspartate, 10 HEPES, and 0.5 3,4-diaminopyridine; and the pipette solution was composed of (in mM) 130 *N*-methyl-D-glucamine, 1.15 EGTA, 10 HEPES, and 1 $Ca(OH)_2$ with and without 2 Mg^{2+}-ATP. Both solutions were adjusted to 290–300 mosM with sucrose and pH 7.4 with methane sulfonic acid. $[Ca^{2+}]_i$ was estimated as 100 nM (5a). The pipette resistance was 2–5 MΩ. Data were obtained with a HEKA EPC9 amplifier (Lambrecht/Pfaltz) and sampled on-line with Pulse+Pulsefit software (HEKA) All recordings were made at room temperature (≈25°C). To generate current-voltage relationships, voltage pulses were applied from −100 to +100 mV in 20-mV increments, with 200-ms duration for each voltage step and a 2-s interval between steps. The holding potential between each step was 0 mV. The experimental protocols were established as follows: *1*) vehicle control (DMSO in patch pipette; n = 10 experiments), *2*) thapsigargin control (1 μM thapsigargin in patch pipette; n = 15 experiments), *3*) ML-9 plus thapsigargin (15 μM ML-9 pretreatment for 10–30 min, thapsigargin in patch pipette; n = 13 experiments), *4*) jasplakinolide plus thapsigargin (1 μM jasplakinolide pretreatment for 4 h, thapsigargin in patch pipette; n = 4 experiments), and *5*) cytochalasin D (10 mM cytochalasin D in patch pipette; n = 10 experiments).

Estimation of diffusive capacity. RPAECs were seeded onto Transwell inserts (6.5-mm diameter, 0.4-mm pore size; Costar) at a density of 8.5 × 10^5 cells/ml in a final volume of 100 μl of DMEM plus 10% FBS. The inserts were placed into 24-well plates containing 600 μl of growth medium, and the cells were allowed to grow for 5 days with one change of medium. After confluence was achieved, the growth medium in the upper chamber was replaced with 100 μl of a 1 mg/ml FITC-dextran (mol wt 10,000) solution in Krebs-Henseleit physiological salt solution (PSS). The insert was then moved to a fresh lower well containing 600 μl of PSS. The cells were equilibrated with these solutions at 37°C in a CO_2 incubator for 10 min. After equilibration, the Transwell insert was placed into another lower chamber containing 600 μl of PSS, and the FITC-dextran was allowed to diffuse across the monolayer for 30 min. This procedure was repeated three times so that a total time of 2 h for assessing monolayer integrity was employed. Samples from the lower chamber (50 μl) were taken in triplicate and placed in 96-well cluster plates for measuring fluorescent intensity (Perkin-Elmer luminescence spectrometer LS 50B) with an excitation of 480 nm and an emission at 530 nm. Fluorescence values were then converted to milligrams of FITC-dextran per milliliter with a standard curve that was generated concurrent with the measurements of monolayer integrity. With these values, diffusive capacity (PS) was calculated by determining the net rate of FITC-dextran flux (J_s) generated for each concentration difference (ΔC) across the monolayer with the equation PS = J_s/(ΔC). PS is expressed in nanoliters per minute.

Statistical methods. Data are reported as means ± SE. Comparisons were made with either unpaired Student's t-test or one-way analysis of variance with repeated measures as appropriate. A Student-Newman-Keuls post hoc test was applied. Differences were considered significant at $P < 0.05$.

RESULTS

Regulation of calcium release by MLCK. Ins(1,4,5)P_3 receptors possess putative ankyrin binding domains predicted to associate the receptor with cytoskeletal elements held under tension in endothelial cells (3, 7, 20, 38, 39). We therefore tested whether inhibiting MLCK, which has previously been shown to decrease endothelial cell tension, would alter the kinetics of calcium release. Figure 1 demonstrates that acute ap-

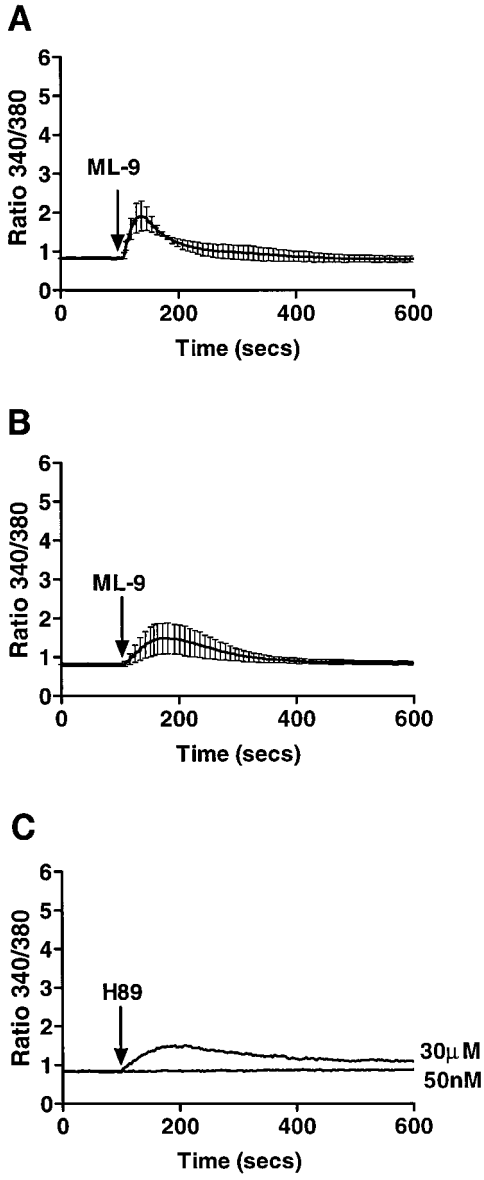

Fig. 1. Acute inhibition of myosin light chain kinase (MLCK) increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat pulmonary artery endothelial cells (RPAECs). *A*: 100 μM ML-9 applied directly to confluent RPAECs incubated in an extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) of 2 mM produced a transient increase in $[Ca^{2+}]_i$ that returned to baseline levels ($n = 5$ experiments). $P < 0.05$. *B*: reducing $[Ca^{2+}]_o$ from 2 mM to 100 nM did not prevent ML-9 from increasing $[Ca^{2+}]_i$ ($n = 8$ experiments). $P < 0.05$. *C*: representative trace showing that application of H-89 to RPAECs at a concentration that specifically inhibits protein kinase A activity (50 nM) did not increase $[Ca^{2+}]_i$, but addition of H-89 at a concentration that also inhibits MLCK activity (30 μM) transiently increased $[Ca^{2+}]_i$. Ratio 340/380, ratio of 340- to 380-nm fluorescence.

plication of the MLCK inhibitor ML-9 to confluent fura 2-AM-loaded RPAECs produced a transient increase in $[Ca^{2+}]_i$ in the presence (2 mM; Fig. 1*A*, Table 1) and relative absence (100 nM; Fig. 1*B*) of extracellular calcium, indicating that ML-9 stimulates calcium release. Similar results were obtained with other MLCK inhibitors including W-7, which caused a peak increase in $[Ca^{2+}]_i$ from baseline values of 137 ± 2 nM (ratio 340/380 = 0.9 ± 0.01) to 1.8 ± 0.86 (ratio 340/380; $P < 0.05$; $n = 9$ experiments). The protein kinase (PK) A inhibitor H-89 did not alter $[Ca^{2+}]_i$ at concentrations specific for PKA but increased $[Ca^{2+}]_i$ when used at concentrations that reportedly inhibit MLCK (Fig. 1*C*). Similarly, inhibition of PKC activity with chelerythrine did not alter $[Ca^{2+}]_i$ (data not shown). These data therefore implicate MLCK, but not PKA or PKC, in the regulation of calcium release in RPAECs.

Because the Ins(1,4,5)P_3-sensitive calcium pool is depleted after sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibition (31), we utilized thapsigargin to assess whether ML-9 increased $[Ca^{2+}]_i$ by stimulating calcium release from an Ins(1,4,5)P_3-sensitive calcium pool. Although application of thapsigargin in a low extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) produced a transient increase in $[Ca^{2+}]_i$ from 0.7 ± 0.02 to 1.6 ± 0.13 (ratio 340/380; $P < 0.05$; $n = 7$ experiments), pretreatment of the cells incubated in 2 mM $[Ca^{2+}]_o$ with ML-9 nearly abolished the thapsigargin-induced calcium release [from 0.9 ± 0.03 to 1.1 ± 0.05 (ratio 340/380); P = not significant (NS) from baseline]. Similarly, thapsigargin pretreatment prevented ML-9 from increasing $[Ca^{2+}]_i$ (ratio 340/380 = 0.7 ± 0.03; P = NS from baseline; $n = 5$ experiments), suggesting that ML-9 and thapsigargin target a similar Ins(1,4,5)P_3-sensitive pool. To confirm this possibility, RPAECs were microinjected with heparin to inhibit the Ins(1,4,5)P_3 receptor (30). In physiological concentrations of $[Ca^{2+}]_o$, baseline $[Ca^{2+}]_i$ ratios were similar in heparin-injected (ratio 340/380 = 0.9 ± 0.03; $n = 6$ cells) and PBS-injected or noninjected cells (ratio 340/380 = 0.9 ± 0.03; $n = 5$ cells), demonstrating good cell viability. In control cells, application of ML-9 increased $[Ca^{2+}]_i$ to 1.6 ± 0.6 (ratio 340/380; $P < 0.05$; $n = 6$ cells) and the subsequent addition of thrombin resulted in an abrupt peak increase in $[Ca^{2+}]_i$ to 3.4 ± 0.5 (ratio 340/380; $P < 0.05$; $n = 6$ cells; Fig. 2*A*). Heparin microinjection prevented both the ML-9- and thrombin-induced increase in $[Ca^{2+}]_i$ (Fig. 2, *B* and *C*). Thus these data demonstrate that ML-9 promotes calcium release through the Ins(1,4,5)P_3 receptor.

Table 1. *Pharmacological tools and their respective cellular targets*

Agent	Target
Thapsigargin	Inhibit SERCA
Thrombin	Activate thrombin receptor
ML-9	Inhibit MLCK
W-7	Calmodulin antagonist
H-89	
50 nM	Inhibit PKA
30 μM	Inhibit MLCK
Chelerythrine	PKC antagonist
Heparin	Inhibit Ins(1,4,5)P_3 receptor
Cytochalasin D	Disrupt F-actin
Jasplakinolide	Stabilize F-actin

SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; MLCK, myosin light chain kinase; PKA, protein kinase A; PKC, protein kinase C; Ins(1,4,5)P_3, inositol 1,4,5-triphosphate.

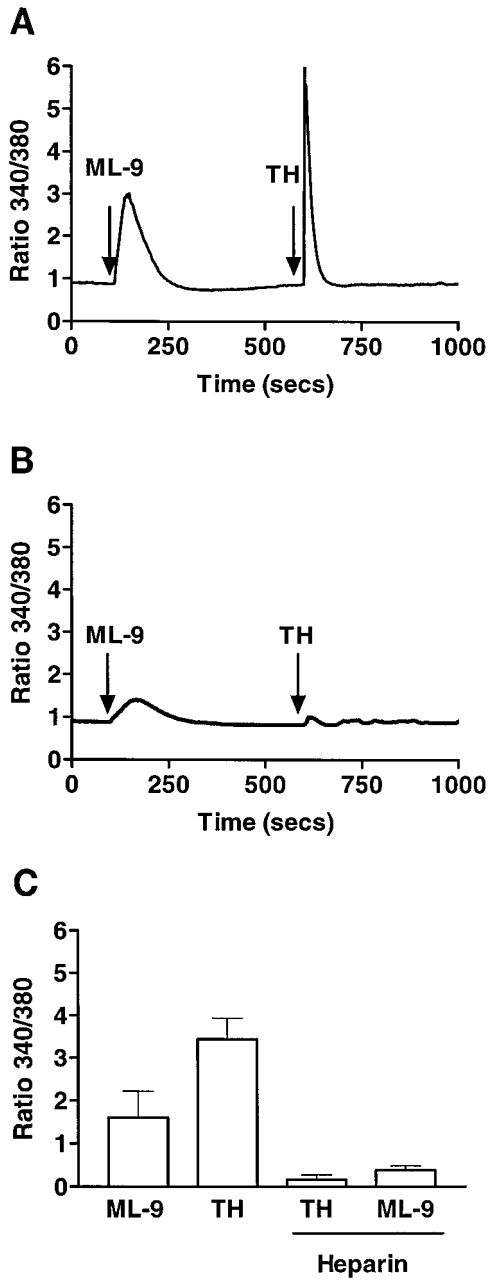

Fig. 2. Inhibition of MLCK causes calcium release from inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]-sensitive calcium pools. *A*: representative trace demonstrating that application of 15 μM ML-9 increased $[Ca^{2+}]_i$. Subsequent addition of thrombin (TH; 7 U/ml) produced a characteristic spike in $[Ca^{2+}]_i$ due to calcium release from Ins(1,4,5)P_3-sensitive calcium pools. *B*: treatment of heparin (5 U/ml)-microinjected cells with ML-9 (100 μM) and thrombin (7 U/ml) exhibited significantly diminished $[Ca^{2+}]_i$ responses. *C*: average peak $[Ca^{2+}]_i$ responses to ML-9 and thrombin, demonstrating that heparin microinjection significantly diminished Ins(1,4,5)P_3-dependent sensitivity ($n = 6$ experiments). $P < 0.05$.

Role of MLCK in activation of store-operated calcium entry. MLCK regulates actomyosin interaction and the inward centripetal tension in endothelial cells, although it is unclear whether MLCK-dependent cell tension effects activation of store-operated calcium entry. We therefore next examined whether inhibition of MLCK regulates store-operated calcium entry (37). Figure 3*A* demonstrates that thapsigargin produced a slowly developing, sustained increase in $[Ca^{2+}]_i$. However, ML-9 pretreatment significantly attenuated thapsigargin-dependent calcium release (ratio 340/380 = 1.6 ± 0.1 without ML-9, $n = 7$ experiments, vs. ratio 340/380 = 0.2 ± 0.05 with ML-9, $n = 8$ experiments; $P < 0.05$) and abolished the calcium entry response. Because ML-9 reduced thapsigargin-dependent calcium release, the calcium stores were likely not depleted, suggesting inhibition of calcium entry could be due to either preservation of the intracellular calcium pool or disruption of a mechanism gating the store-operated calcium entry channel. To address this issue, thapsigargin was applied first to activate store-operated calcium entry and then ML-9 was added (Fig. 3*C*). Addition of ML-9 immediately reduced $[Ca^{2+}]_i$, suggesting that MLCK regulates the activation state of a membrane channel. To confirm this idea, thapsigargin was applied to RPAECs incubated in nominally calcium-free medium (100 nM) followed by readdition of extracellular calcium (2 mM). Thapsigargin stimulated a transient, calcium release-dependent increase in $[Ca^{2+}]_i$. Replenishing $[Ca^{2+}]_o$ resulted in a sustained, calcium entry-dependent increase in $[Ca^{2+}]_i$ that was immediately reduced after application of ML-9 (Fig. 3*D*).

The ML-9-dependent decrease in $[Ca^{2+}]_i$ observed in Fig. 3, *C* and *D*, could be due to inhibition of a calcium entry channel, stimulation of calcium extrusion, or membrane depolarization. We addressed this issue using whole cell electrophysiology in single RPAECs in voltage-clamp mode. Solutions were designed to isolate a store-operated calcium entry current as Moore et al. (18) have previously described. Although cells exhibited only a slight leak current under unstimulated conditions, inclusion of thapsigargin in the patch pipette activated a calcium current that at positive voltages was inwardly rectifying (Fig. 4, *A* and *B*). This current was similar to the previous report by Moore et al. in these cells. Furthermore, exclusion of Mg-ATP from the patch pipette abolished the store-operated calcium entry current (Fig. 4*C*). Similarly, pretreatment with ML-9 to cells supplied with ATP abolished the response to thapsigargin (Fig. 4*D*), suggesting that MLCK regulates a calcium entry channel likely due to a phosphorylation-dependent event.

Activation of MLCK evokes enzyme translocation from the cytosol to the F-actin cytoskeleton where it promotes actomyosin interaction and increases centripetally directed tension (9, 10, 20, 34, 38). We therefore examined whether the configuration of F-actin represented an important determinant of store-operated calcium entry. Initial studies utilized cytochalasin D to disrupt F-actin filaments and eliminate RPAEC inward tension (Fig. 5). Cytochalasin D activated an inward calcium current with biophysical properties resembling a thapsigargin-sensitive store-operated calcium entry current (Table 2). To further address this idea, jasplakinolide was utilized to stabilize F-actin. Pretreatment with jasplakinolide eliminated the re-

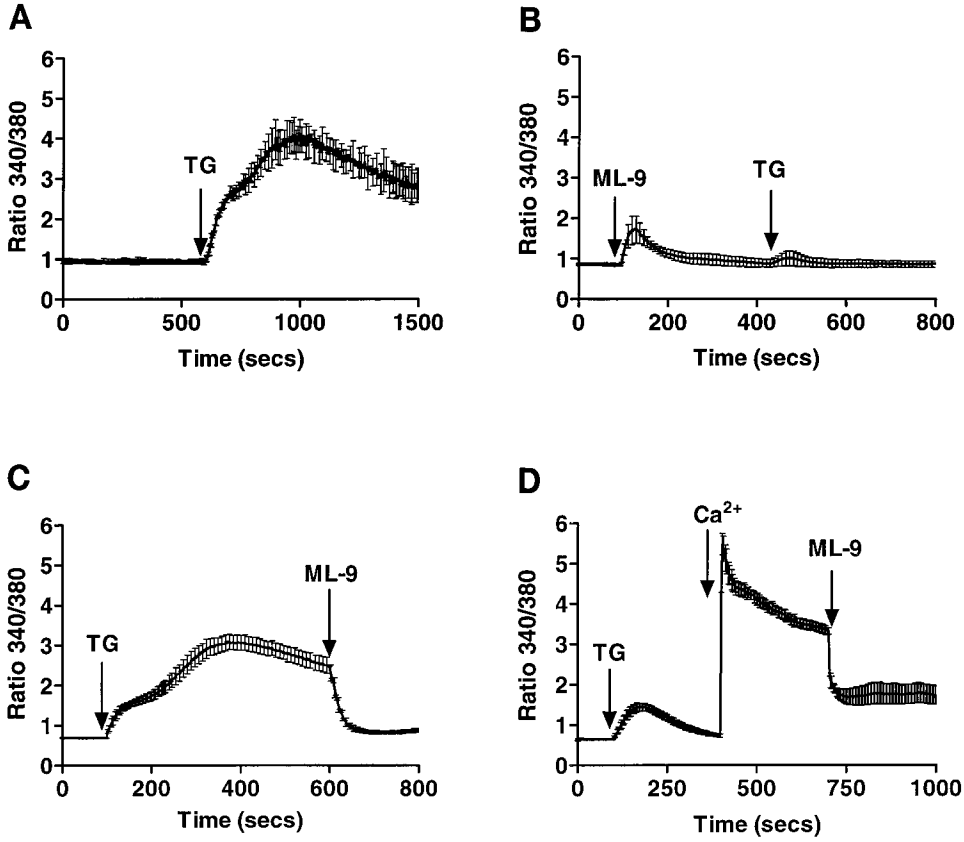

Fig. 3. Inhibition of MLCK inactivates store-operated calcium entry. *A*: thapsigargin (TG; 1 μM) produced a slowly developing, sustained increase in $[Ca^{2+}]_i$ (n = 6 experiments). *B*: ML-9 (100 μM) pretreatment caused a transient increase in $[Ca^{2+}]_i$ and prevented thapsigargin (1 μM)-induced calcium release and activation of store-operated calcium entry (n = 5 experiments). *C*: activation of store-operated calcium entry with 1 μM thapsigargin was immediately reduced after application of ML-9 (100 μM; n = 4 experiments). *D*: application of thapsigargin (1 μM) to RPAECs incubated in 100 nM $[Ca^{2+}]_o$ produced a transient increase in $[Ca^{2+}]_i$ that returned to baseline value. Restoring $[Ca^{2+}]_o$ to 2 mM resulted in a rapid and sustained increase in $[Ca^{2+}]_i$ because calcium entry occurs through store-operated calcium entry channels. ML-9 (15 μM) immediately inactivated store-operated calcium entry and returned $[Ca^{2+}]_i$ to near baseline levels (n = 6 experiments).

sponse to thapsigargin, suggesting that MLCK may gate store-operated calcium entry channels through the regulation of actomyosin-based tension.

We performed studies to address whether the action of ML-9 was due to its inhibitory effect on MLCK. Figure 6, *A* and *B*, demonstrates the dose-dependent inhibition of store-operated calcium entry by ML-9, with an IC_{50} of 4.6 μM, similar to its calculated IC_{50} for MLCK (3.8 μM). Moreover, peak $[Ca^{2+}]_i$ responses to thapsigargin are plotted in Fig. 6*C* and demonstrate that although inhibition of PKA or PKC does not alter the $[Ca^{2+}]_i$ response to thapsigargin, inhibition of MLCK with ML-9, W-7, or H-89 each significantly reduced the $[Ca^{2+}]_i$ response to thapsigargin. Thus it is most likely that the effect of ML-9 is through its inhibition of MLCK activity.

MLCK and endothelial cell permeability. Inhibition of MLCK activity prevents the increase in RPAEC permeability induced by G_q-coupled agonists like thrombin, although it is unclear whether MLCK inhibition has similar salutary effects on permeability changes induced by activation of store-operated calcium entry. RPAEC monolayers exhibited a constitutive diffusive capacity to a 23-Å FITC-dextran (mol wt 10,000) tracer that increased 27% after application of thapsigargin (Fig. 7*A*). Although the increase in permeability was greatest 30 min after the application of thapsigargin, only a slight increase in permeability was apparent 2 h after treatment, suggesting that barrier function improved over the time course evaluated (Fig. 7*B*). Consistent with our previous reports (4, 13, 18, 29), the thapsigargin-induced increase in permeability required 2 mM $[Ca^{2+}]_o$, indicating that activation of store-operated calcium entry was the stimulus for barrier disruption (Fig. 7, *C* and *D*).

Because ML-9 inhibited calcium entry, we tested its effect on thapsigargin-induced barrier disruption. In the presence of 2 mM $[Ca^{2+}]_o$, ML-9 produced a 20% increase in endothelial cell permeability and did not prevent thapsigargin from further increasing diffusive capacity by 45% (Fig. 8*A*). Both ML-9 and thapsigargin plus ML-9 treatments increased permeability to the greatest extent at 30 min (Fig. 8*B*). Permeability induced by ML-9 recovered by 60 min. However, permeability induced by treatment of thapsigargin plus ML-9 did not fully recover, suggesting that MLCK activity is required for restoration of barrier function. Sensitivity to ML-9 was increased when studies were conducted with low $[Ca^{2+}]_o$. In the presence of 100 nM $[Ca^{2+}]_o$, ML-9 increased RPAEC permeability by 108% (Fig. 8*C*); this increase in permeability did not reverse (Fig. 8*D*). Similar to our prior studies (4, 13, 18, 29), however, a further thapsigargin-induced increase in permeability was prevented by reducing the electrochemical membrane calcium gradient (Fig. 8, *C* and *D*). Thus the data support the idea that in low $[Ca^{2+}]_o$ MLCK is barrier protective.

DISCUSSION

Although activation of MLCK promotes endothelial cell permeability, the link between enzyme activation

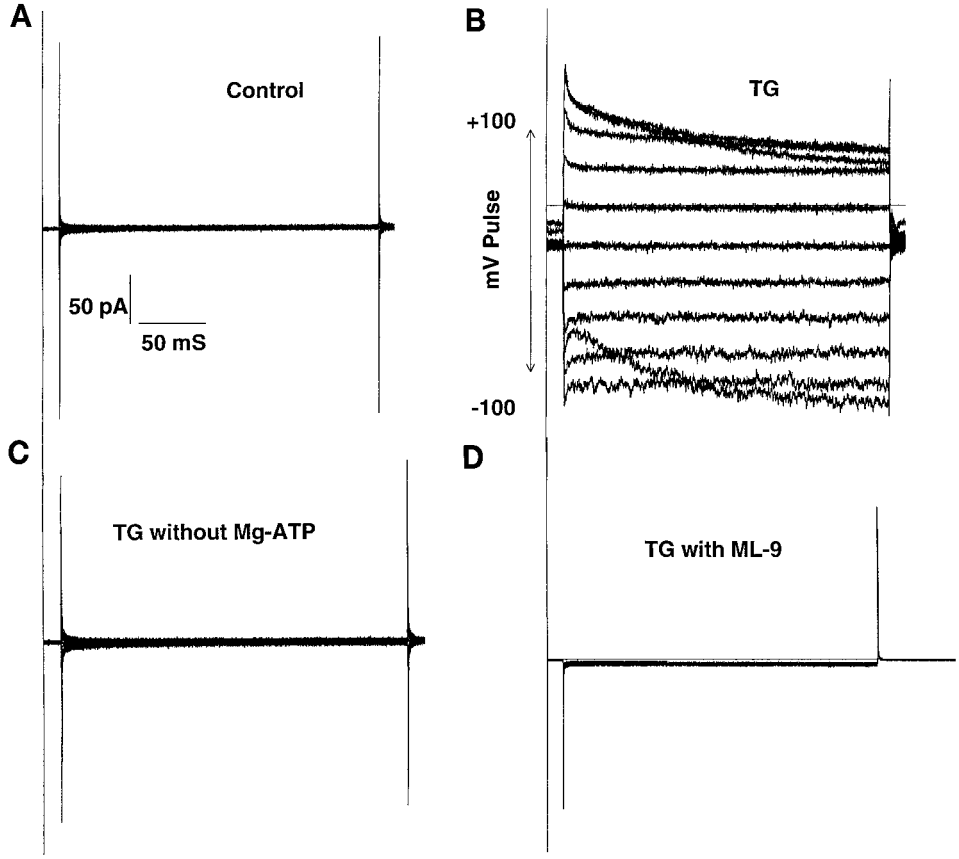

Fig. 4. Thapsigargin activation of a store-operated calcium entry current is dependent on MLCK. Shown are representative traces of current voltage (*I-V*) plots at a voltage range of −100 to +100 mV. *A*: voltage-clamped cells exhibited only a slight leak current under unstimulated conditions. *B*: inclusion of 1 μM thapsigargin in the patch pipette induced an inward calcium current that at positive voltages was inwardly rectifying. *C*: excluding Mg-ATP from the internal solution prevented thapsigargin (1 μM) from activating the store-operated calcium entry current. *D*: pretreating RPAECs with 15 μM ML-9 for 10 min similarly prevented thapsigargin (1 μM) from activating an inward calcium current.

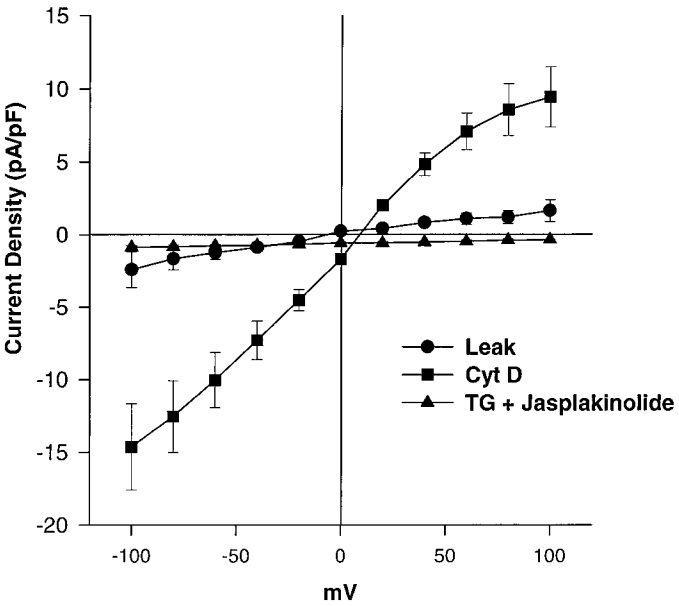

Fig. 5. F-actin regulation of store-operated calcium entry. Shown are representative traces from *I-V* plots at a voltage range of −100 to +100 mV. Inclusion of cytochalasin D (Cyt D; 10 μM) in the patch pipette activated an inward calcium current that was inwardly rectifying at positive voltages. Stabilization of F-actin with jasplakinolide (1 μM) prevented thapsigargin (1 μM) from activating a store-operated calcium entry current.

and generation of intercellular gaps is incompletely understood. Similarly, activation of store-operated calcium entry is sufficient to increase endothelial cell permeability, but the mechanism(s) responsible for activation of the membrane channel is unknown. Our present studies tested the hypothesis that MLCK activation by inflammatory calcium agonists regulates calcium entry important for control of endothelial cell barrier function.

The MLCK inhibitor ML-9 was utilized to assess kinase regulation of endothelial cell store-operated calcium entry and barrier function. ML-9 inhibits ATP binding to MLCK, with an IC_{50} of 3.8 μM, similar to its IC_{50} for regulation of store-operated calcium entry. Moreover, neither PKA nor PKC inhibitors influenced

Table 2. *Thapsigargin- and cytochalasin D-induced whole cell current profiles*

	Thapsigargin	Cytochalasin D
E_{rev}, mV	+12	+10
Current density, pA/pF	7.63 ± 1.00	10.0 ± 1.90
Slope conductance, nS	1.64	1.14

Values are averages for reversal potential (E_{rev}) and slope conductance and means ± SE for current density at −60 mV; n = 8 experiments for thapsigargin (1 μM)-induced and 5 experiments for cytochalasin D (10 μM)-induced whole cell currents generated in rat pulmonary artery endothelial cells.

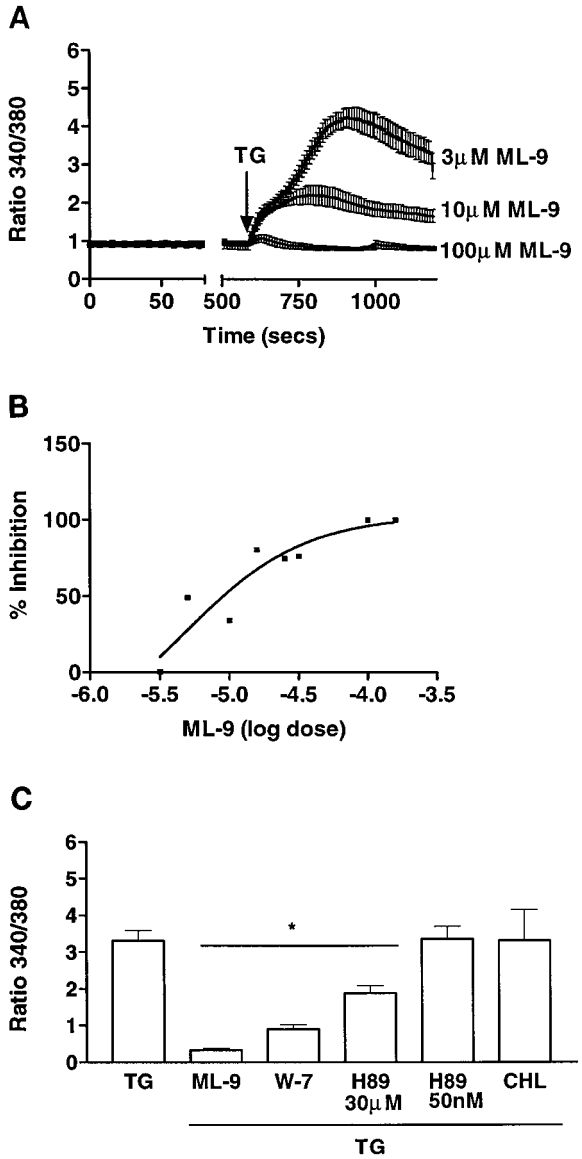

Fig. 6. Store-operated calcium entry is regulated by MLCK. *A*: pretreatment with ML-9 produced a dose-dependent inhibition of the calcium response to thapsigargin. *B*: utilizing the $[Ca^{2+}]_i$ response to thapsigargin as in *A*, the calculated IC_{50} of ML-9 is 4.6 μM, similar to its direct IC_{50} for MLCK (3.8 μM). *C*: peak response to thapsigargin was attenuated with inhibitors of MLCK, including ML-9 (100 μM), W-7 (calcium/calmodulin antagonist; 500 μM), and H-89 (30 μM; $n = 5$ experiments/group). $P < 0.05$ vs. thapsigargin. However, inhibition of neither protein kinase A with H-89 (50 nM) nor protein kinase C with chelerythrine (CHL; 0.66 μM) prevented thapsigargin from increasing $[Ca^{2+}]_i$ ($n = 4$ experiments/group). P = not significant (NS) compared with thapsigargin. *Significantly different from control, $P < 0.05$.

store-operated calcium entry, suggesting that ML-9 did not alter $[Ca^{2+}]_i$ responses through either of these kinases. Our findings, however, cannot rule out the possibility that ML-9 inhibits other, currently unidentified kinases in addition to MLCK.

Calcium signaling. Initial studies utilizing ML-9 indicated that it induces a transient rise in $[Ca^{2+}]_i$ due to calcium release from a thapsigargin- and heparin-sensitive intracellular store. Two known intracellular actions of heparin could account for these observations (30). The most likely effect and most widely accepted action of heparin are via its direct binding to the Ins(1,4,5)P_3 receptor at the Ins(1,4,5)P_3 binding site. In this context, our data suggest that ML-9 alters gating characteristics of the Ins(1,4,5)P_3 receptor in its constitutive environment, under basal levels of $[Ca^{2+}]_i$, Ins(1,4,5)P_3, and calmodulin (2, 21), to transiently increase calcium permeability. However, heparin may also uncouple receptor activation of G_q proteins and thus interrupt Ins(1,4,5)P_3 production. In this context, our data suggest that ML-9 could stimulate Ins(1,4,5)P_3 production. We do not currently know whether ML-9 alters inositol polyphosphate metabolism. Future studies will be required to more completely address how heparin specifically inhibits calcium release from the Ins(1,4,5)P_3 receptor.

Recent studies (11, 36, 37) have indicated that ML-9, likely by inhibiting MLCK activity, prevents activation of store-operated calcium entry. Our data support these previous findings, although we observed that ML-9 reduced both thapsigargin-stimulated calcium release and calcium entry. This finding suggested that ML-9 may prevent activation of store-operated calcium entry by interfering with the ability of thapsigargin to deplete the calcium store. We therefore conducted studies in which thapsigargin was utilized to first activate store-operated calcium entry before ML-9 was applied. Under these conditions, ML-9 immediately reduced $[Ca^{2+}]_i$, consistent with the idea that MLCK influences the activation state of a membrane calcium channel, although stimulation of calcium extrusion through the plasmalemmal Ca^{2+}-ATPase or Na^+/Ca^{2+} exchanger could not be eliminated.

To further address whether MLCK activity regulates a membrane calcium channel directly, patch-clamp studies were undertaken in which stimulation of calcium entry currents could be specifically studied without the confounding influence of calcium extrusion mechanisms. Although thapsigargin stimulated a calcium entry current similar to that in these and other cells as described in a previous report (18), reducing Mg-ATP in the internal solution eliminated activation of a store-operated calcium entry current. Because channel rundown is not normally observed over the time course of our experiments (18), these data indicate that a phosphorylation event is required for channel activation. This phosphorylation event is ML-9 sensitive, implicating MLCK in control of store-operated calcium entry.

The mechanism linking calcium store depletion to activation of a membrane calcium current remains elusive. Inhibition of store-operated calcium entry by ML-9 implicates involvement of the actin- and myosin-based contractile apparatus in this mechanism. Prior studies (12, 23, 25) have implicated F-actin in control of calcium signaling and, in particular, activation of a store-operated calcium entry current. Indeed, one important role of F-actin may be to maintain a close physical association between the endoplasmic reticulum and the cell membrane (23). Cytochalasin D disrupts F-actin and immedi-

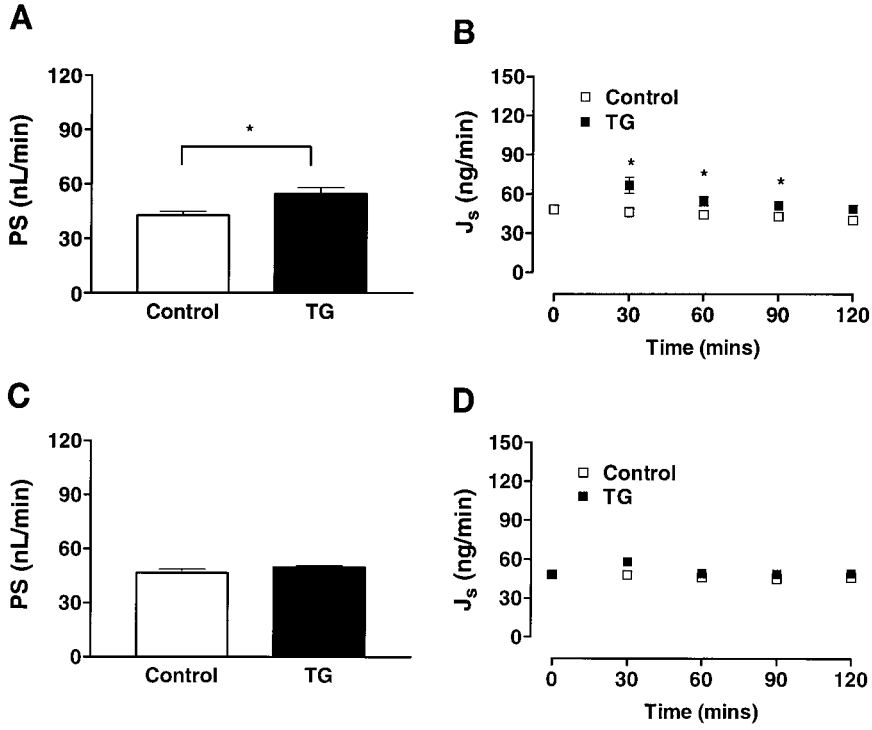

Fig. 7. Calcium entry is required for thapsigargin to increase RPAEC permeability. *A*: confluent RPAEC monolayers exhibited constitutive flux to a FITC-dextran (mol wt 10,000) tracer that was increased with thapsigargin (1 μM; n = 24 experiments). Measurements were made at 1 h. PS, diffusive capacity. $P < 0.05$. *B*: when evaluated over a 2-h time course, control monolayers exhibited similar, stable rates of macromolecular flux (J_s). The thapsigargin-induced increase in permeability was greatest at the 30-min time point and recovered toward control value by 2 h (n = 24 experiments), indicating a reduction in barrier diffusive capacity. P = NS vs. control. *C*: in 100 nM $[Ca^{2+}]_o$, thapsigargin (1 μM) did not increase permeability (n = 4 experiments). Measurements were made at 1 h. P = NS vs. control. *D*: in 100 nM $[Ca^{2+}]_o$, both control and thapsigargin-treated cells exhibited similar diffusive capacities over a 2-h time course (n = 4 experiments). P = NS. *Significantly different from control, $P < 0.05$.

ately eliminates endothelial cell tension, increasing the distance between the endoplasmic reticulum and the plasmalemma. In voltage-clamped RPAECs, inclusion of cytochalasin D in the patch pipette activated a calcium current with biophysical properties resembling the calcium current activated by thapsigargin. Stabilizing F-actin with jasplakinolide eliminated the thapsigargin-induced calcium entry current. Thus our data support the ideas that *1*) F-actin conformation is an important determinant of the store-operated calcium entry current and *2*) MLCK may control activation of store-operated calcium entry through stimulation of actomyosin-based tension. However, our findings are not consistent with recent observations (6, 22, 25, 40) in other cell types that F-actin

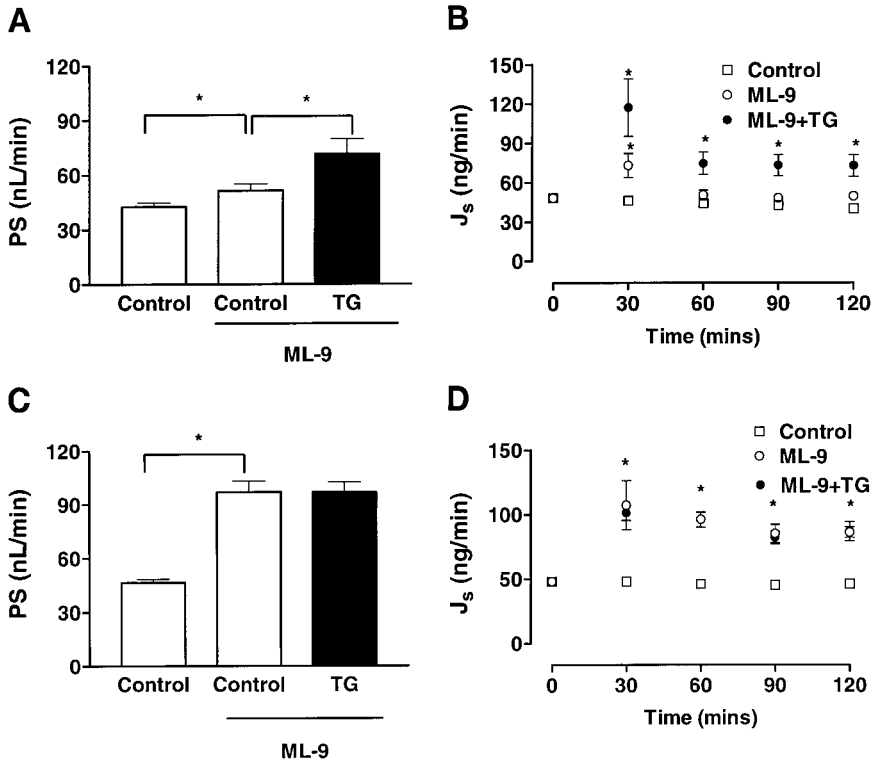

Fig. 8. Inhibition of MLCK increases permeability when $[Ca^{2+}]_o$ is reduced to 100 nM. *A*: in 2 mM $[Ca^{2+}]_o$, ML-9 (100 μM) slightly increased RPAEC diffusive capacity to the FITC-dextran (mol wt 10,000) tracer (n = 15 experiments). $P < 0.05$. However, ML-9 did not prevent thapsigargin (1 μM) from further increasing permeability (n = 10 experiments). P = NS. Measurements were made at 1 h. *B*: both ML-9 (100 μM) and the combined treatment of ML-9 (100 μM) with thapsigargin (1 μM) produced their greatest increase in permeability at 30 min (n = 15–24 experiments/group). $P < 0.05$. The increase in permeability was reduced by 2 h, indicating a reduction in diffusive capacity. ML-9-induced permeability increased returned to control values by 90 min. P = NS vs. control. *C*: in 100 nM $[Ca^{2+}]_o$, ML-9 (100 μM) produced a large increase in permeability. Thapsigargin did not further increase permeability (n = 4–8 experiments/group). Measurements were made at 1 h. P = NS vs. ML-9. *D*: evaluation of permeability over a 2-h time course revealed minimal restoration of diffusive capacity, suggesting that ML-9-induced permeability did not reverse (n = 8 experiments/group). $P < 0.05$. *Significantly different from control, $P < 0.05$.

disruption does not influence activation of store-operated calcium entry. The reasons for these disparate findings are unclear, although two clear differences between the studies are apparent. In prior reports, cytochalasin D had not been included in the patch pipette but, rather, was pretreated. Thus the acute response to cytochalasin D may differ substantially from its long-term application. Additionally, prior studies have not utilized endothelial cells. Although speculative, mechanically sensitive cells like endothelial cells may possess a greater reliance on cytoskeletal control of store-operated calcium entry than do other less mechanically sensitive cell types.

Endothelial cell permeability. Although an important role for MLCK in control of endothelial cell barrier function is well established, its mechanism of action is still incompletely understood (7, 9, 10, 14, 27, 38, 39). Specifically, it is unclear whether a MLCK-dependent increase in centripetally directed tension is sufficient to generate intercellular gaps. Our current data indicate that in addition to stimulating an inward centripetal tension that pulls cells apart, MLCK could control calcium entry at the membrane and thus influence signal amplification through calcium-sensitive targets involved in endothelial cell barrier function. We therefore performed studies to address the link between MLCK, calcium entry, and regulation of RPAEC permeability.

Consistent with previous reports (4, 13), activation of store-operated calcium entry was sufficient to increase endothelial cell permeability. The magnitude of this effect was greatest at 30 min and decreased in severity over a 2-h time course, indicating that endothelial cell barrier function improved to near control values. Prior studies did not assess whether thapsigargin-induced barrier disruption was reversible; in fact, these data were surprising considering that prolonged exposure to thapsigargin induces cell apoptosis (32). Mechanisms of intercellular gap repair are poorly understood. Thus it is not presently clear whether repair of the monolayer in our experiments occurred due to resolution of gap-promoting stimuli, activation of repair mechanisms, or both. A prior study (26) indicated that $[Ca^{2+}]_i$ stimulation of MLC_{20} phosphorylation is transient, peaking within 1 min and returning to baseline levels by 15–30 min. Similarly, calcium stimulation of phosphatase (PP2b) activity has been implicated in decreasing MLC_{20} phosphorylation over prolonged time periods (33). These prior studies suggest that resolution of gap-promoting stimuli may contribute to resealing barrier function. However, our studies demonstrated that ML-9 prevented barrier restoration in the presence of thapsigargin at time points when MLC_{20} phosphorylation had returned to baseline levels. Thus although our data suggest that the resealing of intercellular gaps proceeds via an ML-9-sensitive mechanism, the link between MLCK, MLC_{20} phosphorylation, and actomyosin interaction in mediating this process is currently unclear.

Reducing $[Ca^{2+}]_o$ to 100 nM eliminated thapsigargin-induced increases in permeability, confirming that barrier disruption required calcium entry across the cell membrane. These data are consistent with previous reports linking calcium entry to barrier disruption (4, 13, 29). Reducing $[Ca^{2+}]_o$ is sufficient to reorganize centrally localized F-actin while maintaining its peripheral rim (18). Moreover, in low $[Ca^{2+}]_o$, thapsigargin neither induces stress fibers nor increases MLC_{20} phosphorylation like it does when stimulation of calcium entry is permitted (18). We have interpreted these data to suggest that calcium entry is a critical amplification signal regulating endothelial barrier function.

In our current studies, ML-9 prevented thapsigargin from stimulating calcium entry but did not prevent thapsigargin from increasing permeability; rather, inhibition of MLCK activity promoted the thapsigargin-dependent increase in macromolecular flux. MLCK inhibition has previously been shown (27) to either eliminate or partially attenuate permeability induced by G_q agonists that activate store-operated calcium entry. MLCK inhibition did not have similar protective effects against the direct $[Ca^{2+}]_i$-elevating agent ionomycin (8). In this case, ionomycin disrupted the endothelial cell barrier by decreasing cAMP content, stimulating tyrosine kinase activity, and reducing phosphotyrosine incorporation of p125 focal adhesion kinase. Thapsigargin has previously been shown (29) to substantially decrease cAMP content, although its effect on tyrosine kinase activity and phosphotyrosinated substrates has not been evaluated in this context.

Considering that ML-9 abolished thapsigargin-induced calcium entry, our data unmask a previously undetermined mechanism of permeability. This mechanism of barrier regulation was further supported in studies with low $[Ca^{2+}]_o$ where ML-9 alone was sufficient to induce a large increase in permeability. Thus the data confirm two distinct mechanisms of barrier disruption: a first mechanism that is dependent on activation of store-operated calcium entry and a second mechanism that is exacerbated by low $[Ca^{2+}]_o$ and occurs after ML-9 treatment.

In conclusion, our present studies were predicated around the idea that endothelial cell tension, established by the function of MLCK, importantly dictates calcium signaling and endothelial cell barrier function. Our findings in RPAECs indicate that ML-9 promotes calcium release from an Ins(1,4,5)P_3 receptor and inhibits activation of store-operated calcium entry channels. Even though activation of store-operated calcium entry is sufficient to increase endothelial cell permeability and inhibition of MLCK prevents calcium entry, the inhibition of MLCK activity does not prevent thapsigargin from increasing permeability. Indeed, inhibition of MLCK disrupts endothelial barrier function, unmasking a novel mechanism regulating the endothelial cell barrier. Future studies will be required to assess how the distribution of forces within endothelial cells is altered after MLCK inhibition, particularly in low $[Ca^{2+}]_o$, to further address this mechanism of barrier control.

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