Regulation of Thrombin-Mediated Endothelial Cell Contraction and Permeability

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ABSTRACT **A variety of physical forces exist in a dynamic equilibrium in the vascular endothelium (EC) monolayer and serve to maintain EC responsiveness while preserving the integrity of the EC monolayer and barrier properties. Thrombin has potent effects on EC permeabilities disrupting the equilibrium between tethering forces (cadherins, focal adhesion plaque) and forces that increase centripetal tension primarily via myosin light chain (MLC) phosphorylation. Like other EC effects, thrombin-induced MLC kinase (MLCK) activation is dependent upon receptor proteolysis, Ca^{2+} mobilization, and activation of protein kinase C (PKC). While EC gap formation is central to barrier dysfunction and dependent upon activation of MLCK, (which phosphorylates MLC) an obligatory event in smooth muscle cell contraction, little is known regarding the events that reverse inflammatory responses, halt the contractile response, and initiate relaxation. However, as these events likely include MLC dephosphorylation, further examination of the processes that regulate MLC protein phosphatase activity, focal intercellular junctions, and extracellular matrix adhesions is needed. These investigations should yield new information as to how receptor occupancy is transduced into specific cellular responses, such as increased permeability, which promotes pathological vascular processes such as tissue edema formation and organ dysfunction.**

Keywords: **Thrombin, permeability, myosin light chain kinase, protein phosphatase, focal adhesion kinase**

Investigations into the interaction of the multifunctional serine protease thrombin with the vascular endothelium (EC) has revealed an exciting panorama of cellular responses that have increased our basic understanding of the endothelium. Over the past decade important pro- and anticoagulant properties as well as pro- and anti-inflammatory properties of the activated endothelium have been delineated. In this brief review, we focus upon proinflammatory signal-transducing events evoked by thrombin, which result in the formation of intercellular gaps coupled to the loss of a semiselective vascular barrier to circulatory proteins (i.e., EC barrier dysfunction).

IN VIVO AND IN VITRO EFFECT OF THROMBIN ON VASCULAR PERMEABILITY

Our laboratory has extensively studied the development of tissue edema *in vivo* utilizing a sheep lung lymph model of high-permeability pulmonary edema.[1–3] We have extended our initial *in vivo* observations to an *in vitro* model of EC permeability,[4–6] to demonstrate that thrombin reversibly increases albumin clearance across confluent bovine pulmonary EC monolayers.[4] Vascular endothelium barrier dysfunction that was elicited by thrombin was found to correlate with actin microfilament redistribution indicative of contraction and intercellular gap formation.[3–8] These observations led to detailed examination of signal-transducing mechanisms involved in thrombin-induced EC activation from the initial interaction with its specific cell receptor, to the intermediate second messenger–mediated activation of the contractile apparatus, and finally to the terminal events (i.e., gap formation and barrier dysfunction).

Thrombin Receptor Proteolysis

Thrombin-induced EC activation requires thrombin to maintain active proteolytic activity.[3,5,6,9,10] and (as discussed elsewhere in this issue) occurs through a novel mechanism involving proteolysis at a critical arginine

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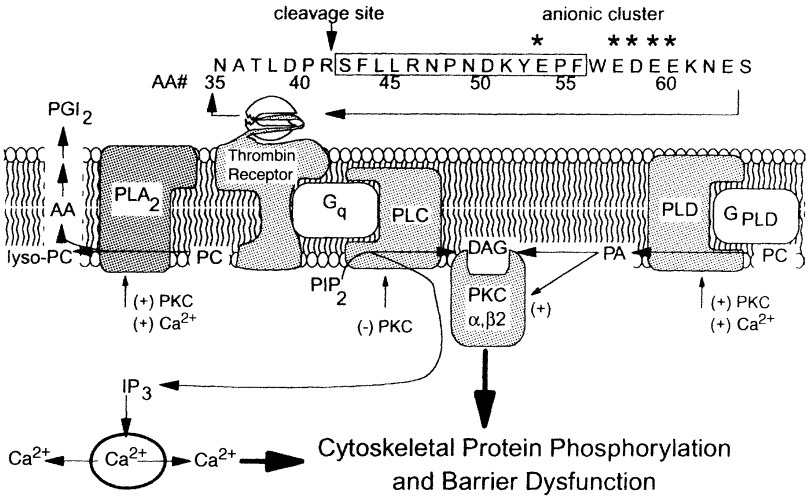

FIG. 1. Potential pathways of endothelial cell activation by thrombin. Proteolysis of the thrombin receptor by thrombin occurs at a critical arginine residue (R41), generating a new NH_2 terminus that functions as a tethered ligand. The boxed amino acids correspond to the amino acid sequence of TRAP 14, the synthetic thrombin receptor–activating peptide. The schema indicates that at least three membrane phospholipases are now recognized to be activated by thrombin receptor cleavage. Receptor proteolysis is coupled by a bacterial toxin-insensitive G protein (Gq), leading to PI-phospholipase C (PLC)–catalyzed hydrolysis of PIP_2 and subsequent production of DAG and inositol triphosphate (IP_3). The Ca^{2+} secretagogue IP_3 mobilizes Ca^{2+}, and DAG activates protein kinase C (PKC). Although PKC exerts feedback inhibition of PLC, an increase in $[Ca^{2+}]_i$ and PKC activation are essential to thrombin-induced phospholipase A_2 and phospholipase D hydrolysis of membrane phosphatidylcholine (PC) generating the release of free arachidonate (AA) and phosphatidic acid (PA). Phosphatidic acid potentially functions as an intracellular second messenger to directly activate PKC[49] or is hydrolyzed by phosphatidic acid phosphatase to DAG, producing sustained activation of PKC. An increase in PKC activity and the rise in $[Ca^{2+}]_i$ are also critical events that participate in thrombin-induced cytoskeletal protein phosphorylation, reorganization of the contractile apparatus with contraction, and barrier dysfunction.

(R^{41}) residue (Fig. 1) of the extracellular portion of the seven transmembrane domain thrombin receptor.[10] We have shown that synthetic thrombin receptor-activating peptides corresponding to the initial 5– to 14–amino acid residues (SFLLR ...) of the new NH_2-terminus of the human thrombin receptor are sufficient to increase Ca^{2+}, prostaglandin I_2 (PGI_2) synthesis, and platelet-derived growth factor mRNA expression in human EC, and to induce barrier dysfunction in both bovine and human EC.[10,11] This suggests that the cleaved thrombin/receptor is coupled by a G protein to phospholipase C (PLC)-catalyzed production of the protein kinase C (PKC) activator, DAG, and the Ca^{2+} secretagogue inositol triphosphate (IP_3)[3,12,13] and functions as a tethered ligand to induce signaling events involved in EC barrier dysfunction[10] (Fig. 1).

ROLE OF Ca^{2+}, PKC, AND cAMP IN EC BARRIER DYSFUNCTION

Thrombin-induced PKC activation and $Ca^{2+}{}_i$ mobilization occur in less than 1 minute.[8,14,15] Both signaling responses are essential for hydrolysis of phosphatidylcholine, generation of arachidonate and phosphatidic acid[9,15] and to thrombin-induced barrier dysfunction (Fig. 1).[3,7,14–18] We recently characterized the presence of both Ca^{2+}-sensitive and -independent PKC isotypes in EC by Western blot analysis. Bovine EC primarily contains α and β_2 PKC isotypes with smaller quantities of ξ and δ. Human EC is similar, except that δ is undetectable. After phorbol 12-myristate 13-acetate (PMA) stimulation, both α and β_2 translocate rapidly to the plasma membrane and nucleus and are profoundly downregulated by 18-hour PMA, while ξ and δ are neither translocated nor downregulated. Activation of PKC by PMA does not increase $[Ca^{2+}]_i$[5,15] but produces a 2- to 3-fold increase in bovine pulmonary artery endothelial cell (BPAEC) permeability compared to a 5-fold increase with thrombin.[7,8] The Ca^{2+} ionophores, A23187 and ionomycin, enhanced PMA-induced barrier dysfunction (5-fold increase), suggesting both PKC and Ca^{2+} are required for the full thrombin effect.[7,11] Both thrombin and PMA induced time- and PKC-dependent EC contraction and phosphorylation of the actin-, myosin-, and Ca^{2+}/calmodulin (CaM)-binding protein caldesmon$_{77}$ as well as the intermediate filament, vimentin, *in situ* and *in vitro*.[8] These studies suggest a central role of PKC in EC gap formation and barrier dysfunction.

Cholera toxin regulates adenylate cyclase activity in EC via adenosine diphosphate (ADP) ribosylation of G_s, a stimulatory G protein.[9,11,19] In contrast to its augmentation of thrombin-induced phospholipase D activity,[9] cholera toxin pretreatment reduced basal albumin clearance, significantly attenuated α-thrombin- and PMA-mediated barrier dysfunction, and abolished EC gap formation.[11,19,20]

The adenylate cyclase activator, forskolin or cyclic adenosine monophosphate (cAMP) analogues were also protective,[7,11] suggesting that cAMP/protein kinase A (PKA) modulates thrombin-induced EC contraction gap formation and barrier dysfunction. Cyclic adenosine monophosphate inhibition of thrombin-mediated EC permeability, like histamine,[21,22] does not involve decreased IP_3 production, reduced $[Ca^{2+}]_i$, or inhibition of PKC activity.[11]

ROLE OF MYOSIN LIGHT CHAIN PHOSPHORYLATION IN EC PERMEABILITY

Bioactive agonists, such as thrombin, increase EC permeability via a diffusive pathway with increased paracellular transport (i.e., solute exchange occurs primarily at intercellular junctions). Actin and myosin, which comprise ~16% of EC proteins,[8,23] are involved in maintaining the flattened EC morphology, in EC resistance to sheer stress, and in control of the paracellular pathway of EC permeability by modulating EC gap formation. Increased EC centripetal forces (promoting gap formation) occur via the cyclical interaction of myosin with actin with the myosin monomer composed of two heavy chains ~200 kDa each, and two pairs of light chains of 20 kDa (MLC_{20}) and 17 kDa (Fig. 2). The NH_2-terminal half of myosin forms the globular heads, each containing the adenosine triphosphate (ATP)- and actin-binding site that mediates the actin-activated Mg-ATPase activity of myosin.[24] The CO_2H-terminal half of myosin forms a dimeric coiled helical rod that self-associates to form bipolar filaments. A rise in $[Ca^{2+}]_i$ is required for the CaM-dependent activation of myosin light chain kinase (MLCK),[24] whose phosphorylation of MLC_{20} initiates contractile activity by the movement of actin and myosin filaments past one another. In permeabilized bovine EC, ATP, Ca^{2+}, MLCK, and CaM were absolute requirements for EC retraction/contraction that was accompanied by MLC phosphorylation.[25,26] In "skinned" porcine EC monolayers and in isolated permeabilized sheers of human EC, gap formation required ATP and occurred only when free myosin binding sites were available on actin filaments.[23] Bovine EC displays constitutive wrinkling of silicone rubber matrix and further wrinkles when cells are exposed to vasoactive mediators,[21,23] again indicating the presence of dynamic centripetal tension. Thus, EC gap formation involves actomyosin-dependent contractile mechanisms similar to smooth muscle where MLC phosphorylation correlates with the initial rate of tension development. The level of MLC_{20} phosphorylation in EC has been suggested to reflect the amount of isometric tension exerted on the underlying extracellular matrix.[27]

THROMBIN-MEDIATED MYOSIN LIGHT CHAIN PHOSPHORYLATION IN BOVINE AND HUMAN EC

With this background in mind, we hypothesized that thrombin-mediated EC gap formation and barrier dysfunction are the consequences of MLCK-mediated MLC_{20} phosphorylation and subsequent contraction.[7,11] After successfully separating phosphorylated from nonphosphorylated MLC_{20} by urea gel electrophoresis combined with Western blotting with a MLC-specific antibody,[3,5] as well as by 2-dimensional gel electrophoresis,[5] we confirmed that in both bovine and human EC, MLC_{20} is constitutively phosphorylated with ~0.4 mol phosphate/

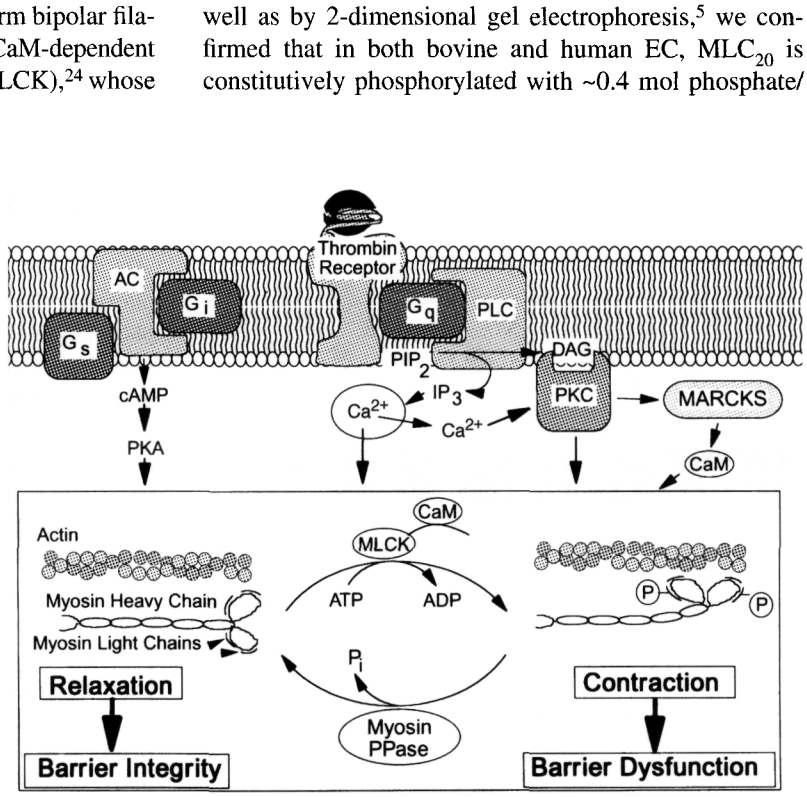

FIG. 2. Postulated mechanisms of endothelial cell permeability. Current concepts of agonist-induced vascular permeability invoke an increase in vascular endothelium (EC) gap formation, which allows enhanced paracellular transport. Cytosolic Ca^{2+}, via the Ca^{2+}-binding protein calmodulin (CaM), activates Ca^{2+}-CaM–dependent myosin light chain kinase (MLCK). Modeling events in smooth muscle cells and phosphorylation of myosin light chains may initiate endothelial cell contraction by promoting actin-myosin interaction and cross-bridge cyclin and subsequent increases in EC permeability. In this scheme, MLC dephosphorylation by myosin-specific phosphatases (PPase) promote actomyosin dissociation, cellular relaxation, and restoration of barrier function.

mol MLC_{20}.[7,27] Both thrombin[7] and histamine[27] increase MLC_{20} phosphorylation (predominantly present as diphosphorylated MLC), which peaks at 60 seconds with 60% to 80% of MLC species phosphorylated.[7,11] By 30 minutes these have returned to control levels, indicating either subsequent inactivation of MLCK, significant induction of myosin protein phosphatase (PPase) activity, or both. Thrombin has further been shown to increase EC centripetal tension from 0.65 to 1.3×10^5 dyne/cm^2,[28] and the increase in MLC_{20} phosphorylation and isometric tension is consistent with the hypothesis that activation of signal transduction pathways mediates an increase to a new level of "latch state" isometric tension through the cytoskeleton. These effects of thrombin on bovine or human EC were mimicked by the 7–amino acid thrombin receptor activating peptide, TRAP-7, with substantial dose-dependent rises in diphosphorylated MLC (Fig. 3).

The kinetics of these key thrombin-mediated cellular effects indicate that a prompt rise in $Ca^{2+}{}_i$ precedes maximal MLC phosphorylation and subsequent decreases in electrical resistance as well as increases in monolayer permeability, which plateaus by 10 minutes.[7] The kinase responsible for the rapid rise in the level of phosphorylated MLC is undoubtedly MLCK, as independent pretreatment with the intracellular Ca^{2+} chelator, BAPTA-AM,[7] the MLCK inhibitors ML-7 or KT 5926, or two antagonists of the Ca^{2+}/CaM signal-transducing pathway, W-7 or trifluoperazine, significantly attenuated thrombin-induced MLC phosphorylation in BPAEC and HUVEC.[7] In companion experiments, EC pretreated with MLCK inhibitor KT 5926, or 100 μM TFP (30 minutes) also attenuated the thrombin-induced permeability response,[7] providing further strong evidence that thrombin-mediated, Ca^{2+}-dependent MLC phosphorylation via MLCK is operational in the development of gap formation and subsequent barrier dysfunction.

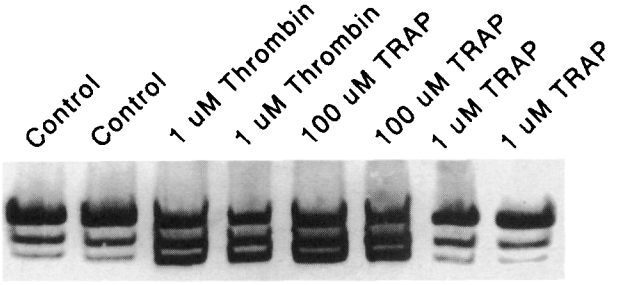

FIG. 3. Effect of thrombin and thrombin receptor–activating peptide (TRAP) on myosin light chain (MLC) phosphorylation in bovine pulmonary artery endothelial cell monolayers. Shown is an immunoblot of MLC separated on urea gel electrophoresis. Confluent endothelial cells were treated with either vehicle (lanes 1 and 2), 1 μM thrombin for 2 minutes (lanes 3 and 4), 100 μM TRAP-7 (lanes 5 and 6), or 1 μM TRAP-7 (lanes 7 and 8) for 2 minutes. Thrombin caused a dramatic shift in MLC from the nonphosphorylated to the phosphorylated species. This effect was mimicked by TRAP in the dose-dependent manner.

ROLE OF PKC/PKA IN REGULATION OF THROMBIN-REDUCED MLC PHOSPHORYLATION

Unlike smooth muscle cells and human platelets,[29,30] PMA (2 to 30 min) failed to alter EC MLC_{20} phosphorylation.[7] Although MLC phosphorylation was not increased by PMA, we next determined whether PKC activation by thrombin may participate in the regulation of MLC phosphorylation. Protein kinase C activity was inhibited with either H-7 or staurosporine or by PKC downregulation via prolonged PMA treatment (24 h, 100 nM), strategies that accomplish PKC inhibition in EC[14,15] and attenuate thrombin-induced barrier dysfunction.[3,8,18] Both strategies resulted in ~50% inhibition of the thrombin-induced MLC phosphorylation observed at 2 minutes, suggesting a role for PKC in regulating MLCK activity elicited by thrombin.[7] Cholera toxin partially blocked constitutive and thrombin-induced MLC phosphorylation (40% inhibition),[7,11] and additional agents that increase cAMP had similar inhibitory effects on MLC phosphorylation.[7,11] These data suggest that thrombin is a potent activator of EC MLCK and this activation is regulated by PKC and PKA. Although the actual mechanism of PKC regulation of MLCK is unknown, thrombin produces a time-dependent phosphorylation of the p81 CaM-binding protein, myristoylated alanine-rich C kinase substrate (MARCKS) in HUVEC.[31] When CaM-bound MARCKS is phosphorylated by PKC *in vitro*, CaM is released and becomes available to CaM-dependent MLCK,[32] thus facilitating MLCK activity. Recently, we have discovered a high-molecular-weight MLCK isoform in embryonic tissues and have cloned the isoform present in cultured endothelium.[33] Further structure/function studies of this enzyme will reveal significant information regarding its regulation by serine, threonine, and tyrosine kinases.

MYOSIN-SPECIFIC PPase ACTIVITY IN EC

Myosin light chain kinase deactivation/MLC dephosphorylation rapidly follows maximal MLC phosphorylation induced by thrombin,[7] with MLC dephosphorylation nearly complete by 30 minutes. To further assess EC Ser/Thr PPase activities, control and thrombin-stimulated (10 min) BPAEC homogenates were added to a reaction mixture containing either ^{32}P-phosphorylase A or ^{32}P-MLC. Increasing the dilution (10- to 100-fold) of either control or thrombin stimulated homogenates in the mixture produced an increase in PPase-specific activity (maximal at 1:250 dilution) reflecting dilution of endogenous PPase inhibitors.[34] The addition of calyculin (inhibitor of $PPases_{1 \text{ and } 2A}$) resulted in >95% dose-dependent inhibition of total PPase activity using either ^{32}P-phosphorylase A or ^{32}P-rabbit skeletal muscle MLC as PPase substrates. In contrast, when okadaic acid was added to EC homogenates, only 30% inhibition of total ^{32}P-phosphorylase A

PPase activity was observed at concentrations specific for $PPase_{2A}$ (<10 nM).[35] This suggests that ~70% of total phosphorylase A PPase activity in EC is due to type 1 PPase activity. The contention that the PPase responsible for myosin dephosphorylation is likely a type 1 PPase and not a type 2A[36,37] is further supported by two additional observations from our laboratory. The first observation was that immunoreactive type 2A PPase, but not type 1 PPase, was virtually absent from a myosin-enriched pellet fraction, whereas both PPases were readily observed (by Western blotting) in the myosin-depleted supernatant fraction.[35] Our second observation was that up to 100 nM okadaic acid, a concentration that preferentially inhibits type 2A PPase activity, had no effect on either basal or thrombin-induced phosphorylation, permeability, and intercellular gap formation, whereas calyculin A produced dose- and time-dependent increases in both basal and thrombin-induced MLC phosphorylation and albumin permeability and significantly increased EC gap formation[38] (Table 1). Finally, although further studies are needed, we failed to observe significant induction of PPase activity in thrombin-stimulated EC homogenates when compared to controls. This suggests that the rapid dephosphorylation of MLC that occurs after thrombin may be more the consequence of MECK deactivation combined with high endogenous PPase activity. However, further studies are needed to fully characterize PPase regulation of myosin dephosphorylation and structure/function activities.

EFFECT OF THROMBIN ON EC "TETHERING" FORCES

In opposition to permeability-promoting contractile forces produced by MLC phosphorylation, the endothelium maintains semiselectivity to macromolecule permeation and resistance to paracellular transport via several distinct modalites that can be modulated by bioactive agents such as thrombin. Table 2 lists potential mechanisms by which thrombin produces EC barrier dysfunction. In addition to contraction, a second potential mechanism is transcellular vasicular transport, which is reported in experimental data but unlikely to be a major contributor to EC permeability.[39,40] A third potential contributory mechanism may be an alteration in the highly negatively charged glycocalyx, which provides electrostatic repelling of protein-cell interactions. A fourth mechanism by which EC barrier dysfunction may occur in response to thrombin is via alterations in the apparent tight junctions that exist between the confluent endothelium and are similar to Z0-1 (zone occludens)–like connections observed in tight epithelial junctions. Adherens junctions are mediated by specialized interjunctional adhesive proteins known as cadherins that form homotypic connection between ECs and are anchored to the actin cytoskeleton.[41,42] Cadherins with homology to N-cadherin and P-cadherin have been identified in endothelium.[43] Optimal cadherin function is Ca^{2+}-dependent, as Ca^{2+} chelation of the extracellular environment with EGTA or intracellular Ca^{2+} chelation with BAPTA produces significant EC gap formation and barrier dysfunction.[7] In addition, antibodies directed against N-cadherin, a predominant endothelial cadherin, blocked the restoration of endothelial integrity in a calcium-repletion assay.[44] Finally, focal adhesions, which exert a similar "tethering" force between the cell and the extracellular matrix (ECM), represent another potential site of thrombin-evoked permeability responses[45] (Fig. 4). In these structures, the role of transmembrane receptors for ECM proteins is performed by integrins in EC basolateral membranes that are linked to the actin-based cytoskeleton by the focal adhesion plaque, a complex structure that includes the actin-binding proteins vinculin, talin, paxillin, and α-actinin.[46] In addition to these structural proteins, a variety of important cellular enzymes, including **p125fak** protein kinase, colocalize with focal adhesion plaques.[47] Prior work from our laboratory has focused upon the focal adhesion plaque as a putative site of gap formation. These studies indicate that thrombin induces a rapid and profound rearrangement of focal adhesion plaques as detected by vinculin immunofluorescence[48,49] in association with increased FAK antiphosphorylation. The signaling pathways evoked by thrombin to produce these changes are unclear. Significant changes in the level of serine/threonine phosphorylation of vinculin, talin, or paxillin were not observed.[50] However, significant tyrosine kinase or phosphatase activities may participate in the reorganization of the focal adhesion plaque elicited by other bioactive agonists.

TABLE 1. Effect of Phosphatase Inhibitors on the Contractile Responses of Intact Endothelial Cells[35]

	Calyculin (10 nM, 1 h)	*Okadaic Acid (100 nM, 1 h)*
Myosin light chain phosphorylation	Greater than 3-fold increase	No effect
Permeability	Greater than 3-fold increase	No effect
Gap formation	Increased	No effect

TABLE 2. Potential Mechanisms of Endothelial Cell Barrier Dysfunction Induced by Thrombin

1. Increased contractile forces
2. Increased transcellular vesicular permeability
3. Alteration in membrane electrostatic charges
4. Decreased intercellular junctional connections
5. Decreased endothelial cell–extracellular matrix adhesive forces

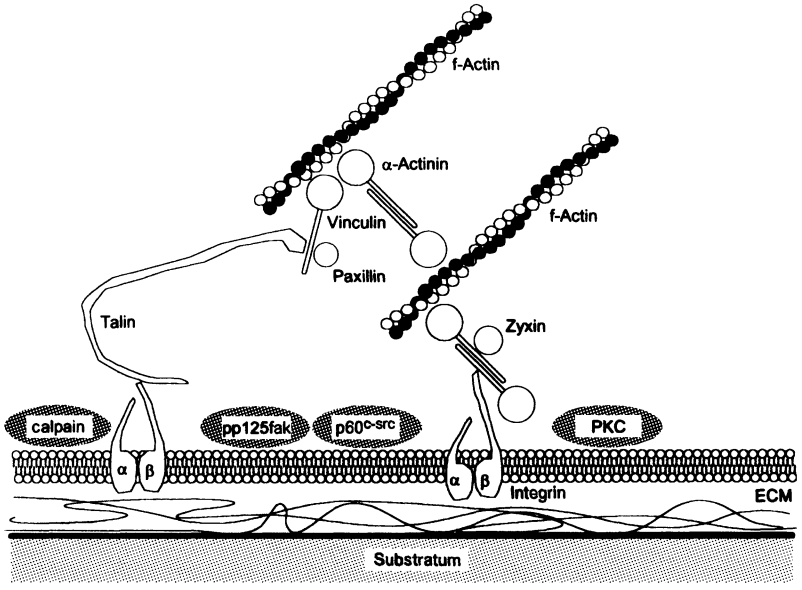

FIG. 4. Schematic representation of the arrangement of proteins within the focal adhesion plaque. The focal adhesion plaque is a cytoplasmic protein complex comprised of such proteins as talin, vinculin, α-actinin, paxillin, and zyxin that mediates the linkage of integrin extracellular matrix receptors to the filamentous f-actin cytoskeleton. The importance of these structures in cellular signaling and organization is suggested by the colocalization of several important cellular signaling proteins, including protein kinase C (PKC), calcium-dependent protease (calpain, and the tyrosine kinases pp60c-src and focal adhesion kinase (p125fak), with the focal adhesion plaque complexes.

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