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 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-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 (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 assessed for endothelial morphology (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\(^{2+}\)-sensitive fluorescent dye 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 \(\mu\)M fura 2-AM, 0.003% pluronic acid, and 2 mM or 100 nM Ca\(_{Cl}\). 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 nM Ca\(_{Cl}\)) for an additional 20 min. After deesterification, [Ca\(^{2+}\)]i was estimated with an Olympus IX79 inverted microscope at \(\times 400\) 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\(^{2+}\)-bound (340-nm) to Ca\(^{2+}\)-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 \(^{35}\)S)methionine (555 \(\mu\)Ci/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 \(\mu\)M), or thrombin (7 U/ml) in Ca\(^{2+}\)-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 \(\mu\)l 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 \(\mu\)l 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).

**Assessment of endothelial cell shape change and intercellular gap formation.** BPAECs were seeded onto Transwell inserts (6.5-mm diameter, 0.4-\(\mu\)m pore size; Costar) at a density of 2.27 \(\times\) 10\(^4\) cells/mm\(^2\) in a final volume of 100 ml of DMEM plus 10% fetal bovine serum. The inserts were placed into 24-well plates containing 600 \(\mu\)l of growth medium, and the cells were allowed to grow for 5 days. After the cells achieved confluence, the growth medium in the upper chamber was replaced with 100 ml of a 1 mg/ml FITC-dextran solution in Krebs-Henseleit PSS. The insert was then moved to a fresh lower well containing 600 \(\mu\)l of PSS. The cells were equilibrated with these solutions at 37°C in a CO\(_2\) incubator for 15 min. After equilibration, the Transwell insert was placed into another lower chamber containing 600 \(\mu\)l 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 of the lower chamber (50 \(\mu\)l) were taken in triplicate and placed in 96-well cluster plates for measuring fluorescence intensity (Perkin-Elmer luminescence spectrometer LS 50B) excitation at 480 nm and 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, in nanoliters per minute) was calculated by determining the net rate of FITC-dextran flux (J\(_{PS}\) ) generated for each concentration difference (AC) across the monolayer with the equation PS = J/AC.

**Statistical methods.** Data are reported as means \(\pm\) SE. Comparisons were made with either paired or unpaired Student's t-test or one-way or two-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**

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

Figure 1, C and D, shows that the thrombin-induced 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 Ca$^{2+}$-release response to thrombin was abrogated, although microinjected cells still displayed an increased [Ca$^{2+}$]$_i$, that was sustained. This finding suggested that thrombin opened a membrane-Ca$^{2+}$ influx pathway independent of an Ins(1,4,5)P$_3$-mediated Ca$^{2+}$ store depletion event (Fig. 1C). However, when BPAEC SOCs were activated with thapsigargin and then challenged with thrombin, no additional change in [Ca$^{2+}$]$_i$ 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$^{2+}$ influx in BPAECs.

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 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 [Ca$^{2+}$]$_o$. 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 Ca$^{2+}$, the basal stoichiometry decreased 15% to 0.33 ± 0.05 mol phosphate/mol MLC$_{20}$, suggesting that basal leak of Ca$^{2+}$ across the endothelial plasmalemma contributed to MLC$_{20}$ phosphorylation status. Prevention of 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 [Ca$^{2+}$]$_o$. These data indicate that, at least over the time period studied, 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 Ca$^{2+}$ entry.

Activation of store-operated 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 \([Ca^{2+}]_o\) 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 extracellular Ca\(^{2+}\), thrombin increased permeability 40% under these conditions, suggesting that its effect was independent of store-operated Ca\(^{2+}\) entry.

Because thrombin initiates cell signaling through proteolytic cleavage of its receptor, the permeability response in low extracellular Ca\(^{2+}\) could reflect proteolysis of receptor-independent substrates. To examine this possibility, the direct receptor agonist, thrombin receptor-activating peptide (TRAP; Calbiochem-Novabiochem), was tested. Figure 5, A and B, shows that TRAP produced a transient peak in \([Ca^{2+}]_i\) followed by a sustained plateau that resembled the response initiated by thrombin. Similar to Fig. 4, thrombin increased diffusive capacity by 65 and 95% in the presence and absence, respectively, of extracellular Ca\(^{2+}\) (Fig. 5B). TRAP increased permeability by 275%, but this effect was eliminated in low extracellular Ca\(^{2+}\). Thus permeability increased by both receptor-independent (e.g., thapsigargin) and -dependent (e.g., TRAP) activation of store-operated Ca\(^{2+}\) entry is sufficient to increase macromolecular permeability.

**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 \([Ca^{2+}]_i\) (21, 22, 27, 33, 36). As described here and consistent with previous observations (1, 14, 21, 22), the thrombin-induced increase in
[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
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 Gq 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 Gq 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\(^{2+}\) 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-Å dextran macromolecules across BPAEC monolayers under control conditions and after thapsigargin (1 μM) 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.

**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 μM; 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-Å dextran macromolecules across BPAEC monolayers in the presence (2 mM) and relative absence (100 nM) of extracellular Ca\(^{2+}\). However, TRAP (100 μM) only increased permeability in the presence of 2 mM extracellular Ca\(^{2+}\). *Significantly different from respective control group, P < 0.05.
Future studies will be required to comprehensively discriminate between the effect of MLC20 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 Ca2+ agonists (12, 27).

In summary, our present studies addressed key issues relating to Ca2+ regulation and control of endothelial cell shape and barrier function. Receptor-coupled, G protein-linked inflammatory mediators may activate Ca2+ influx pathways under dual regulation by plasmalemmal effectors and the filling state of internal Ca2+ pools. Our data also extend the previous observation that store-operated Ca2+ entry can initiate intercellular gap formation, in part, by an associated transient increase in MLC20 phosphorylation. Furthermore, in addition to store-operated Ca2+ 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 Ca2+ entry, MLCK activity, and MLC20 phosphorylation status as related to endothelial Ca2+ homeostasis and barrier function (29).

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