Receptor-dependent activation of store-operated calcium entry increases endothelial cell permeability

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Moore, Timothy M., Natalie R. Norwood, Judy R. Creighton, Pavál Babal, George H. Brough, D. Michael Shasby, and Troy Stevens. Receptor-dependent activation of store-operated calcium entry increases endothelial cell permeability. Am J Physiol Lung Cell Mol Physiol 279: L691–L698, 2000.—The present study evaluated the necessity of store-operated Ca2+ entry in mediating thrombin-induced 20-kDa myosin light chain (MLC20) phosphorylation and increased permeability in bovine pulmonary arterial endothelial cells (BPAECs). Thrombin (7 U/ml) and thapsigargin (1 μM) activated Ca2+ entry through a common pathway in confluent BPAECs. Similar increases in MLC20 phosphorylation were observed 5 min after thrombin and thapsigargin challenge, although thrombin produced a sustained increase in MLC20 phosphorylation that was not observed in response to thapsigargin. Neither agonist increased MLC20 phosphorylation when Ca2+ influx was inhibited. Thrombin and thapsigargin induced inter-endothelial cell gap formation and increased FITC-dextran (molecular radii 23 Å) transfer across confluent BPAEC monolayers. Activation of store-operated Ca2+ entry was required for thapsigargin and thrombin receptor-activating peptide to increase permeability, demonstrating that activation of store-operated Ca2+ entry is coupled with MLC20 phosphorylation and is associated with intercellular gap formation and increased barrier transport of macromolecules. Unlike thrombin receptor-activating peptide, thrombin increased permeability without activation of store-operated Ca2+ entry, suggesting that it partly disrupts the endothelial barrier through a proteolytic mechanism independent of Ca2+ signaling.

thapsigargin; thrombin; myosin light chain kinase; receptor-operated calcium channels; lung

ELEVATED CYTOSOLIC Ca2+ concentration ([Ca2+]i) promotes inter-endothelial cell gap formation and increased vascular permeability (7, 9, 11, 12, 15–17, 19, 21–23, 25, 26, 35, 38, 41). Both Ca2+ release from intracellular stores and Ca2+ influx across the cell membrane contribute to an increase in [Ca2+]i, (1, 2, 25, 30–32, 34, 42). Ca2+ release is accomplished by generation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P3], which stimulates Ins(1,4,5)P3 receptors located predominantly in the smooth endoplasmic reticulum membrane (1, 2, 32). Ca2+ influx may be accomplished by a number of different mechanisms (1, 14, 15, 17, 24, 26, 28, 30) including 1) membrane potential-regulated Ca2+ leak, 2) Na+/Ca2+ exchange, 3) activation of mechanosensitive cation channels, 4) ligand stimulation of receptor-coupled cation channels (ROCs), and 5) activation of capacitative or store-operated Ca2+ entry channels (SOCs). In regard to endothelial barrier properties, recent data indicate that Ca2+ influx in response to agents activating SOCs promotes intercellular gap formation and increases transport of macromolecules across pulmonary arterial endothelial monolayers (4, 5, 19, 24, 25, 41). The specific molecular target affected by Ca2+ entry via SOCs to regulate permeability is unclear.

Endothelial myosin light chain (MLC) kinase (MLK) can be activated by Ca2+/calmodulin to initiate phosphorylation of 20-kDa MLC (MLC20), leading to actomyosin interaction and endothelial tension development (9, 10, 13, 20, 27, 36, 37, 44, 45). It is unknown whether Ca2+-stimulated MLK activation and MLC20 phosphorylation are regulated in a preferential manner by Ins(1,4,5)P3-mediated Ca2+ release from intracellular stores or Ca2+ influx across the plasmalemma. However, a recent report (43) indicated that Ca2+ entry associated with SOC activation may be linked to MLC20 phosphorylation (43). This observation coupled with the known role of MLK in permeability regulation suggests that a complex relationship exists among MLC20 phosphorylation status, Ca2+ influx through SOCs, and endothelial barrier function.

Thrombin is a receptor-coupled inflammatory agonist that elicits Ins(1,4,5)P3-dependent Ca2+ release coincident with Ca2+ influx in endothelial cells (14, 28). In addition, thrombin promotes endothelial cell shape change and in vitro barrier disruption in part by influencing MLK activity and MLC20 phosphorylation (21, 22, 27, 33, 36). Our present studies tested whether thrombin-induced MLC20 phosphorylation and endothelial barrier disruption are regulated by Ca2+ influx through SOCs. Our data indicate that Ca2+ influx is...
coupled to increased MLC_{20} phosphorylation in response to thrombin, although the influx pathway activated by thrombin is receptor gated as well as store operated. Although direct activation of the thrombin receptor increased permeability dependent on store-operated Ca^{2+} entry, thrombin also increased permeability through a proteolytic mechanism.

**METHODS**

**Isolation and culture of bovine pulmonary artery endothelial cells.** Bovine pulmonary artery endothelial cells (BPAECs) were isolated and cultured according to methods previously described (39). In addition, cells were purchased from Clonetics (San Diego, CA). All cells were routinely identified as endothelial by morphological assessment (“cobblestone” appearance at confluence), factor VIII antigen staining, and uptake of acetylated 1,1’-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate-labeled low-density lipoprotein. Cells were studied between passages 6 and 10.

**[Ca^{2+}]_{i} estimation with fura 2 epifluorescence.** BPAECs were seeded onto chambered glass coverslips (Nalge Nunc, Naperville, IL) and grown to confluence. [Ca^{2+}]_{i} was estimated with the Ca^{2+}_{i}-sensitive fluorescent fura 2-AM (Molecular Probes, Eugene, OR) according to methods previously described (19, 25, 40, 41). Briefly, BPAECs were washed with 2 ml of a HEPES (Fisher Scientific, Atlanta, GA)-buffered physiological salt solution (PSS) containing (in g/l) 6.9 NaCl, 0.35 KCl, 0.16 KH_{2}PO_{4}, 0.141 MgSO_{4}, and 2.0 d-glucose and 25 mM HEPES. The loading solution (2 ml) consisted of PSS plus 3 μM fura 2-AM, 0.003% pluronic acid, and 2 mM or 100 mM CaCl_{2}. BPAECs were loaded for 20 min in a CO_{2} incubator at 37°C. The cells were washed again with PSS (2 ml) and treated with deesterification medium (PSS plus 2 mM or 100 mM CaCl_{2}) for an additional 20 min. After deesterification, [Ca^{2+}]_{i} was estimated with an Olympus IX70 inverted microscope at ×400 and a xenon arc lamp photomultiplier system (Photon Technologies, Monmouth Junction, NJ). Data were acquired with PTI Felix software. Epifluorescence (signal averaged) was measured from three to four endothelial cells in a confluent monolayer, and the changes in [Ca^{2+}]_{i} are reported as the fluorescence ratio of the Ca^{2+}_{i}-bound (340-nm) to Ca^{2+}_{i}-unbound (380-nm) excitation wavelengths emitted at 510 nm.

**MLC_{20} phosphorylation assay.** BPAECs were seeded in T-75 flasks and grown to confluence. MLC_{20} phosphorylation was assessed as previously described (8, 36) with minor modifications. Briefly, confluent BPAECs were transferred to micropore filters and labeled with 1.5 ml of 35S)methionine (555 μCi/ml) in D/Val MEM with 20% calf bovine serum for 48 h at 37°C and 5% CO_{2}. After a 5- or 15-min incubation with vehicle control, thapsigargin (1 μM), or thrombin (7 U/ml) in Ca^{2+}_{i}-containing (2 mM) or -depleted (100 nM) PSS, the reactions were stopped by snap-freezing in a dry ice-methanol bath, and the cells were lysed. The lysate was sedimented at 1,000 g for 5 min at 4°C, and the supernatant was incubated with 20 μl of rabbit anti-human platelet myosin antibody (2 mg/ml) at 4°C for 1 h. The mixture was centrifuged at 100,000 g for 1 min, and the pellet was collected, washed with lysing buffer, and recentrifuged. The pellet was then washed with 0.5 ml of a 50:50 mixture of lysing buffer and PBS. The pellet was resuspended in 35 μl of urea lysing buffer for two-dimensional gel electrophoresis to isolate unphosphorylated and phosphorylated MLC_{20} isoforms. Isoelectric focusing and second dimension SDS-PAGE were performed as described (36). The resulting bands were quantitated by densitometry. MLC_{20} exhibited un-, mono-, and diphosphorylated states. Stoichiometry of moles phosphate per mole of MLC_{20} was calculated as described (36).

**RESULTS**

The Ca^{2+} influx pathway activated by thrombin is under dual regulation by receptor coupling and store depletion. Changes in [Ca^{2+}]_{i}, elicited by thrombin compared with those elicited by thapsigargin, an activator of SOCs, were assessed in confluent fura 2-loaded BPAECs (Fig. 1). Figure 1A shows that thrombin produced a change in [Ca^{2+}]_{i} characterized by an abrupt and transient “spike” in the fluorescence ratio, representing a rapid Ca^{2+} release from intracellular stores. After the spike, a sustained elevation in [Ca^{2+}]_{i} above baseline was observed. In addition, the average sustained [Ca^{2+}]_{i} level demonstrated some variability over time as indicated by the wide SE range, which was attributed to oscillatory changes in [Ca^{2+}]_{i}, at different rates of cycling for individual cells in the measurement field (40). Thapsigargin elicited a more gradual increase
in \([\text{Ca}^{2+}]_{\text{i}}\) that likewise remained elevated above basal levels on activation of SOCs. To assess the \(\text{Ca}^{2+}\) influx component of the thrombin response, experiments were repeated with fura 2-loaded BPAECs incubated in a low extracellular \(\text{Ca}^{2+}\) concentration \((\text{[Ca}^{2+}]_{\text{o}}\); Fig. 1B). Reducing \([\text{Ca}^{2+}]_{\text{o}}\) to 100 nM slightly attenuated the thapsigargin-induced \(\text{Ca}^{2+}\) release and did not affect the peak \([\text{Ca}^{2+}]_{\text{i}}\), response to thrombin. However, the sustained elevation in \([\text{Ca}^{2+}]_{\text{i}}\), seen previously for both agonists was absent, thereby indicating that \(\text{Ca}^{2+}\) influx was a vital component to the total change in \([\text{Ca}^{2+}]_{\text{i}}\), generated in response to thapsigargin and thrombin.

Figure 1, C and D, shows that the thrombin-induced \(\text{Ca}^{2+}\) influx occurs through a pathway under dual regulation by store depletion and receptor gating. With a heparin microinjection, the Ins(1,4,5)P_3-dependent \(\text{Ca}^{2+}\)-release response to thrombin was abrogated, although microinjected cells still displayed an increased \([\text{Ca}^{2+}]_{\text{i}}\), that was sustained. This finding suggested that thrombin opened a membrane-\(\text{Ca}^{2+}\) influx pathway independent of an Ins(1,4,5)P_3-mediated \(\text{Ca}^{2+}\) store depletion event (Fig. 1C). However, when BPAEC SOCs were activated with thapsigargin and then challenged with thrombin, no additional change in \([\text{Ca}^{2+}]_{\text{i}}\) could be observed (Fig. 1, D and E). Thus these data suggest that redundant mechanisms related to receptor coupling as well as to store-operated events regulate thrombin-induced \(\text{Ca}^{2+}\) influx in BPAECs.

\(\text{Ca}^{2+}\) influx is coupled to MLC_20 phosphorylation. We observed two major isoforms of MLC in BPAECs; the smaller isoform was 16 kDa, and the larger more basic isoform was \(~20\) kDa. Similar to human umbilical vein endothelial cells and platelets, the 16-kDa isoform existed at two distinct isoelectric points (data not shown).

The 20-kDa isoform exhibited three distinct isoelectric points (data not shown). The 16-kDa isoform was \(\sim16\) kDa, and the larger more basic isoform was \(\sim20\) kDa. Similar to human umbilical vein endothelial cells and platelets, the 16-kDa isoform existed at two distinct isoelectric points (data not shown). The 20-kDa isoform exhibited three distinct isoelectric points corresponding to un-, mono-, and diphosphorylated states. Figure 2 illustrates the effects of thrombin and thapsigargin on MLC_20 phosphorylation state with respect to time and \([\text{Ca}^{2+}]_{\text{o}}\). Basal stoichiometry of 0.39 ± 0.06 mol phosphate/mol MLC_20 increased 135 and 120% 5 min after application of thrombin and thapsigargin, respectively. Whereas the response to SOC activation with thapsigargin was transient, the response to thrombin persisted at 15 min. When studies were repeated in 100 nM extracellular \(\text{Ca}^{2+}\), the basal stoichiometry decreased 15% to 0.33 ± 0.05 mol phosphate/mol MLC_20, suggesting that basal leak of \(\text{Ca}^{2+}\) across the endothelial plasmalemma contributed to MLC_20 phosphorylation status. Prevention of \(\text{Ca}^{2+}\) entry effectively eliminated the thrombin- and thapsigargin-induced increase in MLC_20 phosphorylation, thereby clamping endothelial MLC_20 phosphorylation levels below values normally existing in the presence of physiological \([\text{Ca}^{2+}]_{\text{o}}\). These data indicate that, at least over the time period studied, \(\text{Ca}^{2+}\) influx was tightly coupled to the level of MLC_20 phosphorylation induced by SOC activation alone (e.g., thapsigargin) and by thrombin-stimulated \(\text{Ca}^{2+}\) entry.

Activation of store-operated \(\text{Ca}^{2+}\) entry increased endothelial cell permeability. Figure 3 shows phase-contrast micrographs for BPAEC monolayers challenged with thrombin or thapsigargin. Although untreated
(control) cells, in either the presence or absence of extracellular Ca\(^{2+}\), displayed close cell-cell apposition without apparent intercellular gaps (Fig. 3, A and B). Thrombin challenge of BPAEC monolayers for 5 min resulted in obvious gap formation regardless of \([\text{Ca}^{2+}]_o\) studied (Fig. 3, C and D). Thapsigargin likewise elicited gap formation (Fig. 3E) but only when \([\text{Ca}^{2+}]_o\) was conducive to \(\text{Ca}^{2+}\) influx in response to the opening of SOCs (Fig. 3F). Figure 4 illustrates that the gap formation produced by thrombin and store-operated \(\text{Ca}^{2+}\) entry was sufficient to allow for an increase in diffusive capacity to macromolecular FITC-dextran. In the presence of 2 mM extracellular \(\text{Ca}^{2+}\), permeability was increased 60% with thrombin and 35% with thapsigargin. Although the increase in permeability to thapsigargin was eliminated in 100 nM \(-\text{Calcium}\), suggesting that activation of store-operated \(\text{Ca}^{2+}\) entry is required for the agonists to induce phosphorylation over the time course studied \((n = 5 \text{ monolayers/group})\). \(\text{MLC}_{20}\) phosphorylation is expressed as mol phosphate/mol \(\text{MLC}_{20}\). *Significantly different from respective control group, \(P < 0.05\).

**DISCUSSION**

Store-operated \(\text{Ca}^{2+}\) influx in pulmonary artery endothelium is alone sufficient to stimulate endothelial cell shape change and increase macromolecular permeability (4, 5, 19, 24, 25, 41). However, humoral inflammatory agonists do not selectively activate store-operated \(\text{Ca}^{2+}\) entry without first binding receptors coupled to \(\text{G}\) proteins, thereby activating multiple intracellular signaling pathways. Thus the role of store-operated \(\text{Ca}^{2+}\) influx in permeability regulation should be investigated further in conjunction with receptor-coupled inflammatory mediators. Thrombin is one such mediator coupled to \(\text{G}_q\) proteins that stimulates cultured pulmonary artery endothelial cell shape change to increase macromolecular permeability by activating multiple, parallel intracellular signaling pathways concurrent with increased \([\text{Ca}^{2+}]_i\) (21, 22, 27, 33, 36). As described here and consistent with previous observations (1, 14, 21, 22), the thrombin-induced increase in

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**Fig. 2.** Activation of store-operated \(\text{Ca}^{2+}\) entry promoted but did not sustain 20-kDa myosin light chain (\(\text{MLC}_{20}\)) phosphorylation. A, control, thapsigargin (1 \(\mu\)M), and thrombin (7 U/ml) treatments for 5 min in 2 mM extracellular \(\text{Ca}^{2+}\). Top: myosin bands obtained from 2-dimensional gel electrophoresis for the un- (A), mono- (A'), and diphosphorylated (A") forms of \(\text{MLC}_{20}\). Bottom: area achieved by each product. There was an increase in mono- and diphosphorylated forms of \(\text{MLC}_{20}\) after activation of store-operated \(\text{Ca}^{2+}\) entry. B: thapsigargin (1 \(\mu\)M) increased \(\text{MLC}_{20}\) phosphorylation for 5 but not for 15 min, whereas thrombin (7 U/ml) promoted \(\text{MLC}_{20}\) phosphorylation for at least 15 min. Studies were carried out in the presence of 2 mM extracellular \(\text{Ca}^{2+}\) [positive (+) Control]. C: neither thapsigargin nor thrombin increased \(\text{MLC}_{20}\) phosphorylation in the relative absence of extracellular \(\text{Ca}^{2+}\) (100 nM; -Calcium), suggesting that activation of store-operated \(\text{Ca}^{2+}\) entry is required for the agonists to induce phosphorylation over the time course studied \((n = 5 \text{ monolayers/group})\). \(\text{MLC}_{20}\) phosphorylation is expressed as mol phosphate/mol \(\text{MLC}_{20}\). *Significantly different from respective control group, \(P < 0.05\).
[Ca\(^{2+}\)]_i occurs in response to Ins(1,4,5)P_3-mediated Ca\(^{2+}\) release and sustained Ca\(^{2+}\) influx, which are distinguished with the Ca\(^{2+}\) fluorophore fura 2 (14, 21, 22, 40). With respect to Ca\(^{2+}\) release, our data indicate that extracellular Ca\(^{2+}\) levels do not influence peak responses to thrombin. However, the sustained increase in [Ca\(^{2+}\)]_i elicited by thrombin is dependent on Ca\(^{2+}\) influx because a nominally Ca\(^{2+}\)-free extracellular environment prevents this increase from occurring.

Endothelial cells possess several discernible Ca\(^{2+}\) entry pathways (1, 2, 7, 26, 28), and electrophysiological characterization of Ca\(^{2+}\) and other cationic currents suggests that multiple Ca\(^{2+}\) entry channels may be activated by thrombin (28). Presently, the molecular identities of Ca\(^{2+}\) entry pathway(s) in endothelial cells are poorly understood. The transient receptor potential (trp) gene family is likely responsible for forming membrane Ca\(^{2+}\) channels (3, 24, 25, 28), although there are

Fig. 3. Activation of store-operated Ca\(^{2+}\) entry promoted inter-endothelial cell gap formation as shown in phase-contrast micrographs of BPAEC monolayers (original magnification, \(\times 100\)). A: [Ca\(^{2+}\)]_o = 2 mM (control). B: [Ca\(^{2+}\)]_o = 100 nM. C: 5-min postthrombin treatment. [Ca\(^{2+}\)]_o = 2 mM. D: 5-min postthrombin treatment. [Ca\(^{2+}\)]_o = 100 nM. E: 5-min postthapsigargin treatment. [Ca\(^{2+}\)]_o = 2 mM. F: 5-min postthapsigargin treatment. [Ca\(^{2+}\)]_o = 100 nM. Arrowheads, gap formation at cell-cell adhesion sites after addition of agonist. Results are representative of 3–5 experiments/treatment.
many uncertainties concerning Trp proteins. It is unclear whether Trp proteins are responsible for the Ca^{2+} release-activated current measured in nonexcitable cells (6, 18). Likewise, it is not known how Trp channels expressed in endothelial cells, principally Trp1 and Trp4, are specifically gated in terms of G protein coupling and Ca^{2+} store regulation (3, 6, 26, 28).

A recent work (3) indicated that some Trp monomeric isoforms exhibit preferential regulation by G_q protein activation, whereas others demonstrate regulation by store depletion. That report also suggested that Ca^{2+} influx pathways in nonexcitable cells are composed of Trp proteins forming multimeric channel complexes, consistent with the notion that Trp channel complexes composed of both G_q protein-regulated monomers and store-operated monomers could be dually regulated. Our present data support this concept as evidenced by the results from the heparin microinjection experiments and from the experiments where BPAECs were challenged with thrombin after thapsigargin pretreatment. When Ins(1,4,5)P_3-mediated Ca^{2+} store depletion was prevented by heparin blockade of the Ins(1,4,5)P_3 receptor and the store-operated Ca^{2+} influx pathway was inhibited, an increase in [Ca^{2+}]_i equivalent to that in the absence of heparin blockade was observed. Thus thrombin activated a receptor-coupled Ca^{2+} influx channel that mediated the sustained increase in [Ca^{2+}]_i in the absence of Ins(1,4,5)P_3-dependent store depletion.

This receptor-coupled pathway may not be distinct from the store-operated pathway; e.g., store depletion and G protein stimulation may activate the same pool of membrane Ca^{2+} influx channels. When the BPAEC store-operated pathway was stimulated with thapsigargin, the level of sustained [Ca^{2+}]_i generated was not altered by thrombin. Had a different and distinctly regulated population of membrane Ca^{2+} channels sensitive to thrombin been activated, the overall level of [Ca^{2+}]_i would have increased, especially in combination with thapsigargin-induced sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibition. One possible explanation for these data is that the receptor-coupled Ca^{2+} entry pathway [elucidated during heparin blockade of the Ins(1,4,5)P_3 receptor] is the same pathway stimulated by store depletion. Ca^{2+} entry in BPAECs, therefore, would occur via a channel complex under redundant regulation by membrane G proteins and internal store filling coupled to the Ins(1,4,5)P_3 signaling cascade. We do not rule out an alternative possibility that activation of a distinct population of membrane SOCs with thapsigargin causes simultaneous or time-dependent inhibition of receptor-coupled Ca^{2+} influx.
store-operated Ca\textsuperscript{2+} entry and intracellular events downstream from Ca\textsuperscript{2+} entry leading to shape change and permeability alterations. We hypothesized that store-operated Ca\textsuperscript{2+} entry-induced increases in endothelial permeability involved activation of the Ca\textsuperscript{2+}/calmodulin-regulated MLCK and subsequent MLC\textsubscript{20} phosphorylation, leading to increased actomyosin interactions and tension development (9, 13, 43, 44). Consistent with this hypothesis, we observed that activation of store-operated Ca\textsuperscript{2+} entry by thapsigargin was tightly coupled to a significant increase in MLC\textsubscript{20} phosphorylation, suggestive of an increase in MLCK activity; Ca\textsuperscript{2+} influx was associated with a similar degree of MLC\textsubscript{20} phosphorylation stimulated by thrombin.

The thrombin-induced increase in MLC\textsubscript{20} was sustained for at least 15 min. This particular finding differed from the thapsigargin-induced MLC\textsubscript{20} phosphorylation profile, showing a dramatic increase in phosphorylation that dissipated to near basal levels after 15 min. These data are consistent with the recent findings of Shasby et al. (36), who demonstrated that thrombin-mediated signaling inhibits protein phosphatase 1 activity, resulting in a prolonged increase in MLC\textsubscript{20} phosphorylation. This signaling response is unique to thrombin compared with other receptor-dependent (e.g., histamine) and -independent (e.g., ionomycin, thapsigargin, Staphylococcus aureus α-toxin clamped) Ca\textsuperscript{2+} agonists. Thus G\textsubscript{q} protein-coupled inflammatory agonists increase and maintain MLCK activity and MLC\textsubscript{20} phosphorylation via activation of several Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent processes, consistent with a previous work (10) indicating that endothelial MLCK activity is a function of multiple signal inputs.

Finally, the present studies indicated that activation of store-operated Ca\textsuperscript{2+} entry in response to thapsigargin and TRAP is sufficient to increase endothelial cell permeability. However, the role of MLC\textsubscript{20} in linking store-operated Ca\textsuperscript{2+} entry to barrier disruption remains unclear. Both thrombin and thapsigargin increased permeability over a 1-h time course, even though MLC\textsubscript{20} phosphorylation had returned to near baseline values in response to thapsigargin. Although these data would support a role for MLC\textsubscript{20} phosphorylation in the initial response to activation of store-operated Ca\textsuperscript{2+} entry, MLC\textsubscript{20} phosphorylation was not associated with the prolonged permeability response. Future studies will be required to comprehensively discriminate between the effect of MLC\textsubscript{20} phosphorylation with increased centripetally directed tension and the loss of cell-cell and cell-matrix adhesion in the regulation of the sustained permeability response to Ca\textsuperscript{2+} agonists (12, 27).

In summary, our present studies addressed key issues relating to Ca\textsuperscript{2+} regulation and control of endothelial cell shape and barrier function. Receptor-coupled, G protein-linked inflammatory mediators may activate Ca\textsuperscript{2+} influx pathways under dual regulation by plasmalemmal effectors and the filling state of internal Ca\textsuperscript{2+} pools. Our data also extend the previous observation that store-operated Ca\textsuperscript{2+} entry can initiate intercellular gap formation, in part, by an associated transient increase in MLC\textsubscript{20} phosphorylation. Furthermore, in addition to store-operated Ca\textsuperscript{2+} entry, thrombin activates proteolytic signaling pathways that can act independently to produce changes in pulmonary endothelial cell shape and increased permeability. Subsequent work is necessary to fully elucidate the exact nature of the relationship between endothelial store-operated Ca\textsuperscript{2+} entry, MLCK activity, and MLC\textsubscript{20} phosphorylation status as related to endothelial Ca\textsuperscript{2+} homeostasis and barrier function (29).

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