Role of CaM kinase II and ERK activation in thrombin-induced endothelial cell barrier dysfunction

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THE ENDOTHELIAL SERVES as a semiselective permeability barrier between the blood and underlying tissues. The vascular integrity is important for preservation of organ function, whereas compromise of endothelial cell barrier leads to increased permeability, a cardinal feature of inflammation. Endothelial cell barrier integrity is critically dependent on cytoskeletal elements and the dynamic equilibrium between tethering forces, mediated by cell-cell and cell-extracellular matrix contacts, and contractile forces driven by an actomyosin motor (11). It is generally assumed that the primary permeability pathway across the vessel wall occurs via a paracellular route and is closely associated with the formation of intercellular gaps (11). Actomyosin interaction is involved in both endothelial cell retraction and gap formation and depends on myosin light chain (MLC) phosphorylation catalyzed by myosin light chain kinase (MLCK) (53, 54).

Edemagenic factors such as the serine protease thrombin, generated during activation of the coagulation cascade, directly increase vascular permeability in vivo and in vitro (14, 36), indicating the relevance of in vitro models of thrombin-induced endothelial cell permeability. Thrombin induces endothelial cell activation and cellular responses via activation of its G protein-coupled receptors, increases intracellular Ca2+ as a direct result of phospholipase C activation, and increases phospholipase A2, phospholipase D, and protein kinase C activities (16, 19, 27). These signals result in the rearrangement of the endothelial actin cytoskeleton. We have previously shown a critical role for MLCK activation and MLC phosphorylation in thrombin-induced actin cytoskeleton rearrangement, gap formation, and increases in endothelial cell permeability (15). However, contractile forces in this model of endothelial cell permeability are unlikely to be entirely modulated by MLCK-dependent mechanisms of endothelial cell contraction. This is suggested by the reproducible observation that the time response curve of thrombin-mediated permeability and MLC phosphorylation is not tightly correlative, with a relatively sustained level of permeability while MLC phosphorylation is decreasing (15), findings reminiscent of the latch state postulated to exist in slowly contracting smooth muscle.

One potential MLCK-independent contractile mechanism may involve the cytoskeletal protein caldesmon, a major actin-, myosin-, tropomyosin-, and calmodulin-binding protein, which is involved in the regulation of smooth muscle and nonmuscle contraction (46). Caldesmon is expressed from a single gene and spliced to yield either a high-molecular-weight smooth muscle-specific isoform (120–150 kDa) or a low-molecular-weight (70–80 kDa) isoform, widely distributed in non-
Caldesmon exhibits inhibitory function toward actin–tropomyosin-activated myosin ATPase activity, which can be reversed by calmodulin (CaM) binding in a Ca$^{2+}$-dependent manner and/or by Ser/Thr phosphorylation (37). Caldesmon can be phosphorylated by CaM kinase II, a multifunctional Ca$^{2+}$/CaM-dependent Ser/Thr protein kinase, with phosphorylation sites at both NH$_2$ and COOH termini (30), and by extracellular signal-regulated kinases (ERK) at two major COOH terminus sites, Ser$^{789}$ and Ser$^{798}$ (2, 9), based on the numbering of the mammalian high-molecular-weight caldesmon sequence (29). Phosphorylation of smooth muscle caldesmon by CaM kinase II leads to dissociation of myosin from caldesmon, reduces the binding of caldesmon to actin, and reverses inhibition of the myosin Mg$^{2+}$-ATPase (26, 39, 40, 47). The effect of phosphorylation of smooth muscle caldesmon by ERK is less well understood but reported to slightly attenuate its interaction with actin (8) and to a lesser extent by ERK is less well understood but reported to slightly attenuate its interaction with actin (8) and to release actomyosin interaction (10, 21, 22, 52). These studies in smooth muscle using high-molecular-weight caldesmon isoforms are applicable to nonmuscle cells, given the close structural and functional relationship between the caldesmon isoforms. In nonmuscle cells, caldesmon cross-links actin and myosin and is involved in cell contractility, cell division, assembly of actin stress fibers, and interferes with the formation of focal adhesions (7, 23, 25, 46, 55, 57).

We recently reported that thrombin-induced CaM kinase II activation modulates endothelial cell permeability in an MLCK-independent fashion (5), analogous to smooth muscle thin filament-dependent contractile regulation (12). Phosphorylation by CaM kinase II alters the function of a variety of substrates (6), including MAPK (ERK) activities (1). This is of particular interest since we have shown that ERK activation occurs via sequential Ras, Raf-1, and MEK activities and regulates phorbol ester-induced endothelial cell barrier dysfunction (50). However, the interrelationship between ERK and CaM kinase II activities in thrombin-mediated endothelial cell permeability remains unclear.

In the present study, we examined this linkage and explored the role of caldesmon, a potential cytoskeletal target in thrombin-stimulated signaling pathways. Our data indicate that ERK activation is involved in endothelial cell permeability induced by thrombin and proceeds in a CaM kinase II-dependent fashion. CaM kinase II/ERK inhibition attenuates thrombin-induced phosphorylation of caldesmon and reverses thrombin-induced dissociation of caldesmon-myosin complex. Furthermore, the inhibition of the CaM kinase II/ERK signaling pathway leads to impairment of thrombin-induced stress fiber formation and attenuated endothelial cell barrier dysfunction. These results provide new insights into nonmuscle contractile regulation and endothelial barrier regulation.

**Materials and Methods**

**Reagents.** Bovine thrombin was obtained from Sigma (St. Louis, MO); KN-93 and U0126 were purchased from Calbiochem (La Jolla, CA) and Promega (Madison, WI), respectively; antibody to MLC was produced in rabbit against purified baculovirus-expressed and purified smooth muscle MLC by Biodesign International (Kennebunk, ME); antibodies to caldesmon and CaM kinase II were purchased from Sigma; anti-ERK and phosphospecific anti-ERK MAPK antibodies were obtained from New England Biolabs (Beverly, MA); diphosphospecific anti-MLC antibodies were raised against MLC phosphorylated on Thr$^{18}$ and Ser$^{19}$, MLCK sites of phosphorylation as described (38); and phosphospecific anti-caldesmon antibodies were kindly provided by Dr. L. P. Adam (Boston, MA). All other reagents came from commercial sources as specified in the text.

**Cell culture.** Bovine pulmonary artery endothelial cells were obtained frozen at 16 passages from American Type Culture Collection (CCL 209; Manassas, VA) and were utilized at passages 19–24. Endothelial cells were cultured in complete media and maintained at 37°C in a humidified atmosphere of 5% CO$_2$/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 the appropriate size flasks or dishes.

**Transendothelial electrical resistance.** Endothelial cells were seeded onto evaporated gold microelectrodes and grown to confluence as we have previously described (20). Endothelial cells formed a confluent monolayer that covered the microelectrodes connected to an electrical cell-substrate impedance system (Applied Biophysics, Troy, NY). Resistance values from each microelectrode (measured in ohms) were normalized as the ratio of measured resistance to baseline resistance and plotted vs. time.

**Western immunoblotting and immunoprecipitation of caldesmon.** After being treated, endothelial cell monolayers grown in 35-mm dishes were rinsed with ice-cold PBS, lysed with 2× SDS sample buffer, and boiled for 5 min. Extracts were separated on SDS-PAGE, transferred to nitrocellulose (30 V, 18 h), and reacted with antibody of interest. Immunoreactive proteins were visualized using an enhanced chemiluminescent detection system. The relative intensities of the protein bands were quantified by scanning densitometry. The comparisons of two means were performed using Student’s t-test. Differences in two groups are considered statistically significant when P < 0.05. For immunoprecipitation under either denaturing or nondenaturing conditions, confluent endothelial cells (~10$^6$) were rinsed with PBS, then lysed with the addition of either boiling lysis buffer (1% SDS, 1 mM sodium vanadate, 10 mM Tris·HCl, pH 7.4) or ice-cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, and protease inhibitors). A total of 20 μl of protein G-Agarose (Calbiochem), 400 μl of H$_2$O, 400 μl of immunoprecipitation buffer, and 100 μl of total endothelial cell lysate were combined and incubated for 30 min at 4°C, followed by centrifugation for 5 min. The supernatant fraction was retrieved, and 10–20 μg of monoclonal antibody to caldesmon was added and incubated for 1 h at 4°C. Approximately 20–30 μl of protein G-Agarose was added to each tube and incubated for additional 30 min, followed by centrifugation for 1 min. Pellets were washed three times with immunoprecipitation buffer, resuspended in 2× SDS sample buffer, and boiled for 5 min. Samples were subjected onto SDS-PAGE,
transferred to nitrocellulose (30 V, 18 h), and analyzed by Western immunoblotting.

Detection of caldesmon phosphorylation. Thrombin-induced caldesmon phosphorylation was assessed either in caldesmon immunoprecipitates obtained from 32P-labeled endothelial cells or using previously characterized (9, 10, 13) phosphospecific anti-caldesmon antibodies raised against peptide sequences surrounding the two major ERK-catalyzed phosphorylation sites: Ser286 [PDXGKS(PO4)PAPKPG] and Ser789 [CQSVDKVTSP(PO4)PTKV], based on the numbering of the human high-molecular-weight caldesmon sequence (29).

MLC phosphorylation. Endothelial cell MLC phosphorylation was analyzed by SDS-PAGE followed by Western immunoblotting with diphosphospecific anti-MLC antibodies as we have recently reported (42).

Endothelial cell transfection with adenovirus encoding constitutively active α-CaM kinase II. A cDNA for constitutively active mutant of α-CaM kinase II (generated by site-directed mutagenesis of amino acids Thr286 and Val287 to aspartic acid) was placed downstream of a cytomegalovirus (CMV) promoter in a replication-deficient adenovirus as described (3). An adenovirus with CMV promoter but without insertion for the adenovirus as described (18). An adenovirus with CMV promoter but without insertion for the adenovirus as described (18). An adenovirus with CMV promoter but without insertion for the adenovirus as described (18).

Preparation of subcellular fractions. Bovine pulmonary artery endothelial cells (80–100%) were fractionated into cytosolic, membrane, and nuclear/cytoskeleton fractions as previously described (43). Cells were rinsed with PBS and incubated in ice-cold cytosolic buffer (0.01% digitonin, 10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 5 mM EDTA, 5 μM phallolidin) and protease inhibitory cocktail (1:500 diluted, Calbiochem) with agitation for 10 min at 4°C. Soluble cytosolic fraction was collected, dishes were rinsed with cytosolic buffer without protease inhibitors, and the residual material was extracted with membrane buffer (0.5% Triton X-100, 10 mM PIPES, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 3 mM EDTA, 5 μM phallolidin, and protease inhibitory cocktail) with agitation for 20 min at 4°C. Soluble (membrane) fraction was collected, and protein material remaining on dish (nuclear/cytoskeletal fraction) was scraped in SDS buffer (0.5% Triton X-100, 0.5% SDS, 10 mM Tris–HCl, pH 6.8, and protease inhibitory cocktail), shortly sonicated (3 times), and boiled at 100°C for 5 min. Aliquots of samples from subcellular fractions were used for measurements of protein concentration by BCA assay (Pierce, Rockford, IL). Equal protein amounts of samples were subjected onto SDS-PAGE, transferred to nitrocellulose, and tested by Western immunoblotting with specific antibodies.

Immunofluorescence microscopy. Immunofluorescence microscopy studies were performed as we have previously described (18). After being treated, endothelial cells grown on gelatinized coverslips were rinsed with PBS, fixed in 3.7% paraformaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 for 10 min. Cells were then washed with PBS, blocked with PBS-Tween 20 (0.5%; PBS-T) containing 2% BSA for 30 min, and incubated with primary antibodies. After being washed with PBS-T, cells were incubated with corresponding fluorochrome-conjugated secondary antibodies and 1 U/ml of Texas red-X phalloidin (Molecular Probes, Eugene, OR). Coverslips were mounted on slides with Slow-Fade mounting medium (Molecular Probes) and analyzed using a Nikon Eclipse TE 300 microscope. Images were captured by Sony Digital Photo camera DKC 5000.
RESULTS

Thrombin-induced ERK activation in bovine pulmonary artery endothelial cells. We first examined ERK activation in confluent bovine pulmonary artery endothelial cells treated with thrombin (100 nM). Thrombin induces rapid ERK activation, defined by time-dependent increases in phospho-ERK immunoreactivity (maximal at 5 min), which was abolished by pretreatment with U0126 (10 μM, 30 min), a specific MEK inhibitor (Fig. 1). These results were confirmed by experiments in which thrombin-induced ERK activation was assessed by immunofluorescence microscopy analysis using phospho-ERK-specific antibodies in conjunction with changes in the actin cytoskeleton using fluorochrome-conjugated phalloidin. These studies revealed significant thrombin-induced actin stress fiber and intercellular gap formation, with clear evidence of ERK activation (Fig. 2).

We have previously demonstrated thrombin to potently increase Ca2+ and the activity of the Ca2+/CaM-dependent protein kinase II (5, 19). To explore whether thrombin-induced ERK activation is a downstream event of CaM kinase II activities (1, 33), endothelial cells were pretreated with KN-93, a specific CaM kinase II inhibitor (10 μM, 30 min), and challenged with thrombin (100 nM, 5 min). As shown in Fig. 3, A and C, thrombin-induced ERK activation was attenuated by KN-93 pretreatment, indicating that ERK activation lies downstream of the CaM kinase signaling cascade. Additional experiments using KN-92, an inactive analog of KN-93, showed that pretreatment with KN-93 (10 μM, 30 min) but not with KN-92 (10 μM, 30 min) attenuates thrombin-induced ERK activation (data not presented). To confirm this linkage between CaM kinase II and ERK activation, we infected bovine pulmonary artery endothelial cells with either adenoviral-based constitutively active α-CaM kinase II or empty vector constructs at ~20 MOI and analyzed our results after 30 h. As shown in Fig. 3, B and D, constitutively active CaM kinase II produced sustained ERK activation, whereas the empty vector construct failed to do so. Together, these results show that in bovine macrovascular endothelium, thrombin induces substantial ERK activation in a time-dependent manner and that ERK activation lies downstream of CaM kinase II activities.

Role of CaM kinase II and ERK activities in thrombin-induced endothelial cell barrier dysfunction, MLC phosphorylation, and actin stress fiber formation. We have previously shown that inhibition of CaM kinase II activation attenuates thrombin-induced endothelial cell permeability (5). Taking into account the linkage between CaM kinase II and ERK activities, we next examined whether ERK inhibition attenuates thrombin-induced decreases in electrical resistance across bovine endothelial cell monolayers. Confluent bovine
pulmonary artery endothelial cells grown on gold microelectrodes to measure transendothelial electrical resistance were pretreated with U0126 (10 μM) and, after stabilization, treated with thrombin (100 nM). As shown in Fig. 4A, MEK inhibition with U0126 attenuates the magnitude of the decline in electrical resistance induced by thrombin as well as delays the onset of barrier dysfunction. We have previously demonstrated that thrombin-induced permeability involves MLCK-dependent actomyosin interaction. To investigate the role of ERK activation in this mechanism, we assessed the potential activation of MLCK by ERK.

**Fig. 3.** Calmodulin (CaM) kinase II-dependent ERK activation. A and C: effect of CaM kinase II inhibition on thrombin-induced ERK activation. Endothelial cells pretreated with either vehicle (DMSO) or KN-93 (10 μM, 30 min) and challenged with thrombin (100 nM, 10 min) were analyzed for ERK activation by Western immunoblotting using phosphospecific anti-ERK antibodies (n = 4). Thrombin-induced ERK activation is attenuated by KN-93 pretreatment. Error bars are means ± SD. *P < 0.05 compared with thrombin. B and D: effect of constitutively active α-CaM kinase II on ERK phosphorylation. Endothelial cells were infected with either control or recombinant adenovirus encoding constitutively active α-CaM kinase II as described in MATERIALS AND METHODS (n = 3). Recombinant, but not control, adenovirus causes sustained ERK activation reflected by phospho-ERK immunoreactivity. Cont, control.

**Fig. 4.** Effect of ERK inhibition on thrombin-induced declines in electrical resistance and myosin light chain (MLC) diphosphorylation. A: measurements of normalized electrical resistance across endothelial cell monolayer grown on gold microelectrodes (n = 3). Endothelial cells were rinsed with medium 199 to remove serum, incubated to stabilize basal electrical resistance, and then pretreated with either vehicle (DMSO) or U0126 (10 μM; started at arrow a), followed by treatment with either vehicle or thrombin (100 nM; started at arrow b). ERK inhibition attenuates thrombin-induced decreases in transendothelial electrical resistance. B and C: endothelial cells were pretreated with either vehicle or U0126 (10 μM, 30 min), treated with either vehicle or thrombin (100 nM, 5 min), and analyzed by Western immunoblotting using diphosphospecific MLC antibodies (n = 4). Thrombin-induced increases in the level of MLC diphosphorylation were not altered by ERK inhibition. ppMLC, diphosphorylated MLC; Error bars are means ± SD. *P < 0.05 compared with control. **P > 0.05 compared with thrombin.
after thrombin. Confluent bovine pulmonary artery endothelial cells pretreated with U0126 (10 μM, 30 min) and treated with thrombin (100 nM, 10 min) were subjected to SDS-PAGE, followed by Western immunoblotting with diphospho-MLC-specific antibodies. Significant alterations in the level of diphosphorylated MLC species in the presence of ERK inhibition were not detected (Fig. 4, B and C), suggesting that ERK activation is not likely involved in MLCK-dependent thrombin-induced endothelial cell barrier dysfunction. Control experiments using U0124 (10 μM, 30 min), an inactive analog of U0126, also failed to alter the levels of MLC diphosphorylation (data not shown).

We next addressed whether the CaM kinase II/ERK pathway is actually involved in the endothelial cell actin cytoskeletal changes that occur in response to thrombin, using immunofluorescence microscopy analysis. Inhibition of either CaM kinase II by KN-93 or ERK activation by U0126 significantly attenuates thrombin-induced actin stress fiber formation (Fig. 5), findings that are consistent with the attenuation of thrombin-induced declines in electrical resistance observed after either KN-93 (5) or U0126 pretreatment (Fig. 4). However, infection of bovine pulmonary artery endothelial cells with either constitutively active α-CaM kinase II or empty adenoviral construct did not

Fig. 5. Involvement of CaM kinase II/ERK signaling cascade in thrombin-induced stress fiber formation as visualized by immunofluorescence microscopy. Photomicrographs demonstrate endothelial cells stained for actin with Texas red-X phalloidin. Endothelial cells were pretreated with either vehicle (AA), KN-93 (AB and AD; 10 μM, 30 min), or U0126 (BB and BD; 10 μM, 30 min) and challenged with thrombin (AC and BC, AD and BD; 100 nM, 10 min). CaM kinase II as well as ERK inhibition significantly attenuates thrombin-induced stress fiber formation.

Fig. 6. Thrombin-induced translocation and CaM kinase II/ERK-dependent phosphorylation of caldesmon. A and E: endothelial cells treated with thrombin (100 nM, 10 min) were fractionated, as described in MATERIALS AND METHODS (n = 3). Thrombin induces translocation of immunoreactive caldesmon from the cytosolic to the cytoskeletal fraction. Error bars are means ± SD. *P < 0.05 compared with control. B and F: endothelial cells were labeled with [32P]orthophosphate for 2 h in phosphate-free medium (GIBCO BRL), followed by pretreatment with either vehicle, KN-93 (10 μM, 30 min), or U0126 (10 μM, 30 min). After thrombin challenge (100 nM, 10 min), caldesmon immunoprecipitation under denaturing conditions was performed, as described in MATERIALS AND METHODS (n = 4). Thrombin-induced caldesmon phosphorylation is attenuated by either CaM kinase II or ERK inhibition. Error bars are means ± SD. *P < 0.05 compared with control. **P < 0.05 compared with thrombin. C and G: endothelial cells treated with either vehicle or thrombin (100 nM, 10 min) were analyzed for ERK-dependent caldesmon phosphorylation at Ser789 using phosphospecific anti-caldesmon antibodies (n = 4). Thrombin-induced caldesmon phosphorylation at Ser789 is abolished by ERK inhibition. Error bars are means ± SD. *P < 0.05 compared with control. **P < 0.05 compared with thrombin. D and H: endothelial cells were infected with either control or recombinant adenovirus encoding constitutively active α-CaM kinase II, as described in MATERIALS AND METHODS (n = 3). Recombinant adenovirus produced sustained ERK activation and ERK-dependent caldesmon phosphorylation at Ser789 as visualized by phosphospecific anti-caldesmon antibodies. Control adenovirus did not induce either ERK activation or ERK-dependent caldesmon phosphorylation (data not shown). CaD, caldesmon; pCaD, phosphocaldesmon; moi, multiplicity of infection.
produce actin stress fiber formation (data not shown), suggesting that CaM kinase II activities are necessary but not sufficient for actin cytoskeleton remodeling. Collectively, these results indicate that thrombin-induced endothelial cell permeability involves activation of the CaM kinase II-dependent ERK signaling cascade, which modulates endothelial cell MLCK-independent actin stress fiber formation.
Role of CaM kinase II and ERK activities in thrombin-induced caldesmon phosphorylation and dissociation of caldesmon-myosin complex. We next performed differential detergent subcellular fractionation of thrombin-stimulated bovine pulmonary artery endothelial cells as described in MATERIALS AND METHODS. Thrombin (100 nM, 10 min) induced translocation of caldesmon from cytosol to membrane/cytoskeletal fractions (Fig. 6, A and E), consistent with the observed colocalization of caldesmon with stress fibers formed in nonmuscle cells (7, 55). To determine whether caldesmon represents a target for the CaM kinase II/ERK signaling pathway relevant to the thrombin model of endothelial cell permeability, confluent ³²P-labeled endothelial cells were pretreated with either KN-93 (10 μM, 30 min) or U0126 (10 μM, 30 min) and challenged with thrombin (100 nM, 10 min), followed by caldesmon immunoprecipitation. As shown in Fig. 6 (B and F), thrombin-induced caldesmon phosphorylation was attenuated by both CaM kinase II and ERK inhibition. Site-specific phosphorylation of caldesmon by ERK was tested using antibodies specific to the conserved ERK-dependent phosphorylation sites on mammalian caldesmon (Ser²⁷⁸⁹ and Ser²⁷⁸⁹, based on the human high-molecular-weight caldesmon sequence) (29). Caldesmon phosphorylation at Ser²⁷⁵ was not detected, whereas phosphorylation at Ser²⁷⁹ was induced by thrombin and abolished by U0126 pretreatment (Fig. 6, C and H), confirming phosphorylation of the low-molecular-weight caldesmon isoform by ERK in thrombin-challenged macrovascular bovine endothelium. Moreover, thrombin-induced caldesmon phosphorylation at Ser²⁷⁹ was attenuated by pretreatment with KN-93 (10 μM, 30 min) but not by pretreatment with its inactive analog KN-92 (10 μM, 30 min). In addition, CaM kinase II/ERK-dependent caldesmon phosphorylation at Ser²⁷⁹ was confirmed by endothelial cell infection with recombinant adenovirus encoding constitutively active α-CaM kinase II. Constitutively active, but not empty vector adenovirus, induced sustained ERK activation and ERK-dependent caldesmon phosphorylation at Ser²⁷⁹ (Fig. 6, D and H). Given that caldesmon phosphorylation appears important to stress fiber formation (Fig. 5), we next identified the intracellular distribution of caldesmon phosphorylated at the Ser²⁷⁹ site in resting cells and after thrombin challenge by immunofluorescence microscopic analysis. As shown in Fig. 7, in nonstimulated cells, phosphocaldesmon is diffusely distributed, with intense nucleus staining (where phospho-ERK is found; see Fig. 2). In contrast, in thrombin-stimulated endothelium, phosphocaldesmon is prominently colocalized with actin stress fibers, again consistent with a role of phosphorylated caldesmon in stress fiber formation. In smooth muscle, phosphorylation of caldesmon by CaM kinase II reverses inhibition of actin-tropomyosin-activated myosin ATPase activity and prevents the binding of myosin to caldesmon (39, 40, 47). We next verified in nonstimulated resting bovine endothelium that myosin is present in caldesmon nondenaturing immunoprecipitates (Fig. 8). Thrombin (100 nM, 10 min) produces a loss of myosin in caldesmon immunoprecipi-

Fig. 7. Role of ERK-dependent caldesmon phosphorylation in thrombin-induced stress fiber formation as visualized by immunofluorescence microscopy. Endothelial cells cultured on glass coverslips were treated with either vehicle (A and C) or thrombin (100 nM, 10 min; B and D) and used for immunofluorescence microscopy analysis. Photomicrographs show endothelial cells double stained for actin with Texas red-X phalloidin (A and B) and caldesmon phosphorylated at Ser²⁷⁹ by phosphospecific anti-caldesmon antibodies (C and D). Note increases in the amount of phosphorylated caldesmon as well as its colocalization with actin stress fibers after thrombin challenge.

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phosphorylation of threonine and tyrosine residues in a TXY motif highly specific for the ERK1/2 dual specificity MAPK kinase (MEK1/2), although other pathways of ERK activation may take place (45). In our experiments, pretreatment with U0126, a specific MEK inhibitor, abolished thrombin-induced ERK activation, indicating that the predominant pathway of ERK activation after thrombin is via phosphorylation by MEK.

In general, MAPK activity is regulated through three-tiered cascades involving MEK kinase (which may be activated by small GTP-binding proteins), MEK, and MAPK (45). We previously demonstrated that phorbol ester-induced ERK activation in endothelial cells occurs via a signaling cascade containing Ras-Raf1-MEK1/2-ERK1/2, a sequence that represents a Ca2+-independent pathway of ERK activation (50), although ERK activation via Ca2+-dependent mechanisms is also reported (1). We have also shown that thrombin induces Ca2+ elevation (19) and activation of CaM kinase II (5), a multifunctional serine/threonine kinase mediating numerous Ca2+-dependent signaling mechanisms (6). Therefore, we tested the possibility that CaM kinase II mediates thrombin-induced ERK activation in endothelial cells. Pharmacological inhibition of CaM kinase II demonstrated that ERK1/2 activation is dependent on the activation of CaM kinase II, a finding confirmed by endothelial cell infection with a constitutively active adenoviral α-CaM kinase II construct. Although the mode of action of CaM kinase II in ERK activation is largely unknown, these results suggest that Ca2+-dependent regulation of endothelial cell permeability may be mediated by CaM kinase II/ERK activation. Indeed, measurements of transendothelial electrical resistance indicate that inhibition of either CaM kinase II or ERK activation attenuates thrombin-induced decreases in electrical resistance (Ref. 5 and present study), which is consistent with the above linkage between CaM kinase II and ERK.

Our previous results demonstrated that CaM kinase II activities do not significantly alter the levels of MLC phosphorylation in thrombin-stimulated endothelial cells, supporting the putative role for CaM kinase II in MLCK-independent contractile regulation and permeability (5). Similarly, our present data fail to demonstrate significant changes in thrombin-induced MLC diphosphorylation levels due to ERK inhibition. Although we again speculate that CaM kinase II/ERK activities alter endothelial cell permeability in an MLCK-independent fashion, we cannot completely exclude any interrelationship between CaM kinase II, ERK, and MLCK activities, since it was shown that smooth muscle MLCK activity may be regulated by ERK phosphorylation (34, 38). In addition, phosphorylation of MLCK by CaM kinase II may either lead to a decrease in Ca2+-sensitivity of MLC phosphorylation (48, 49) or exert a stimulatory effect on smooth muscle force development and maintenance of tension (33, 44). Whether this posttranslational mechanism exists for the endothelial cell MLCK isoform, which we have recently defined to be modified by both Ser/Thr (17) and Tyr phosphorylation (4), is largely unknown.

**FIG. 8.** A and B: effect of CaM kinase II/ERK inhibition on thrombin-induced myosin dissociation from nondenaturing caldesmon immunoprecipitates (n = 4). Endothelial cells pretreated with either vehicle (DMSO), KN-93 (10 μM, 30 min), or U0126 (10 μM, 30 min) were challenged with thrombin (100 nM, 10 min), followed by caldesmon immunoprecipitation under nondenaturing conditions, as described in MATERIALS AND METHODS. Thrombin-induced myosin dissociation from caldesmon immunoprecipitates was reversed by either KN-93 or U0126 pretreatment. Error bars are means ± SD. *P < 0.05 compared with control. **P < 0.05 compared with thrombin.
Caldesmon is an important regulatory protein of smooth muscle and nonmuscle contraction (46) with tissue-specific localization. In smooth muscle, caldesmon is found in thin filaments, whereas in nonmuscle cells, it is a component of stress fibers (7, 55). Stress fibers have been shown to be truly contractile organelles of nonmuscle cells, containing actin, myosin, α-actinin, calmodulin, MLCK, and focal adhesion proteins (32), an environment where caldesmon may exert its structural and functional properties, such as actomyosin cross-linking and regulation of actin-activated myosin ATPase activity. Assembled stress fibers are presumed to be under tension (31), thus exhibiting actomyosin interaction. Therefore, during stress fiber formation, caldesmon should be preferably recruited in phosphorylated or in Ca^{2+}/calmodulin-bound form, which is in accordance with our data demonstrating caldesmon phosphorylation and its translocation to the cytoskeletal fraction after thrombin treatment. Thrombin-stimulated endothelial cells show prominent stress fiber formation, leading (as proposed) to increased permeability via cell contraction and formation of intercellular gaps (11, 15). Our results demonstrate that inhibition of CaM kinase II/ERK activities partially attenuates thrombin-induced permeability as well as thrombin-induced stress fiber formation, indicating the important role for CaM kinase II and ERK in stress fiber formation and endothelial cell permeability. These results as well as colocalization of phosphocaldesmon within stress fibers also suggest the importance of CaM kinase II/ERK-dependent phosphorylation of caldesmon for stress fiber assembly.

It has been previously demonstrated that phosphorylation of smooth muscle caldesmon by CaM kinase II occurs at multiple sites, including NH_{2}- and COOH-terminal sites, which leads to disinhibition of actin-activated myosin ATPase activity and prevents caldesmon-myosin interaction (39, 40, 47). It has been suggested that phosphorylation of caldesmon within the strong myosin-binding NH_{2}-terminal domain (35, 37, 46, 51) results in steric changes that prevent caldesmon-myosin interaction, thereby allowing actomyosin cross-bridge cycling to occur. We propose that these properties facilitate recruitment of caldesmon phosphorylated by CaM kinase II into the forming stress fiber, whereas inhibition of CaM kinase II should have the opposite effect. ERK-dependent phosphorylation of mammalian caldesmons occurs within highly conservative sequences at two major sites, Ser^{759} and Ser^{789} (2, 9), based on the numbering of human high-molecular-weight caldesmon (29), although additional ERK-dependent phosphorylation sites have not been excluded (9). Our results suggest that ERK-dependent phosphorylation sites are important for caldesmon-myosin interaction. The relatively weak myosin-binding site that exists in the COOH-terminal part of caldesmon (28, 56) contains the known ERK-dependent phosphorylation sites and thus may affect caldesmon-myosin interaction within the COOH terminus. In a manner analogous to caldesmon-myosin interaction within the NH_{2}-terminal domain, dissociation of myosin from the COOH-terminal portion of the caldesmon molecule should potentiate actomyosin interaction, whereas ERK inhibition should reverse this effect. The role of ERK-dependent caldesmon phosphorylation in the regulation of actomyosin interaction, however, is controversial (10, 21, 22, 24, 41, 52) with potential variables, including different laboratory techniques, tissue and species specificity, source of enzyme, or different inhibitory activities of caldesmon, depending on its phosphorylation status. In addition, because the localization of ERK-dependent phosphorylation sites is within the relatively weak COOH-terminal myosin-binding site, the effect of ERK-dependent caldesmon phosphorylation on actomyosin interaction should be less than the phosphorylation of amino acid residues that affect the NH_{2}-terminal myosin-binding site. Nevertheless, together, these studies provide further mechanistic evidence for the regulatory role for CaM kinase II/ERK signaling cascade in the thrombin model of endothelial cell permeability and implicate novel MLCK-independent mechanisms of nonmuscle cytoskeletal regulation via CaM kinase II/ERK-dependent stress fiber formation and caldesmon phosphorylation.

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