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, Paváł Babal, George H. Broughe, 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 artery 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-triphosphate [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 (MLCK) 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 MLCK 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 MLCK 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 MLCK 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

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coupled to increased MLC20 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’,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 an Olympus IX70 inverted microscope at \(\times400\) and a xenon arc lamp photomultiplier (Elmer luminescence spectrometer LS 50B) excitation at 480 nm and emission at 530 nm. Fluorescence values were then determined by 10.220.33.1 on April 6, 2017 http://ajplung.physiology.org/ Downloaded from

Assessment of endothelial cell shape change and intercellular gap formation. BPAECs were seeded onto 35-mm plastic culture dishes and grown to confluence. The cells were washed twice with PBS and once with PSS plus 2 mM or 100 nM CaCl\(_2\) and then equilibrated with 2 ml of PSS for 2 min before the experiment was started. At the end of each experiment, the monolayers were fixed in 3% glutaraldehyde for 10 min and prepared for microscopy as previously described (19, 25). Gap formation was assessed by a pathologist blinded to the experimental protocols, and micrographs were taken of representative areas in the monolayer.

**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 [Ca\textsuperscript{2+}]\textsubscript{i} that likewise remained elevated above basal levels on activation of SOCs. To assess the Ca\textsuperscript{2+} influx component of the thrombin response, experiments were repeated with fura 2-loaded BPAECs incubated in a low extracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}])\textsubscript{o}; Fig. 1B). Reducing [Ca\textsuperscript{2+}]\textsubscript{o} to 100 nM slightly attenuated the thapsigargin-induced Ca\textsuperscript{2+} release and did not affect the peak [Ca\textsuperscript{2+}]\textsubscript{i} response to thrombin. However, the sustained elevation in [Ca\textsuperscript{2+}]\textsubscript{i} seen previously for both agonists was absent, thereby indicating that Ca\textsuperscript{2+} influx was a vital component to the total change in [Ca\textsuperscript{2+}]\textsubscript{i} generated in response to thapsigargin and thrombin.

Figure 1, C and D, shows that the thrombin-induced Ca\textsuperscript{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\textsubscript{3} depletion event (Fig. 1C). However, when BPAEC SOCs were activated with thapsigargin and then challenged with thrombin, no additional change in [Ca\textsuperscript{2+}]\textsubscript{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 Ca\textsuperscript{2+} influx in BPAECs.

Ca\textsuperscript{2+} influx is coupled to MLC\textsubscript{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 corresponding to un-, mono-, and diphosphorylated states. Figure 2 illustrates the effects of thrombin and thapsigargin on MLC\textsubscript{20} phosphorylation state with respect to time and [Ca\textsuperscript{2+}]\textsubscript{o}. Basal stoichiometry of 0.39 ± 0.06 mol phosphate/mol MLC\textsubscript{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 Ca\textsuperscript{2+}, the basal stoichiometry decreased 15% to 0.33 ± 0.05 mol phosphate/mol MLC\textsubscript{20}, suggesting that basal leak of Ca\textsuperscript{2+} across the endothelial plasmalemma contributed to MLC\textsubscript{20} phosphorylation status. Prevention of Ca\textsuperscript{2+} entry effectively eliminated the thrombin- and thapsigargin-induced increase in MLC\textsubscript{20} phosphorylation, thereby clamping endothelial MLC\textsubscript{20} phosphorylation levels below values normally existing in the presence of physiological [Ca\textsuperscript{2+}]\textsubscript{o}. These data indicate that, at least over the time period studied, Ca\textsuperscript{2+} influx was tightly coupled to the level of MLC\textsubscript{20} phosphorylation induced by SOC activation alone (e.g., thapsigargin) and by thrombin-stimulated Ca\textsuperscript{2+} entry.

Activation of store-operated Ca\textsuperscript{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 [Ca\(^{2+}\)]\(_{o}\) studied (Fig. 3, C and D). Thapsigargin likewise elicited gap formation (Fig. 3E) but only when [Ca\(^{2+}\)]\(_{o}\) was conducive to 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 Ca\(^{2+}\) entry was sufficient to allow for an increase in diffusive capacity to macromolecular FITC-dextran. In the presence of 2 mM extracellular Ca\(^{2+}\), permeability was increased 60% with thrombin and 35% with thapsigargin. Although the increase in permeability to thapsigargin was eliminated in 100 nM thapsigargin, suggesting that activation of store-operated Ca\(^{2+}\) entry is required for the agonists to induce phosphorylation over the time course studied (n = 5 monolayers/group). MLC\(_{20}\) phosphorylation is expressed as mol phosphate/mol MLC\(_{20}\). *Significantly different from respective control group, P < 0.05.

**DISCUSSION**

Store-operated 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 Ca\(^{2+}\) entry without first binding receptors coupled to G proteins, thereby activating multiple intracellular signaling pathways. Thus the role of store-operated Ca\(^{2+}\) influx in permeability regulation should be investigated further in conjunction with receptor-coupled inflammatory mediators. Thrombin is one such mediator coupled to 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 [Ca\(^{2+}\)]\(_{i}\). (1, 14, 21, 22), the thrombin-induced increase in
$[\text{Ca}^{2+}]_i$ occurs in response to $\text{Ins}(1,4,5)P_3$-mediated $\text{Ca}^{2+}$ release and sustained $\text{Ca}^{2+}$ influx, which are distinguished with the $\text{Ca}^{2+}$ fluorophore fura 2 (14, 21, 22, 40). With respect to $\text{Ca}^{2+}$ release, our data indicate that extracellular $\text{Ca}^{2+}$ levels do not influence peak responses to thrombin. However, the sustained increase in $[\text{Ca}^{2+}]_i$ elicited by thrombin is dependent on $\text{Ca}^{2+}$ influx because a nominally $\text{Ca}^{2+}$-free extracellular environment prevents this increase from occurring.

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

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**Fig. 3. Activation of store-operated $\text{Ca}^{2+}$ entry promoted inter-endothelial cell gap formation as shown in phase-contrast micrographs of BPAEC monolayers (original magnification, ×100).**

A: $[\text{Ca}^{2+}]_o = 2$ mM (control). B: $[\text{Ca}^{2+}]_o = 100$ nM. C: 5-min postthrombin treatment. $[\text{Ca}^{2+}]_o = 2$ mM. D: 5-min postthrombin treatment. $[\text{Ca}^{2+}]_o = 100$ nM. E: 5-min postthapsigargin treatment. $[\text{Ca}^{2+}]_o = 2$ mM. F: 5-min postthapsigargin treatment. $[\text{Ca}^{2+}]_o = 100$ 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 dualy 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\(^{2+}\)]\(_i\) generation 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\(^{2+}\) in-

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Fig. 4. Thapsigargin increased endothelial cell permeability by activating store-operated Ca\(^{2+}\) entry, although thrombin increased permeability independent of its sustained [Ca\(^{2+}\)]\(_i\), response. Diffusive capacity (PS) values for 23-A dextran macromolecules across BPAEC monolayers under control conditions and after thapsigargin (1 \(\mu\)M) or thrombin (7 U/ml) challenge in the presence (+) and absence (−) of [Ca\(^{2+}\)]\(_o\), were made 60 min after addition of Ca\(^{2+}\) agonists. *Significantly different from respective control group, \(P < 0.05\).

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Fig. 5. Thrombin receptor-activating peptide (TRAP) increased endothelial cell permeability through activation of store-operated Ca\(^{2+}\) entry. A: representative tracing indicating that TRAP (100 \(\mu\)M; sequence: H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH) increased [Ca\(^{2+}\)]\(_i\). B: summary of 5 experiments. Peak and plateau responses to TRAP are similar in magnitude to those to thrombin. \(P = \) not significant. C: thrombin increased the PS of 23-A dextran macromolecules across BPAEC monolayers in the presence (2 mM) and relative absence (100 nM) of extracellular Ca\(^{2+}\). However, TRAP (100 \(\mu\)M) only increased permeability in the presence of 2 mM extracellular Ca\(^{2+}\). *Significantly different from respective control group, \(P < 0.05\).
flux channels, which would also account for our experimental observations. A clear determination of a redundancy in channel regulation can only be achieved with subsequent identification and characterization of membrane proteins forming native endothelial Ca\(^{2+}\) channels and development of specific inhibitors to both receptor-coupled and store-operated Ca\(^{2+}\) entry events.

Even though Ca\(^{2+}\) entry in response to thrombin occurs through receptor- and store-operated pathways, a clear link between the store-operated pathway and endothelial cell shape change has previously been elucidated (4, 5, 19, 24, 25, 41). What has not been made clear, however, is the relationship between store-operated Ca\(^{2+}\) entry and the intracellular events downstream from Ca\(^{2+}\) entry leading to shape change and permeability alterations. We hypothesized that store-operated Ca\(^{2+}\) entry-induced increases in endothelial permeability involved activation of the Ca\(^{2+}\)/calmodulin-regulated MLCK and subsequent MLC\(_{20}\) 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\(^{2+}\) entry by thapsigargin was tightly coupled to a significant increase in MLC\(_{20}\) phosphorylation, suggestive of an increase in MLCK activity; Ca\(^{2+}\) influx was associated with a similar degree of MLC\(_{20}\) phosphorylation stimulated by thrombin.

The thrombin-induced increase in MLC\(_{20}\) was sustained for at least 15 min. This particular finding differed from the thapsigargin-induced MLC\(_{20}\) 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\(_{20}\) phosphorylation. This signaling response is unique to thrombin compared with other receptor-dependent (e.g., histamine) and -independent (e.g., ionomycin, thapsigargin, Staphylococcus aureus \(\alpha\)-toxin clamped) Ca\(^{2+}\) agonists. Thus G\(_{q}\) protein-coupled inflammatory agonists increase and maintain MLCK activity and MLC\(_{20}\) phosphorylation via activation of several Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-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\(^{2+}\) entry in response to thapsigargin and TRAP is sufficient to increase endothelial cell permeability. However, the role of MLC\(_{20}\) in linking store-operated Ca\(^{2+}\) entry to barrier disruption remains unclear. Both thrombin and thapsigargin increased permeability over a 1-h time course, even though MLC\(_{20}\) phosphorylation had returned to near baseline values in response to thapsigargin. Although these data would support a role for MLC\(_{20}\) phosphorylation in the initial response to activation of store-operated Ca\(^{2+}\) entry, MLC\(_{20}\) phosphorylation was not associated with the prolonged permeability response. Future studies will be required to comprehensively discriminate between the effect of MLC\(_{20}\) 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\(^{2+}\) agonists (12, 27).

In summary, our present studies addressed key issues relating to Ca\(^{2+}\) regulation and control of endothelial cell shape and barrier function. Receptor-coupled, G protein-linked inflammatory mediators may activate Ca\(^{2+}\) influx pathways under dual regulation by plasmalemmal effectors and the filling state of internal Ca\(^{2+}\) pools. Our data also extend the previous observation that store-operated Ca\(^{2+}\) entry can initiate intercellular gap formation, in part, by an associated transient increase in MLC\(_{20}\) phosphorylation. Furthermore, in addition to store-operated Ca\(^{2+}\) 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\(^{2+}\) entry, MLCK activity, and MLC\(_{20}\) phosphorylation status as related to endothelial Ca\(^{2+}\) homeostasis and barrier function (29).

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