Myosin Phosphatase and Cofilin Mediate cAMP/cAMP-dependent Protein Kinase-induced Decline in Endothelial Cell Isometric Tension and Myosin II Regulatory Light Chain Phosphorylation* [S]

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This study determined the effects of increased intracellular cAMP and cAMP-dependent protein kinase activation on endothelial cell basal and thrombin-induced isometric tension development. Elevation of cAMP and maximal cAMP-dependent protein kinase activation induced by 10 μM forskolin, 40 μM 3-isobutyl-1-methylxanthine caused a 50% reduction in myosin II regulatory light chain (RLC) phosphorylation and a 35% drop in isometric tension, but it did not inhibit thrombin-stimulated increases in RLC phosphorylation and isometric tension. Elevation of cAMP did not alter myosin light chain kinase catalytic activity. However, direct inhibition of myosin light chain kinase with KT5926 resulted in a 90% decrease in RLC phosphorylation and only a minimal decrease in isometric tension, but it prevented thrombin-induced increases in RLC phosphorylation and isometric tension development. We showed that elevated cAMP increases phosphorylation of RhoA 10-fold, and this is accompanied by a 60% decrease in RhoA activity and a 78% increase in RLC phosphatase activity. Evidence is presented that it is this inactivation of RhoA that regulates the decrease in isometric tension through a pathway involving cofilin. Activated cofilin correlates with increased F-actin severing activity in cell extracts from monolayers treated with forskolin/3-isobutyl-1-methylxanthine. Pretreatment of cultures with tautomycin, a protein phosphatase type 1 inhibitor, blocked the effect of cAMP on 1) the dephosphorylation of cofilin, 2) the decrease in RLC phosphorylation, and 3) the decrease in isometric tension. Together, these data provide in vivo evidence that elevated intracellular cAMP regulates endothelial cell isometric tension and RLC phosphorylation through inhibition of RhoA signaling and its downstream pathways that regulate myosin II activity and actin reorganization.

Endothelial cells lining blood vessels form a continuous layer that constrains proteins and blood elements to the vascular lumen. Disruption of the continuous endothelial barrier leads to an increase in permeability and development of edema, a hallmark of acute and chronic inflammation. Chemical or inflammatory mediators activate signaling pathways that cause endothelial cells to contract forming gaps between adjacent cells leading to an increase in vascular permeability. Several laboratories studying methods to inhibit endothelial cell contraction and vascular permeability have demonstrated that elevation of intracellular cAMP augments barrier function and prevents increases in permeability to a wide range of inflammatory agonists. The primary pathway implicated in preventing increases in permeability is believed to occur through the activation of adenylyl cyclase, accumulation of intracellular cAMP, and activation of PKA. Even though there are numerous reports (1–5) implicating cAMP/PKA in enhancing barrier function and inhibiting endothelial cell contraction, the mechanism by which cAMP/PKA protects vascular integrity is poorly understood.

CAMP is a primary mediator of the biological effects of β-adrenergic hormones, including hormone-induced relaxation of smooth muscle tissue (6–9). In 1984 de Lanerolle et al. (10) proposed, as a mechanism for smooth muscle relaxation, that increases in cAMP lead to activation of PKA, which phosphorylates MLCK inhibiting its catalytic activity and causing a decrease in myosin II activity. Since this hypothesis was proposed, two general classes of MLCKs, nonmuscle and smooth muscle MLCK, have been identified. The nonmuscle or larger form ranges in size between 206 and 220 kDa (11–14), and the smooth muscle or smaller form ranges in size between 125 and 155 kDa (15–18). Both classes of MLCKs have identical biochemical properties in vitro and exhibit overlapping tissue distribution patterns (11, 19, 20).

Numerous studies have shown that smooth muscle MLCK, which has multiple sites that can be phosphorylated by several kinases such as PKA, protein kinase C, and Ca2+/CaM-dependent kinase II in vitro at two residues, site A (Ser992) and site B (Ser1005), in the C-terminal region near the calmodulin binding domain (8, 21, 22). Phosphorylation at these sites results in desensitization of MLCK to activation by Ca2+ or CaM. In addition, inhibition of MLCK activity by p21-activated kinase has been reported (23, 24). However, little information is available documenting the effects of phosphorylation on the nonmuscle form of MLCK.

In nonmuscle cells, elevated cAMP has been shown to cause the following: 1) alterations in cell morphology; 2) inhibition of cell motility; 3) disruption of actin filaments; 4) pericyte relaxation in a silicone rubber wrinkling assay; and 5) fibroblast relaxation in a collagen gel assay (1, 25–29). In endothelial cells, preliminary studies have shown that elevations in cAMP correlate with an increase in MLCK phosphorylation (13, 30) and a decrease in RLC phosphorylation. Nonmuscle MLCK has...
been shown to contain putative PKA consensus phosphorylation sites, and PKA has been reported to inhibit MLCK activity in vitro. Based on these initial observations, it has been inferred that the enhanced barrier function induced by cAMP results from PKA phosphorylation of MLCK and inhibition of MLCK activity leading to decreased RLC phosphorylation and endothelial cell relaxation. For the last 15 years there has been considerable controversy regarding the physiological importance of MLCK phosphorylation by PKA. It is clear that CAMP modulates endothelial barrier function and prevents increases in vascular permeability to inflammatory mediators. That barrier protection and inhibition of endothelial cell contraction are a result of PKA phosphorylation of MLCK remains unsubstantiated.

The Rho family of GTPases, which includes Cdc42, Rac, and Rho, has been shown to play a pivotal role in dynamic rearrangements of the cytoskeleton. Rho cycles between the GDP-bound inactive state and GTP-bound active state and has been implicated in formation of stress fibers, cell motility, cell adhesion, smooth and nonmuscle cell contraction, neurite retraction, and cell cycle progression (reviewed in Refs. 31–33). Upon binding to GTP, Rho interacts with and activates multiple downstream effectors such as mDia, Rho kinase, and phosphatidylinositol 4,5-kinase, which are involved in actin polymerization, formation of focal adhesion/stress fibers, and cell contraction. RhoA activity is tightly controlled by activating factors such as the guanine nucleotide exchange factors that catalyze the exchange of GDP for GTP and by the following inactivating factors: GTPase-activating proteins that enhance hydrolysis of GTP to GDP. In resting cells RhoA is also regulated by RhoGDI (guanosine nucleotide dissociation inhibitor) that binds GDP-Rho and sequesters the complex within the cytoplasm and prevents its activation. In addition, recent evidence has emerged suggesting the PKA/cGMP-dependent protein kinase pathways act as negative regulators of RhoA function (34–36).

*Clostridium botulinum* exoenzyme C3 catalyzes ADP-ribosylation of RhoA at Asn41 (37) and exerts its effects on cell morphology and F-actin stress fibers. Inhibition of Rho by C3 leads to changes in cell morphology and loss of stress fibers similar to those observed in cells treated with either dibutyryl cAMP or forskolin. These observations led to the hypothesis that cAMP and C3 may share a common pathway. Lang et al. (36) studying the effects of cAMP and C3 on HK cells documented that both agents produced similar effects on cell morphology and motility. This observation led to the demonstration that PKA catalyzes phosphorylation of RhoA on Ser188, and this phosphorylation inhibits RhoA activity establishing an alternative pathway for terminating RhoA signaling. Since these initial reports, studies have confirmed that the RhoA signaling cascade is regulated by PKA phosphorylation establishing an alternative pathway for regulating Rho GTPases (35, 36, 38). Although evidence has accumulated establishing PKA as a modulator of RhoA activity, the downstream effects of PKA inhibition of RhoA remain unidentified.

Studies presented in this report characterize the effect of elevated intracellular cAMP on basal and agonist-stimulated isometric tension in confluent endothelial cell monolayers. We provide evidence that in endothelial cells the decrease in RLC phosphorylation and the drop in isometric tension upon elevation of cAMP are not mediated via PKA phosphorylation of MLCK (35, 36), but through RhoA inactivation, which alters the activation of effector proteins within the Rho/Rho kinase signaling cascade. Furthermore, evidence is presented showing that both myosin phosphatase and cofilin activity increase in the presence of RhoA inactivation leading to the decrease in RLC phosphorylation, disruption of F-actin, and the drop in endothelial cell isometric tension observed upon elevation of cAMP.

**Materials and Methods**

**Cell Culture**—The bovine pulmonary artery endothelial (BPAE) cell line isolated and characterized by Del Vecchio and Smith (39) was obtained from the American Type Culture Collection (CLL-209). Cells were grown and maintained in minimal essential media (MEM) supplemented with 2 mM glutamine, 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. BPAE monolayers used in these studies were 6 days post-confluent.

**Cell Labeling**—BPAE monolayers were washed in phosphate-free MEM containing 0.25% bovine serum albumin and labeled with [32P]orthophosphoric acid as described previously (40). Monolayers were incubated in low phosphate media containing 10 μM Fsk, 40 μM IBMX (Fsk/IBMX) and 75 μCi/ml [32P]orthophosphoric acid for the duration of the experiment.

**Protein Purification**—Recombinant rabbit smooth muscle MLCK (MLCK155) was expressed in SF-9 cells (23, 41); recombinant nonmuscle myosin II RLC was expressed in BL21 (DE3) bacteria (41) and purified as described previously (42).

**Antibodies**—Polyclonal antibodies specific to MLCK155 were generated against purified full-length protein. Antibodies specific to nonmuscle myosin II RLC were generated to recombinant light chain (41) coupled to tuberculin PPD (Statens Seruminstitut, Denmark) with glutaraldehyde. Polyclonal antibodies were raised to purified MLCK155 in New Zealand White rabbits, and the IgG fractions were purified from pooled rabbit serum. MLCK155 antibodies were affinity-purified as described by Goeckeler and Wysolmerski (40). A monoclonal antibody raised to chicken gizzard MLCK was purchased from Sigma (M7905; clone K36). Affinity-purified rabbit polyclonal coflin (catalog number 3312) and phosphocofilin (Ser3; catalog number 3311) peptide antibodies were purchased from Cell Signaling Technology. Rabbit polyclonal antibody to RhoA (catalog number SC-178) was from Santa Cruz Biotechnology. A phospho-RhoA-Ser188 peptide antibody (catalog number ST1035) was from Calbiochem. Rabbit polyclonal antibody to LIM kinase (catalog number L2290) was from Sigma, and a phospho-LIM kinase 1/2 peptide antibody (catalog number NSB1076) was from Novus Biologicals, Inc.

**Measurement of Intracellular cAMP and Protein Kinase A Activity**—BPAE monolayers grown on 35 × 10-mm dishes were incubated with 0.5, 1, 5, 10, 20, 30, or 50 μM Fsk alone and then with 10 μM Fsk and 20, 40, 80, 100, and 500 μM or 1 mM IBMX for 10 min. To determine CAMP levels, the cultures were washed with 1 ml of MEM, 0.25% bovine serum albumin containing 40 μM IBMX and flooded with 0.5 ml of working lysis reagent. Samples were processed, and cAMP was measured by using the CAMP Biotrack EIA system (Amersham Biosciences catalog number RP1 225).

To measure PKA activity, monolayers were flooded with 100 μl of ice-cold PKA homogenization buffer (50 mM Tris, 25 mM K2HPO4, 25 mM KH2PO4, 5 mM EDTA, 5 mM EGTA, 1 mM IBMX, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml le-1-tosylamide-2-phenylethyl choloromethyl ketone, 1-chloro-3-tosylamido-7-ami-no-2-heptanone, aprotinin, leupeptin, and pepstatin, pH 7.2). PKA activity was measured in supernatants immediately after cell lysis using a protein kinase A assay (Transbio Corp., Baltimore, MD) following the manufacturer’s instructions. cAMP concentration and PKA activity were normalized to total protein.

**Isometric Tension Measurements**—Isometric tension measurements were performed as described in detail previously (40, 43). BPAE cells were seeded onto precast collagen gels and maintained at 37 °C in a humidified 5% CO2 atmosphere. Monolayers were confluent within 2–3 days after seeding and used for experiments after basal isometric tension stabilized (5–7 days after confluence).
Analysis of Myosin Light Chain Phosphorylation—Myosin II RLC phosphorylation states were separated by urea/glycerol gel electrophoresis (44). Control and treated monolayers were flooded with 1 ml of ice-cold 10% trichloroacetic acid containing 10 mM DTT and processed as described previously (23, 45). The pellets were dissolved in 80 μl of 9.5 M urea, 10 mM DTT, 20 mM Tris, 23 mM glycine, and 0.04% bromophenol blue, pH 8.8.

Urea/glycerol PAGE and Western blotting were carried out as described previously (41). Myosin II RLCs were detected by ECL methodology (Amersham Biosciences). X-ray films were developed at various time intervals to obtain an exposure within the linear range of the films. Myosin RLC quantitation was performed as described previously (40).

MLCK Immunoprecipitation and Activity Measurements—MLCK210 was immunoprecipitated from either [32P]orthophosphoric labeled or unlabeled monolayers with a rabbit polyclonal MLCK antibody (227423) as described previously (23). Immunoprecipitates were electrophoresed on 7.5% SDS gels, transferred to PVDF membrane, probed with a mouse monoclonal to gizzard smooth muscle MLCK, and detected by ECL. PVDF membranes were air-dried and exposed to PhosphorImager plates for detection of 32PO4 incorporation in MLCK. To account for differences in recovery of immunopurified MLCK, autoradiographs and corresponding Western blot ECL films were each quantitated by two-dimensional laser densitometry and MLCK phosphorylation expressed as a ratio of 32PO4 densitometric units to Western blot densitometric units. All values were normalized to untreated controls.

To measure catalytic activity, MLCK210 was immunoprecipitated from unlabeled BPAE monolayers treated with 10 μM Fsk, 40 μM IBMX for 5 and 30 min and used in an in vitro phosphorylation assay. Protein G beads containing MLCK210 were resuspended in 50 μl of phosphorylation buffer (25 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 150 mM KCl, 1 mM CaM, 5 mM CaCl2, 1 mM RLC) containing 125 μM [γ-32P]ATP and incubated at 30 °C for 30 min. Assays were terminated by transferring 10 μl of reaction mixture to P81 papers that were immediately immersed in 10% trichloroacetic acid, 2% Na2P2O7 for 10 min. The papers were washed in 1% trichloroacetic acid, 2% Na2P2O7, and the phosphorylated product was quantitated by liquid scintillation counting. The amount of immunopurified kinase in each reaction mixture was quantitated by Western blot using an affinity-purified rabbit polyclonal MLCK155 antibody. Known amounts of recombinant MLCK155 were electrophoresed in 7.5% SDS gels, transferred to PVDF membrane, and probed with affinity-purified MLCK155 antibody to generate a standard curve, which was used to compute the amount of immunopurified kinase in each reaction mixture. MLCK activity is expressed as picomoles of 32PO4 incorporated per pmol of RLC/ng of MLCK210.

RhoA Phosphorylation and Activity Assay—To determine whether RhoA was phosphorylated in response to elevated cAMP, treated and control monolayers were flooded with 100 μl of 100 °C Laemmli sample buffer, scraped up, sonicated to shear DNA, heated for 10 min at 100 °C, and centrifuged at 22,000 × g for 10 min to remove cell debris. Equivalent samples were electrophoresed on 12% SDS gels, transferred to PVDF membrane, and probed with a 1:1000 dilution of affinity-purified rabbit polyclonal antibody to RhoA or phospho-Ser180-RhoA. Western blots of phospho-RhoA and total RhoA were detected by ECL methodology. X-ray films were quantitated by laser densitometry, and the phospho-RhoA densitometric units were divided by the RhoA densitometric units for each time point. For each experiment, all phospho-RhoA values were then normalized to the control value and expressed as a ratio of control.

BPAE cells were plated at a density of 1 × 10⁶ cells per 100-mm dish and used 6 days post-confluence. The amount of activated GTP-bound RhoA was determined following the methods described by Ren et al. (46, 47). Samples were electrophoresed on 12% gels, transferred to PVDF membranes, and probed with a 1:1000 dilution of a rabbit polyclonal antibody to RhoA. Western blots of GTP-bound RhoA and total cell RhoA were visualized with ECL detection reagents. X-ray films were developed at various time intervals to obtain an exposure within the linear range of the film. Films were scanned in a Personal Densitometer (Amersham Biosciences) and data analyzed as outlined by Ren et al. (46, 47).

Protein Phosphatase Activity Assay—BPAE monolayers were either untreated or incubated with 10 μM Fsk, 40 μM IBMX for 1, 2.5, 5, and 15 min, and the media were aspirated and monolayers snap-frozen. Dishes were placed on a 4 °C copper block to thaw, flooded with 400 μl of homogenization buffer (50 mM Tris, 0.1 mM EDTA, 5 mM β-mercaptoethanol containing protease inhibitors), scraped up, and homogenized in a glass/glass tissue homogenizer, and spun at 8,000 × g for 10 min. 50-μl aliquots of the cell extracts were snap-frozen in liquid N2 for storage at −70 °C. Cell pellets were dissolved in 25 μl of 0.1% SDS, and protein determinations were performed using a Pierce BCA protein assay after chloroform/methanol extraction as outlined by Wessel and Flugge (48). For determination of phosphatase activity, untreated (control) and experimental cell extracts were diluted 20-fold in phosphatase assay buffer (20 mM Tris, 0.2 mM EDTA, 0.5 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, pH 7.0), and equivalent amounts of protein were added to a 50-μl reaction mixture containing 0.5 μM 32PO4-labeled nonmuscle myosin II RLC (41) and incubated at 30 °C for 15 min. Reactions were terminated by adding equal volumes of ice-cold 20% trichloroacetic acid. Proteins were precipitated on ice for 1 h, and the samples washed three times with 100% acetone, and the pellets were dissolved in SDS sample buffer. 32PO4-RLCs were analyzed on 12% SDS gels, stained with Coomassie Blue, dried, and exposed to PhosphorImager plates. Data are expressed as the ratio of phosphorylated RLC in Fsk/IBMX-treated samples/phosphorylated RLC in untreated cell extracts. Dephosphorylation of 32PO4-labeled RLC by untreated cell extracts is therefore 1.0, and the dephosphorylation of 32PO4-labeled RLC by Fsk/IBMX-treated cell extracts is used as a measure of phosphatase activity.

Phospho-LIM Kinase and Phosphocofilin Determinations—Treated and control monolayers were processed and quantitated as outlined above under RhoA phosphorylation and probed with a 1:1000 dilution of affinity-purified rabbit polyclonal antibody to phospho-LIM kinase, LIM kinase, Ser3-phosphocofilin, or cofilin. For each experiment, all phosphoprotein densitometric units were divided by total protein densitometric units for each time point and then normalized to the control value and expressed as a ratio of control.

Actin Filament Depolymerization Assay—Depolymerization of actin filaments by cell extracts from Fsk/IBMX-treated monolayers was quantitated using an F-actin disassembly assay (49). Actin was polymerized in MKEI buffer (20 mM imidazole-HCl, pH 7.0, 100 mM KCl, 2 mM MgCl2, 1 mM EGTA) for 30 min at room temperature. F-actin at a concentration of 5 μM (containing 10% pyrene-labeled actin) was diluted 25-fold into G buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.1 mM DTT, 0.2 mM CaCl2, 0.005% NaN3) containing a 1:20 dilution of cell extract at 25 °C. The decrease in pyrene fluorescence accompanying actin depolymerization was monitored in an Aminco Bowman luminescence spectrometer as outlined by Bryan (49). Phalloidin saturated pyrene-actin filaments were prepared by addition of 20 μM phalloidin.

BPAE cell lysates were prepared by lysing monolayers in 300 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 5.5 mM EGTA, 0.5 mM MgCl2, 0.5 M urea).
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mm ATP, 1 mM DTT containing protease inhibitors) at the desired time intervals after treatment with Fsk/IBMX. Extracts were spun at 22,000 × g for 10 min, and supernatants were snap-frozen in liquid N2. For depolymerization assays, extracts were diluted 1:20 in G buffer in a quartz cuvette prior to addition of pyrene-labeled F-actin.

RESULTS

CAMP Elevation and PKA Activation—BPAE monolayers were incubated in forskolin, a direct activator of adenylate cyclase, in concentrations ranging from 1 to 50 μM for 5 min, and cellular CAMP levels were measured by radioimmunoassay. A dose-dependent increase in cAMP occurred with maximal levels, 5-fold that of controls, induced at a concentration of 10 μM forskolin. BPAE monolayers were then incubated with 10 μM forskolin in the presence of increasing concentrations of the phosphodiesterase inhibitor, IBMX, which inhibits CAMP degradation. A concentration of 40 μM IBMX sustained maximal CAMP levels for up to 60 min (data not shown). Based upon these results, 10 μM Fsk, 40 μM IBMX (Fsk/IBMX) was used in all subsequent experiments.

CAMP levels were measured in confluent BPAE monolayers following treatment with Fsk/IBMX for 30 s, 60 s, and 90 s and 2.5, 5, 10, 15, and 30 min. Untreated monolayers contain 5.4 ± 2.5 pmol of CAMP/mg of protein. Addition of Fsk/IBMX caused a rapid increase in CAMP to 45.3 ± 9.0 pmol/mg protein within 1 min, which remained 7–8-fold above unstimulated cells over 30 min. PKA activity was determined by a [32P]ATP-based assay using a synthetic Leu-Arg-Arg-Ala-Leu-Gly (Kemptide) as the substrate. BPAE cellular PKA activity increased 3-fold within 1 min after Fsk/IBMX treatment and remained elevated over 30 min. The PKA substrate, VASP, is phosphorylated (supplemental Fig. S1) in response to elevated CAMP, indicating that the increase in PKA activity is not a result of post-homogenization activation.

Isometric Tension and Myosin Light Chain Phosphorylation—The effect of CAMP on basal isometric tension was determined in BPAE monolayers grown on pre-cast collagen gels as described previously (40). A stable basal tension of 58–60 dynes (Fig. 1A) develops within 4–5 days after seeding, and monolayers are used within 24 h of reaching a constant basal tension. Fig. 1A is a representative isometric tension tracing of a BPAE monolayer treated with Fsk/IBMX. Isometric declines immediately and rapidly within the first 3–4 min, decreases steadily over the next 8 min, and establishes a new base-line tension of 41 dynes (a 33% reduction) within 10 min. Isometric tension reproducibly declined to 32–35% of the pretreatment base line and remained at these levels for the duration of the experiments.

The effect of Fsk/IBMX treatment on BPAE RLC phosphorylation was analyzed by glycerol/urea gel electrophoresis and Western blotting. Fig. 1B shows a representative blot from a time course of BPAE RLC phosphorylation after incubation with Fsk/IBMX. Untreated control monolayers had a basal level of RLC phosphorylation of 0.33 mol of PO4/mol of RLC. During the first 90 s of incubation with Fsk/IBMX, RLC phosphorylation decreases by 46% to 0.18 mol of PO4/mol of RLC (Fig. 1B, and TABLE ONE). Myosin RLC phosphorylation exhibits a similar reduction over the duration of the experiment decreasing 38% compared with control monolayers after 30 min. TABLE ONE shows the quantitative data from four time course experiments.

Effect of Elevated CAMP on Agonist-induced Isometric Tension and Myosin RLC Phosphorylation—To test the hypothesis that CAMP-mediated decreases in myosin RLC phosphorylation are the result of inhibition of MLCK activity, BPAE monolayers were pretreated with Fsk/IBMX and then stimulated with thrombin, an agonist that has been shown to activate MLCK in endothelial cells (1, 40). Fig. 2A shows a typical isometric tension tracing generated by monolayers preincubated with Fsk/IBMX and then treated with 2 units/ml thrombin. Pretreatment with Fsk/IBMX for 30 min resulted in a drop in tension to 35% of base-line levels to 37 dynes within 10–15 min. Addition of 2 units/ml thrombin caused a rapid increase in isometric tension within the first

| Effect of CAMP on myosin RLC phosphorylation |
|---------------------------------------------|
| BPAE monolayers were treated with Fsk/IBMX for the designated time intervals. Proteins were trichloroacetic acid-precipitated, and the phosphorylation states of myosin II were analyzed by 12% glycerol/urea gel electrophoresis as described under "Materials and Methods." RLC phosphorylation states were identified using an anti-myosin II RLC antibody (Fig. 1B), and the percentage of monophosphorylated (U) monophosphorylated (P1), and diposphorylated (P2) myosin RLC was determined by laser two-dimensional densitometry. The stoichiometry of RLC phosphorylation (mol of phosphate/mol of RLC) was calculated from the percentage of monophosphorylated and diposphorylated RLC as described previously (40). Each point is the mean ± S.E. of four separate experiments. |
| Control | 30 s | 60 s | 90 s | 2.5 min | 5 min | 15 min | 30 min |
|---------|-----|------|-----|--------|------|-------|-------|
| U       | 72 ± 1.1 | 85 ± 0.7 | 85 ± 1.9 | 86 ± 2.3 | 85 ± 1.6 | 86 ± 1.5 | 83 ± 3.3 | 82 ± 2.3 |
| P1      | 23 ± 0.7 | 12 ± 0.4 | 13 ± 1.9 | 13 ± 1.9 | 14 ± 1.5 | 12 ± 1.3 | 15 ± 3.1 | 16 ± 2.1 |
| P2      | 5 ± 0.4 | 3 ± 0.4 | 2 ± 0.7 | 1 ± 0.5 | 1 ± 0.5 | 2 ± 0.5 | 2 ± 0.4 | 2 ± 0.7 |
| mol of PO4/mol of RLC | 0.33 ± 0.02 | 0.18 ± 0.01 | 0.17 ± 0.02 | 0.16 ± 0.03 | 0.17 ± 0.02 | 0.15 ± 0.02 | 0.18 ± 0.04 | 0.21 ± 0.03 |

FIGURE 1. Effect of CAMP on isometric tension and BPAE RLC phosphorylation. A representative tracing of isometric tension produced by BPAE monolayers incubated with 10 μM Fsk, 40 μM IBMX. A rapid drop in tension occurs within 2 min. A new base-line tension is established within 10 min, representing a 33% reduction from pretreatment levels. Addition of 2 μg cytochalasin D (Cyto D) rapidly abolishes tension in the monolayer. B. RLC phosphorylation was analyzed by glycerol/urea gel electrophoresis as outlined under "Materials and Methods." Monolayers incubated with Fsk/IBMX showed a time-dependent decrease in RLC phosphorylation. Quantitative data from four time course experiments are presented in TABLE ONE. Lane 1, vehicle control; lanes 2–8, treatment with Fsk/IBMX for 30, 60, and 90 s and 2.5, 5, 10, 15, and 30 min, respectively. U, unphosphorylated; P1, monophosphorylated; P2, diposphorylated RLC.
BPAE monolayers. Thrombin induced a rapid increase in isometric tension from 0.33 mol of PO₄/mol of RLC (Fig. 2C, lane 1, TABLE TWO), whereas monolayers pretreated with Fsk/IBMX showed a 40% reduction to 0.22 mol of PO₄/mol of RLC (Fig. 2C, lane 2; TABLE TWO). Thirty seconds (Fig. 2C, lane 3; TABLE TWO) after the addition of thrombin to Fsk/IBMX-pretreated monolayers, there is a 64% increase in RLC phosphorylation from 0.22 mol of PO₄/mol RLC to 0.61 mol of PO₄/mol of RLC as indicated by a decrease in unphosphorylated light chain with an accompanying increase in both mono- and diphosphorylated light chains (Fig. 2C, lane 3). By 60 s a maximal level of phosphorylation of 0.75 mol of PO₄/mol of RLC was reached, and then phosphorylation slowly declined over the ensuing 15 min to 0.26 mol of PO₄/mol of RLC. Thrombin-induced RLC phosphorylation in Fsk/IBMX-pretreated monolayers occurred within the same time frame and with approximately the same magnitude as monolayers treated with thrombin alone (data not shown).

Correlation of RLC phosphorylation with isometric tension development (Fig. 2A) shows that maximal phosphorylation occurs within the first 60 s, whereas tension develops more slowly reaching maximal levels by 4 min. The rise in tension therefore paralleled the rise in phosphorylation, although maximal phosphorylation preceded maximal tension development. Within 5 min both tension and phosphorylation began to decline with phosphorylation decreasing more rapidly than tension.

**MLCK₂₁₀ Phosphorylation and Activity Measurements**—We have shown recently (11) that cultured large and small vessel endothelial cells contain the 210-kDa nonmuscle MLCK but not the smaller smooth muscle form. BPAE cells used in this study express only MLCK₂₁₀. To examine more directly the effects of cAMP on the state of phosphorylation and catalytic activity of MLCK₂₁₀, two sets of experiments were performed. In the first set, cultures were labeled with [³²P]orthophosphoric acid and incubated in Fsk/IBMX for 0, 5, 15, and 30 min. The cells were lysed, and MLCK₂₁₀ was immunoprecipitated and quantitated by Western blot (supplemental Fig. S2A). The blot was subsequently exposed to a PhosphorImager screen to determine the incorporation of ³²PO₄ into MLCK₂₁₀ (supplemental Fig. S2B). To account for differential recovery of MLCK₂₁₀ during immunoprecipitation, phosphorimages and the corresponding Western blots were analyzed by laser densitometry, and data were expressed as a ratio of phosphorylated MLCK₂₁₀/immunoblot densitometric units as outlined under "Materials and Methods." Immunoprecipitates from control monolayers show a basal level of MLCK₂₁₀ phosphorylation (supplemental Fig. S2B, lane 1). After 5-, 15-, and 30-min incubations in Fsk/IBMX, no significant increase in ³²PO₄ incorporation into MLCK₂₁₀ occurred (supplemental Fig. S2B, lanes 2–4).

To assess catalytic activity, MLCK₂₁₀ was immunopurified from monolayers treated with Fsk/IBMX for 5 and 30 min, and enzymatic activity was determined as outlined under "Materials and Methods." Immunopurified MLCK₂₁₀ from untreated monolayers incorporated 0.26 ± 0.02 pmol ³²PO₄/pmol of RLC/ng enzyme. The activity determinations obtained from MLCK₂₁₀ immunopurified from Fsk/IBMX-treated cells (supplemental Fig. S2B, lanes 2–4) show that cAMP has no effect on MLCK₂₁₀ catalytic activity.

**Effects of the MLCK Inhibitor, KT5926, on Isometric Tension and Light Chain Phosphorylation**—The data thus far established that cAMP causes a reduction in basal tension and myosin RLC phosphorylation in BPAE cells but did not support the involvement of MLCK₂₁₀ in mediating these effects. In the next set of experiments, the effects of direct inhibition of MLCK₂₁₀ on BPAE cell RLC phosphorylation and basal and agonist-stimulated isometric tension were determined. Fig. 3A

1–2 min and reached a peak of 67 dynes by 4 min. Maximal tension was maintained for ~5 min but declined over the succeeding 20 min. Fig. 2B is a representative tension tracing showing the effect of thrombin on BPAE monolayers. Thrombin induced a rapid increase in isometric tension, which reached a maximum of 129 dynes in 3–4 min and was sustained at this level over 50 min.

BPAE monolayers were pretreated with Fsk/IBMX for 30 min, and myosin RLC phosphorylation was assessed after the addition of 2 units/ml thrombin. Unstimulated control cultures have a basal level of phosphorylation of 0.33 mol of PO₄/mol of RLC (Fig. 2C, lane 1; TABLE TWO), whereas monolayers pretreated with Fsk/IBMX showed a 40% reduction to 0.22 mol of PO₄/mol of RLC (Fig. 2C, lane 2; TABLE TWO). Thirty seconds (Fig. 2C, lane 3; TABLE TWO) after the addition of thrombin to Fsk/IBMX-pretreated monolayers, there is a 64% increase in RLC phosphorylation from 0.22 mol of PO₄/mol RLC to 0.61 mol of PO₄/mol of RLC as indicated by a decrease in unphosphorylated light chain with an accompanying increase in both mono- and diphosphorylated light chains (Fig. 2C, lane 3). By 60 s a maximal level of phosphorylation of 0.75 mol of PO₄/mol of RLC was reached, and then phosphorylation slowly declined over the ensuing 15 min to 0.26 mol of PO₄/mol of RLC. Thrombin-induced RLC phosphorylation in Fsk/IBMX-pretreated monolayers occurred within the same time frame and with approximately the same magnitude as monolayers treated with thrombin alone (data not shown).

Correlation of RLC phosphorylation with isometric tension development (Fig. 2A) shows that maximal phosphorylation occurs within the first 60 s, whereas tension develops more slowly reaching maximal levels by 4 min. The rise in tension therefore paralleled the rise in phosphorylation, although maximal phosphorylation preceded maximal tension development. Within 5 min both tension and phosphorylation began to decline with phosphorylation decreasing more rapidly than tension.

**MLCK₂₁₀ Phosphorylation and Activity Measurements**—We have shown recently (11) that cultured large and small vessel endothelial cells contain the 210-kDa nonmuscle MLCK but not the smaller smooth muscle form. BPAE cells used in this study express only MLCK₂₁₀. To examine more directly the effects of cAMP on the state of phosphorylation and catalytic activity of MLCK₂₁₀, two sets of experiments were performed. In the first set, cultures were labeled with [³²P]orthophosphoric acid and incubated in Fsk/IBMX for 0, 5, 15, and 30 min. The cells were lysed, and MLCK₂₁₀ was immunoprecipitated and quantitated by Western blot (supplemental Fig. S2A). The blot was subsequently exposed to a PhosphorImager screen to determine the incorporation of ³²PO₄ into MLCK₂₁₀ (supplemental Fig. S2B). To account for differential recovery of MLCK₂₁₀ during immunoprecipitation, phosphorimages and the corresponding Western blots were analyzed by laser densitometry, and data were expressed as a ratio of phosphorylated MLCK₂₁₀/immunoblot densitometric units as outlined under "Materials and Methods." Immunoprecipitates from control monolayers show a basal level of MLCK₂₁₀ phosphorylation (supplemental Fig. S2B, lane 1). After 5-, 15-, and 30-min incubations in Fsk/IBMX, no significant increase in ³²PO₄ incorporation into MLCK₂₁₀ occurred (supplemental Fig. S2B, lanes 2–4).

To assess catalytic activity, MLCK₂₁₀ was immunopurified from monolayers treated with Fsk/IBMX for 5 and 30 min, and enzymatic activity was determined as outlined under "Materials and Methods." Immunopurified MLCK₂₁₀ from untreated monolayers incorporated 0.26 ± 0.02 pmol ³²PO₄/pmol of RLC/ng enzyme. The activity determinations obtained from MLCK₂₁₀ immunopurified from Fsk/IBMX-treated cells (supplemental Fig. S2B, lanes 2–4) show that cAMP has no effect on MLCK₂₁₀ catalytic activity.

**Effects of the MLCK Inhibitor, KT5926, on Isometric Tension and Light Chain Phosphorylation**—The data thus far established that cAMP causes a reduction in basal tension and myosin RLC phosphorylation in BPAE cells but did not support the involvement of MLCK₂₁₀ in mediating these effects. In the next set of experiments, the effects of direct inhibition of MLCK₂₁₀ on BPAE cell RLC phosphorylation and basal and agonist-stimulated isometric tension were determined. Fig. 3A
TABLE TWO

Stoichiometry of BPAE myosin RLC phosphorylation

BPAE monolayers were treated as indicated. Experiments were terminated by the addition of 10% trichloroacetic acid, and RLC phosphorylation was analyzed by gelcylinder/urea electrophoresis as outlined under “Materials and Methods.” The stoichiometry of RLC phosphorylation was calculated as described previously (40). Monolayers were pretreated with Fsk/IBMX for 30 min prior to stimulation with thrombin. Elevation of cAMP and PKA activation did not prevent thrombin-induced RLC phosphorylation. Each time point is the mean ± S.E. of four separate experiments. ND indicates not determined.

| Condition                  | 0 s    | 60 s   | 90 s   | 2.5 min | 5 min   | 15 min  | 30 min  |
|----------------------------|--------|--------|--------|---------|---------|---------|---------|
| Control                    | 0.02   | 0.04   | 0.06   | ND      | 0.75    | 0.03    | ND      |
| 2 units/ml thrombin        | 0.02   | 0.16   | 0.16   | 0.10    | ND      | ND      | ND      |
| 10 μM Fsk, 40 μM IBMX      | 0.06   | 0.08   | 0.13   | 0.10    | 0.08    | ND      | ND      |
| 10 μM Fsk, 40 μM IBMX + 2 units/ml thrombin | 0.02   | 0.08   | 0.12   | 0.10    | 0.08    | ND      | ND      |

 shows a typical isometric tension tracing of BPAE monolayers incubated with 100 nM KT5926, a highly selective and potent inhibitor of MLCK (50). A 7-dyne decrease in tension occurred within the first 5 min. This modest decrease was maintained for an additional 10 min before tension slowly returned to base-line levels. Concentrations of up to 1 μM KT5926 produced the same minimal alteration in basal isometric tension (data not shown). Quantitative data of the effects of KT5926 on myosin RLC phosphorylation from four time course experiments are presented in TABLE THREE. Monolayers incubated with 100 nM KT5926 showed a rapid time-dependent decrease in the extent of RLC phosphorylation from 0.33 mol of PO₄/mol of RLC to 0.03 mol of PO₄/mol of RLC by 30 min, which represents a 90% decrease from control values.

The effect of KT5926 on agonist-stimulated tension development and RLC phosphorylation was also determined. As shown in Fig. 3B, monolayers treated with 100 nM KT5926 develop a characteristic 7–10-dyne reduction in basal tension over 25–30 min. Addition of 2 units/ml thrombin (in the continued presence of KT5926) elicited a modest 4–6-dyne increase in tension within the first 5 min, which was maintained for the duration of the experiment. In other experiments, tension generation in response to thrombin failed to equal the base-line tension established at the start of the experiment (data not shown). The effect of KT5926 on thrombin-induced myosin RLC phosphorylation is shown in Fig. 3C and TABLE FOUR. KT5926 alone for 30 min decreases RLC phosphorylation from 0.31 mol of PO₄/mol of RLC to 0.08 mol of PO₄/mol of RLC (Fig. 3C, lanes 1 and 2, respectively). Sixty seconds after addition of thrombin (Fig. 3C, lane 4), RLC phosphorylation increased to 0.11 mol of PO₄/mol of RLC, and by 2.5 min (Fig. 3C, lane 5) a maximum level of phosphorylation of 0.16 mol of PO₄/mol of RLC was seen. This small increase was exclusively because of monophosphorylation of myosin RLC. Monolayers incubated with thrombin for as long as 30 min in the presence of KT5926 (Fig. 3C, lane 8) showed no further increase in RLC phosphorylation and no diphosphorylated light chain was detected. For comparison, monolayers treated with thrombin only for 2.5 min (Fig. 3C, lane 3) exhibit a marked increase in light chain phosphorylation with both mono- and diphosphorylated RLCs present (TABLE FOUR).

In the next set of experiments, MLCK₂₁₀ was inhibited, and the effects of elevated cAMP on isometric tension and RLC phosphorylation was then determined. As shown in Fig. 4, monolayers treated with KT5926 show approximately a 10-dyne decrease in basal tension. Upon addition of Fsk/IBMX, an immediate rapid drop in tension occurred resulting in a 70% reduction in tension within 2–3 min. Tension declined to a new base line over the next 10 min. Addition of cytochalasin D resulted in no further reduction in tension (data not shown). Fig. 4, inset, shows a representative Western blot illustrating the effects of KT5926 and Fsk/IBMX on RLC phosphorylation. Untreated monolayers have a basal level of RLC phosphorylation of 0.24 ± 0.01 mol of PO₄/mol of RLC (Fig. 4, inset, lane 1). KT5926 reduced basal RLC phosphorylation to 0.10 ± 0.03 mol of PO₄/mol of RLC (Fig. 4, inset, lane 2). Elevation of cAMP for 1 and 5 min in monolayers pretreated with KT5926 did not abolish RLC phosphorylation but caused an additional small decrease in RLC phosphorylation to 0.05 ± 0.02 mol of PO₄/mol of RLC and 0.06 ± 0.02 mol of PO₄/mol of RLC respectively (Fig. 4, inset, lanes 3 and 4). Together, these data suggested a mechanism other than inhibition of RLC phosphorylation accounted for the drop in tension upon up-regulation of cAMP.

RhoA Phosphorylation and RhoA Activation—The state of myosin II RLC phosphorylation is dependent on the rate of dephosphorylation by myosin light chain phosphatase, a protein phosphatase type 1 (PP1) phosphatase (32). Recent studies have shown that PKA phosphorylates RhoA at Ser¹₈₈, and this phosphorylation results in decreased binding of RhoA to Rho kinase, an enzyme that has been shown to regulate the activity of myosin light chain phosphatase (38).

In order to determine whether cAMP affected RhoA phosphorylation in BPAE cells, monolayers were incubated in Fsk/IBMX for 0, 5, 15, and 30 min, and RhoA phosphorylation was analyzed as outlined under “Materials and Methods.” Control monolayers exhibit basal levels of RhoA phosphorylation on Ser¹₈₈ (Fig. 5A). Incubation of monolayers in Fsk/IBMX for 5 min resulted in a 4.6-fold increase in RhoA phosphorylation at Ser¹₈₈. Maximal phosphorylation, 10-fold above control, occurred by 15 min and was maintained for the 30-min duration of the experiment. These results demonstrate that RhoA is phosphorylated at Ser¹₈₈ in response to cAMP in endothelial cells.

We next sought to determine whether RhoA phosphorylation at Ser¹₈₈ altered the state of RhoA activity in BPAE monolayers. To assess RhoA activation, an RBD assay was performed on BPAE monolayers by utilizing the RBD fragment from the RhoA effector Rhotekin as outlined by Ren et al. (46). Fig. 5B depicts representative Western blots as well as quantitative data from four time course experiments demonstrating the effects of cAMP on basal and thrombin-stimulated RhoA activity. BPAE monolayers were pretreated with Fsk/IBMX for 30 min, and Rho activity was assessed after addition of 2 units/ml thrombin. Unstimulated monolayers incubated in media containing vehicle only (0.01% Me₂SO) for 30 min indicate that control monolayers exhibit basal RhoA activity (Fig. 5B, inset, lane 1), whereas monolayers pretreated with Fsk/IBMX show a 65% reduction in GTP-bound RhoA (lane 2) compared with controls (lane 1). Sixty seconds after adding thrombin to Fsk/IBMX-pretreated cultures, GTP-bound RhoA increased by 65% (Fig. 5B, inset, lane 3). In contrast, thrombin stimulation of BPAE monolayers preincubated in media alone for 60 s induced a 400% increase in GTP-bound RhoA. Taken together, these data indicate that increased levels of cAMP induce RhoA phosphorylation, inhibiting RhoA activity which prevents activation of the Rho/Rho kinase pathway in BPAE cells.

Numerous agonists have been shown to affect cellular functions through Rho and its downstream effectors. The Rho effector, Rho kinase, regulates myosin II and cell contraction by catalyzing phosphorylation of the regulatory subunit of myosin phosphatase by inhibiting its catalytic activity, which results in an indirect increase in RLC phosphorylation. Antagonism of the Rho/Rho kinase pathway by inhibition
of either RhoA or Rho kinase uncouples a key regulatory cascade that maintains the balance between phosphorylation/dephosphorylation events. Our data have established that treatment with Fsk/IBMX induced phosphorylation of RhoA and inhibition of RhoA activation. Because RhoA activation has been linked to myosin phosphatase regulation, we sought to determine whether cAMP affected myosin light chain phosphatase activity. For quantification of myosin phosphatase activity, $^{32}$PO$_4$-labeled nonmuscle myosin II RLCs were used as substrate. Quantitative data from three time course experiments are presented in Fig. 6. Treatment with Fsk/IBMX for 60 s caused a rapid increase in phosphatase activity as shown by a 50% increase in release of $^{32}$PO$_4$ from labeled RLC substrate (Fig. 6, inset, lane 2). An additional slight increase in phosphatase activity occurred over the ensuing 14 min (Fig. 6, inset, lanes 3–5).

In the next set of experiments, the effects of direct inhibition of myosin phosphatase on BPAE RLC phosphorylation and isometric tension were determined. The PP1 phosphatase inhibitor, tautomycin (51, 52), was used to determine whether inhibition of myosin phosphatase activation prevented the decrease in isometric tension and RLC phosphorylation elicited by cAMP. At 10 nM concentration, tautomycin is a specific inhibitor of PP1 but not PP2 phosphatases. Fig. 7A shows a typical isometric tension tracing produced by BPAE monolayers preincubated with 10 nM tautomycin and subsequently treated with Fsk/IBMX. Tautomycin induced a slow rise in tension reaching a peak tension of 56 dynes by 10 min. A delay of 3–5 min occurred before a change in isometric tension was detected, and tension steadily increased over the next 5–10 min, reaching a peak tension within 12–15 min. Addition of Fsk/IBMX in the continuous presence of 10 nM tautomycin caused a 2–4-dyne reduction in tension within 2–5 min, and the tension was maintained at this level for the duration of the experiment. Inhibition of PP1 phosphatase prevented the rapid decline in isometric tension caused by cAMP.

To determine whether tautomycin also prevented the decrease in RLC phosphorylation elicited by cAMP, BPAE monolayers were pre-treated with 10 nM tautomycin and myosin RLC phosphorylation analyzed after addition of Fsk/IBMX in the presence of inhibitor. Untreated monolayers have a base-line phosphorylation of 0.31 ± 0.02 mol of PO$_4$/mol of RLC (Fig. 7B, lane 1). In monolayers treated with Fsk/IBMX alone, phosphorylation decreases to 0.17 ± 0.03 mol of PO$_4$/mol of RLC (Fig. 7B, lane 2), whereas tautomycin alone results in a greater than 2-fold increase in phosphorylation to 0.71 ± 0.05 mol of PO$_4$/mol of RLC (Fig. 7B, lane 3). Monolayers pretreated with 10 nM tautomycin for 15 min followed by addition of Fsk/IBMX for an additional 15 min in the presence of tautomycin showed levels of phosphorylation (0.76 ± 0.03 mol of PO$_4$/mol of RLC; Fig. 7B, lane 4) comparable with tautomycin treatment alone. These results demonstrate that increased phosphatase activity is necessary for the decrease in isometric tension and RLC phosphorylation and that inhibition of myosin phosphatase blocks the effects induced by cAMP.

**cAMP Effect on LIM Kinase and Cofilin**—Our data indicate that a signaling cascade downstream of the Rho/Rho kinase is responsible for the decline in tension upon elevation of cAMP. Our next objective was to identify which downstream pathway(s) was activated by cAMP. One major pathway downstream from Rho/Rho kinase is the LIM kinase to ADF/cofilin pathway. ADF/cofilins (53–55) are a family of proteins that exert their effects through RhoGTPase-activated GTPase reorganization. Rho/Rho kinase catalyzes phosphorylation and activation of LIM kinase, which in turn phosphorylates and inactivates cofilin. Active cofilin severs and depolymerizes F-actin, whereas inactive phosphocofilin most likely stabilizes F-actin by decreasing subunit turnover rates. Thus the Rho/Rho kinase signaling pathway affects actin dynamics both by inhibiting the severing activity of cofilin and by stabilizing F-actin. To determine whether cofilin activity was altered in response to cAMP, we assessed cofilin activity in treated and control monolayers.

Monolayers were incubated with Fsk/IBMX for the indicated time intervals, and changes in phospho-LIM kinase and phosphocofilin were analyzed and quantitated as described under “Materials and Methods.” Untreated monolayers exhibit a constitutive level of LIM kinase phosphorylation (Fig. 8A, lane 1). A 46% decline in phosphorylation...
TABLE THREE
Effect of KT5926 on RLC phosphorylation

| Time (min) | Control | 30 s | 60 s | 90 s | 2.5 min | 5 min | 15 min | 30 min |
|------------|---------|------|------|------|---------|-------|--------|--------|
| 100 nM KT5926 | 0.33 ± 0.06 | 0.21 ± 0.04 | 0.13 ± 0.04 | 0.06 ± 0.05 | 0.06 ± 0.04 | 0.04 ± 0.04 | 0.03 ± 0.03 | 0.03 ± 0.03 |

TABLE FOUR
Effect of KT5926 and thrombin on RLC phosphorylation

| Time (min) | Control | KT5926 30 min | Thrombin 2.5 min | KT5926 Thr 60 s | KT5926 Thr 2.5 min | KT5926 Thr 5 min | KT5926 Thr 15 min | KT5926 Thr 30 min |
|------------|---------|---------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| U          | 73 ± 4  | 92 ± 0        | 23 ± 4          | 88 ± 4          | 86 ± 2          | 87 ± 5          | 86 ± 2          | 87 ± 10         |
| P1         | 23 ± 3  | 8 ± 1         | 20 ± 2          | 11 ± 2          | 12 ± 2          | 13 ± 2          | 12 ± 1          | 11 ± 8          |
| P2         | 4 ± 1   | 0             | 57 ± 4          | 1 ± 2           | 2 ± 1           | 1 ± 3           | 2 ± 0           | 2 ± 2           |

| Mol of PO4/mol of RLC | 0.31 ± 0.04 | 0.08 ± 0.02 | 1.33 ± 0.08 | 0.11 ± 0.06 | 0.16 ± 0.04 | 0.15 ± 0.08 | 0.16 ± 0.02 | 0.15 ± 0.13 |

FIGURE 4. Effect of MLCK210 inhibition on cAMP-induced decrease in isometric tension and RLC phosphorylation. Representative tracing of isometric tension produced by BPAE monolayer pretreated with 100 nM KT5926 followed by incubation with 10 μM Fsk, 40 μM IBMX in the continued presence of KT5926. KT5926 caused a 10-dyne reduction in tension within 5 min. An immediate drop in tension occurs upon the addition of Fsk/IBMX. Inset, monolayers were pretreated with 100 nM KT5926 for 30 min and then stimulated for 60 s and 5 min with Fsk/IBMX (in the presence of KT5926), and RLC phosphorylation was analyzed by glycerol/urea gel electrophoresis. Lane 1, control monolayer; lane 2, monolayer incubated with KT5926 alone for 30 min; lane 3, monolayer pretreated with KT5926 for 30 min and then stimulated with Fsk/IBMX for 60 s; lane 4, monolayer pretreated with KT5926 for 30 min and then stimulated with Fsk/IBMX for 5 min.

TABLE FIVE
Effect of KT5926 and thrombin on RLC phosphorylation

| Time (min) | Control | KT5926 30 min | Thrombin 2.5 min | KT5926 Thr 60 s | KT5926 Thr 2.5 min | KT5926 Thr 5 min | KT5926 Thr 15 min | KT5926 Thr 30 min |
|------------|---------|---------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| U          | 73 ± 4  | 92 ± 0        | 23 ± 4          | 88 ± 4          | 86 ± 2          | 87 ± 5          | 86 ± 2          | 87 ± 10         |
| P1         | 23 ± 3  | 8 ± 1         | 20 ± 2          | 11 ± 2          | 12 ± 2          | 13 ± 2          | 12 ± 1          | 11 ± 8          |
| P2         | 4 ± 1   | 0             | 57 ± 4          | 1 ± 2           | 2 ± 1           | 1 ± 3           | 2 ± 0           | 2 ± 2           |

| Mol of PO4/mol of RLC | 0.31 ± 0.04 | 0.08 ± 0.02 | 1.33 ± 0.08 | 0.11 ± 0.06 | 0.16 ± 0.04 | 0.15 ± 0.08 | 0.16 ± 0.02 | 0.15 ± 0.13 |

FIGURE 5. RhoA phosphorylation and RhoA activity. A, BPAE monolayers were incubated with 10 μM Fsk, 40 μM IBMX for 5, 15, and 30 min. Cell lysates were analyzed by Western blot for Ser188-phospho-RhoA and total RhoA. Fsk/IBMX induced an increase in RhoA phosphorylation at residue Ser188. B, Western blot (inset) and quantitative data demonstrate RhoA activity. BPAE monolayers were treated as follows: vehicle control (lane 1); 10 μM Fsk, 40 μM IBMX treatment for 30 min (lane 2); Fsk/IBMX treatment for 30 min followed by addition of 2 units/ml thrombin for 60 s (lane 3); 60 s of incubation with 2 units/ml thrombin alone (lane 4). Cell lysates were incubated with GST-RBD fusion protein, electrophoresed, transferred to PVDF membranes, and probed with a RhoA antibody. Rho activity was determined as the amount of RBD-bound active RhoA normalized to the amount of total RhoA in the cell lysate. Incubation with Fsk/IBMX inhibits basal RhoA activity as well as thrombin-induced RhoA activation. Cont, control.
incubated in the presence of 10 mM Fsk, 40 μM IBMX for the desired time intervals, and cell homogenates were prepared as outlined under “Materials and Methods.” For determination of phosphatase activity, equivalent amounts of protein were added to a 50-μl reaction mixture containing 0.5 μM 32PO4-labeled recombinant myosin II RLC and incubated at 30 °C for 15 min. The reaction was terminated by addition of an equal volume of 20% trichloroacetic acid, and precipitated protein samples were washed with 100% acetone and analyzed on 12% SDS gels. Dried gels were exposed to PhosphorImager plates for quantification of 32PO4-labeled RLC. Fsk/IBMX treatment caused a rapid increase in phosphatase activity as indicated by the release of 32PO4 from labeled RLC. Inset: lane 1, vehicle control; lanes 2–4, treatment with Fsk/IBMX for 1, 2.5, 5, and 15 min, respectively.

FIGURE 7. Effect of phosphatase inhibition on cAMP-induced decreases in isometric tension and RLC phosphorylation. A, representative isometric tension profile produced by BPAE cells incubated with 10 nM tautomycin followed by treatment with Fsk/IBMX. Tautomycin induces a gradual rise in tension that plateaus within 15 min and prevents the decline in tension caused by elevated cAMP. B, monolayers were pretreated with 10 nM tautomycin for 15 min and then stimulated for 15 min with Fsk/IBMX (in the presence of tautomycin), and RLC phosphorylation was analyzed by glycerol/urea gel electrophoresis. Lane 1, unstimulated control; lane 2, monolayer incubated with Fsk/IBMX for 15 min; lane 3, monolayer stimulated with 10 nM tautomycin for 30 min; lane 4, cells pretreated with 10 nM tautomycin for 15 min prior to addition of 10 μM Fsk/40 μM IBMX for an additional 15 min. Elevating cAMP reduces RLC phosphorylation by 50% compared with control, whereas tautomycin causes greater than a 2-fold increase in RLC phosphorylation as evident by the shift from unphosphorylated RLC to the monophosphorylated and diphosphorylated RLC (lane 3). Pretreatment with tautomycin protects against Fsk/IBMX-induced decrease in RLC phosphorylation (lane 4).

FIGURE 6. Effects of Fsk/IBMX on BPAE phosphatase activity. BPAE monolayers were incubated in the presence of 10 μM Fsk, 40 μM IBMX for the desired time intervals, and cell homogenates were prepared as outlined under “Materials and Methods.” For determination of phosphatase activity, equivalent amounts of protein were added to a 50-μl reaction mixture containing 0.5 μM 32PO4-labeled recombinant myosin II RLC and incubated at 30 °C for 15 min. The reaction was terminated by addition of an equal volume of 20% trichloroacetic acid, and precipitated protein samples were washed with 100% acetone and analyzed on 12% SDS gels. Dried gels were exposed to PhosphorImager plates for quantification of 32PO4-labeled RLC. Fsk/IBMX treatment caused a rapid increase in phosphatase activity as indicated by the release of 32PO4 from labeled RLC. Inset: lane 1, vehicle control; lanes 2–4, treatment with Fsk/IBMX for 1, 2.5, 5, and 15 min, respectively.

FIGURE 8. Effects of cAMP on LIM kinase and cofilin phosphorylation. Monolayers were treated with Fsk/IBMX for 1, 5, 15, and 30 min (lanes 2–5, respectively), and lysates were analyzed by Western blot for phospho-LIM kinase (A) and Ser3-phosphocofilin (B). Lane 1, A and B vehicle control, 0.05% Me2SO; C, endothelial cell monolayers were pretreated with 10 nM tautomycin for 15 min and then incubated with Fsk/IBMX for the desired time intervals. Cell extracts were analyzed for Ser3-phosphocofilin and total cofilin. Lane 1, vehicle control, 0.05% Me2SO; lane 2, 30 min of incubation with 10 nM tautomycin only; lanes 3–6, 15 min of incubation with tautomycin followed by 10 μM Fsk, 40 μM IBMX in the presence of tautomycin for 1, 5, 15, and 30 min, respectively. D, quantitative data illustrating the effects of cAMP and tautomycin on Ser3-phosphocofilin. Data shown represent results from four separate experiments. Results are expressed as relative phosphocofilin levels, the ratio of densitometric units of treated monolayers to densitometric units of control (Cont) monolayer.

FIGURE 9. Effect of phosphatase inhibition on cAMP-induced decreases in isometric tension and RLC phosphorylation. A, representative isometric tension profile produced by BPAE cells incubated with 10 nM tautomycin followed by treatment with Fsk/IBMX. Tautomycin induces a gradual rise in tension that plateaus within 15 min and prevents the decline in tension caused by elevated cAMP. B, monolayers were pretreated with 10 nM tautomycin for 15 min and then stimulated for 15 min with Fsk/IBMX (in the presence of tautomycin), and RLC phosphorylation was analyzed by glycerol/urea gel electrophoresis. Lane 1, unstimulated control; lane 2, monolayer incubated with Fsk/IBMX for 15 min; lane 3, monolayer stimulated with 10 nM tautomycin for 30 min; lane 4, cells pretreated with 10 nM tautomycin for 15 min prior to addition of 10 μM Fsk/40 μM IBMX for an additional 15 min. Elevating cAMP reduces RLC phosphorylation by 50% compared with control, whereas tautomycin causes greater than a 2-fold increase in RLC phosphorylation as evident by the shift from unphosphorylated RLC to the monophosphorylated and diphosphorylated RLC (lane 3). Pretreatment with tautomycin protects against Fsk/IBMX-induced decrease in RLC phosphorylation (lane 4).

Actin Depolymerizing Activity—In a final set of experiments, we sought to determine whether cAMP up-regulated F-actin depolymerizing activity in BPAE cell extracts. Cultures were incubated with Fsk/IBMX for 1, 5, and 30 min, monolayers lysed, and the effects on actin dynamics measured in an assay that monitors the disassembly of F-actin. Elevated cAMP increases the rate of actin filament disassembly upon dilution below its critical concentration (Fig. 9). Extracts from buffer-treated control cultures exhibit a low level of actin depolymerization compared with pyrene-labeled F-actin alone (Fig. 9A). The extent of depolymerizing activity in untreated controls varied slightly from experiment to experiment. The extent and rate of actin filament depolymerization increase in monolayers treated with Fsk/IBMX for 1, 5, and 30 min, respectively, indicating more effector protein was activated with time (Fig. 9A). Thus, CAMP results in up-regulation of actin depolymerizing activity, which correlates with dephosphorylation/activation of cofilin (Fig. 8).

In an attempt to ascertain if cofilin was the severing protein responsible for cAMP-induced F-actin depolymerizing activity, we determined...
Because cofilin has the ability to both induce severing of F-actin as well as accelerate the rate of actin depolymerization, we sought to determine whether Fsk/IBMX treatment caused a change in F-actin content. As shown in supplemental Fig. S3, monolayers treated with Fsk/IBMX exhibited no significant change in F-actin content, suggesting that the change in pyrene fluorescence induced by Fsk/IBMX-treated cell extracts (Fig. 9) results from cofilin severing activity and is not due to an enhanced rate of depolymerization.

DISCUSSION

This study analyzes the role of cAMP in regulating endothelial cell isometric tension and myosin II RLC phosphorylation. We have demonstrated that an increase in intracellular cAMP and activation of PKA result in the following: 1) a decrease in endothelial basal isometric tension and RLC phosphorylation but not an inhibition of thrombin-induced tension development and RLC phosphorylation; 2) no change in MLCK210 catalytic activity; 3) phosphorylation of RhoA and inhibition of RhoA activation and activity; 4) up-regulation of myosin II phosphatase activity; 5) an increase in F-actin severing activity; and 6) depolymerization and thereby activation of cofilin. Furthermore, tautomycin, a PP1 phosphatase inhibitor, blocks the decreases in isometric tension and RLC phosphorylation as well as the depolymerization and activation of cofilin induced by cAMP. Collectively, these results directly implicate the Rho/Rho kinase signaling cascade as the major signaling pathway responsible for regulating basal isometric tension and RLC phosphorylation, and these results identify myosin phosphatase and cofilin as the downstream effectors that mediate the decline in endothelial cell isometric tension in response to elevated cAMP.

Agents that elevate cAMP are known to relax vascular smooth muscle (6–8). The majority of studies in smooth muscle have focused on cAMP effects on lowering intracellular Ca2+ and/or its effect on altering MLCK catalytic activity (9). Investigators examining the role of cAMP in endothelial permeability found no alterations in Ca2+ homeostasis, suggesting that the mechanism of action of cAMP is downstream of Ca2+ entry (2). To identify the mechanism responsible for the cAMP action on endothelial cells, we monitored the effects of Fsk/IBMX treatment on basal and agonist-induced tension development and RLC phosphorylation. In agreement with previous work (13, 58), we found Fsk/IBMX treatment resulted in a rapid, sustained elevation of cAMP and maximal activation of PKA, which correlates with a decline in tension and a decrease in RLC phosphorylation. Recent reports have documented a decline in RLC phosphorylation upon elevation of cAMP and attributed this decrease to PKA-catalyzed phosphorylation and inhibition of MLCK (1, 58–60). Identification of PKA consensus sequences in nonmuscle MLCK (19, 59) contributed additional support to this idea. Although this hypothesis, akin to that proposed for regulation of smooth muscle relaxation (6, 7, 10), is attractive, strong supportive in vivo evidence is lacking.

Our results demonstrate that unstimulated monolayers exhibit constitutive phosphorylation of MLCK210. Treatment with Fsk/IBMX causes no significant increase in MLCK210 phosphorylation nor a change in catalytic activity. In vitro, PKA phosphorylates MLCK and decreases its catalytic activity by reducing its affinity for Ca2+/CaM (7, 10). Initial studies examining an in vivo role for PKA-mediated phosphorylation and inhibition of MLCK in smooth muscle have yielded conflicting results (8, 10). However, more detailed examination of the Ca2+/CaM activation properties and MLCK catalytic activity in smooth muscle tissue has shown that cAMP/PKA activation has no effect on MLCK activation or catalytic activity (8, 61).

Although data from several studies in endothelial cells have led investigators to evoke the PKA phosphorylation theory for the regulation of...
MLCK activity to explain the decrease in RLC phosphorylation and enhanced barrier function in response to elevated cAMP, neither MLCK phosphorylation nor changes in MLCK catalytic activity were measured in these studies. A more recent study measured MLCK phosphorylation and activity and reported that cholera toxin (13) induced a 2.5-fold increase in MLCK phosphorylation and a 4-fold decrease in MLCK activity. It is difficult to compare this study with ours in view of the different agents employed to elevate cAMP and the use of a synthetic peptide antibody to MLCK (D119) (12, 15), which reacts more avidly with myosin IIA (11) than with MLCK. Furthermore, normalizaton of experimental samples was not undertaken to account for differential recovery of MLCK during immunoprecipitation reactions. It is conceivable that the difference reported for MLCK activity could be accounted for by variable recovery of immunoprecipitated kinase.

To probe more directly the role of MLCK in isometric tension development and constitutive RLC phosphorylation, the enzyme was directly inhibited with KT5926, a highly selective and potent inhibitor of MLCK that interacts with the enzyme at its catalytic site (50). KT5926, in contrast to Fsk/IBMX, induced a precipitous drop in RLC phosphorylation, yet caused only a small (8–10 dyne) decrease in basal tension. It inhibited thrombin-induced tension development and RLC phosphorylation. These results indicate that the cAMP-induced decline in tension occurs by a mechanism independent of MLCK inactivation and further demonstrate that agonist-induced tension is dependent on MLCK-catalyzed activation of myosin II, consistent with results observed in agonist-stimulated smooth muscle preparations (62, 63). Thus the results obtained from MLCK phosphorylation experiments, MLCK activity assays, and inhibitor studies do not support the PKA phosphorylation hypothesis for regulation of BPAE MLCK.

In addition, cAMP elicited a rapid drop in tension and RLC phosphorylation in a cell line that possesses no MLCK isoforms (45). Therefore, we conclude that PKA-mediated inhibition of MLCK cannot be the mechanism mediating the decline in BPAE isometric tension and RLC phosphorylation in response to elevated cAMP.

A member of the Ras superfamily, RhoA, has been implicated as the molecular switch involved in the regulation of nonmuscle and smooth muscle contraction (32, 33). RhoA regulates cellular tension through concerted activities of its RhoA-dependent effectors Dia (64, 65) and Rho kinase (31). Its major effector, Rho kinase, changes cellular tension either indirectly by inhibiting myosin phosphatase activity or directly through phosphorylation of myosin II RLC (32, 33). Studies in vitro and in vivo (35, 36, 38, 67) have shown that PKA phosphorylates RhoA on Ser188, and this phosphorylation has been proposed as a molecular mechanism by which cAMP directly inactivates RhoA or RhoA effectors. Fsk/IBMX-treated endothelial cell monolayers showed a 10-fold increase in RhoA phosphorylation within 5 min. Concomitant with the increase in RhoA phosphorylation, cAMP caused a 65% inhibition of RhoA activity in control cultures and 200% inhibition in thrombin-stimulated monolayers. These results are consistent with previous reports (36, 38) showing that agents that elevate cAMP or cell-permeable analogs of cAMP induce RhoA phosphorylation and inhibit RhoA activity. Essler et al. (68) failed to demonstrate RhoA phosphorylation in human umbilical vein endothelial cells in response to cholera toxin and concluded that cAMP interferes with Rho signaling by a PKA-independent mechanism, but in a more recent study, Qiao et al. (69) showed that cAMP inhibited thrombin-induced RhoA activation in microvascular endothelial cells. Studies focusing on the ability of PKA/cGMP-dependent protein kinase to induce smooth muscle relaxation have established PKA-catalyzed phosphorylation of RhoA as a negative regulator of the GTPase signaling cascade demonstrating a critical role for RhoA in governing cellular tension (34, 70).

Of the many Rho effector proteins that are activated by GTP-RhoA, it is Rho kinase that most likely plays a pivotal role in governing cellular tension because of its ability to affect both the state of RLC phosphorylation and actin filament stability. Active Rho kinase catalyzes phosphorylation of the myosin targeting subunit (MYPT1) of myosin light chain phosphatase causing inhibition of its activity; active Rho kinase also directly phosphorylates myosin RLC. Phosphorylation of either substrate results in a net increase in RLC phosphorylation, myosin II activation, and contraction. Actin filament stability is achieved through Rho kinase-catalyzed phosphorylation and activation of LIM kinase which in turn phosphorylates and inhibits the actin severing and depolymerizing activity of coflin. Thus antagonism of the Rho kinase pathway has two major consequences: 1) up-regulation of myosin phosphatase activity, and 2) activation of the severing and depolymerizing activity of cofilins.

The experimental data presented in this report showed that Fsk/IBMX up-regulated myosin phosphatase activity by 76% above unstimulated controls by 15 min. This increase in myosin phosphatase activity correlates with the decline in basal BPAE RLC phosphorylation and isometric tension, strongly suggesting that the decline in RLC phosphorylation mediates the drop in tension. Studies have shown that agents that interfere with RhoA function block lysosphosphatic acid-induced RLC phosphorylation in fibroblasts (45, 71) and prevent thrombin-induced increases in human umbilical vein endothelial cell permeability and RLC phosphorylation (72). These effects were reported to be mediated through enhanced myosin phosphatase activity. Essler et al. (72) confirmed these observations by showing that agents that elevate cAMP increase myosin phosphatase activity to a similar degree as occurs by direct inhibition of RhoA or suppression of Rho kinase activity with Y27632. BPAE monolayers treated to elevate cAMP then stimulated with thrombin fail to maintain maximal tension development and RLC phosphorylation. In smooth muscle, the sustained phase of tension development correlates with activation of the RhoA/Rho kinase pathway; phosphorylation and inhibition of myosin phosphatase with agents that inhibit this pathway are known to prevent sustained force development (32). We suggest that the failure of Fsk/IBMX-treated monolayers to sustain maximal tension in response to thrombin stimulation results in part from the inhibition of RhoA activity and the increased myosin phosphatase activity. In addition to up-regulating phosphatase activity, PKA-catalyzed phosphorylation of MYPT1 has been proposed as a direct control mechanism for regulating myosin phosphatase activity. Muranyi et al. (73) documented phosphorylation of MYPT1 by PKA but showed that this phosphorylation has no effect on the phosphatase activity and suggested that the effects of PKA on smooth muscle relaxation are indirect.

Although it is generally accepted that either direct or indirect up-regulation of myosin phosphatase activity and the resultant dephosphorylation of myosin RLC is responsible for smooth muscle and nonmuscle relaxation, our data indicate that another pathway(s) works in conjunction with myosin phosphatase to cause the decline in endothelial tension. Our results from direct inhibition of MLCK in support this idea because inhibition of MLCK results in a 90% decline in RLC phosphorylation with only a minimal drop in isometric tension. The rapid drop in tension with minimal change in RLC phosphorylation that occurs upon addition of Fsk/IBMX to monolayers pretreated with KT5926 also lends support to this hypothesis.

A priori, coflin, a RhoA effector responsible for regulating actin filament stability, was the most likely candidate to account for the observed decrease in tension in response to cAMP. To evaluate directly the role of coflin in regulating tension, we assessed the state of coflin activation in BPAE

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2 Z. M. Goeckeler and R. B. Wysolmerski, unpublished results.
monolayers. Within 60 s of Fsk/IBMX treatment, LIM kinase was inactivated, cofilin activated, and activation correlated with the decline in isometric tension. By 30 min, 80% of BPAE cofilin was active, and the majority of central stress fibers were disrupted with aggregates of F-actin localized at the cell periphery in agreement with previous reports (3, 4, 28). Although F-actin undergoes significant cellular redistribution, monolayers treated with Fsk/IBMX show minimal change in total F-actin content suggesting actin filament disruption results from F-actin severing activity and is not due to an enhanced rate of depolymerization. Taken together, these data suggest active cofilin mediates severing and redistribution of F-actin. Our results are consistent with studies showing cAMP induces rapid dephosphorylation/activation of cofilin and disruption of actin filaments in cortical neurons (74), rat parotid slices (75), HT4 cells (76), and thyroid cells (77). Depolymerization assays showed that F-actin severing activity was turned on in response to cAMP, and this activity in endothelial cell extracts was phallolidin-sensitive, identifying cofilin as the severing protein that mediates actin filament disruption. Results reported in this study are in agreement with Chan et al. (56), who showed epidermal growth factor stimulation up-regulates cofilin severing activity in MTLn3 cells lysates, and the work of Bailly et al. (77), who demonstrated a decrease in filament length in ultrastructural studies of epidermal growth factor-treated cells. Gehler et al. (78) recently reported that BDNF promoted activation of ADF/cofilin by dephosphorylating ADF/cofilin. The severing activity of ADF/cofilin was necessary to mediate the effects of BDNF because a nonsevering mutant of ADF/cofilin blocked the effects of BDNF on filopodial dynamics. These investigators also showed that the ADF/cofilin effects were independent of myosin II activity and that the two pathways had additive effects on filopodial dynamics. Cofilin activation results in actin reorganization by severing actin filaments and/or accelerating the rate of actin depolymerization. Even though the relative importance of these two effects on actin filaments is controversial (79–81), recent studies suggest that cofilin severing activity is more important for motility and extension of nerve growth cones (82), lamellipodial protrusions (56, 83), and yeast cell growth (84, 85). Together, these data support cAMP stimulation of cofilin dephosphorylation/activation turning on the severing activity of cofilin with the resulting severance of actin filaments, loss of cellular tension, and fall in isometric tension.

Tautomycin, a P1 phosphatase inhibitor, prevents RLC dephosphorylation, phosphocofilin activation, and the decline in isometric tension in response to cAMP, suggesting that a P1 phosphatase not only regulates myosin activation but also cofilin activity. Even though slingshot phosphatase (SSH1L; see Ref. 86) and chronophin (87, 88) have been proposed as the key regulators of cofilin activation, our data indicate that the P1 phosphatase plays a prominent role in cofilin regulation in response to cAMP. In vitro studies with purified protein phosphatases have shown that phosphocofilin is a substrate for both P1 and P2A phosphatases (75, 90). Meberg et al. (74) showed calycin A, an inhibitor of phosphatase P1 and P2A, prevented cAMP-induced dephosphorylation of cofilin in HT4 cells. In human lymphocytes, protein phosphatases P1 and P2A have been shown to associate with and dephosphorylate cofilin (90, 91). However, recent in vitro studies have shown that phosphocofilin is protected from phosphatase-mediated dephosphorylation at Ser3 by protein 14-3-3ζ, suggesting the P1 and P2A phosphatase effects on cofilin may be indirect (92). Furthermore, other studies have shown that SSH1L phosphatase is regulated by phosphorylation (93) and that Ca2+ -induced cofilin activation is mediated indirectly by P2B phosphatase dephosphorylating and activating the SSH1L phosphatase (94). Because the targeting subunit of P1 phosphatase has the potential to interact with multiple substrates (66, 89, 95), it is conceivable that an MYPT1 isoform or the as-yet unidentified targeting subunit is responsible for mediating dephosphorylation of cofilin by regulating SSH1L phosphatase. Irrespective of the mechanism, treatment with tautomycin prevents cofilin dephosphorylation/activation and the decline in isometric tension.

In summary, this study has sought to address the link between cAMP and the Rho-GTPase pathway to explain the decline in isometric tension and RLC phosphorylation in response to cAMP in endothelial cells. The data presented here provide evidence that PKA modulates the cAMP response by phosphorylating RhoA and preventing downstream activation of RhoA effectors. Therefore, we propose the following integrated model to explain the mechanism by which cAMP mediates the decline in BPAE isometric tension. Elevation of intracellular cAMP results in activation of PKA, which phosphorylates RhoA and inhibits RhoA activation preventing downstream activation at multiple levels within the Rho kinase cascade. Inhibition of RhoA activity prevents Rho kinase-catalyzed phosphorylation and inhibition of myosin phosphatase as well as phosphorylation and activation of LIM kinase. Inactivation of LIM kinase and increased phosphatase activity both contribute to cofilin dephosphorylation and thus increased severing of F-actin. At the same time, inhibition of RhoA activity directly decreases myosin RLC phosphorylation by Rho kinase and augments myosin phosphatase activity. We conclude the effects of cAMP occur at multiple levels through coordinated kinase inhibition and phosphatase activation. The combined effects of the unregulated phosphatase activity and severing of actin filaments are responsible for the decline in isometric tension and RLC phosphorylation in response to cAMP (Fig. 10).

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