Role of cAMP-dependent protein kinase A activity in endothelial cell cytoskeleton rearrangement

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Liu, Feng, Alexander D. Verin, Talai Borbiev, and Joe G. N. Garcia. Role of cAMP-dependent protein kinase A activity in endothelial cell cytoskeleton rearrangement. Am J Physiol Lung Cell Mol Physiol 280: L1309–L1317, 2001.—To examine signaling mechanisms relevant to cAMP/protein kinase A (PKA)-dependent endothelial cell barrier regulation, we investigated the impact of the cAMP/PKA inhibitors Rp diastereomer of adenosine 3’,5’-cylic monophosphorothioate (Rp-cAMPS) and PKA inhibitor (PKI) on bovine pulmonary artery and bovine lung microvascular endothelial cell cytoskeleton reorganization. Rp-cAMPS as well as PKI significantly increased the formation of actin stress fibers and intercellular gaps but did not alter myosin light chain (MLC) phosphorylation, suggesting that the Rp-cAMPS-induced contractile phenotype evolves in an MLC-independent fashion. We next examined the role of extracellular signal-regulated kinases (ERKs) in Rp-cAMPS- and PKI-induced actin rearrangement. The activities of both ERK1/2 and its upstream activator Raf-1 were transiently enhanced by Rp-cAMPS and linked to the phosphorylation of the well-known ERK cytoskeletal target caldesmon. Inhibition of the Raf-1 target ERK kinase (MEK) either attenuated or abolished Rp-cAMPS- and PKI-induced ERK activation, caldesmon phosphorylation, and stress fiber formation. In summary, our data elucidate the involvement of the p42/44 ERK pathway in cytoskeletal rearrangement evoked by reductions in PKA activity and suggest the involvement of significant cross talk between cAMP- and ERK-dependent signaling pathways in endothelial cell cytoskeletal organization and barrier regulation.

Raf-1; mitogen-activated protein kinase; caldesmon

PULMONARY VASCULAR ENDOTHELIUM functions as a semiselective tissue barrier between circulating blood components and the interstitium. During acute lung injuries, increases in circulating cytokines, bioactive agents, and biophysical forces such as stretch or shear stress lead to increased endothelial cell permeability, formation of intercellular gaps, and life-threatening edema. Much less is known regarding mechanisms of barrier restoration; however, studies using whole animals, isolated perfused and ventilated lungs, and cultured endothelial cell models have shown that increased concentrations of intracellular cAMP and augmented cAMP-dependent protein kinase A (PKA) activity enhance and restore endothelial barrier function (1, 2, 12, 14, 15, 28, 31, 33). cAMP is generated from intracellular ATP by adenylyl cyclase, a family of membrane-bound enzymes containing at least nine isoforms that vary in their sensitivities to activation by the stimulatory heterotrimeric GTP regulatory protein Gs, to inhibition by Gi (6, 43), and to Ca2+ and protein kinase C (PKC) (42). A key cellular target for cAMP is PKA, whose two regulatory subunits bind cAMP and induce a conformational change that produces subunit dissociation from the catalytic subunits, resulting in enzymatic activation (23, 44). Cholera holotoxin, via ADP ribosylation of Gαs, increases adenylyl cyclase activity and the synthesis of cAMP. These events dramatically reduce basal endothelial cell permeability, prevent thrombin-, phorbol 12-myristate 13-acetate (PMA)-, and pertussis toxin-induced permeability and gap formation (14, 31, 33) and reverse thrombin-induced permeability and gap formation (31). Both forskolin, a well-known direct adenylyl cyclase activator, and the cAMP analog dibutyryl cAMP also provide barrier protection, indicating that the cAMP barrier protective effects are accomplished specifically via its target, PKA activation (4, 11). Although cGMP analogs were ineffective in these studies of barrier protection, indicating that cross-activation of cGMP-dependent kinase was unlikely (31), the role of cGMP in barrier protection remains controversial (50).

We and others have demonstrated that endothelial cell barrier regulation is highly dependent on the actomyosin cytoskeleton, regulated through both myosin light chain kinase (MLCK)-dependent and -independent signaling pathways (12, 37). For example, thrombin-induced endothelial cell contraction and permeability involve the phosphorylation of myosin light chains (MLC) catalyzed by a novel nonmuscle MLCK isoform (12, 13, 45, 47). In contrast, endothelial cell barrier dysfunction produced by PKC activation is not correlated with MLCK activities but evolves via a signaling pathway that includes the activation of extracellular signal-regulated kinases (ERKs) and phosphorylation of endothelial cell cytoskeletal targets such
as caldesmon (31, 40, 46). PKA phosphorylates endothelial MLCK, thereby reducing MLCK activity, leading to decreased basal level MLC phosphorylation (12, 13), and directly phosphorylates many actin-binding proteins such as adducin, dematin, and filamin, resulting in reduced actin bundling (3, 20, 26, 48).

R<sub>p</sub> diastereomer of adenosine 3′,5′-cyclic monophosphorothioate (R<sub>p</sub>-cAMPS) and R<sub>p</sub> diastereomer of 8-bromoadenosine 3′,5′-cyclic monophosphorothioate (R<sub>p</sub>-8-BrcAMPS) are nonhydrolyzable diastereomers of (1,25) are nonhydrolyzable diastereomers of (-cyclic monophosphorothioate) and (-cyclic monophosphate) diastereomers of (-cyclic monophosphorothioate) and (-cyclic monophosphate) diastereomers of (-cyclic monophosphorothioate) and (-cyclic monophosphate). Both agents inhibit PKA activity in human microvascular and macrovascular endothelial cells and other cell types in vitro and in vivo at concentrations ranging from 10 to 500 μM (22, 35). PKA inhibitor (PKI) is a NH<sub>2</sub>-terminal myristoylated peptide that specifically and directly inhibits PKA catalytic activity (29). Although it is established that elevated cAMP and PKA activity enhances endothelial cell barrier function, the effect of reduced intracellular PKA on endothelial cell signaling mechanisms involved in barrier regulation remain unclear. In this study, we used the specific PKA inhibitors R<sub>p</sub>-cAMPS, R<sub>p</sub>-8-BrcAMPS, and PKI to determine the effect of decreased PKA activity on the endothelial cell cytoskeleton and further investigated the involvement of mitogen-activated protein kinase (MAPK) signaling pathways in R<sub>p</sub>-cAMPS- and PKI-induced endothelial cell cytoskeletal rearrangement.

**MATERIAL AND METHODS**

**Reagents.** Endothelial cells were cultured in medium 199 (M199; Life Technologies, Rockville, MD) supplemented with 20% (vol/vol) colostrum-free bovine serum (Irvine Scientific, Santa Ana, CA), 15 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, MA), 1% antibiotic and antimycotic (10,000 U/ml penicillin, 10 mg/ml streptomycin, and 25 μg/ml amphotericin B; Life Technologies), and 0.1 mM nonessential amino acids (Life Technologies). R<sub>p</sub>-cAMPS, R<sub>p</sub>-8-BrcAMPS, and PKI were purchased from Calbiochem (La Jolla, CA). MLC antibody was produced in rabbits against baculovirus-expressed and purified smooth muscle MLC by Biodesign International (Kennebunk, ME), and rabbit anti-phospho-p44/42 ERK was purchased from New England Biolabs (Beverly, MA). Phospho-caldesmon Ser<sup>788</sup> antibody was kindly provided by Dr. Leonard P. Adam (Bristol-Myers Squibb, Princeton, NJ). Unless specified, all other reagents were obtained from Sigma (St. Louis, MO).

**Bovine endothelial cell culture.** Bovine pulmonary artery endothelial cells were purchased from American Type Culture Collection (Manassas, VA) and utilized at passages 19–24. Bovine lung microvascular endothelial cells were purchased from Cell Systems (Kirkland, WA) and used at passages 3–9. Cells were cultured and maintained in complete medium at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air. Endothelial cells grew to contact-inhibited monolayers with the typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin and resuspended in fresh culture medium and placed in appropriate size flasks or dishes.

**MLC phosphorylation assay.** Assays were performed as we previously described in detail (12, 38). Briefly, after endothelial cell monolayers were incubated with either PKA inhibitors or vehicle controls, cells were lysed in 10% trichloroacetic acid, and the precipitates were homogenized and subjected to urea-polyacrylamide gel electrophoresis followed by immunoblotting with anti-MLC antibody. Immunoreactive proteins were detected using the enhanced chemiluminescence detection system (ECL; Amersham, Little Chalfont, UK), and the separated unphosphorylated, monophosphorylated, and diphosphorylated forms of MLC were quantified by laser scanning densitometry.

**Western immunoblotting.** After treatment, endothelial cell monolayers grown in 35-mm dishes were rinsed with ice-cold PBS, lysed with 100 μl of 2% SDS sample buffer (25), scraped into 1.5-ml microcentrifuge tubes, and boiled immediately for 5 min. Extracts (10 μl) were separated on 12% SDS-PAGE and transferred to nitrocellulose (30 V, 18 h). After being blocked with PBS-T (PBS with 0.1% Tween 20) containing 5% nonfat milk for 1 h, nitrocellulose blots were reacted with primary antibodies diluted in PBS-T containing 5% BSA for 1 h, washed with PBS-T (3 × 10 min), incubated with peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, 1:10,000 dilution, Sigma; or goat anti-mouse IgG, 1:10,000 dilution, Bio-Rad Laboratories, Richmond, CA) diluted in PBS-T with 5% nonfat milk for 1 h, and again washed with PBS-T (3 × 10 min). Finally, immunoreactive proteins were detected using ECL. The relative intensities of the protein bands were quantified by scanning densitometry.

**Raf-1 kinase activity assay.** Raf-1 kinase activity was determined using a commercially available kit (Upstate Biotechnology, Lake Placid, NY) according to manufacturer’s recommendations with minor modifications. Confluent endothelial cells grown in 60-mm dishes were treated with either R<sub>p</sub>-8-BrcAMPS (200 μM) or vehicle control for 10 min after 18 h of serum starvation in M199. The cells were lysed on ice in lysis buffer (500 μl; 50 mM Tris, pH 7.5, 5 mM EDTA, 1 mM EGTA, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 0.1% β-mercaptoethanol, and 0.1% Triton X-100) including a 1:500 diluted protease-inhibitory cocktail (200 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 160 mM aprotinin, 10 μM leupeptin, and 2 μM pepstatin A; Calbiochem, La Jolla, CA) for 30 min. Cell lysates were transferred into 1.5-ml Eppendorf tubes, debris was removed by a 10-min centrifugation at 16,000 g (4°C), and the supernatants were incubated with 4 μg of sheep anti-human c-Raf kinase COOH-terminal antibodies on ice for 2 h, followed by incubation with 100 μl of PBS-prewashed and lysis buffer-equilibrated protein G Sepharose slurry (containing 30% protein G Sepharose 4 fast flow; Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h at 4°C with gentle agitation. Protein G Sepharose with immunoprecipitated Raf-1 was washed and incubated with inactive glutathione S-transferase (GST)-mitogen-activated protein (MAP) kinase kinase 1 (MEK1), inactive GST-p42 MAP kinase in the kinase assay buffer containing magnesium, ATP, and an inhibitor cocktail to inhibit other serine/threonine kinases. The activated p42 MAP kinase was then used to phosphorylate myelin basic protein (MBP) in the presence of [γ-<sup>32</sup>P]ATP. The radiolabeled substrate was allowed to bind to P81 phosphocellulose paper, and the radioactivity per minute was measured in a scintillation counter. In parallel, sheep IgG was used in separate immunoprecipitation reactions to control nonspecific binding of cellular proteins with the primary antibody. This nonspecific radioactive count (in counts/min) was subtracted from the counts per minute generated by anti-c-Raf antibody, resulting in the relative Raf-1 kinase activities (in counts/min).
Detection of ERK activation. Endothelium grown in 35-mm dishes was rinsed three times with M199 and incubated with Rp-cAMPS, Rp-8-BrcAMPS (200 μM), or PKI (20 μM) for the indicated periods of time in M199 (700 μl) in a 37°C incubator with 5% CO2. After being rinsed with ice-cold PBS, cells were preincubated with 2× SDS sample buffer (100 μl), scraped into 1.5-ml Eppendorf tubes, and boiled for 5 min. The cell lysates (10 μl) were subjected to 12% SDS-PAGE, followed by Western blotting. ERK1/2 phosphorylation was detected with 1 μg/ml of rabbit anti-phospho-p44/42 MAPK, total ERKs were detected with monoclonal anti-pan ERK antibody (50 ng/ml, Transduction Laboratories, Lexington, KY). In some experiments, cells were preincubated with 50 μM PD-98059 or 10 μM UO-126 for 30 min before Rp-cAMPS or PKI treatment.

Measurement of caldesmon phosphorylation. PKA inhibition-induced caldesmon phosphorylation was assessed using two complementary methods. First, the composite phosphorylation of caldesmon was measured in caldesmon immunoprecipitates obtained from 32P-labeled cells. Endothelial cell monolayers in 60-mm dishes were serum starved in PO 43−/−/−/− free DMEM for 16 h followed by radioactive labeling with 200 μCi/ml of [γ-32P]orthophosphate for 4 h. After being rinsed with PO 43−/−/−/− free DMEM, cells were incubated with Rp-8-BrcAMPS (200 μM) or vehicle controls for either 10 or 30 min. After a brief rinse with PBS, cells were lysed in boiling lysis buffer (100 μl) containing 10 mM Tris, pH 7.4, 1 mM sodium orthovanadate, and 1% SDS. Cell lysates were transferred into microcentrifuge tubes and boiled for 5 min. Cell debris was removed by centrifugation at 16,000 g for 5 min, and supernatants were transferred into 1.5-ml Eppendorf tubes containing 10 μl of monoclonal anti-caldesmon, 300 μl of H2O, and 400 μl of 2× immunoprecipitation buffer (20 mM Tris, pH 7.4, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.4 mM sodium orthovanadate, 2% Triton X-100, and 1% NP-40). The mixture was incubated on ice for 1 h followed by incubation with 100 μl of preequilibrated protein G Sepharose slurry (containing 30% protein G Sepharose 4 fast flow, Amersham) for 1 h at 4°C with gentle agitation. After being washed (4×) with immunoprecipitation buffer, the immunoprecipitated complex was resuspended in 2× SDS sample buffer (40 μl) and boiled for 5 min. The supernatant (10 μl) was subjected to electrophoresis on 12% SDS-PAGE, and protein was transferred to nitrocellulose and exposed to a phosphorothioate (Rp) diastereomer of adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS) on endothelial cell actin stress fiber and intercellular gap formation in endothelium. We have recently reported that decreased PKA activity by Rp-cAMPS treatment resulted in significant decreases in electrical resistance across bovine pulmonary artery endothelial cell monolayers, indicating an increase in endothelial cell permeability (32). To study the mechanisms involved in PKA-mediated barrier regulation, we first investigated the effect of reduced PKA activity on endothelial cell cytoskeletal organization. Endothelial cells were incubated with Rp-cAMPS or its highly cell-permeable form Rp-8-BrcAMPS and stained with Texas Red-X phalloidin to detect filamentous actin (F-actin). Whereas stress fiber and gap formation were not present in vehicle-treated cells (Fig. 1A), Rp-cAMPS treatment (200 μM, 15 min) significa-
cyclic monophosphorothioate (8-BrcAMPS; 200 μM) for 10 min (lane 2) and 60 min (lane 3), or thrombin (Thr, 100 nM) for 10 min (lane 4) or pretreated with Rp-cAMPS (200 μM) for 30 min followed by thrombin (100 nM, 10 min, lane 5). Cells were lysed and trichloroacetic acid precipitated, and the unphosphorylated (un-P) and mono (mono-P)- and diphosphorylated (di-P) forms of MLC were separated by urea-polyacrylamide gel electrophoresis, detected by immunoblotting and quantified by densitometry. The stoichiometry (in mol/mol) was calculated as we previously described (12). Neither basal nor stimulated MLC phosphorylation was significantly altered by reductions in protein kinase A (PKA) activity. Data are representative of 3 independent experiments.

Fig. 2. Effect of Rp-cAMPS on endothelial cell myosin light chain (MLC) phosphorylation. Bovine endothelial cell monolayers were incubated with vehicle (lane 1), Rp-cAMPS (Rp, 200 μM) for 10 min (lane 2) and 60 min (lane 3), or thrombin (Thr, 100 nM) for 10 min (lane 4) or pretreated with Rp-cAMPS (200 μM) for 30 min followed by thrombin (100 nM, 10 min, lane 5). Cells were lysed and trichloroacetic acid precipitated, and the unphosphorylated (un-P) and mono (mono-P)- and diphosphorylated (di-P) forms of MLC were separated by urea-polyacrylamide gel electrophoresis, detected by immunoblotting and quantified by densitometry. The stoichiometry (in mol/mol) was calculated as we previously described (12). Neither basal nor stimulated MLC phosphorylation was significantly altered by reductions in protein kinase A (PKA) activity. Data are representative of 3 independent experiments.

cantly increased the intensity of F-actin staining, the number of actin stress fibers, and the number of paracellular gaps in endothelial cell monolayers (Fig. 1B). These findings are similar to thrombin treatment (100 nM, 10 min, Fig. 1C), which also increased both stress fiber formation and the number of gaps. However, whereas thrombin reduced F-actin staining at the cortical ring, Rp-cAMPS induced strong actin polymerization of the peripheral band at this site. These results indicate that reductions in PKA activity with Rp-cAMPS and with Rp-8-BrcAMPS (data not shown) produce actin reorganization via a mechanism that likely differs from that evoked by thrombin and are highly consistent with an important role for PKA in endothelial barrier regulation.

MLC phosphorylation is not significantly increased by Rp-cAMPS. Our laboratory has previously shown that the phosphorylation status of MLC is a key determinant of endothelial cell contraction and intercellular gap formation (12, 31) and transendothelial migration of neutrophilic leukocytes (15). Therefore, to study the signaling pathways involved in Rp-cAMPS-induced cytoskeleton reorganization, we examined the phosphorylation status of MLC after Rp-cAMPS treatment in endothelial cell monolayers. Interestingly, despite significant actin stress fiber formation, inhibition of endothelial cell PKA activity by Rp-cAMPS (200 μM, 10 and 60 min) or by Rp-8-BrcAMPS (data not shown) failed to significantly alter MLC phosphorylation, whereas thrombin (100 nM, 10 min) as a positive control induced dramatic increases in diphosphorylated MLC (Fig. 2). Furthermore, pretreatment with Rp-cAMPS (200 μM, 30 min) did not affect thrombin-induced MLC phosphorylation, suggesting that the endothelial cell cytoskeleton reorganization mediated by reductions in PKA activity occurs independently of MLCK activity.

Role of ERK signaling pathway in endothelial cell stress fiber formation induced by decreased PKA activity. We have recently shown that ERK1/2 activation is a key event in PKC-induced endothelial cell barrier dysfunction (46), which, similar to Rp-cAMPS, evolves via a MLCK-independent mechanism. We next examined the role of the ERK signaling cascade in Rp-cAMPS-induced actin reorganization. Rp-cAMPS and Rp-8-BrcAMPS (200 μM) induced significant ERK phosphorylation in a time-dependent manner (Fig. 3), reaching maximum ERK activation at 10 min and falling thereafter to diminished levels after 30 min. Pretreatment with PD-98059 (50 μM, 30 min), a spe-
cific ERK kinase (MEK-1) inhibitor, completely abolished Rp-cAMPS-induced ERK activation without affecting the total amount of ERK protein (Fig. 4A), indicating that activation of ERKs by Rp-cAMPS occurred specifically via MEK-1 activity. Furthermore, a NH₂-terminal myristoylated PKA-inhibitory peptide, PKI, which specifically and directly inhibits PKA catalytic activity (29), was used to confirm that the effects of Rp-cAMPS are exclusively attributed to PKA inhibition. Consistent with Rp-cAMPS effects in bovine macrovascular endothelial cells, PKI (20 μM, 10 min) dramatically enhanced ERK activation in bovine microvascular endothelial cells (Fig. 4B, top), which was distinguished by anti-pan ERK antibody because phosphorylated ERK migrates slower on acrylamide gels (Fig. 4B, bottom). Like Rp-cAMPS, PKI-induced ERK activation also was abolished by specific MEK-1 inhibition (UO-126; Fig. 4B). PKI (20 μM) also produced stress fiber and intercellular gap formation in endothelial cell monolayers, which were significantly attenuated by UO-126 (Fig. 4C). Similarly, inhibition of MEK-1 by PD-98059 partially blocked Rp-cAMPS-induced stress fiber formation in endothelial cells (data not shown). These data suggest that the actin polymerization avidly enhanced by inhibition of PKA activity is ERK dependent. Reduced PKA activity also increased the activity of the upstream MEK-activating Raf-1 kinase as measured by the phosphorylation of MBP through the Raf-1-MEK1-ERK2 kinase cascade. As shown in Fig. 5, the incubation of endothelial cells with Rp-8-BrCAMP (200 μM, 10 min) produced a 2.6-fold increase in Raf-1 activity, consistent with previous reports that the elevation of cellular cAMP levels downregulates Raf-1 kinase activity (8, 27). These data provide further confirmation that inhibition of PKA activity activates the ERK pathway. Consistent with these data, elevation of cAMP level and PKA activity by forskolin, which increases cellular cAMP by activating adenylyl cyclase, reduced basal ERK phosphoryla-

**Fig. 4.** Involvement of ERK kinase (MEK)/ERK signaling pathway. A: ERK phosphorylation in endothelial cells preincubated with PD-98059 (PD, 50 μM, 30 min) or vehicle control and then treated with Rp-cAMPS (200 μM, 10 min) or phorbol 12-myristate 13-acetate (PMA) as control. ERK phosphorylation was completely abolished by PD-98059. Ab, antibody. B: PKA-inhibitory peptide (PKI, 20 μM, 10 min) also significantly increased ERK phosphorylation, which was abolished by UO-126 (10 μM, 30 min). C: effect of PKI on bovine lung microvascular endothelial cell cytoskeletal organization in the absence and presence of MEK inhibition. Endothelial cell monolayers were treated with PKI (20 μM, 15 min) or vehicle in the absence and presence of UO-126 (10 μM, 30 min) in M199, fixed, permeabilized, and stained with 1 U/ml of Texas Red-X phalloidin to visualize F-actin. a, Vehicle control; b, PKI alone; c, UO-126 alone; d, UO-126 pretreatment followed by incubation with PKI. Reduction of PKA activity by PKI increased F-actin polymerization and stress fiber and gap formation. Inhibition of MEK activity by UO-126 significantly attenuated PKI-induced stress fiber and gap formation. Arrowheads indicate intercellular gaps. Data shown are representative of 3 independent experiments.

**Fig. 5.** Effect of Rp-cAMPS on Raf-1 kinase activity. Bovine endothelial cell monolayers were serum starved for 18 h and incubated with Rp-8-BrCAMP (200 μM, 10 min). Raf kinase was immunoprecipitated from cell lysates by anti-c-Raf kinase antibody and incubated with inactive MEK1, inactive p42 MAPK to sequentially activate MEK1, and p42 MAPK. The activated p42 MAPK was then used to phosphorylate myelin basic protein (MBP) in the presence of γ-32P]ATP. The radioactivities of the 32P-labeled MBP were counted in a scintillation counter. The Raf-1 kinase activities after Rp-8-BrCAMP were compared with the basal Raf-1 activities. Data are presented as percentage of control (means ± SE, n = 3, P < 0.01). Inset: raw data showing average counts/min (CPM) from a typical experiment performed in triplicate. Inhibition of PKA activity significantly increased Raf-1 kinase activity.
tion (16) and caused F-actin dissolution (data not shown). These findings confirm an important association between PKA and ERK activities and cytoskeletal regulation.

Role of caldesmon phosphorylation in Rp-cAMPS- and PKI-induced endothelial cell cytoskeletal reorganization. Although our findings suggest that ERK may be involved in endothelial cell barrier regulation by PKA, the mechanisms of ERK-mediated cytoskeletal rearrangement remain unclear. However, phosphorylation of the thin filament-associated cytoskeletal protein caldesmon, a known ERK target, has been postulated as an important event in the regulation of smooth muscle contraction (34, 49). Furthermore, thrombin-, PMA-, and pertussis toxin-mediated endothelial cell barrier dysfunction are associated with caldesmon phosphorylation and redistribution to new actin filaments (16, 40, 46). Therefore, to further study the mechanisms of PKA inhibition-induced endothelial cell cytoskeletal rearrangement and cell contraction, we investigated the effect of reduced PKA activity on caldesmon phosphorylation. First, 32P-labeled endothelial cells were treated with Rp-8-BrCAMPs (200 μM, 10 and 30 min), and the composite caldesmon phosphorylation in immunoprecipitates from cell lysates was determined by autoradiography. Inhibition of PKA significantly enhanced caldesmon phosphorylation both at 10 min (80% increase) and at 30 min (120% increase, Fig. 6A). To define ERK involvement in caldesmon phosphorylation, Rp-cAMPs-treated endothelial cell lysates were immunoblotted with an antibody generated against the phosphopeptide 781CQSVDKVTSPKV785, which contains the specific ERK phosphorylation site Ser789 present in mammalian h-caldesmon (21). As shown in Fig. 6B, caldesmon phosphorylation, induced by PKA inhibition and detected by the ERK-specific antibody, was significantly increased after 10 min (70% increase) and was sustained through 60 min. Furthermore, inhibition of PKA activity by PKI evoked significant caldesmon phosphorylation. Inhibition of PKA by PKI evoked significant caldesmon phosphorylation. Reduction in MEK activity by UO-126 completely blocked PKI-induced caldesmon phosphorylation. Data are representative of 3 independent experiments.

Fig. 6. Reduction in PKA activity increases caldesmon phosphorylation. A: bovine endothelial cell monolayers were labeled with 32P-orthophosphate (4 h) and treated with Rp-8-BrCAMPs (Rp, 200 μM, 10 and 30 min). Total caldesmon (CaD) was immunoprecipitated under denaturing conditions using monoclonal anti-caldesmon antibody. After electrophoresis with 12% SDS-PAGE, the immunoprecipitates were transferred to nitrocellulose followed by phosphorimaging and autoradiography. Top: autoradiography of a representative experiment showing the increase in caldesmon phosphorylation evoked by Rp-8-BrCAMPs. Bottom: increase of caldesmon phosphorylation in 3 independent experiments (means ± SE, *P < 0.05). B: ERK-specific caldesmon phosphorylation detected by Western blotting. Bovine endothelial cells incubated with Rp-cAMPs (200 μM, 10, 30, and 60 min) were lysed and immunoblotted with an anti-caldesmon antibody that specifically recognizes ERK phosphorylation site Ser789 of caldesmon. Top: immunoblot of a representative experiment showing ERK-mediated caldesmon phosphorylation. Bottom: increase of caldesmon phosphorylation in 3 independent experiments (means ± SE, *P < 0.05). C: ERK-specific caldesmon phosphorylation produced by PKI. Bovine endothelial cells were incubated with MEK inhibitor UO-126 (UO, 10 μM, 30 min) or vehicle, followed by incubation with PKI (20 μM, 10 min). Cells were lysed and immunoblotted with the ERK-specific anti-phospho-caldesmon antibody. Inhibition of PKA by PKI evoked significant caldesmon phosphorylation. Reduction in MEK activity by UO-126 completely blocked PKI-induced caldesmon phosphorylation. Data are representative of 3 independent experiments.
There is substantial evidence that activation of cAMP-dependent PKA enhances vascular barrier integrity, whereas decreased cAMP is correlated with impaired barrier function (12, 31, 41, 42). This implies that PKA has an important role in vascular barrier regulation by modulating the balance between endothelial cell contractile and tethering forces in some manner, although the precise signaling pathways involved remain unclear. Our data support this central role of PKA in barrier regulation and strongly implicate MAPK activity as a relevant signaling paradigm. Three specific PKA antagonists (Rp-cAMPS, Rp-8-BrcAMPS, and PKI) were utilized to explore the potential mechanisms of PKA in endothelial cell permeability regulation and were found to produce dramatic actin cytoskeletal rearrangement and intercellular gap formation, indicating cell contraction and barrier dysfunction. The actin rearrangement after PKI and Rp-cAMPS is neither reminiscent of PMA, which produces cortical actin dissolution without stress fiber formation (46), nor thrombin, where the stress fibers are thick and accompanied by cortical actin disappearance (12). Previous studies in our laboratory have shown that increased PKA activity by cholera toxin significantly reduces MLCK activity via enhanced MLCK phosphorylation (13). PKA inhibition by Rp-cAMPS, however, failed to affect basal or thrombin-induced MLC phosphorylation. These results suggest that the endothelial cell barrier dysfunction mediated by reductions in PKA activity does not involve a direct effect on MLCK activity, findings that are similar to other specific models of vascular permeability that evolve in a MLC-independent fashion (16, 46). Consistent with two PKC-dependent models of endothelial cell barrier dysfunction produced by pertussis toxin and PMA, where the activation of MAPK signaling pathways has been implicated (16, 46), we found that inhibition of PKA activity transiently increased MAPK phosphorylation, especially ERK1/2 MAPK, and facilitated subsequent actin rearrangement and stress fiber formation. The mechanism by which inhibition of PKA increases ERK activity is unclear but involves an increase in the activity of Raf-1 kinase, the enzyme immediately upstream of MAPK kinase (MEK). This is consistent with the notion that basal cAMP production and PKA activity inhibit the MAPK signaling cascade by phosphorylating Raf-1 and, presumably, reducing Raf-1 binding affinity to Ras-GTP and directly decreasing Raf-1 catalytic activity (5, 8, 19, 27, 51). It is most likely that reduction in this basal PKA activity allows the accumulation of active Raf-1 and sequential increases in MEK and ERK MAPK activities. Our results are similar to the findings of D’Angelo et al. (8), who found that basal PKA activity constitutively suppresses activation of Raf-1/ERK in bovine brain capillary endothelial cells. Our data extend this observation in several ways but most importantly suggest that basal PKA activity in bovine lung endothelium is not only sufficient to suppress the ERK signaling pathway but is also necessary for the maintenance of endothelial cell barrier integrity (32, 41).

One potentially important endothelial cytoskeletal target for ERK phosphorylation is nonmuscle caldesmon, a 77-kDa actin-, myosin- and calmodulin-binding protein hypothesized to function as a molecular switch in the regulation of nonmuscle and smooth muscle contraction (39) by modulating the dynamics of actin filament organization (34, 52). Studies in bovine endothelium have shown that thrombin- and PMA-mediated endothelial barrier dysfunction is temporally linked and directly associated with caldesmon phosphorylation and redistribution (40). Furthermore, MAPK-induced smooth muscle caldesmon phosphorylation has been suggested to reverse the inhibitory effects of caldesmon on cross-bridge cycling, thereby allowing actomyosin contraction (7, 9, 17, 18). Although this remains controversial (24, 30), antisense oligodeoxynucleotide strategies to deplete caldesmon from smooth muscle significantly attenuated agonist-induced tension development (10). In the present study, using antibodies specifically immunoreactive with ERK-mediated caldesmon phosphorylation sites, we demonstrated that reduction of PKA activity stimulated caldesmon phosphorylation, implicating a role of ERK-dependent caldesmon phosphorylation in PKA barrier regulation. Our data also indicate, however, that additional pathways exist that link PKA and actin stress fiber formation since complete MEK inhibition attenuated agonist-induced tension development (10). In the present study, using antibodies specifically immunoreactive with ERK-mediated caldesmon phosphorylation sites, we demonstrated that reduction of PKA activity stimulated caldesmon phosphorylation, implicating a role of ERK-dependent caldesmon phosphorylation in PKA barrier regulation. Our data also indicate, however, that additional pathways exist that link PKA and actin stress fiber formation since complete MEK inhibition attenuated agonist-induced tension development (10).

In summary, our study demonstrates the critical role of cAMP and PKA activity in the regulation of endothelial cell cytoskeleton reorganization. Pharmacological reduction in PKA activity is sufficient to induce actin stress fiber formation, leading to cell contraction mediated, at least in part, by the activation of a Raf-1/ERK signaling pathway. Although the exact ERK targets that produce endothelial cell cytoskeletal rearrangement resulting in paracellular gap formation remain unclear, our data support an active role of caldesmon in this process.

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