Role of CPI-17 in the regulation of endothelial cytoskeleton

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Kolosova, Irina A., Shwu-Fan Ma, Djanyak M. Adyshev, Peyi Wang, Motoi Ohba, and Alexander D. Verin. Role of CPI-17 in the regulation of endothelial cytoskeleton. Am J Physiol Lung Cell Mol Physiol 287: L970–L980, 2004. First published July 2, 2004; doi:10.1152/ajplung.00398.2003.—We have previously shown that myosin light chain (MLC) phosphatase (MLCP) is critically involved in the regulation of agonist-mediated endothelial permeability and cytoskeletal organization (Verin AD, Patterson CE, Day MA, and Garcia JC. Am J Physiol Lung Cell Mol Physiol 269: L99–L108, 1995). The molecular mechanisms of endothelial MLCP regulation, however, are not completely understood. In this study we found that, similar to smooth muscle, lung microvascular endothelial cells expressed specific endogenous inhibitor of MLCP, CPI-17. To elucidate the role of CPI-17 in the regulation of endothelial cytoskeleton, full-length CPI-17 plasmid was transiently transfected into pulmonary artery endothelial cells, where the background of endogenous protein is low. CPI-17 had no effect on cytoskeleton under nonstimulating conditions. However, stimulation of transfected cells with direct PKC activator PMA caused a dramatic increase in F-actin stress fibers, focal adhesions, and MLC phosphorylation compared with untransfected cells. Inflammatory agonists histamine and, to a much lesser extent, thrombin were capable of activating CPI-17. Histamine caused stronger CPI-17 phosphorylation than thrombin. Inhibitory analysis revealed that PKC more significantly contributes to agonist-induced CPI-17 phosphorylation than Rho-kinase. Dominant-negative PKC-α abolished the effect of CPI-17 on actin cytoskeleton, suggesting that the PKC-α isoform is most likely responsible for CPI-17 activation in the endothelium. Depletion of endogenous CPI-17 in lung microvascular endothelial cell significantly attenuated histamine-induced increase in endothelial permeability. Together these data suggest the potential importance of PKC/CPI-17-mediated pathway in histamine-triggered cytoskeletal rearrangements leading to lung microvascular barrier compromise.

myosin phosphatase; myosin light chain phosphorylation; endothelial barrier

REORGANIZATION OF THE CYTOSKELETON plays many crucial roles in endothelial cell (EC) migration, adhesion, and contraction. Actomyosin-driven contraction, in turn, is a key event in the regulation of endothelial barrier function. EC contraction is initiated by Ser/Thr phosphorylation of the 20-kDa regulatory myosin light chain (MLC), which is tightly linked to F-actin filament reorganization (15, 17). The inflammatory agonists thrombin and histamine produce rapid increases in MLC phosphorylation, actomyosin interaction, stress fiber formation, and increased endothelial permeability (41).

MLC phosphorylation mechanism involves two key enzymes: Ca2+/calmodulin-dependent MLC kinase (MLCK) and MLC phosphatase (MLCP). MLCK has been detected in many tissues (14), including endothelium (16). Less is known about endothelial MLCP. Smooth muscle MLCP is a holoenzyme composed of three subunits: a catalytic subunit of 38 kDa that was identified as protein phosphatase 1 (PP1) catalytic subunit δ-isoform (PP1Cδ) (18) and two noncatalytic subunits of 21 and 110–130 kDa (1, 36). The larger one, called myosin phosphatase targeting subunit 1 (MYPT1), binds to the catalytic subunit and targets it to MLC, providing substrate specificity (21). The function of the smaller subunit is unknown. Studies by our laboratory (43) and others (4) have demonstrated the involvement of PP1 in the regulation of EC gap formation and barrier function in pulmonary endothelium. Furthermore, only the PP1Cδ isoform was associated with the actomyosin complex (44). Immunochemical data and inhibitory analysis indicate the involvement of PP1 in maintaining the normal cytoskeletal structure in quiescent umbilical vein endothelium (35). MYPT1 has also been recently found in EC (20). These findings suggest that smooth muscle cells (SMC) and EC share a common MLC dephosphorylation mechanism involving MLCP holoenzyme.

Rho-kinase (RhoK) and protein kinase C (PKC) have been proposed to mediate the inhibition of smooth muscle MLCP, leading to increased MLC phosphorylation in response to various agonists (for a review see Ref. 38). Phosphorylation of the MYPT1 regulatory site (Thr695 in chicken MYPT1) by RhoK induces inhibition of MLCP activity (22). The regulation of MLCP activity in EC seems to include a Rho-dependent signaling pathway. The permeability-augmenting activity of RhoA in response to thrombin appears to be mainly mediated by RhoK, which phosphorylates and inactivates MLCP (7). In contrast to RhoK, PKC has not been shown to phosphorylate MLCP subunits in vivo. However, in vascular smooth muscle, activation of PKC by phorbol ester leads to inhibition of MLCP, increase in MLCP phosphorylation, and contraction (28). A number of experimental facts suggest that CPI-17 (for PKC-potentiated inhibitory protein of 17 kDa) is involved in PKC-dependent inhibition of MLCP. CPI-17 is a soluble globular protein described as a specific inhibitor for MLCP (11). Initially, CPI-17 was assumed to be present in smooth muscle tissues only, but recently it was found in platelets (45) and brain (5). Phosphorylation of CPI-17 at Thr38 by PKC enhances its inhibitory potency 1,000-fold (12). The phosphorylation of purified MLCP toward MLC is completely inhibited by phosphorylated CPI-17 (34). Histamine, phenylephrine, GTPyS, and phorbol ester caused in vivo phosphorylation of CPI-17 at Thr38 (23). Histamine-induced phosphorylation of CPI-17 in smooth muscles appeared to be catalyzed by 10.220.33.3 on November 6, 2017 http://ajplung.physiology.org/ Downloaded from http://ajplung.physiology.org/ view/66/6

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by PKC-α and -δ isoforms (10). These data raise the probability that CPI-17 represents an alternative mechanism for inhibition of MLCP (different from MYPT1 phosphorylation).

Little is known about the physiological role of CPI-17. A recent study by Woodsme and coworkers (46) demonstrated that the expression pattern of CPI-17 among different smooth muscle tissues correlates with their extent of PKC-induced contraction, implying that CPI-17 is a key component in modulating smooth muscle tone. Phosphorylation of CPI-17 by PKC may contribute to MLCP inhibition during agonist-induced platelet secretion (45) and cerebellar long-term synaptic depression (9). There are a limited number of studies demonstrating the direct role of CPI-17 in cytoskeletal remodeling.

Overexpression of CPI-17 in fibroblasts lacking endogenous protein caused abnormal accumulation of cortical F-actin fibers and significantly retarded spreading of fibroblasts on fibronectin with elevated levels of MLC phosphorylation (13).

In the present study, we explored a possible role of CPI-17 in the remodeling of endothelial cytoskeleton in response to inflammatory agonists and direct activation of PKC by phorbol ester. We used two complementary approaches: overexpression of recombinant CPI-17 in EC, and depletion of endogenous CPI-17 using a small interfering RNA (siRNA) technique. Our results suggest that a PKC/CPI-17 pathway may be involved in the regulation of MLC phosphorylation, reorganization of microfilaments and focal adhesions, and histamine-induced barrier dysfunction.

MATERIALS AND METHODS

Materials. PMA, Y-27632, Ro-31-8220, bisindolylmaleimide, and rottlerin were purchased from Calbiochem (San Diego, CA). thrombin, histamine, monoclonal antivinculin antibody, and monoclonal anitactin antibody were purchased from Sigma (St. Louis, MO); mono- and polyconal anti-myc and polyclonal anti-PKC-α antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CPI-17 and antiphospho-CPI-17 (Thr38) antibodies were from Upstate (Lake Placid, NY). Monoclonal pan-ERK antibody was from BD Biosciences (San Diego, CA). Texas red-conjugated phalloidin, goat anti-mouse Alexa 488 or Alexa 546, goat anti-rabbit Alexa 488 or Alexa 546, and goat anti-mouse Alexa 350 antibodies were purchased from Molecular Probes (Eugene, OR). Phosphospecific (Thr19/Ser13) anti-MLC antibody was generously provided by Dr. M. Crow (Johns Hopkins University).

Cell cultures. Human pulmonary artery EC (HPAEC), human umbilical vein EC (HUVEC), and human lung microvascular EC (HLMVEC) were purchased from Clonetics (Walkersville, MD) and were cultured in EBM-2 complete medium (Clonetics). HPAEC were utilized at passages 5–10, HUVEC at passages 2–3, and HLMVEC at passages 4–8.

Total RNA isolation. Total RNA from cultured endothelial and HeLa cells was isolated using silica gel-based membrane with the RNeasy kit from Qiagen (Valencia, CA). Total RNA from human uterus was purchased from Clontech (Palo Alto, CA).

Cloning of endothelial CPI-17. First-strand cDNA was synthesized from total RNA using either random hexamer or oligo(dT) primers (Advantage RT-for-PCR kit, Clontech). PCR amplification was carried out using Advantage 2 Polymerase Mix (Clontech). The designed primers were based on human expressed sequence tag (EST) sequence (accession no. A128172) highly homologous to porcine smooth muscle CPI-17: 5'-ATGGCGCTTCGACGGCGTG-3' (sense primer) and 5'-AGAGTGCAAGGGGTTCAAGGGTGAG-3' (antisense primer). PCR product was cloned into pcR4-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced. For a subcloning of the CPI-17 coding region into mammalian expression vector, KpnI and XbaI restriction sites were created at 5' and 3'-ends, respectively, by PCR. CPI-17 DNA was inserted into Myc-tagged pcDNA3.1 mammalian expression vector (Invitrogen) using the abovementioned restriction sites.

Real-time RT-PCR analysis. Transcript levels of CPI-17, MYPT1, and PP1 were measured in 96-well microtiter plates using ABI Prism 7700 Sequence Detector Systems (PerkinElmer/Applied Biosystems, Foster City, CA). TaqMan GAPDH Control Reagents were used as internal controls for normalization. Quantification primers and probes were designed using Primer Express (PerkinElmer/Applied Biosystems, Foster City, CA). For CPI-17 (accession no. AB056508): sense, 5'-CTGAGCCGCCGATGGGA-3' (380–396); antisense, 5'-GGCTTCTCTTCTCCTCTCT-3' (438–446); probe, 5'-CAGACATGCCGAGATCAGCTA-3' (399–425). For MYPT1 (accession no. AF458859): sense, 5'-TGGGACATGTGATGATGAG-3' (755–779); antisense, 5'-CGTTTTTCACTAGGAGCTTTT-3' (860–884); probe, 5'-CAACAAATGGGTGCAAACACGCTTG-3' (783–808). For PP1 (accession no. AF092905): sense, 5'-TGCTAACAGCACTGTGTTAACCT-3' (909–932); antisense, 5'-ACTACATCTCTACCCAGCATTAC-3' (964–987); probe, 5'-TTTTTCACT-CCCCATAATGCTGTCG-3' (916–939).

All the probes were labeled with 6-carboxy tetramethylrhodamine at the 5'-end and FAM or JOE at the 3'-end for target and GAPDH, respectively. Fluorescent signals were generated during each PCR cycle via the 3'-end extension reaction of TaqMan Gold to provide specific transcript expression levels. For the reaction, 25 ng of total RNA and TaqMan Gold RT-PCR Core Reagents Kit (PerkinElmer/Applied Biosystems, Foster City, CA) were mixed together, and RT-PCR cycle parameters were 48°C for 30 min followed by 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 60 s. Threshold cycle (Ct), which correlates inversely with the target mRNA levels, was set as the cycle at which each fluorescent signal was first detected above background. Initial experiments demonstrated identical real-time amplification efficiencies of target and reference gene, which is a prerequisite for the relative quantification, 2^-ΔΔCt, method, used here (27).

The expression level of each target gene was calculated by standardizing the target gene copy number with the GAPDH copy number in a sample. Purity and specificity of all products were confirmed by omitting the RT or template and by appropriate size and single melting temperature. Analysis of results is based on three independent experiments. Specific mRNA transcript levels were expressed as fold difference.

Depletion of endogenous CPI-17 in EC. To reduce the content of endogenous CPI-17, HLMVEC were treated with CPI-17-specific siRNA duplex, which guides sequence-specific degradation of the homologous mRNA (6). siRNA was ordered from Qiagen in purified, desalted, 2'-deprotected duplex form. Duplex of sense 5'-AC- CUGUGUAGAGUCUUCACUTT-3' and antisense 5'-UTGUGAG- CAGCUCCUGAAGU-5' siRNA was selected with the Qiagen Silence RNA Design Tool. Nonspecific RNA duplex FL-Luciferase GL2 (Dharmacon Research, Lafayette, CO) was used as a control treatment. HLMVEC were plated on 60-mm dishes to yield 70% confluence, and the transfection of siRNA was performed with the GeneSilencer transfection reagent (Gene Therapy Systems, San Diego, CA) to result in a final RNA concentration of 100 nM. Forty-eight hours later, cells were harvested and used for total RNA isolation. To estimate the amount of CPI-17 RNA in HLMVEC after siRNA treatment, the semiquantitative RT-PCR method was used. Total RNA (0.5 μg) was subjected to PCR in a 25-μl reaction mixture using reagents from Superscript One Step RT-PCR kit (Invitrogen). To amplify the 268-bp part of CPI-17 cDNA, 200 nM each of the following primers were used: sense, 5'-CGGATGGAAGCCGACAT-3' antisense, 5'-AGGAGAAGTGCAAGGGTCA-3'. The amplified part of DNA corresponds to nucleotides 388–655 in CPI-17 sequence from GenBank.
Bank (accession no. AB056508). 18S cDNA was amplified using 40 nM primers from TaqMan Gold RT-PCR Core Reagents Kit (Perkin-Elmer/Applied Biosystems). The PCR products were analyzed by agarose gel electrophoresis.

For transendothelial resistance measurement, cells were plated in electrode well and transfected with siRNA as described.

**Endothelial monolayer resistance measurement.** The electrical resistance of EC monolayers was measured with the electrical cell impedance sensor technique as previously described (42). In this system (Applied Biophysics, Troy, NY), the cells are cultured on a small gold electrode (10⁻⁴ cm²) in complete medium. The cells act as insulating particles, and the total resistance across the monolayers is composed of the resistance between the ventral cell surface and the electrode and the resistance between cells. A 4,000-Hz AC signal with 1-V amplitude was applied to the EC through a 1-MΩ resistor, creating an approximate constant current (1 μA). The lock-in amplifier attached to the electrodes detected changes in both magnitude and phase of the voltage appearing across the EC monolayers. Electrical resistance increased immediately after the cells attached to and covered the electrodes, and the resistance achieved a steady state when the EC became confluent. Thus experiments were conducted after the electrical resistance achieved a steady state. Resistance data were normalized to the initial voltage and plotted as a normalized resistance.

**Transfection of EC with expression vector.** To transfect EC with pcDNA3.1 expression vector, FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) was used. We followed the transfection protocol suggested by the manufacturer with a DNA:FuGENE ratio of 1:3. HPAEC (50–70% confluence) were plated on coverslips in 12-well dishes and incubated with DNA/FuGENE 6 mixture prepared as follows: 50 μl Opti-MEM I medium, 0.5 μg DNA, and 1.5 μg FuGENE. The mixture was incubated at room temperature for 30 min and added to the dishes with 1 ml Opti-MEM I medium. After 4 h of incubation, the medium was changed to complete medium, and the cells were allowed to grow for 12–18 h before experimental treatments.

**Construction of dominant-negative PKC-α adenovirus and EC infection.** Dominant-negative mutant of PKC-α (DN-PKC-α) was generated by substitution of arginine for lysine at the ATP binding site of PKC-α as described previously (31). DN-PKC-α cDNA was inserted into pACXawt cosmid, generating a recombinant adenovirus vector. Purification and titration of the adenovirus were performed as previously described (30). We infected 70% confluent HPAEC with adenovirus vector of LacZ or DN-PKC-α with multiplicities of infection of 10, 20, and 50 and incubated them for 24 h.

**Immunofluorescent microscopy and image analysis.** Cultured cells grown on glass coverslips were fixed in 3.7% formaldehyde solution in phosphate-buffered saline (PBS) for 10 min at 4°C and washed three times with PBS. The cells were permeabilized with 0.2% Triton X-100 in PBS supplemented with 0.1% Tween 20 (PBST) for 5 min, washed three times with PBS, and blocked with 2% BSA in PBST for 20 min. Incubation with specific antibody diluted with blocking solution was performed for 1 h at room temperature. Anti-c-Myc was used for immunofluorescent detection of Myc-tagged CPI-17. Specific antibodies were used for detection of vinculin, diphospho-MLC, and PKC-α. After three washes with PBS, cells were incubated with appropriate secondary antibody (1:200) conjugated with fluorescent dye Alexa 488 (green) or Alexa 594 (red) or Alexa 350 (blue) for 1 h at room temperature. Actin microfilaments were stained with Texas red-labeled phallolidin for 1 h at room temperature.

After immunostaining, the glass slides were analyzed using a Nikon video-imaging system (Nikon Instech) consisting of a phase-contrast inverted microscope Nikon Eclipse TE2000 connected to digital camera and image processor (Hamamatsu Photonics). Quantification of diphospho-MLC immunostaining was performed using MetaVue 4.6 (Universal Imaging, Downington, PA). Staining intensity was thresholded in each image, and analyzed cells were manually marked out. The ratio of the thresholded area to the whole cell area was determined. The values were statistically processed using SigmaPlot 7.1 (SPSS Science, Chicago, IL) software.

**RESULTS**

**Endothelial CPI-17.** CPI-17, one of the regulators of MLCP activity in SMC (10, 23, 26), has also been identified in platelets (45) and brain (5). In our attempt to find out whether CPI-17 is present in EC, we designed primers based on human EST sequence (accession no. AI128172) highly homologous to porcine smooth muscle CPI-17. Using RNA purified from HPAEC as a template, we were able to clone and sequence a 520-bp product that turned out to be an open-reading frame encoding a protein composed of 147 amino acids. This protein was identical to larger isoform of CPI-17 found in human aorta (47). We did not obtain any PCR product corresponding to the smaller (120 amino acid) CPI-17 isoform reported previously (47).

As an inhibitory component of MLCP, CPI-17 is expected to act in conjunction with other components of MLCP holoenzyme. To quantify the relative amounts of CPI-17 and MLCP subunits in EC, we used real-time PCR as described in MATERIALS AND METHODS. Figure 1A shows that micro- and macrovascular EC were equal in terms of the amount of PP15 and MYPT1. In contrast, the amount of CPI-17 in microvascular EC was considerably larger than in macrovascular EC. Compared with smooth muscle (uterus), HLMVEC express less CPI-17. However, a smaller amount of CPI-17 in HLMVEC corresponded to a smaller amount of both catalytic (PP15) and regulatory (MYPT1) subunits of MLCP. Furthermore, the

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**Fig. 1. Real-time PCR analysis of myosin phosphatase-related components in endothelium and smooth muscle.** The values are represented as a relative expression of specific mRNA. The ratio in human pulmonary artery endothelial cells (HPAEC) is taken as a calibrator. A: expression of CPI-17, myosin phosphatase targeting subunit 1 (MYPT1), and protein phosphatase (PP) 15 relative to GAPDH. HUVEC, human umbilical vein endothelial cells; HLMVEC, human lung microvascular endothelial cells; B: expression of CPI-17 mRNA relative to other components of myosin phosphatase.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CPI-17/GAPDH</th>
<th>MYPT1/GAPDH</th>
<th>PP15/GAPDH</th>
<th>mRNA Ratio</th>
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</thead>
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<td>1</td>
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<tr>
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<td>1.1</td>
<td>0.9</td>
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<td>98.6</td>
<td>3.3</td>
<td>4.9</td>
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**A**

**B**
relative amount of CPI-17 mRNA in HLMVEC was only 1.3 times less than in uterus, if plotted as a ratio between CPI-17 and PP1 (Fig. 1B). In contrast, in both HPAEC and HUVEC, this number was 30 times less than in uterus. These data suggest that mechanisms of MLCP regulation are different in macrovascular and microvascular EC. Similar to smooth muscle, CPI-17 may play an important role in regulation of microvascular endothelial cytoskeleton.

CPI-17 induced cytoskeletal reorganization in endothelium. To investigate the direct effect of CPI-17 on endothelial cytoskeleton, HPAEC were transiently transfected with expression vector carrying the CPI-17 coding sequence tagged with c-Myc epitope. Macrovascular EC were chosen for these experiments, since the endogenous level of CPI-17 in these cells is low. Figure 2 shows immunofluorescent images of HPAEC expressing recombinant CPI-17. Overexpressed CPI-17 did not cause any noticeable changes in EC cytoskeleton in nonstimulated cells (Fig. 2, A–D). Exposure of cells to a direct PKC activator, PMA, however, produced a dramatic increase in the amount of both stress fibers and focal adhesions in CPI-17-transfected cells (Fig. 2, E–H). Stress fibers and focal adhesion plaques in those cells also looked thicker. These data suggest that PKC is directly involved in CPI-17 activation in endothelium.

HPAEC pretreated with RhoK inhibitor Y-27632 exhibited complete dissolution of stress fibers and focal adhesions (Fig. 2, I–L). In these conditions, CPI-17-overexpressing cells were no different from nontransfected cells. Stimulation with PMA, however, restored thick focal adhesions and actin bundles in transfected cells (Fig. 2, M–P). Thus inhibition of myosin

![Image](https://example.com/image.png)

**Fig. 2.** Effect of CPI-17 on microfilaments and focal adhesions. HPAEC were transfected with Myc-tagged CPI-17. The cells were treated with either vehicle or Rho-kinase (RhoK) inhibitor Y-27632 (5 μM, 30 min) and then with either vehicle or PMA (0.1 μM, 30 min). After the treatment the cells were double-stained to visualize CPI-17 simultaneously with either actin or vinculin. Anti-c-Myc and antivinculin antibodies were used for immunofluorescent detection of CPI-17 and vinculin, respectively. Actin microfilaments were stained with Texas red-labeled phalloidin. Double-stained images are shown in parallel. Transfected cells are indicated with arrowheads. CPI-17 overexpression affected neither actin structures (A, B) nor focal adhesions (C, D) in nonstimulated cells. PMA caused both thickening of microfilaments (E, F) and enlargement of focal adhesion plaques (G, H) in transfected cells. Treatment with Y-27632 caused dissolution of stress fibers (I, J) and focal adhesions (K, L); however, PMA treatment restored cytoskeletal structures in CPI-17-transfected cells (M–P).
phosphatase by CPI-17 may lead to enhancement of cytoskeletal elements (stress fibers and focal adhesions) via a Rho-independent mechanism. Immunofluorescent staining demonstrates that distribution patterns of actin and CPI-17 look similar, which may suggest colocalization of CPI-17 with both stress fibers and polymerized actin in the cortical area (Fig. 3).

It has been shown that PKC might be involved in CPI-17 phosphorylation in smooth muscles (10) and platelets (45). Our data demonstrate that general PKC inhibitors bisindolylmaleimide and Ro-31-8220 abolished the effect of CPI-17 on the endothelial cytoskeleton (Fig. 4A). Rottlerin, which is more specific toward PKC-δ, did not cause any effect (Fig. 4A). The inactive mutant of PKC-α, which we introduced into the cells by employing adenovirus (Fig. 4B), inhibited the effect of CPI-17. PMA treatment did not induce the formation of thick actin bundles in cells simultaneously overexpressing both CPI-17 and mutant PKC-α (Fig. 4C). These data indicate that PKC-α may be involved in CPI-17 phosphorylation/activation in EC.

Histamine is capable of activating CPI-17 and PKC in endothelium. Inflammatory agonists histamine and thrombin have been reported to cause stress fiber formation and an increase in MLC phosphorylation in the endothelium (15, 41). On the other hand, CPI-17 is evidently involved in histamine-induced contraction in smooth muscles (10) as well as in thrombin-induced platelet secretion (45). We were interested, therefore, in elucidating the possible role of these agonists in CPI-17 activation in EC. Figure 5 shows that both histamine and thrombin increased the level of diphospho-MLC in HLMVEC. Basal and agonist-induced MLC phosphorylation was strongly attenuated by RhoK inhibitor Y-27632. Interestingly, only histamine- but not thrombin-induced MLC phosphorylation was attenuated by treatment with PKC inhibitor Ro-31-8220. These data suggest that RhoK activity is essential for MLC phosphorylation induced by histamine and thrombin. However, PKC plays a noticeable role only in the histamine response. Accordingly, immunofluorescent staining demonstrated (Fig. 6A) that CPI-17-transfected cells exhibit a higher MLC phosphorylation level only after histamine, but not thrombin, treatment. PMA, as expected, greatly increased MLC phosphorylation in transfected cells. Quantitative analysis (Fig. 6B) demonstrated that the intensity of diphospho-MLC staining in CPI-17-transfected cells was slightly higher (1.25-fold) than in nontransfected cells even in unstimulated conditions. This may be explained by the presence of some amount of the phosphorylated active form of CPI-17 (as shown on Fig. 7). Histamine, thrombin, and PMA increased diphospho-MLC staining in transfected cells 4-, 6.2-, and 1.2-fold, respectively, compared with neighboring nontransfected cells. It may be concluded that histamine and PMA, but not thrombin, are able to trigger CPI-17 activation leading to MLCP inhibition and increased MLC phosphorylation. It should be pointed out, however, that thrombin produces a higher level of MLC phosphorylation than histamine or PMA. Therefore, this high background may mask the effect of thrombin on CPI-17 activity.

The direct study of CPI-17 phosphorylation confirmed the different potencies of these agonists with respect to CPI-17 activation. PMA caused the strongest CPI-17 phosphorylation, and histamine appeared to be more potent than thrombin (Fig. 7). Inhibition of PKC with Ro-31-8220 diminished CPI-17 phosphorylation to basal levels or lower. Although agonist-induced CPI-17 phosphorylation was attenuated by RhoK inhibitor Y-27632, it was still higher than the basal level. CPI-17 phosphorylation therefore depends on both PKC and RhoK activity. PKC, however, seems to contribute more. Interestingly, Y-27632 slightly inhibited PMA-induced CPI-17 phosphorylation, which suggests that PMA treatment led to RhoK activation. As seen in Fig. 8, in accordance with immunoblotting data, Ro-31-8220 greatly decreased diphospho-MLC immunostaining in histamine-treated, but not thrombin-treated, cells (for a comparison see Fig. 6). Ro-31-8220 also completely inhibited the effect of CPI-17 on MLC phosphorylation in histamine- and PMA-stimulated cells. After the inhibition of RhoK by Y-27632, the amount of diphospho-MLC in cells was drastically reduced, but CPI-17 still was able to enhance MLC phosphorylation after activation with either stimulus (Fig. 8). Thrombin, however, was less potent since it only worked in cells expressing larger amounts of CPI-17 (Fig. 8, M and N). These data indicate that not only the direct activator of PKC, phorbol ester, but also more physiologically relevant inflammatory agonists histamine and, to a lesser extent, thrombin are capable of activating CPI-17 in endothelium. However, since histamine-induced MLC phosphorylation is PKC dependent, but thrombin-induced MLC phosphorylation is not, histamine but less likely thrombin may be a physiological activator of CPI-17 in human endothelium.

CPI-17 depletion attenuates histamine-induced endothelial barrier dysfunction. Because our data demonstrate that a relatively large amount of CPI-17 is present in human microvascular EC and that its overexpression in macrovascular EC causes tremendous changes in actin cytoskeleton, MLC phosphorylation, and focal adhesions after EC stimulation with histamine and PMA, we hypothesized that endogenous CPI-17 is important for human microvascular endothelial barrier function. To test this hypothesis we reduced the content of endogenous CPI-17 by the gene silencing method (6). This technique was successfully used for CPI-17 depletion in cerebellar cells (9). In our experiment treatment of HLMVEC with CPI-17-specific siRNA caused a significant reduction in the amount of CPI-17 mRNA compared with the nonspecific RNA treatment, as determined by semiquantitative RT-PCR (Fig. 9A). Accord-
Fig. 4. PKC inhibitors and dominant-negative (DN)-PKC-α abolished effect of CPI-17 on actin cytoskeleton. 

**A:** HPAEC were transfected with Myc-tagged CPI-17. The cells were treated for 30 min with 5 μM of bisindolylmaleimide (BIM), Ro-31-8220, or rottlerin and then with PMA (0.1 μM, 30 min). After the treatment cells were double-stained for c-Myc and actin. Double-stained images are shown in parallel.

**B:** overexpression of DN-PKC-α in HPAEC. Cells were infected with indicated amount of adenovirus, incubated for 48 h, and subjected to immunoblotting with PKC-α-specific antibody.

**C:** HPAEC were infected with adenovirus [multiplicity of infection (MOI) = 50] carrying either DN-PKC-α or LacZ. Twenty-four hours later cells were transfected with CPI-17 plasmid and allowed to grow for additional 12 h. Then cells were treated either with vehicle or PMA (0.1 μM, 30 min). After the treatment, cells were triple-stained with anti-c-Myc, anti-PKC-α, and Texas red-labeled phalloidin to simultaneously visualize CPI-17, PKC-α, and actin. Triple-stained images are shown in parallel. The cells expressing both CPI-17 and DN-PKC-α lost their ability to form thick actin bundles after PMA treatment.

Fig. 5. Phosphorylation of myosin light chain (MLC) in HLMVEC treated with inflammatory agonists. Cells were incubated in serum-free medium for 1 h and then treated with either histamine or thrombin for 5 min. Then cells were placed on ice, washed with PBS, and lysed with Laemmli buffer. Diphospho-MLC (ppMLC) was detected in the lysates by immunoblotting (left). The same membrane was reprobed with antitubulin antibody. The image was scanned and quantified using ImageQuant 5.2 software. Values are presented as an average ratio + SE of ppMLC signal to tubulin signal and represent an n of 3 experiments (right). C, control; Ro, Ro-31-8220; Y, Y-27632.
ingly, the amount of CPI-17 protein expression was reduced (Fig. 9B). To examine the effect of CPI-17 depletion on EC barrier function, we monitored electrical resistance across EC monolayers [transendothelial electrical resistance (TER)] in CPI-17-depleted cells without stimulation and after stimulation with histamine (Fig. 9C) and thrombin (Fig. 9D). Both histamine and thrombin decreased TER of HLMVEC with a maximal decrease at 20 min after stimulation. Restoration of TER after thrombin was slower than after histamine. Furthermore, histamine subsequently increased electrical resistance to above the basal level. This phenomenon was previously reported by Moy et al. (29). Histamine-induced decrease in TER was

Fig. 6. Overexpression of CPI-17 caused increased MLC phosphorylation in cells stimulated with PMA and histamine. A: HPAEC were transfected with Myc-tagged CPI-17. The cells were treated with histamine (5 μM, 5 min), thrombin (0.1 μM, 5 min), or PMA (0.1 μM, 30 min) as indicated. After the treatment cells were double-stained to visualize c-Myc simultaneously with ppMLC. Double-stained images are shown in parallel. CPI-17-transfected cells (arrowheads) showed more ppMLC compared with nontransfected cells after the stimulation with histamine (c, d) or PMA (g, h). B: quantitative analysis of ppMLC staining using MetaVue 4.6 software. The data are presented as a ratio of the staining intensity in CPI-17-transfected cells to the staining intensity in neighboring untransfected cells (15–20 cells were analyzed in each group).

Fig. 7. PMA and agonists induce CPI-17 phosphorylation. HPAEC were transfected with Myc-tagged CPI-17. The cells were treated with vehicle, PKC inhibitor Ro-31-8220 (5 μM, 30 min), or RhoK inhibitor Y-27632 (5 μM, 30 min), and then with histamine (5 μM, 5 min), thrombin (0.1 μM, 5 min), or PMA (0.1 μM, 30 min) as indicated. After the treatment cells were lysed in Laemmli buffer and subjected to immunoblotting using phospho-specific anti-CPI-17 antibody (left). The same membrane was reprobed with anti-c-Myc antibody. The image was scanned and quantified using ImageQuant 5.2 software. Values are presented as an average ratio ± SE of phospho-CPI-17 signal to c-Myc signal and represent an n of 3 experiments (right).
attenuated in CPI-17-depleted cells compared with control cells. In contrast, thrombin-induced permeability was unaffected by CPI-17 depletion. Thus CPI-17 is a part of a histamine-triggered signaling pathway leading to lung microvascular barrier compromise.

DISCUSSION

Similar to smooth muscle, endothelial cells are equipped with contractile apparatus, which is involved in cell shape changes, motility, and transendothelial permeability regulation. The process of EC contraction and intercellular gap formation appears to be controlled by actin-myosin interaction via Ca\(^{2+}\)/CaM-dependent MLC phosphorylation (15). Inhibition of MLCP via a Rho-dependent mechanism contributes to increased MLC phosphorylation, endothelial contraction, and permeability (7, 8). An additional factor that influences barrier function is the activation of PKC (for a review see Ref. 37). In this study, we investigated the possible role of endogenous inhibitor of MLCP CPI-17 in MLC phosphorylation and cytoskeletal rearrangements caused by inflammatory agonists and the direct PKC activator PMA.

We have found that CPI-17 was present to a greater extent in microvascular than in macrovascular EC. Although CPI-17 in microvascular EC was not as abundant as in smooth muscle (uterus), the smaller amount of CPI-17 in HLMVEC correlated with smaller amounts of other MLCP components. Further-
Fig. 9. Effect of CPI-17 depletion on decrease in transendothelial electrical resistance (TER) induced by histamine. HLMVEC were treated either with CPI-17 small interfering (si) RNA or nonspecific (ns) RNA for 48 h as described in MATERIALS AND METHODS. A: depletion of CPI-17 RNA after treatment with siRNA. Total RNA was isolated and 288 bp CPI-17 cDNA was amplified. Ribosomal 18S cDNA was amplified as an internal control. B: CPI-17 antibody was used for Western immunoblotting to detect CPI-17 protein depletion. Pan-ERK was stained as a control. C, D: TER registered after siRNA treatment. The cells were incubated in serum-free medium for 1 h followed by challenge with either 5 μM histamine (C) or 20 nM thrombin (D). Each bar represents pooled values from 3 experiments (n = 6). CPI-17 depletion significantly attenuated the effect of histamine on TER.

more, the CPI-17/PP1 mRNA ratio in HLMVEC was close to the ratio in uterus (Fig. 1B). The amount of CPI-17 in microvascular EC, which is less than in smooth muscle, may reflect the fact that contractile machinery in EC is generally less developed. It is beyond doubt, however, that contractile properties of EC are important component of endothelial function.

To investigate how CPI-17 may be involved in cytoskeletal regulation in endothelium, we overexpressed CPI-17 in HPAEC where the endogenous background was lower compared with microvascular EC. Overexpressed CPI-17 affected neither MLC phosphorylation nor cytoskeleton in unstimulated cells. However, it caused dramatic changes in cells treated with direct PKC activator PMA. Numerous thick actin filaments and focal adhesion plaques were formed throughout the cell. This effect is different from the effect observed earlier in fibroblasts, where CPI-17 overexpression enhanced actin filaments only in the cortical area and without any stimulation (13).

It is known that lung macro- and microvascular EC differ from each other both morphologically and functionally even within one organ (39). The higher expression of CPI-17 in microvascular EC may contribute to these differences. Pulmonary microvascular EC exhibit increased numbers of focal adhesion sites per intercellular junction compared with macrovascular EC (33). The higher expression of CPI-17 in microvascular EC and the ability of CPI-17 to enhance focal adhesions may contribute to the adhesive microvascular phenotype.

Phosphorylation of CPI-17 is considered to serve as a triggering mechanism of its activation (23). Although in vitro Thr38 of CPI-17 can be phosphorylated by multiple kinases, including PKC (11) and RhoK (25), little is known about kinases participating in CPI-17 phosphorylation in vivo probably due to the unavailability of specific inhibitors. In a previously published study of Kitazawa and colleagues (23), inhibitors of RhoK (Y-27632) and PKC (GF-109203X) reduced smooth muscle contraction and CPI-17 phosphorylation in parallel, suggesting that CPI-17 functions downstream of both RhoK and PKC. Our direct study of agonist-induced CPI-17 phosphorylation in EC demonstrated that, indeed, both RhoK and PKC may contribute to CPI-17 phosphorylation in EC, since pharmacological inhibition of both kinases attenuated CPI-17 phosphorylation. However, the PKC inhibitor completely eliminated agonist-induced CPI-17 phosphorylation, but the RhoK inhibitor did not (Fig. 7). Therefore, it is most probable that PKC is essential for CPI-17 phosphorylation. RhoK also may affect CPI-17 phosphorylation indirectly, since it has been shown that inhibition of RhoK blocks PKC translocation/activation in endothelial and epithelial cells (19). It has been suggested that in smooth muscle PKC-α and -δ isoforms play a major role in CPI-17 phosphorylation (10). We found that DN-PKC-α abolished the effect of CPI-17 on endothelial cytoskeleton.

Because CPI-17 mRNA is expressed in EC and the protein is potent modulator of cytoskeleton, CPI-17 may play a role in PKC-dependent regulation of endothelial barrier. Besides PMA, two inflammatory barrier-disrupting agonists, histamine and thrombin, were tested as potential activators of CPI-17 in EC. The differences between endothelial responses to histamine and thrombin have long been discussed. Both agonists induce endothelial permeability accompanied by increased MLC phosphorylation. In contrast to the more transient effect of histamine, the reduction of endothelial barrier function induced by thrombin lasts over 1 h and is far beyond the transient rise in cytoplasmic Ca2+ concentration that accompanies the leakage induced by histamine (29). It has been suggested that the thrombin response required additional activation or sensitization steps other than Ca2+/CaM-dependent MLC phosphorylation. RhoK-dependent MYPT1 phosphorylation and inactivation are thought to be major factors contributing to agonist-induced Ca2+ sensitization in smooth muscle (40). In accordance with this hypothesis, several studies indicate that the RhoK/MYPT1 pathway is important for regulation of barrier properties of cultured EC (2, 3, 7, 8). The results obtained from thrombin-stimulated EC in culture suggest that...
RhoA and RhoK affect permeability via modulation of MLC phosphorylation and actomyosin contraction. We found that both thrombin- and histamine-induced MLC phosphorylation was sensitive to RhoK inhibition (Fig. 5).

PKC appears to be involved in agonist-induced increases in endothelial permeability (37). It has been shown that activation of PKC, and PKC-ε in particular, is a part of the thrombin-induced barrier disruption. This PKC activation is linked to modifications of VE-cadherin, p120, and β-catenin and cell-cell junction disassembly (24, 32). In our experiments thrombin apparently caused PKC activation, since thrombin-induced CPI-17 phosphorylation was sensitive to a PKC inhibitor. However, thrombin appeared to be less potent activator of CPI-17 phosphorylation than histamine (Figs. 7, 8). Furthermore, histamine- but not thrombin-induced MLC phosphorylation was PKC dependent (Fig. 5). It is possible that thrombin-induced activation of PKC is sufficient to affect cell-cell junction proteins, but not strong enough to cause significant CPI-17 phosphorylation, leading to MLCP inhibition and increased MLC phosphorylation. We conclude that histamine, and much less likely thrombin, may serve as a physiologically relevant activator of CPI-17 in EC. The fact, that histamine-induced, but not thrombin-induced, endothelial permeability is attenuated in CPI-17-depleted microvascular EC (Fig. 9) supports the hypothesis that the PKC/CPI-17 pathway is involved in endothelial barrier regulation by histamine. Our data suggest that differences between histamine and thrombin responses discussed above may be related, at least in part, to the involvement of CPI-17. Both histamine and thrombin induce endothelial barrier dysfunction via MLCP inhibition and MLC phosphorylation via Rho/RhoK pathway. Besides that, histamine may activate the PKC/CPI-17 pathway. Additional studies are needed to clarify the differences in agonist-mediated MLCP regulation in micro- and macrovascular endothelium.

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