Protein phosphatase 2B inhibitor potentiates endothelial PKC activity and barrier dysfunction

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Protein phosphatase 2B inhibitor potentiates endothelial PKC activity and barrier dysfunction. Am J Physiol Lung Cell Mol Physiol 281: L546–L555, 2001.—Serine/threonine (Ser/Thr) protein phosphatases (PPs) are implicated in the regulation of vascular endothelial permeability (4, 38, 44, 45), although the precise cellular and molecular mechanisms are not well understood. Treatment of endothelial cells with inhibitors of PP1 and PP2A (i.e., okadaic acid and calyculin A) resulted in increases in endothelial permeability that were accompanied by increased myosin light chain (MLC) phosphorylation (44). The increased MLC phosphorylation is believed to promote endothelial contraction, which provides the increased centripetal tension required to decrease endothelial cell-cell adhesion, resulting in increased permeability. However, in one study (4), the okadaic acid- or calyculin A-mediated increased endothelial permeability occurred without a concomitant increase in MLC phosphorylation, suggesting that the Ser/Thr PPs may also regulate endothelial barrier function independent of MLC phosphorylation. Furthermore, in human epidermal cells, these Ser/Thr PP inhibitors induced hyperphosphorylation of β-catenin, an adherens junction-associated protein, which was accompanied by loss of cell-cell contact (37). Although these studies suggest that Ser/Thr PPs promote endothelial barrier function, it remains unclear whether Ser/Thr PPs play a physiological role in limiting endothelial barrier dysfunction caused by inflammatory mediators.

A potential target of Ser/Thr PPs that may be important in endothelial barrier function regulation is the family of protein kinase C (PKC) isoforms. It is known that at least for the classic PKC-α and PKC-β (6, 15, 22), and possibly for the atypical PKC-ζ (41), phosphorylation controls their intrinsic catalytic activity such that their dephosphorylation results in increased kinase activity. Purified Ser/Thr PPs (i.e., PP1 and PP2A) have been shown to inhibit recombinant PKC-α activity (34). In various experimental models with intact cells, okadaic acid inhibited dephosphorylation of the PKC-α, PKC-ζ, and PKC-λ isoforms, resulting in increased phosphotransferase activity of the isoforms (14, 43). At present, little is known...
regarding the regulation of PKC by Ser/Thr PPs in endothelial cells.

The PKC signaling pathway is a well-documented mechanism in the regulation of mediator-induced increases in vascular endothelial permeability as demonstrated in several experimental models (19, 24, 25, 28). The PKC family consists of at least 12 known Ser/Thr protein kinases, which are classified primarily by structure, function, and cofactor requirements for activation. These isoforms are distributed to different intracellular sites, are selectively sensitive to PKC inhibitors and downregulation, and have different selectivity for substrates, suggesting that the isoforms are function specific (20, 31). Our laboratory (29, 48) and others (2, 9) have shown that the classic PKC-α and -β isoforms, which are the primary Ca2+ -dependent isoforms expressed by endothelial cells, may comprise the more important isoforms in the regulation of endothelial permeability. The novel PKC-ε and the atypical PKC-ζ and -λ isoforms are also present in endothelial cells (Lum, unpublished observations); however, their role in barrier function regulation has not been defined.

In this study, we investigated the role of Ser/Thr PPs in the regulation of PKC in endothelial cells and the relationship to mediator-induced endothelial barrier dysfunction. PKC activation was determined by phosphotransferase activity and phosphorylation of PKC-α and -β isoforms, and transendothelial resistance was measured as an index of barrier function. The results indicate that the PP2B inhibitor FK506 (but not okadaic acid and calyculin A) potentiated the thrombin-induced increase in PKC phosphotransferase activity and phosphorylation of the PKC-α isoform in BPMECs. FK506 also inhibited the recovery of the thrombin-mediated decrease in transendothelial resistance. Downregulation of PKC rescued the FK506-mediated inhibition of the recovery. The findings support the thesis that PP2B may play an important physiological role in the recovery of endothelial barrier dysfunction to normal, which may occur through the regulation of PKC in endothelial cells.

METHODS

Cell Culture

BPMECs (Vec Technologies, Rensselaer, NY) were maintained in culture in MCDB 131 medium supplemented with 10% fetal bovine serum, 10 ng/ml of human epidermal growth factor, 1 µg/ml of hydrocortisone, 90 ng/ml of heparin, and antibiotics. BPMECs, obtained at a population doubling of 10, were grown and subcultured to population doublings between 18 and 22 for study. The cells incorporated acetylated low-density lipoprotein and exhibited the expected morphological endothelial phenotype.

PKC Activity

Cell collection. BPMECs were grown in six-well dishes and treated according to the experimental protocol. All subsequent steps were carried out on ice with ice-cold reagents. The cells were washed with phosphate-buffered saline (PBS) and collected by scraping in extraction buffer (0.02 M Tris, 0.5 mM EDTA, and 0.5 mM EGTA) containing protease inhibitors (2.5 mM phenylmethylsulfonyl fluoride, 25 µg/ml of leupeptin, and 25 µg/ml of aprotinin) and 10 mM 2-mercaptoethanol. The collected samples were frozen in liquid N2 until the assay was performed. Samples were lysed by two repeated freeze-thaw cycles and centrifuged for 20 min at 20,000 g at 4°C, and the supernatant was collected as the cytosolic (soluble) fraction. The pellet was resuspended in extraction buffer plus 0.3% Triton X-100, homogenized with a microfuge pestle, and incubated on ice for 30 min with periodic vortexing. The samples were centrifuged for 10 min at 20,000 g at 4°C, and the supernatant was collected as the membrane (particulate) fraction.

Phosphotransferase activity. Phosphotransferase activity of the cell fractions was determined by incorporation of 32P into the PKC consensus peptide substrate (16). The reaction was performed by adding 33 µM unlabeled ATP plus [γ-32P]ATP to the samples in the presence of the specific PKC peptide substrate [Ser25]PKC19–31 (0.1 mg/ml), 0.01% phosphatidyserine, 0.01% dioleylglycerol, 5.4 mM MgCl2, 20 mM Tris base, and 20 mM CaCl2. The mixture was incubated for 5 min before being quenched with ice-cold 75 mM H3PO4. Basal levels of activity were determined by adding the reaction mixture to identical samples with H3PO4 already present. Samples were vacuum filtered through ion-exchange cellulose disks and counted in a scintillation counter. Values were calculated as specific PKC activity (in pmol 32P incorporated·min⁻¹·mg protein⁻¹).

Phosphorylation of PKC

BPMECs were grown to confluence in 60-mm culture dishes and washed twice with warm phosphate-free DMEM. The cells were preloaded with [32P]orthophosphoric acid (0.3 mCi/ml) in the same medium for 3 h at 37°C and treated according to experimental protocol. The reaction was stopped by placing the dishes on ice and immediately washing them two times with ice-cold Ca2+- and Mg2+-free PBS, and the cells were collected by scraping in extraction buffer (in mM: 150 NaCl, 1 EDTA, 1 EGTA, 50 Tris-Cl (pH 7.4), 1 NaF, 1 sodium vanadate, 1 pepstatin A, and 1 phenylmethylsulfonyl fluoride; 1% Nonidet P-40; 0.25% sodium deoxycholate; 25 µg/ml of leupeptin; and 25 µg/ml of aprotinin). The cell extract was aspirated through a 21-gauge needle to further disrupt the cells and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was precleared with 1.0 µg of mouse IgG and protein A/G PLUS-agarase for 1 h at 4°C. The precleared lysate (400 µg) was incubated with 0.5 µg of affinity-purified anti-PKC-α or PKC-β monoclonal antibody (MAB) for 1 h at 4°C on a rocker, protein A/G PLUS-agarase was added to each sample, and the incubation was continued overnight. Negative controls were samples without the added primary MAB. The immunocomplex of proteins was collected by centrifugation at 2,500 rpm for 5 min at 4°C, washed four times with PBS, and resuspended in 1× electrophoresis buffer. The samples were boiled for 5 min and centrifuged briefly, and the supernatant was collected for SDS-PAGE as described in Western Blot Analysis. The gel was dried overnight with gel drying film (Promega, Madison, WI) and analyzed with the STORM phosphorimaging system (Molecular Dynamics, Sunnyvale, CA).
Western Blot Analysis

BPMECs were grown to confluence in 60-mm culture dishes. After the experimental treatment, the cells were washed twice with PBS and collected in extraction buffer as described in PKC Activity, and the protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit with bovine serum albumin as a standard (Pierce, Rockford, IL). The cell lysates were loaded at constant protein concentrations, separated by SDS-PAGE in 10% acrylamide, and electrophoresed to a nitrocellulose or polyvinylidene difluoride membrane. The nonspecific binding of antibodies to the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T). The blocked membrane was then incubated with an affinity-purified MAb diluted in TBS-T with 1% nonfat dry milk overnight at 4°C in a rocker. The blot was washed five times with TBS-T and incubated with goat anti-mouse IgG secondary antibody conjugated with horseradish peroxidase. The bands were detected with the enhanced chemiluminescence kit. Specificity of the protein bands was confirmed with protein standards, control cell lysate, or the peptide immunogen as a negative control.

Transendothelial Electrical Resistance

Transendothelial electrical resistance, an index of endothelial barrier function, was determined in real time with the electric cell-substrate impedance sensor (ECIS) system (Applied BioPhysics, Troy, NY) (11, 23, 27). The system consisted of a large gold-plated electrode (2 cm²) and five smaller gold-plated electrodes (10⁻³ cm²) with a 400-µl well fitted above each small electrode. The small and large electrodes were connected to a phase-sensitive lock-in amplifier, and an alternating current was supplied through a 1-MΩ resistor. The blocked membrane was then incubated with an affinity-purified MAb diluted in TBS-T with 1% nonfat dry milk overnight at 4°C in a rocker. The blot was washed five times with TBS-T and incubated with goat anti-mouse IgG secondary antibody conjugated with horseradish peroxidase. The bands were detected with the enhanced chemiluminescence kit. Specificity of the protein bands was confirmed with protein standards, control cell lysate, or the peptide immunogen as a negative control.

Materials

Reagents. Unless indicated, reagents were purchased from Sigma (St. Louis, MO). MCDB 131 medium, DMEM, penicillin-streptomycin, PBS, and Hanks’ balanced salt solution were purchased from Gibco BRL (Life Technologies, Gaithersburg, MD); fetal bovine serum was from HyClone (Logan, UT); the enhanced chemiluminescence kit and [32P]orthophosphoric acid were from Amersham Life Sciences (Arlington Heights, IL); and [γ-32P]ATP was purchased from NEN-DuPont (Wilmington, DE). The BCA kit was from Pierce (Rockford, IL), and protein A/G PLUS-agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). FK506 was purchased from BIOMOL (Plymouth Meeting, PA), and calyculin A and okadaic acid were from Calbiochem (San Diego, CA).

Antibodies. Anti-human PKC-α, PKC-β, PP1α catalytic subunit, PP2A catalytic subunit α, and PP2B catalytic subunit A MAb were purchased from Transduction Laboratories (Lexington, KY); and goat anti-mouse IgG-horseradish peroxidase MAb was purchased from Amersham Life Sciences.

Statistics

Single-sample data were analyzed by two-tailed t-test; a multiple range test (Scheffe’s test) was used for comparisons of experimental groups with a single control group (40).

RESULTS

Expression of PP1, PP2A, and PP2B

The expression of Ser/Thr PPs in BPMECs was determined by Western blot analysis. Results indicated that PP1, PP2A, and PP2B were expressed by these endothelial cells (Fig. 1). PP1 consistently showed bands at ~40 and 50 kDa, which were similar to the positive control A431 cells. PP2A showed a band at ~40 kDa. A band for PP2B was observed at ~60 kDa, as also indicated by the positive control rat brain tissue.

For study, the Ser/Thr PPs in BPMECs were inhibited pharmacologically to evaluate their role in the regulation of thrombin-induced endothelial barrier dysfunction. A range of concentrations of the inhibitors of PP1 and PP2A (calyculin A and okadaic acid) and PP2B (FK506) were evaluated to determine the concentrations that alone did not alter baseline endothelial barrier function. Both calyculin A (IC₅₀: PP1 = 0.40 nM; PP2A = 0.25 nM) and okadaic acid (IC₅₀: PP1 = 49 nM; PP2A = 0.28 nM) are highly selective for PP1 and PP2A, with minimal inhibition of PP2B (26). FK506 selectively inhibits PP2B at nanomolar affinities by forming a complex with FK506-binding protein and does not inhibit PP1, PP2A, or other PPs (21). Based on this initial concentration evaluation, 200 nM okadaic acid, 2 nM calyculin A, and 100 ng/ml of FK506 were shown to not alter baseline transendothelial electrical resistance (Fig. 2A) nor PKC activity (Fig. 2B). Both FK506 and okadaic acid appeared to increase PKC activity slightly, but it was not significant. These concentrations of inhibitors were used for subsequent investigation of the involvement of Ser/Thr PPs in thrombin-mediated endothelial barrier dysfunction.

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Fig. 1. Western blot showing expression of protein phosphatase (PP) 1, PP2A, and PP2B in bovine pulmonary microvascular endothelial cells (BPMECs). Positive controls were A431 cell lysate for PP1 and PP2A and rat brain tissue for PP2B (n = 2 experiments). Nos. at left and right, molecular mass.
Effects of Ser/Thr PP Inhibitors on Thrombin-Induced PKC Activity and Phosphorylation

The proinflammatory mediator thrombin (100 nM) increased PKC phosphotransferase activity (>3-fold) in the membrane cell fraction of BPMECs within 2 min and was sustained for up to 30 min (Fig. 3A). Pretreatment of the cells with 100 ng/ml of FK506 for 15 min potentiated this increased PKC activity (12-fold over control value; Fig. 3B). Pretreatment with 2 nM calyculin A or 200 nM okadaic acid did not potentiate the thrombin-induced increase in PKC activity but tended to lower (not significantly) the increased activity (Fig. 3B).

Because the phosphorylation state of PKC is directly correlated to its intrinsic kinase activity (5, 6, 15, 32), we next investigated whether FK506-mediated potentiation of thrombin-activated PKC activity was related to increased phosphorylation of the PKC enzyme. We determined phosphorylation of the classic PKC isoforms (i.e., PKC-α and -β) in response to thrombin or thrombin plus FK506 because these isoforms have been reported to be important in the regulation of endothelial permeability. Immunoprecipitation results showed that treatment of BPMECs with 100 nM thrombin (5 min) phosphorylated a band corresponding to PKC-α, and that pretreatment with FK506 potentiated the phosphorylation of PKC-α (Fig. 4, A and B). However, neither thrombin treatment alone nor in combination with FK506 pretreatment resulted in a phosphorylated band corresponding to PKC-β (data not shown). Negative controls (absence of the anti-PKC-α or PKC-β MAbs) lacked the specific phosphorylated band.

Effects of Ser/Thr PP Inhibitors on Thrombin-Induced Transendothelial Resistance

We determined whether Ser/Thr PP inhibitors alter the transendothelial electrical resistance across BPMECs in response to thrombin. Stimulation of BPMEC monolayers with 100 nM thrombin rapidly decreased the resistance, which varied between 15 and 25% below baseline (Fig. 5A). The resistance drop returned toward baseline within ~30 min (Fig. 5A).
A. When BPMECs were pretreated with 100 ng/ml of FK506, the thrombin-induced decrease in resistance was not reversed within the period of study (>1 h; Fig. 5B). In contrast, pretreatment with 200 nM okadaic acid or 2 nM calyculin A did not significantly alter the time of recovery as evaluated by the time needed to return to 50% of maximal resistance decrease after thrombin stimulation (Fig. 5C). The effects of the Ser/Thr PP inhibitors on the initial resistance decrease in response to thrombin are shown in Fig. 6. The results indicate that neither FK506 nor okadaic acid pretreatment altered the initial resistance decrease in response to thrombin. However, pretreatment with 2 nM calyculin A significantly enhanced the thrombin-mediated decrease in resistance (Fig. 6).

Relationship of PKC to Recovery From Endothelial Barrier Dysfunction

A continuous prolonged stimulation of cells with submaximal or maximal concentrations of phorbol esters is known to reduce or abolish PKC activity, phorbol ester binding, and PKC immunoreactivity (22). Therefore, phorbol 12-myristate 13-acetate (PMA) was used for the downregulation of PKC to test the involvement of PKC in the FK506-mediated inhibition of recovery of the thrombin-induced resistance decrease. BPMECs were treated with 1.0 μM PMA for 16 h, resulting in the loss of PKC-α but not of PKC-β protein (Fig. 7A) as detected by Western blot analysis. PMA treatment did not downregulate PP1, PP2A, and the thrombin receptor (Fig. 7B), whereas PP2B expression was slightly decreased (Fig. 7B).

PKC downregulation rescued the FK-506 effects on the thrombin response; that is, it protected against the...
FK506-mediated inhibition of recovery (Fig. 8A). Additionally, downregulation did not alter the thrombin-mediated initial resistance decrease but significantly inhibited (~70%) the thrombin-mediated resistance decrease when combined with FK506 pretreatment (Fig. 8B). Because these findings suggest a possible relationship of recovery from barrier dysfunction with decreased PKC activity, we next investigated whether the thrombin-mediated phosphorylation of PKC-α was decreased during the recovery period of the thrombin-mediated resistance decrease. Results indicated that PKC-α phosphorylation was reduced to near control levels by 10 min of thrombin treatment (Fig. 9).

**DISCUSSION**

Findings from the present study indicated that one mechanism by which Ser/Thr PPs may reverse endothelial barrier dysfunction is through the regulation of PKC. The major supportive observations are that 1) the thrombin-induced increase in PKC activity was potentiated by the PP2B inhibitor FK506 but not by inhibitors of PP1 and PP2A, 2) FK506 also inhibited recovery of the normally reversible decrease in trans-endothelial resistance in response to thrombin, and 3) PKC downregulation rescued the FK506-mediated inhibition of recovery.

The results indicated that FK506 but not calyculin A or okadaic acid potentiated the thrombin-induced increased PKC activity. Because the Ser/Thr PP inhibitors are known to be potent and selective inhibitors of the classes of Ser/Thr PPs (26), the findings suggest that primarily PP2B (also known as calcineurin or Ca$^{2+}$- and calmodulin-dependent PP) regulated PKC activity in the control of endothelial barrier function. The lack of potentiation by calyculin A and okadaic acid on PKC activity was likely not related to insufficient inhibitor concentrations because the studies used inhibitor concentrations that were greater than fourfold over the IC$_{50}$. This observation provides novel information demonstrating the selective regulation of PKC by PP2B in endothelial cells. Importantly, the finding that FK506 alone had minimal effects on PKC activity supports the notion that the thrombin response included engagement of PP2B for the regulation of PKC. Although specific functions of PP2B remain relatively unknown, PP2B has been shown to be highly expressed in the brain and a key enzyme in the signal transduction cascade in T-cell activation responses (50). PP2B also has a narrower substrate specificity than the other Ser/Thr PPs, the majority of its most effective substrates being regulators of other protein kinases and phosphatases (39). It will be important to fully understand the specific mechanisms by which PP2B regulates PKC.

Although specific mechanisms by which Ser/Thr PPs regulate PKC are unclear, both direct and indirect pathways may exist to limit activation of PKC. It has been shown that the phosphorylation of PKC by phosphoinositide-dependent kinase-1 (PDK-1) on serine and threonine residues of the activation loop appears to be required for optimal kinase activity (5, 15, 32).
Furthermore, the upstream regulator of PDK-1 is phosphatidylinositol (PI) 3-kinase (10). Therefore, it is conceivable that Ser/Thr PPs may regulate at the level of PDK-1 or PI 3-kinase, which, in turn, determines the phosphorylation state of PKC. However, findings obtained with in vitro PKC assays indicate that both PP1 and PP2A reversibly inhibited PKC activity, implicating direct regulation of PKC by these Ser/Thr PPs (34).

In the present study, it remains to be resolved whether PP2B in endothelial cells regulates PKC-α directly or through PDK-1 and PI 3-kinase.

The FK506-mediated increase in PKC activity and phosphorylation of PKC-α appear to be selective for the endothelium. In monkey kidney CV-1 and mouse NIH/3T3 fibroblasts, PKC-ζ activation occurred after okadaic acid treatment (41). Similarly, okadaic acid caused activation of PKC-ζ and PKC-λ in rat adipocytes (43). These studies indicate that several PKC isoforms are possible regulatory target molecules of Ser/Thr PPs.

Our results showed that FK506 predominantly inhibited the reversal phase and not the initial drop in resistance after thrombin stimulation, suggesting a possible physiological role of PP2B in the regulation of the return of endothelial barrier dysfunction to normal. The finding that FK506 also potentiated the increased PKC activity provides support that this recovery from barrier dysfunction may be mediated through the regulation of PKC. Conceivably, endothelial cells activated by an inflammatory stimulus would present with increased endothelial permeability, which is also associated with PP2B activation, resulting in the limitation of PKC activation responses and the consequent reversal of the increased permeability. Further support for this notion is provided by Verin et al. (44), who reported that thrombin treatment of bovine pulmonary artery endothelial cells resulted in increased PP2B activity that became maximal by 20 min, a time period in which barrier dysfunction was recovering. In this same study, treatment of endothelial cells with deltamethrin, a PP2B inhibitor, inhibited ~50% of the thrombin-mediated dephosphorylation of MLC induced at 60 min, which was accompanied by significant enhancement of the thrombin-mediated increased endothelial permeability (44). These results

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in combination with findings from the present study indicate that PP2B may have multiple targets in the regulation of the reversal of increases in endothelial permeability.

We found that PKC downregulation effectively rescued the FK506-mediated inhibition of recovery from barrier dysfunction. These results provide evidence that the FK506-mediated potentiation of PKC activity was functionally correlated to inhibition of recovery from endothelial barrier dysfunction. Furthermore, the finding that FK506 also potentiated the phosphorylation of PKC-α in response to thrombin implicates this PKC isoform in the regulation of the recovery phase. Additional support for this thesis is the observation that PKC-α phosphorylation had decreased to near basal level by 10 min of thrombin activation, a period when the decreased resistance was returning toward baseline. It is also possible that both novel and atypical classes of PKC regulate endothelial barrier function, and future studies are needed to address whether PPs regulate their activity as well.

The results indicate that the downregulation had a minimal effect in inhibiting the initial decrease in resistance in response to thrombin, suggesting that PKC isoforms resistant to PMA-mediated downregulation and other signaling pathways are involved in this phase of the barrier dysfunction response. However, the finding that PKC downregulation combined with PP2B inhibition prevented ∼70% of the thrombin-mediated resistance decrease was surprising, and the underlying basis is not apparent. We speculate that PP2B may have other interactions with the PKC signaling pathway besides PKC-α. From previous work by Vuong et al. (48) and others (49), selective PKC isoforms have negative feedback regulation. It is possible that PP2B regulates other PKC isoforms that have a negative feedback function, which would contribute to the inhibition of the resistance decrease in response to thrombin. This possibility has yet to be tested in our endothelial cell model.

Downregulation by PMA resulted in the complete loss of the PKC-α protein (but not PKC-β protein). Furthermore, PMA-mediated downregulation appeared to be selective for PKC because expression of PP1, PP2A, and the thrombin receptor was unaltered and PP2B was slightly decreased. Our results are consistent with the idea that PKC isoforms possess different susceptibilities to downregulation (1, 13, 17, 35). Different sensitivities to downregulation have been documented for PKC isoforms belonging to the same class (e.g., PKC-α vs. PKC-β) or between different classes of isoforms (e.g., PKC-α vs. PKC-δ) (1, 13, 35). Although downregulation is generally attributed to proteolytic degradation by endogenous proteases such as calpain, the basis of differential sensitivity has been hypothesized to be related to the presence of Ca²⁺ and various phospholipids such as phosphatidylinositol and phosphatidylserine (18). Furthermore, it is thought that phosphorylation of PKC targets the enzyme for proteolysis. Our finding that PKC-α was phosphorylated and downregulated but that PKC-β was neither phosphorylated nor downregulated supports this thesis.

The observation that calyculin A (equally selective for PP1 and PP2A) enhanced the extent of the thrombin-mediated drop in resistance but that okadaic acid (>100 times more selective for PP2A than PP1) had no effect suggests that PP1 and not PP2A was involved in the regulation of the barrier dysfunction. Because neither calyculin A nor okadaic acid significantly altered the thrombin-induced PKC activity, the present results suggest that the calyculin A-mediated response was likely independent of PKC. PP1, in particular myosin-associated PP1, has been proposed as a key endothelial phosphatase that dephosphorylates MLC, promoting decreased actomyosin contraction in endothelial cells (7, 38, 45, 46). Alternatively, calyculin A has been shown to regulate the phosphorylation state of junctional proteins. In human epidermal cells, both okadaic acid and calyculin A have been shown to induce hyperphosphorylation of β-catenin, a junctional protein associated with cadherins, which was accompanied by the loss of cell-cell contacts (37). In another study, PP2A was shown to regulate β-catenin signaling (36). Thus these observations suggest that PP1 and PP2A are implicated in the regulation of the phosphorylation state of MLC and junctional proteins, contributing to the overall regulation of endothelial barrier dysfunction (3, 12, 33, 42, 47).

There has been little information regarding expression of Ser/Thr PPs in endothelial cells. We observed that BPMECs expressed PP1, PP2A, and PP2B, which is consistent with reports that bovine pulmonary artery endothelial cells also express these three Ser/Thr PPs (44, 45). Together, the findings indicate that in the lung, both conduit and microvascular endothelial cells express Ser/Thr PPs, suggesting their relatively conserved expression across different vascular beds.

In summary, the major findings in the present study demonstrate that 1) the thrombin-induced activation of PKC was potentiated by the PP2B inhibitor FK506 but not by inhibitors of PP1 and PP2A, 2) FK506 also inhibited the recovery phase of the thrombin-induced decrease in transendothelial resistance, and 3) PKC downregulation rescued the FK506-mediated inhibition of recovery. These results provide evidence for the notion that PP2B may play an important physiological role in the regulation of the return of endothelial barrier dysfunction to normal. Furthermore, PKC isoforms, in particular PKC-α, may be critical targets regulated by PP2B for this function.

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