Regulation of endothelial cell barrier function by calcium/calmodulin-dependent protein kinase II

TALAIBEK BORBIEV, ALEXANDER D. VERIN, SHU SHI, FENG LIU, AND JOE G. N. GARCIA

Division of Pulmonary and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21224

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Abstract

Borbiev, Talaibek, Alexander D. Verin, Shu Shi, Feng Liu, and Joe G. N. Garcia. Regulation of endothelial cell barrier function by calcium/calmodulin-dependent protein kinase II. Am J Physiol Lung Cell Mol Physiol 280: L983–L990, 2001.—Thrombin-induced endothelial cell barrier dysfunction is tightly linked to Ca$^{2+}$-dependent cytoskeletal protein reorganization. In this study, we found that thrombin increased Ca$^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II) activities in a Ca$^{2+}$- and time-dependent manner in bovine pulmonary endothelium with maximal activity at 5 min. Pretreatment with KN-93, a specific CaM kinase II inhibitor, attenuated both thrombin-induced increases in monolayer permeability to albumin and decreases in transendothelial electrical resistance (TER). We next explored potential thrombin-induced CaM kinase II cytoskeletal targets and found that thrombin causes translocation and significant phosphorylation of nonmuscle filamin (ABP-280), which was attenuated by KN-93, whereas thrombin-induced myosin light chain phosphorylation was unaffected. Furthermore, a cell-permeable N-myristoylated synthetic filamin peptide (containing the COOH-terminal CaM kinase II phosphorylation site) attenuated both thrombin-induced filamin phosphorylation and decreases in TER. Together, these studies indicate that CaM kinase II activation and filamin phosphorylation may participate in thrombin-induced cytoskeletal reorganization and endothelial barrier dysfunction.

thrombin; filamin; myosin light chain phosphorylation; transendothelial electrical resistance

Thrombin is a multifunctional serine protease that plays a central role in hemostasis by regulating platelet aggregation and blood coagulation, with profound effects on vascular endothelial cell function. Thrombin promotes endothelial cell activation and increases vascular permeability, producing an elevation in intracellular Ca$^{2+}$ as a direct result of phosphatidylinositol-specific phospholipase C activation as well as increases in phospholipase A$_2$, phospholipase D, protein kinase C, and myosin light chain kinase (MLCK) activities (6–9, 16, 17). Potential mechanisms of endothelial cell barrier dysfunction induced by thrombin include increases in contractile forces, decreases in intercellular junctional connections, and reductions in endothelial cell-extracellular matrix adhesive forces (10). Furthermore, thrombin challenge produces dramatic cytoskeletal rearrangements concomitant with phosphorylation of myosin light chains (MLC) (6, 26) and caldesmon (30), an actin-binding protein that mediates smooth muscle contraction and may be involved in the contractile mechanism of endothelial cell barrier regulation. Another actin-binding protein that may also be important for cell shape regulation, cell locomotion, and potentially endothelial cell barrier regulation is the ubiquitously distributed nonmuscle filamin, a 280-kDa homodimer present in the cortical cytoplasm and responsible for three-dimensional actin network formation (12). Filamin provides attachment of filamentous actin to plasma membrane glycoproteins (GP1bα in platelets, FcγR1 in leukocytes), binds directly to cytoplasmic domains of β$_1$- and β$_2$-integrins, and associates with furin, presenilin, and SEK-1, indicating its important regulatory role in actin cytoskeleton organization and multifunctional properties (4, 18, 19, 21, 25, 29, 38, 39). It has been suggested that the translocation of filamin from the endothelial cell membrane to the cytosol may be actually involved in the disassembly of the membrane-cytoskeleton network and subsequent increases in vascular permeability, whereas association of filamin with the membrane-cytoskeleton interface may contribute to the establishment or maintenance of barrier function (13). Filamin function is modulated by cAMP-dependent protein kinase A-mediated phosphorylation, which increases the resistance of filamin to calpain proteolysis (1), whereas Ca$^{2+}$-dependent CaM kinase II-induced filamin phosphorylation decreases its actin filament cross-linking activity (24). Furthermore, filamin phosphorylation induced by bradykinin and H$_2$O$_2$ via protein kinase A and CaM kinase II pathways is associated with redistribution of filamin between cytosol and membrane compartments (13, 35). In this study, we examined whether CaM kinase II activities are involved in thrombin-induced endothelial cell barrier dysfunction and explored filamin as a potential cytoskeletal target for CaM kinase II. Our data indicate that both CaM kinase II and filamin are important participants in vascular barrier regulation.

Address for reprint requests and other correspondence: J. G. N. Garcia, The Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, 4B.77, Baltimore, MD 21224-6801 (E-mail: drgarcia@welch.jhu.edu).

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**MATERIALS AND METHODS**

**Reagents.** Bovine thrombin was obtained from Sigma (St. Louis, MO), KN-93 and ionomycin were purchased from Calbiochem (La Jolla, CA), fura 2-AM was obtained from Molecular Probes (Junction City, OR), antibody to MLC kinase II was purchased from Transduction Laboratories (Lexington, KY), phospho-CaM kinase II antibody was obtained from ABR (Golden, CO), antibody to smooth muscle MLCK was obtained from Sigma, and antibody to human filamin was purchased from Chemicon (Temecula, CA). Synthesized myristoylated and purified peptides were obtained from the Biochemistry and Biotechnology Facility, Indiana University (Indianapolis, IN) and were based on the deduced amino acid sequence of human umbilical vein endothelial cell filamin (11). The CaM site containing filamin peptide contains amino acid residues 2517–2528 of filamin: NH₂-TGPRLVS*-NHSLHE-COOH, with Ser⁴ corresponding to the CaM kinase II phosphorylation site. Control peptide has the sequence of NH₂-SPFEVKVGTECGNQK-COOH representing 564–579 amino acid residues, which is not phosphorylated by CaM kinase II. Both peptides were successfully reported in the investigation of endothelial cell permeability (37).

**Bovine pulmonary artery endothelial cell culture.** Bovine pulmonary artery endothelial cells were obtained frozen at 16 passages from American Tissue Type Culture Collection (CCL 209; Manassas, VA) and were utilized at passages 19–24 (6). Endothelial cells were cultured in complete medium and maintained at 37°C in a humidified atmosphere of 5% CO₂–95% air and grew to contact-inhibited monolayers with the typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin and resuspended in fresh culture medium and passaged to appropriate size flasks or dishes.

**CaM kinase II activity assay.** Endothelial cell monolayers grown in 60-mm dishes were incubated with thrombin for indicated times and lysed in 500 ml of lysis buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na₂VO₃, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, and 1:500 diluted protease inhibitory cocktail (Transduction Laboratories) at 4°C for 1 h with gentle agitation. Pellets were washed three times with immunoprecipitation buffer and resuspended in electrophoresis sample buffer, boiled for 5 min, and centrifuged for 5 min. After centrifugation, the supernatant was incubated with 2.5 mg of CaM kinase II monoclonal antibodies (Transduction Laboratories) at 4°C for 1 h, followed by incubation with 100 µl of protein G Sepharose slurry (containing 30% protein G Sepharose 4 fast flow; Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at 4°C with gentle agitation. Immunoprecipitates were washed twice with lysis buffer, and the CaM kinase II activity was determined by the incorporation of [³²P]orthophosphate into its specific substrate peptide using the CaM kinase II assay kit (Upstate Biotechnology, Lake Placid, NY).

**MLC phosphorylation in endothelium.** Endothelial cell monolayers were analyzed for MLC phosphorylation by urea-PAGE as we have previously described (6), followed by Western immunoblotting with specific anti-MLC antibodies. Intracellular Ca²⁺ determinations assessed by fura 2 fluorescence. Intracellular Ca²⁺ concentration ([Ca²⁺]i) was measured by fluorescence in bovine pulmonary artery endothelial cells loaded with fura 2-AM. Confluent endothelial cell monolayers were removed from 75-cm² tissue flasks and seeded onto glass coverslips. When confluent, the endothelial cells were exposed to loading buffer (Hanks’ buffer supplemented with 0.1% pluronic acid, 1 mM CaCl₂ and 5 µM fura 2-AM) for 20 min at 37°C. After fura 2-AM loading, endothelial cells were washed twice in buffer supplemented with 1 mM Ca²⁺ and then maintained in the same buffer. Continuous monitoring of the cellular fluorescence was performed at 37°C with constant stirring in a Perkin Elmer LS-50 fluorometer by ratio of the 340/380-nm excitation signal.

**Transendothelial electrical resistance.** Endothelial cells were seeded onto evaporated gold microelectrodes and grown to confluence as previously described (34). The endothelial cell monolayer-covered microelectrodes were then connected to an electrical cell substrate impedance system (Applied Biophysics, Troy, NY) and rinsed with medium 199 (GIBCO BRL), and the transendothelial electrical impedance was monitored for ~1 h to establish a baseline resistance. Resistance values from each microelectrode (measured in ohms) were normalized as the ratio of measured resistance to baseline resistance and plotted vs. time.

**Albumin clearance measurement of endothelial cell permeability.** Macromolecule permeability of cultured endothelial cell monolayers was assessed in previously described (5, 6). Gelatinized polycarbonate micropore membranes (Nucleapore, Pleasanton, CA) were mounted on the base of plastic cylinders and endothelial cells and then were seeded on the membranes and grown to confluence. The system consists of two compartments, the upper compartment (luminal) and the lower compartment (abluminal), which are separated by a polycarbonate filter on which the endothelial cell monolayer is grown. The lower compartment was stirred continuously and kept at a constant temperature of 37°C by use of a thermally regulated water bath. BSA (4%) complexed to Evans blue dye was added to the luminal compartment for 1 h to establish the baseline albumin clearance rate, and then samples were taken from the abluminal compartment every 10 min for measurements. Transendothelial cell albumin transport was determined by measuring the absorbance of Evans blue dye present in the abluminal chamber at 620 nm in a spectrophotometer (Vmax Multiplate Reader; Molecular Devices, Menlo Park, CA).

**CaM kinase II, MLCK, and filamin immunoprecipitation and Western immunoblotting.** Confluent endothelial cells were rinsed with PBS, then lysed with the addition of either boiling 1% SDS, 1 mM sodium vanadate, and 10 mM Tris-HCl, pH 7.4, or cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, and protease inhibitors). To a microcentrifuge tube, 50 µl of Omnisorb (Calbiochem), 400 µl of H₂O₂, 400 µl of immunoprecipitation buffer, and 100 µl of total endothelial cell lysate were added and incubated for 30 min at 4°C, followed by centrifugation for 15 min. To supernatants, 10–100 µg of polyclonal or monoclonal antibody were added and incubated for 1 h at 4°C. Omnisorb (50–200 µl; Calbiochem) was added to each tube and incubated for an additional 30 min followed by centrifugation for 1 h. Pellets were washed three times with immunoprecipitation buffer and resuspended in electrophoresis sample buffer, boiled for 5 min, and centrifuged for 5 min, and supernatants were loaded onto SDS-PAGE, transferred to nitrocellulose (30 V for 18 h or 90 V for 2 h) and reacted with antibody of interest. Immunoreactive proteins were detected using enhanced chemiluminescent detection system according to the manufacturer’s directions (Amersham, Little Chalfont, UK). The relative intensities of the protein bands were quantified by scanning densitometry.

**Preparation of subcellular fractions.** Confluent endothelial cells (80–100%) were fractionated into cytosolic, membrane, and nuclear/cytoskeletal fractions as previously described.
CaM kinase II activation is dependent on increases in cytosolic calcium (9), we found that thrombin-induced CaM kinase II activation, confirming complete inhibition of CaM kinase II activity in the presence (+) of Ca\(^{2+}\), whereas in the absence (−) of Ca\(^{2+}\) (chelation with EGTA, 2 mM, 30 min), ionomycin-induced CaM kinase II activity was reduced, suggesting that CaM kinase II activity is Ca\(^{2+}\)-dependent. Error bars are means ± SD. Effect of Ca\(^{2+}\) chelation on thrombin-induced CaM kinase II activity in endothelial cells. Confluent endothelial cells were pretreated with EGTA (2 mM, 30 min) or vehicle control followed by thrombin challenge (100 nM, 15 min). Thrombin-induced CaM kinase II activity was blocked by EGTA pretreatment indicating that CaM kinase II activity is Ca\(^{2+}\)-dependent. Error bars are means ± SD. *P < 0.05 compared with control.
CaM kinase II immunoprecipitates as detected by Western immunoblotting with anti-phospho-CaM kinase II antibodies (Fig. 2, inset). Taken together, these results demonstrate that thrombin and ionomycin induce significant and rapid Ca$^{2+}$-dependent CaM kinase II activation in endothelium.

**Effect of CaM kinase II inhibition on thrombin-induced endothelial cell barrier dysfunction and MLC phosphorylation.** Thrombin is a potent bioactive agonist that produces increased vascular permeability (5, 30). Because thrombin-induced CaM kinase II activation in endothelial cells was completely abolished by the specific CaM kinase II inhibitor KN-93, we next examined the role of CaM kinase II inhibition in thrombin-induced endothelial cell permeability and barrier dysfunction as assessed by alterations in albumin clearance and TER. CaM kinase II inhibition with KN-93 significantly attenuated both thrombin-induced clearance of Evans blue dye-albumin across confluent endothelial cell monolayers grown on polycarbonate filters (Fig. 3) and thrombin-induced declines in TER (Table 1). Together, these data suggest an important role of CaM kinase II in the regulation of thrombin-induced endothelial cell barrier dysfunction.

We have previously shown that thrombin-induced contractile events, gap formation, and barrier dysfunction occur via MLCK-dependent mechanisms, indicating an important role of MLC phosphorylation in this model of vascular permeability (6). We next examined the involvement of CaM kinase II in the MLCK-dependent pathway of thrombin-induced endothelial cell barrier dysfunction. Confluent endothelial cell monolayers pretreated with KN-93 and challenged with thrombin were used for assessing the levels of nonphosphorylated, monophosphorylated, and diphosphorylated MLC in confluent endothelium. CaM kinase II inhibition did not alter the stoichiometry of thrombin-induced MLC-phosphorylated species (Fig. 4A), suggesting that CaM kinase II activity is more likely to be involved in MLCK-independent regulatory pathways that contribute to a thrombin-induced endothelial cell barrier dysfunction. Consistent with these data, immunoprecipitation of MLCK from $^{32}$P-labeled endothelial cell homogenates after KN-93 pretreatment failed to demonstrate significant alterations in thrombin-induced MLCK phosphorylation (Fig. 4B).

**Effect of thrombin-induced filamin phosphorylation and translocation on TER.** We next addressed whether filamin, an actin-binding protein known to be phosphorylated by CaM kinase II in vitro (24), may represent a relevant CaM kinase II target in thrombin-stimulated endothelium. Differential detergent subcellular fractionation revealed bovine endothelial cell filamin to be distributed in both cytosolic (~58%) and membrane (~42%) endothelial cell fractions. Thrombin stimulation of confluent bovine endothelium resulted in rapid but transient translocation of filamin from the cytosolic to the membranous fraction after 1 min, quickly returning to the cytosolic fraction by 5 min (Fig. 5). We pretreated endothelial cells with either CaM peptide (5 µM, 30 min), a synthetic myristoylated filamin peptide that contains a previously described serine phosphorylation site (11) for CaM kinase II within the human endothelial cell filamin sequence (NH$_2$-T$^{2517}$GPRLVS$^{2529}$NHSHEL$^{2325}$COOH) or with a synthetic myristoylated control filamin peptide (NH$_2$-S$^{64}$PFEVKVGTECNQK$^{273}$-COOH), which does not contain a CaM kinase II consensus site. We found that the CaM peptide but not the control peptide significantly attenuated thrombin-stimulated filamin translocation (100 nM, 1 min; Fig. 6A). To explore whether thrombin induces filamin phosphorylation, endothelial cell monolayers were labeled with $[^{32}$P]orthophosphate.
for 2.5 h, pretreated with KN-93, and challenged with thrombin (100 nM, 10 min). Bovine endothelial cell filamin was then immunoprecipitated and detected in homogenates as a single 250-kDa band. Thrombin induced significant filamin phosphorylation that was partially attenuated by KN-93 pretreatment, suggesting that CaM kinase II phosphorylates nonmuscle filamin in vivo (Fig. 6B). Together, these results indicate that thrombin-induced CaM kinase II activation results in nonmuscle filamin phosphorylation and subcellular translocation.

Our next experiments utilized 32P-labeled endothelial cell monolayers previously pretreated with the myristoylated peptides (5 μM; CaM peptide or control) added for 30 min before thrombin stimulation (100 nM, 10 min) and filamin immunoprecipitation. Thrombin-induced filamin phosphorylation was significantly attenuated by the CaM peptide but not by the control peptide (Fig. 6C), thereby confirming the observed inhibition of filamin phosphorylation by KN-93 (Fig. 6B) and identifying a potentially important and physiologically relevant site of filamin phosphorylation by CaM kinase II in situ. To explore the physiological consequences of these events, TER measurements were obtained in endothelial cell monolayers pretreated with either CaM peptide or control peptide (5 μM) followed by thrombin (100 nM) stimulation. The CaM kinase II inhibitory peptide, but not the control peptide, significantly attenuated thrombin-induced decreases in TER (Table 2), consistent with an important role for CaM kinase II and filamin phosphorylation in thrombin-induced endothelial cell barrier dysfunction.

**DISCUSSION**

Our data indicate that thrombin-induced Ca2+ mobilization in bovine pulmonary artery endothelial cells leads to CaM kinase II activation and autophosphorylation. Inhibition of CaM kinase II activity with the specific inhibitor KN-93 attenuated both thrombin-induced increases in albumin clearance and decreases in TER, suggesting an important role for CaM kinase II activities in thrombin-induced endothelial cell barrier dysfunction. We have previously shown that thrombin-induced endothelial cell barrier dysfunction is tightly linked to cytoskeletal rearrangement and stress fiber formation and involves, at least in part, increases in the level of phosphorylated regulatory MLCs catalyzed by CaM kinase II. The phosphorylation of MLCs by CaM kinase II leads to stress fiber formation and cell barrier dysfunction.

**Table 1. Effect of KN-93 on thrombin-induced alterations in transendothelial electrical resistance**

<table>
<thead>
<tr>
<th>Intervention</th>
<th>0 h</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,160 ± 148</td>
<td>1,120 ± 108</td>
<td>1,130 ± 115</td>
<td>1,148 ± 128</td>
<td>1,154 ± 133</td>
</tr>
<tr>
<td>KN-93</td>
<td>1,052 ± 224</td>
<td>967 ± 143</td>
<td>1,027 ± 149</td>
<td>1,098 ± 165</td>
<td>1,135 ± 154</td>
</tr>
<tr>
<td>Thrombin</td>
<td>1,221 ± 149</td>
<td>840 ± 148</td>
<td>694 ± 101*</td>
<td>653 ± 94*</td>
<td>630 ± 86*</td>
</tr>
<tr>
<td>KN-93/thrombin</td>
<td>1,090 ± 212</td>
<td>842 ± 166†</td>
<td>883 ± 178‡</td>
<td>877 ± 167‡</td>
<td>877 ± 136‡</td>
</tr>
</tbody>
</table>

Values are means ± SD of electrical resistance measurements in Ω from 6 transendothelial electrical resistance experiments. Endothelial cell monolayers were rinsed with medium 199 and after ~1 h pretreated with 5 μM KN-93 for 30 min. At time 0, cells were challenged by either 100 nM thrombin or vehicle, and means of electrical resistance were calculated for 0, 1, 2, 3, and 4 h time points. *P < 0.05 between control and thrombin-treated groups. †P > 0.05 and ‡P < 0.05 between thrombin- and KN-93/thrombin-treated groups. Pretreatment with KN-93 attenuated thrombin-induced declines in electrical resistance.

**Fig. 4.** Effect of CaM kinase II inhibition on thrombin-induced MLC and MLCK phosphorylation. MLC phosphorylation was detected by using urea gel electrophoresis, which separates non-, mono-, and diphosphorylated MLC. A: thrombin-induced MLC phosphorylation (100 nM) was not altered by pretreatment with KN-93 (5 μM, for 30 min). Error bars are means ± SD. *P < 0.05 compared with vehicle. B: endothelial cell monolayers were pretreated with KN-93 (5 μM for 30 min) and challenged with thrombin (100 nM for 15 min) followed by MLCK kinase (MLCK) immunoprecipitation as described in MATERIALS AND METHODS. Thrombin-induced MLCK phosphorylation (100 nM) was not changed after KN-93 pretreatment (5 μM, for 30 min).
by MLCK with Rho kinase involvement (6, 10). However, pretreatment with KN-93 before thrombin challenge on endothelial cells does not alter the level of MLC phosphorylation in endothelial cells, indicating that CaM kinase II involvement in endothelial cell barrier regulation is not likely via modifications in thrombin-induced MLCK or Rho kinase activation. Because neither thrombin-induced MLCK nor MLC phosphorylation was inhibited by KN-93 pretreatment, CaM kinase II does not appear to phosphorylate the endothelial cell MLCK isoform (8), as demonstrated in smooth muscle cells (32, 33). Smooth muscle MLCK phosphorylation by CaM kinase II leads to decreases in Ca$^{2+}$ sensitivity and attenuation of kinase activity (32, 33), although other reports suggest a lack of correlation between CaM kinase II activation and parameters of MLC phosphorylation and force in histamine- and KCl-stimulated arterial smooth muscle (28). Because inhibition of CaM kinase II attenuates thrombin-induced endothelial cell barrier dysfunction without altering either MLCK or MLC phosphorylation status, we speculated the existence of additional CaM kinase II-dependent targets affecting EC barrier function.

Assuming an important role for the actin cytoskeleton in maintenance of endothelial cell barrier function, Table 2. Effect of CaM peptide on thrombin-induced decline in transendothelial electrical resistance

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Baseline</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,389 ± 220</td>
<td>1,393 ± 231</td>
<td>1,328 ± 187</td>
<td>1,320 ± 185</td>
<td>1,330 ± 217</td>
</tr>
<tr>
<td>Thrombin</td>
<td>1,388 ± 221</td>
<td>980 ± 108</td>
<td>849 ± 177</td>
<td>767 ± 185</td>
<td>743 ± 181</td>
</tr>
<tr>
<td>Control peptide</td>
<td>1,401 ± 252</td>
<td>968 ± 134*</td>
<td>845 ± 143*</td>
<td>761 ± 182*</td>
<td>718 ± 179*</td>
</tr>
<tr>
<td>Thrombin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control peptide/thrombin</td>
<td>1,383 ± 273</td>
<td>1,227 ± 199†</td>
<td>1,262 ± 281‡</td>
<td>1,102 ± 272§</td>
<td>973 ± 198§</td>
</tr>
</tbody>
</table>

Values are means ± SD of electrical resistance measurements in Ω from transendothelial electrical resistance experiments; n = 4. Endothelial cells were pretreated with either control or Ca$^{2+}$/calmodulin (CaM) peptide (5 µM, 30 min) and at time 0 challenged with 100 nM thrombin. Means of electrical resistance were calculated for baseline and 1, 2, 3, and 4 h time points. *P < 0.05 between control and thrombin-treated groups. †P > 0.05, ‡P < 0.05, and §P < 0.01 between thrombin- and CaM peptide-treated groups. Pretreatment with CaM peptide but not with control peptide attenuated thrombin-induced declines in electrical resistance across endothelial cell monolayers.

Fig. 5. Thrombin-induced filamin translocation. Time course of thrombin-induced filamin redistribution between cytosolic and membrane fractions. Cells were exposed to 100 nM thrombin for 1, 2, and 5 min. Error bars are means ± SD. *P < 0.05 compared with control (0 min). Inset: representative (n = 4) immunoblot of filamin from cytosolic and membrane fractions of endothelial cells treated with thrombin (100 nM) for the indicated times.

Fig. 6. Inhibition of thrombin-induced filamin translocation and phosphorylation by KN-93 and CaM peptide. A: effect of myristoylated peptides on thrombin-induced subcellular filamin distribution. Endothelial cells were pretreated with either control or CaM peptide (5 µM, 30 min) followed by thrombin challenge (100 nM, 1 min). CaM peptide, but not control peptide, prevented thrombin-induced filamin subcellular redistribution. B: endothelial cells were labeled with [32P]orthophosphat e for 2.5 h in phosphate-free medium and then treated with 100 nM thrombin in either the presence or absence of KN-93 pretreatment (5 µM for 30 min). Filamin immunoprecipitates were obtained as described in MATERIALS AND METHODS. Thrombin induced filamin phosphorylation, which was attenuated by KN-93 pretreatment. C: filamin immunoprecipitates were obtained from endothelial cell monolayers as described in MATERIALS AND METHODS. Cells were pretreated with either 5 µM CaM peptide or 5 µM control peptide for 30 min followed by thrombin (100 nM) treatment for 15 min.
potential substrates for CaM kinase II include an abundant number of actin-binding proteins that may affect endothelial cell permeability. For example, caldesmon is a regulatory contractile protein that is phosphorylated by several protein kinases (including CaM kinase II), and thereby promotes actomyosin interaction in smooth muscle (22, 23). Our unpublished data indicate that CaM kinase II is consistently found in caldesmon immunoprecipitates from endothelial cells, suggesting that caldesmon phosphorylation by CaM kinase II after thrombin may participate in modulation of endothelial cell barrier function. In this study, we have focused on another important CaM kinase II target potentially involved in endothelial cell barrier regulation, the actin-binding and cross-linking protein filamin (ABP-280). Filamin is located at the interface between the cytoskeleton and the plasma membrane, where it anchors filamentous actin to plasma membrane proteins. In this spatial locale, filamin is uniquely qualified to control cell shape and cell locomotion, stabilize attachments to other cells and to the substratum, and regulate cellular responses to external stimuli (20). The importance of filamin was demonstrated in human malignant melanoma cell lines that lack ABP-280 (3) and display impaired locomotion and circumferential blebbing of the plasma membrane, whereas reserved expression of ABP-280 restores motility and reduces membrane blebbing. Actin cross-linking properties of filamin are affected by CaM kinase II-dependent phosphorylation, which occurs with a maximal stoichiometry of 1 mol of phosphate per mole of filamin dimer and leads to a decrease in filamin-regulated actin filament cross-linking activity (24). We speculate that filamin phosphorylation causes a shift in the cortical actin filament sol-gel transitions that are critical for cell movement (31) and thus leads to changes in endothelial cell barrier function. Thus regulation of the mechanical properties of the cytosplasmic actin network by filamin phosphorylation may alter endothelial cell permeability. Our TER experiments using a CaM peptide, which functions as a specific competitive inhibitor of nonmuscle filamin phosphorylation by CaM kinase II, strongly suggest that filamin phosphorylation is involved in endothelial cell barrier regulation, although the exact mechanism is not clear and consequent signaling events are not well understood. The subcellular redistribution of filamin appears to be involved in the disassembly of the membrane-cytoskeleton network, loss of junctional integrity between cells, and an increase in vascular permeability (13–15, 35–37). In this case, observed direct association of filamin with integrins (19, 29), which play major roles in cell-cell and cell-extracellular matrix interactions, may be important in endothelial cell barrier regulation. Physiologically relevant filamin subcellular redistribution may also affect protein interactions within the complexes of focal contacts and adherens junctions, triggering correspondent signal transduction processes. Further work is necessary to link changes in filamin phosphorylation and subcellular distribution to endothelial cell barrier regulation.

In conclusion, we have shown that CaM kinase II activation is involved in thrombin-induced endothelial cell barrier dysfunction. CaM kinase II activation does not alter the level of MLC phosphorylation but leads to phosphorylation and translocation of the cytoskeletal actin-binding protein nonmuscle filamin, with subsequent modulation of endothelial cell barrier function, presumably via changes in the mechanical properties of the cytoplasmic actin network. Further examination of filamin-mediated cytoskeletal rearrangement as well as other CaM kinase II cytoskeletal targets may reveal new insights into nonmuscle cytoarchitectural regulation.

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