Endothelial cell retraction is induced by PAK2 monophosphorylation of myosin II

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SUMMARY

The p21-activated kinase (PAK) family includes several enzyme isoforms regulated by the GTPases Rac1 and Cdc42. PAK1, found in brain, muscle and spleen, has been implicated in triggering cytoskeletal rearrangements such as the dissolution of stress fibers and reorganization of focal complexes. The role of the more widely distributed PAK2 in controlling the cytoskeleton has been less well studied. Previous work has demonstrated that PAK2 can monophosphorylate the myosin II regulatory light chain and induce retraction of permeabilized endothelial cells. In this report we characterize PAK2’s morphological and biochemical effect on intact endothelial cells utilizing microinjection of constitutively active PAK2. Under these conditions we observed a modification of the actin cytoskeleton with retraction of endothelial cell margins accompanied by an increase in monophosphorylation of myosin II. Selective inhibitors were used to analyze the mechanism of action of PAK2. Staurosporine, a direct inhibitor of PAK2, largely prevented the action of microinjected PAK2 in endothelial cells. Butanedione monoxime, a non-specific myosin ATPase inhibitor, also inhibited the effects of PAK2 implicating myosin in the changes in cytoskeletal reorganization. In contrast, KT5926, a specific inhibitor of myosin light chain kinase was ineffective in preventing the changes in morphology and the actin cytoskeleton. The additional finding that endogenous PAK2 associates with myosin II is consistent with the proposal that cell retraction and cytoskeletal rearrangements induced by microinjected PAK2 depend on the direct activation of myosin II by PAK2 monophosphorylation of the regulatory light chain.

Key words: PAK, Myosin II, Actin, Contraction, Cdc42

INTRODUCTION

The vascular system is lined by an intact monolayer of endothelial cells whose major function is to serve as a selective barrier to fluid and solute flux across the vascular wall. Breakdown of this selective barrier results in increased permeability and edema formation (Majno and Palade, 1961; Wysolmerski and Lagunoff, 1984; Claudio et al., 1994; McDonald et al., 1999). Since Majno and Palade (1961) presented their initial report describing opening of intracellular junctions, it has been generally accepted that gap formation between endothelial cells evoked by bioactive agents leads to increased vascular permeability. The formation of transient pores within the endothelial cytoplasmic domain creating the equivalent of intercellular gaps remains a possibility (Feng et al., 1999). The molecular events resulting in endothelial cell gap formation are likely to be complex and involve modulation of the balance between cell cohesion, junctional integrity, intrinsic contractile activity and perhaps membrane fusion.

Previously we have proposed that the phosphorylation of myosin II regulatory light chain (RLC) by the Ca²⁺/calmodulin (CaM)-dependent enzyme, myosin light chain kinase (MLCK), mediates endothelial cell contraction and is an essential requirement for one component of increased permeability (Goeckeler and Wysolmerski, 1995). In support of this hypothesis, we have shown that in permeabilized (Wysolmerski and Lagunoff, 1990, 1991) and intact endothelial cell monolayers (Goeckeler and Wysolmerski, 1995), RLC phosphorylation preceded cell retraction and isometric tension development. In both preparations, RLC phosphorylation was dependent on Cu²⁺/CaM and was catalyzed by MLCK. These experiments established a direct correlation between cell retraction, isometric tension and MLCK-mediated RLC phosphorylation. Although the interaction of cytoplasmic Cu²⁺ with CaM has been shown to be the primary determinant for activation of MLCK and RLC phosphorylation, it has become apparent that Ca²⁺/CaM-independent pathways exist for activation of cell retraction/contraction.
Studies from permeabilized smooth muscle preparations indicate that a Ca\(^{2+}/\text{CaM}\)-independent pathway regulated by heterotrimeric G proteins or monomeric Ras-related low molecular mass GTPase is either directly or indirectly responsible for regulation of cell contraction (Kitazawa et al., 1991; Kimura et al., 1996; Otto et al., 1996). Other studies have shown that Rho-associated kinase activated by GTP-Rho can directly phosphorylate isolated myosin light chain and intact myosin in a Ca\(^{2+}/\text{CaM}\)-independent manner (Amano et al., 1996). Experiments using skinned smooth muscle preparations have also documented the ability of Rho-associated kinase to induce smooth muscle contraction and RLC phosphorylation in the absence of Ca\(^{2+}/\text{CaM}\) (Kureishi et al., 1997) lending further support for alternative pathways for activation of cell retraction/contraction.

In nonmuscle cells, Rho, Rac-1 and Cdc42 have been implicated in cytoskeletal reorganization, cell motility, apoptosis and stress responses (Hall, 1994; Narumiya, 1996; Burridge and Chrzanowska-Wodnicka, 1996; Sells and Chernoff, 1997). Activated Cdc42 has been shown to induce actin reorganization in fibroblasts (Kozma et al., 1995); however, the mechanism responsible for this cytoskeletal modification is unknown (Ridley and Hall, 1992; Ridley et al., 1992; Sells and Chernoff, 1997). One of the first Cdc42 target proteins identified was a member of the p21-activated kinase (PAK) family (Sells and Chernoff, 1997). Three mammalian PAK isoforms have been described: the 68 kDa protein, PAK1 (α-PAK; Manser et al., 1994), the 62 kDa protein, PAK2 (γ-PAK, hPAK 65; Teo et al., 1995) and the 65 kDa protein, PAK3 (β-PAK; Manser et al., 1995). PAK2 has been described as a protease-activated kinase (Jakobi et al., 1996) and a H4/S6 kinase (Benner et al., 1995). PAK2 exhibits a widespread tissue distribution, whereas the other two isoforms, PAK1 and PAK3, are largely confined to brain (Hall, 1994; Manser et al., 1995; Teo et al., 1995). The PAKs are highly homologous kinases that preferentially bind the activated form of Cdc42 or Rac1, but not Rho. This association induces a conformational change causing autophosphorylation at multiple sites in the PAKs resulting in activation of their protein kinase activity (Benner et al., 1995; Dennis and Masaracchia, 1993; Manser et al., 1997). Several substrates for the PAKs have been tentatively identified in genetic and biochemical studies and implicated in such processes as actin reorganization and cell motility. However, the exact effectors and mechanism of activation of PAK induced cytoskeletal activities have not been clearly elucidated.

In recent studies we have established that myosin II is a substrate for PAK2 (Ramos et al., 1997; Chew et al., 1998), that PAK2 phosphorylates nonmuscle myosin II RLC in vitro in a Ca\(^{2+}/\text{CaM}\)-independent manner and that PAK2 monophosphorylates myosin II RLC exclusively at Ser-19. Saponin permeabilized endothelial monolayers incubated with Cdc42-activated PAK2 or the active catalytic domain of PAK2 underwent ATP-dependent retraction and actin reorganization (Chew et al., 1998). A role for PAK-mediated phosphorylation in cell retraction is also supported by the evidence that Ca\(^{2+}\)-independent contraction of Triton-skinned smooth muscle cells occurred in response to incubation with either PAK1 or Rho-kinase (Van Eyk et al., 1998). Collectively, these data indicate a role for PAK-induced cell retraction/contraction in response to Cdc42 and Rac1.

In order to define the function of PAK2 in endothelial cells, we microinjected constitutively activate PAK2 into endothelial cells. We further utilized a set of inhibitors, staurosporine, KT5926 and butanedione monoxime (BDM), to provide evidence that the cell retraction and changes in actin distribution we observed after PAK2 microinjection depend on direct activation of myosin II by PAK2.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise indicated. Dextran-conjugated fluorescein dye (M\(_g\) 10x10\(^3\), lysine-fixable) and rhodamine phalloidin were purchased from Molecular Probes Inc. (Eugene, OR). S6-23 was synthesized as previously described (Brandon and Masaracchia, 1991). H4-chimer peptide was synthesized by Genosys (Houston, TX).

Cell culture

The bovine pulmonary artery endothelial (BPAE) cell line established by Del Vecchio and Smith (1981) was obtained from the American Type Culture Collection. Cells were maintained in Eagle’s minimal essential medium (MEM) supplemented with 1 mM glutamine, 10% fetal calf serum, 50 U/ml penicillin, and 50 \(\mu\)g/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO\(_2\)-95% air atmosphere. Cells used in this study were seeded at a density of 400,000 cells/35x10 mm dish 24 hours prior to microinjection.

Microinjection

For microinjection, the cells were placed in MEM without phenol red containing 20 mM Hepes and 0.25% bovine serum albumin. Cell microinjection was performed with a Narishige injector (model IM-188) and a micromanipulator (Model HO-188). Microinjection pipettes were pulled from 1.0 mm glass capillaries using a vertical pipette puller (David Kopf Instruments, model 1M-400,000 cells/35x10 mm dish). For maximal PAK2 protein expression, isopropyl-\(-\text{D-}

Videomicroscopy studies

Videomicroscopy of the injected cells employed Hoffman Modulation Contrast utilizing a ×40 objective. Stage temperature was kept at 37±1°C. Images were captured with a CCD camera (SenSys Photometrics Inc., KAT-1400-G2) at intervals after injection under control of ImageProPlus v3.01 (Media Cybernetics, MD) and saved to a Zip disc (Iomega Corporation, Utah). Individual cell outlines were manually traced in Photoshop v5.0 (Adobe, CA) and cell areas were determined using ImageProPlus v3.01.

Protein purification

The constitutively active human PAK2 DNA construct was obtained from Dr. Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, PA). A single colony of BL-21(DE3) bacteria carrying the constitutive active PAK2 DNA construct was grown at 37°C in 2.5 liter L-Amp broth containing 50 mg/ml ampicillin to an OD\(_{600}\) of 0.8-1.0. For maximal PAK2 protein expression, isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM and incubated for an additional two hours at 37°C. Cells were then harvested by centrifugation at 10,000 g. The cell pellet was
collected, snap frozen in liquid N₂ and stored at –70°C. Packed cells were lysed in 10 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 2.5 mM DTT, 1% Triton X-100, 10 mg/ml DNase and RNase, 10 mg/ml each of leupeptin, PMSF, pepstatin, aprotinin, soybean trypsin inhibitor, and 80 mg/ml benzamidine) per 500 ml bacterial culture. The pellet was resuspended and left on ice for 10 minutes, then sonicated at 4°C for ten 15-second bursts. Cell extracts were spun at 15,000 rpm for 15 minutes. The supernatant was saved and added to 3 ml of washed Talon Resin (Clontech Laboratories, Inc., Palo Alto, CA) and incubated at 4°C for 30 minutes with mild agitation. The resin-bound-protein was collected and washed three times with 4°C lysis buffer. The resin was poured into a small column, washed with lysis buffer, followed by 20 ml lysis buffer containing 5 mM imidazole (pH 8.0). PAK2 was eluted with 30 ml lysis buffer containing 100 mM imidazole (pH 8.0). The fractions containing His-tagged PAK2 were pooled and dialyzed against 2 liters of dialysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl) overnight at 4°C. The final protein concentration was 4 mg/ml by BCA assay. Aliquots were stored at –80°C. Constitutively active mouse PAK1 (mPAK3) was purified as described by Wu et al. (1996).

Human placenta PAK2 was purified as described previously (Dennis and Masaracchia, 1993). H4 was purified as previously described (Eckols et al., 1983). Myosin II regulatory chains and recombinant Cdc42 were expressed in BL21 cells and purified as described (Chew et al., 1998). Smooth muscle MLCK was prepared from chicken gizzards following a modification (Wysolmerski and Lagunoff, 1991) of the procedures outlined by Adelstein and Klee (1981). Unregulated MLCK was prepared by tryptic digestion of MLCK in the presence of bound calmodulin (Adelstein et al., 1981).

**Myosin light chain phosphorylation**

Endothelial cells grown on 3×3 mm cloning coverslips were microinjected with constitutively active PAK2 or buffer as outlined above. Approximately 85% of the cells on each coverslip were microinjected over a 30 minute interval and used for analysis of RLC phosphorylation. Following microinjection, each coverslip was incubated over a 30 minute interval and used for analysis of RLC phosphorylation. The proteins were pelleted by centrifugation; the pellets were washed twice with cold acetone, air dried and dissolved in 12 μl glycerol urea sample buffer. The extent of myosin II RLC phosphorylation was measured by separation of the myosin II RLC phosphorylation states by glycerol/urea polyacrylamide electrophoresis (Chew et al., 1999). Separated RLC were transferred to nitrocellulose and quantitated by immunoblot analysis with antisera raised to nonmuscle phosphorylatable myosin light chain.

In another set of experiments, constitutively active PAK2 was introduced into BPAE cytosol by using the osmotic delivery method developed by Okada and Rechsteiner (1982). Endothelial cells were grown in 12-well plates and constitutively active PAK2 mixed with the osmotic delivery solution was applied to BPAE cells following the manufacturer’s instructions (Gene Tools, LLC, Corvallis, Oregon). Experiments were terminated at the appropriate time interval by flooding cultures with 10% TCA containing 10 mM DTT. Cells were scraped up with a rubber policeman and proteins precipitated for 60 minutes on ice. Samples were washed in acetone, air dried and dissolved in glycerol/urea sample buffer. RLC phosphorylation was assessed as outlined above. To assess the delivery of constitutively active PAK2, paired cultures were lysed in SDS sample buffer, separated by 10% SDS-PAGE, transferred to PVDF membrane and probed with a mouse monoclonal raised to PAK2.

**Cellular inhibitors studies**

Staurosporine (Calbiochem-Novabiochem Corp., San Diego, CA) and KT5926 (Fluka Biochemicals, USA) were dissolved in dimethyl sulfoxide (DMSO) at final concentrations of 2.14 mM and 1 mM, respectively. BDM was prepared fresh at a concentration of 5 mM in tissue culture media. The effect of each of these inhibitors on the activity of PAK2 was determined. Recombinant PAK2 (0.24 μg) or purified human placenta PAK2 (0.47 μg) was activated by incubation at 30°C for 10 minutes in 10 mM Tris, pH 7.4, containing 10 mM MgCl₂, 100 μM [γ-32P]ATP (specific activity ~300), and 10 μg bovine albumin in the presence or absence of recombinant 1 μg Cdc42 which had been charged with 100 μM GTPγS. Inhibitors were added to the reaction mixture and the assay initiated by the addition of protein or peptide substrates. Substrate concentrations were 40 μg H4, 40 μg myelin basic protein, 3 μg nonmuscle MLC, 100 μM S6-23 (AKRRRLSSLRSTSKSESSQK) or 50 μM H4-Site 2 chimeric peptide (VKRISVDPVPAVPGVD). The reaction mixture (50 μl) was incubated at 30°C for 10 minutes. Phosphorylated product was measured on P81 paper as previously described (Eckols et al., 1983).

In some experiments enzyme was activated with trypsin (Dennis and Masaracchia, 1993) followed by autophosphorylation as described above.

For assessing the effects of inhibitors on BPAE cells, the final concentrations of inhibitors were 150 nM KT5926, 50 nM staurosporine and 5 mM BDM. Cells were preincubated for 10 minutes in the presence of staurosporine and KT5926, and 5 minutes in BDM before microinjection.

**Antibodies**

Polyclonal antibodies to purified recombinant PAK2 were raised in New Zealand White rabbits. An immunoglobulin (IgG) fraction was purified from pooled rabbit serum by Ammonium Sulfate Fractionation as described previously (Wysolmerski and Lagunoff, 1991). Mouse monoclonal antibody (mAb) specific for recombinant PAK2 was made by Saint Louis University Hybridoma Center. Ascites fluid containing the PAK2 antibody was purified over a Protein G column and IgG fractions aliquoted and stored at –80°C.

**Immunoprecipitation of PAK2**

BPAE monolayers were washed twice with 37°C PBS and lysed in immunoprecipitation buffer (25 mM Tris-HCl, pH 7.9, 100 mM NaF, 10 mM EGTA, 5 mM EDTA, 250 mM NaCl, 50 mM sodium pyrophosphate, 1% NP-40, 0.5% deoxycholic acid, 10 mg/ml each of leupeptin, aprotinin, pepstatin, and chymostatin, 80 μg/ml benzamidine, 80 μg/ml DTT, and 100 μg/ml soybean trypsin inhibitor). Cell lysates were sonicated twice for 30 second bursts, incubated on ice for 20 minutes and centrifuged at 100,000 g in a Beckman TL-100 ultracentrifuge. BPAE supernatants were added to Protein A-Sepharose precomplexed with rabbit anti-PAK2 antibody and incubated at 4°C for 2 hours. Immunoprecipitates were electrophoresed on 10% SDS gels, transferred to PVDF membrane and incubated with an anti-mouse PAK2 antibody. Immunoreactive bands were visualized with enhanced chemiluminescence reagents (ECL) reagents (Amersham Corp., Arlington Heights, IL).

**Western blotting**

Whole BPAE cell lysates were prepared by washing cells grown on tissue culture dishes with PBS and lysing monolayers with 50 μl of 100°C Laemmli SDS sample buffer. Lysates were heated at 100°C, sonicated for 3 ten seconds bursts, proteins separated by SDS-PAGE, and transferred onto a 0.45 μm PVDF membrane (Millipore, Bedford, MA). Membranes were probed with either rabbit or mouse anti-PAK2 Ab. Secondary HRP-conjugated anti-rabbit or anti-mouse antibodies (Bio-Rad, CA) were used at 1:6,000 dilution. The signal was detected using ECL reagents.

**Rhodamine-phalloidin staining of F-actin**

Cells were washed twice with PBS, fixed in 3% paraformaldehyde in stabilization buffer (127 mM NaCl, 5 mM KCl, 1.1 mM NaH₂PO₄, 0.4 mM KH₂PO₄, 2 mM MgCl₂, 5.5 mM glucose, 1 mM EGTA, 20 mM Pipes, pH 6.5) for 1 hour, and permeabilized with 0.1% (v/v)
Triton X-100 for 10 minutes at room temperature. Cells were stained with rhodamine phalloidin as previously described (Goeckeler and Wysolmerski, 1995) and then coverslipped with 0.1 M n-propylgallate in glycerol:PBS (9:1). Z-series images were collected with a Bio-Rad MRC 1024 confocal microscope.

RESULTS

Endogenous expression of PAK2 in BPAE cells

In order to determine the presence of PAK2 in BPAE cells, cell lysates were probed with three antibodies raised to PAK2: (1) a rabbit polyclonal antibody raised to recombinant PAK2, (2) a mouse monoclonal antibody raised to recombinant PAK2 and (3) a peptide specific polyclonal antibody unique to PAK2 (VKRISVIDPVPAPVDG; Ramos et al., 1997). PAK2 could be detected in whole cell lysates with each of the 3 antibodies. Fig. 1A and B show the presence of a single 60 kDa band detected with the monoclonal antibody and the peptide specific polyclonal antibody, respectively. Immunoprecipitates with the rabbit polyclonal antibody to PAK2 from endothelial cell extracts probed with either the mouse monoclonal antibody (Fig. 1C) or the peptide polyclonal antibody (data not shown) confirmed the presence of PAK2 in the BPAE cells.

Retraction of BPAE cells microinjected with PAK2 and PAK1

Fig. 2 illustrates the time dependent effects of introducing constitutively active PAK2 into endothelial cells by microinjection. The change in cell area (Fig. 2B-E) following microinjection of constitutively active PAK2 (Fig. 2A) was measured from selective frames in the video recording as described in Materials and Methods and the composite results shown in Fig. 2. Within 10 minutes (Fig. 2B) cells injected with PAK2 showed evidence of retraction reducing their surface area by 15%. Retraction was progressive (Fig. 2C-E), and at 15, 30 and 60 minutes the reduction in surface area was 21%, 45% and 67%, respectively (Table 1). Microinjection of constitutively active PAK1 into endothelial cells also caused cell retraction. The time course and extent of PAK1 (Table 1) induced cell retraction was comparable to the retraction induced by PAK2. Cells either globally retracted leaving fine retraction filaments adherent to the substrate or else initially retracted perpendicularly to the long axis of the cell followed by retraction along the long axis. The response of a single cell which was globally retracted by PAK2 microinjection is summarized in Fig. 2F. This composite micrograph shows the outline of the same cell at 0,10, 15, 30 and 60 minutes post injection. Table 1 presents the results of measurements of the footprints of 10 cells recorded at 10, 30 and 60 minute intervals after microinjection of PAK1 or PAK2.

Actin distribution

In uninjected (Fig. 3A) and control injected (Fig. 3B) preconfluent BPAE cells, rhodamine-phalloidin staining demonstrated a well ordered array of parallel stress fibers that established a cell axis. A prominent collection of F-actin was evident along the inner side of the plasma membrane and fine needle-like extensions containing fine actin filaments were occasionally seen along the cell margins. Control cells microinjected with fluorescein conjugated-dextran (Fig. 3B) exhibited no significant differences from uninjected cells (Fig. 3A). Microinjection of constitutively active PAK2 caused remodeling of the F-actin cytoskeleton. Within 10 minutes, F-actin reorganization was observed as evidenced by the loss of the ordered array of parallel filaments and the appearance of small droplets of actin dispersed throughout the cytoplasm (Fig. 3C). For the most part the cortical actin array persisted. Cell retraction was readily documented as an increase in cell height observed in the confocal microscope. The dynamic reorganization of actin filaments progressed over the next 50 minutes. At 30 minutes (Fig. 3D) and 60 minutes (Fig. 3E) after PAK2 microinjection, evident loss of F-actin filaments was associated with increasing numbers of F-actin aggregates, so that by 60 minutes the aggregates were the predominant F-actin form within the cytoplasm (Fig. 3E). Stress fibers began

Table 1. PAK2 and PAK1 induced endothelial cell retraction

<table>
<thead>
<tr>
<th></th>
<th>Control (microinjection buffer)</th>
<th>PAK2</th>
<th>PAK1</th>
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<tbody>
<tr>
<td>% retraction</td>
<td>% retraction</td>
<td>% retraction</td>
<td></td>
</tr>
<tr>
<td>10 minutes</td>
<td>0.2±0.04</td>
<td>14.3±0.90</td>
<td>11.6±0.27</td>
</tr>
<tr>
<td>30 minutes</td>
<td>0.1±0.02</td>
<td>46.6±1.06</td>
<td>52.5±0.67</td>
</tr>
<tr>
<td>60 minutes</td>
<td>0.2±0.04</td>
<td>57.8±0.88</td>
<td>60.1±0.42</td>
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</table>

Preconfluent BPAE cells were microinjected with 1 mg/ml constitutively active PAK2 or PAK1. Footprints from 10 cells were measured at 10, 30, 60 minutes after the microinjection and cell area determined. Percentage retraction was determined by comparing the cell area prior to microinjection with the loss in cell area after microinjection of constitutively active PAK2 or PAK1. The data are presented as means ± s.d. of at least 10 determinations in three separate experiments.
to reform about 1.5 hours (Fig. 3F) after PAK2 microinjection and the droplets of actin concomitantly disappeared. During the recovery period some cells showed prominent membranes lined with F-actin (Fig. 3G). At this time point, the filamentous actin again became the dominant phalloidin-stainable actin structure. By 4 hours after microinjection, cells appeared indistinguishable from control cells (Fig. 3H).

PAK2 induced RLC phosphorylation

Data to this point established that PAK2 induced cell retraction and F-actin reorganization. To determine the effect of microinjection of PAK2 on myosin II RLC phosphorylation, two sets of experiments were performed. In the first approach, cells grown on cloning coverslips were microinjected with constitutively active PAK2 and RLC phosphorylation analyzed by glycerol/urea gel electrophoresis. As illustrated in Fig. 4A, cells microinjected with active PAK2 show an 85% increase in myosin II RLC phosphorylation. Control cells exhibit a basal phosphorylation of 0.2±0.02 mol PO4/mol myosin RLC (Fig. 4A, lane 1). After microinjection of constitutively active PAK2, phosphorylation increases to 0.37±0.02 mol PO4/mol RLC within 35 minutes (Fig. 4A, lane 2). This increase in phosphorylation was exclusively attributable to monophosphorylation of myosin II RLC (P1). No increase in diphosphorylated (P2) RLC occurred. In a second set of experiments, we utilized an osmotic delivery protocol to introduce active PAK2 into the endothelial cell cytoplasm. Cells were treated with delivery solution containing 40 μg/ml PAK2 or buffer control for 30 minutes, proteins TCA precipitated and samples analyzed for RLC phosphorylation as outlined in Materials and Methods. Fig. 4B shows the increase in RLC monophosphorylation after osmotic delivery of active PAK2. In buffer control cells basal phosphorylation is 0.20±0.01 mol PO4/mol RLC (Fig. 4B, lane 1). Treatment with buffer containing only osmotic delivery reagent resulted in no change in RLC phosphorylation (Fig. 4B, lane 2). In contrast, osmotic delivery of constitutively active PAK2 resulted in an increase in RLC phosphorylation to 0.30±0.03 mol PO4/mol RLC (Fig. 4B, lane 3) solely due to monophosphorylation of myosin II RLC; no increase in diphosphorylation was detected. To confirm that PAK2 was successfully delivered to the endothelial cell cytoplasm, cell lysates were probed by western blot with a mouse monoclonal to PAK2. Western blot (Fig. 4C) confirmation that constitutively active PAK2 is present within the cytoplasm (Fig. 4C, lane 2) is possible because constitutively active PAK2 was constructed with a His-tag and migrates with a higher molecular mass on SDS gels than the endogenous endothelial cell PAK2. The data obtained from both of these experimental approaches clearly indicate that constitutively active PAK2 induces an increase in monophosphorylation of myosin II RLC. Analysis of MLC phosphorylation is the mean ± SE of 5 separate experiments.

PAK2 inhibitor studies

The mechanism by which PAK2 induced retraction in endothelial cells was further investigated by inhibiting three specific potential events in the response: protein phosphorylation, myosin II activation and MLCK activation. This experimental strategy was pursued by pretreating cells with staurosporine, a potent nonspecific inhibitor of protein kinases (Watson et al., 1988; Herbert et al., 1990; Godbois et al., 1992), KT5926; a selective inhibitor of MLCK (Nakanishi et al., 1990) and butanedione monoxime (BDM) an agent that inhibits myosin II ATPase activity (Fryer et al., 1988; Herrmann et al., 1993; Cramer and Mitchison, 1995). Staurosporine was found to inhibit PAK2 with all substrates...
tested (Table 2). With H4 as substrate, an IC$_{50}$ of 0.64 nM was observed with Cdc42-GTP$_{S}$-activated enzyme. When PAK2 was activated with trypsin, the observed IC$_{50}$ was 0.49 nM. No significant differences in the observed IC$_{50}$ values were determined when myelin basic protein, or the synthetic peptides S6-23 or H4-Site2 chimer were used as substrates in place of H4. A slightly lower IC$_{50}$ was consistently observed with trypsin-activated enzyme as compared to Cdc42-GTP$_{S}$-activated enzyme with all substrates tested; however, these differences were not statistically significant.

To ensure that neither the MLCK inhibitor KT5926 nor the myosin II inhibitor BDM affected PAK2 activity directly, recombinant PAK2 and placental PAK2 were assayed in the presence of KT5926 or BDM. Results are shown in Table 2. Neither KT5926 at a concentration of 150 nM nor 5 mM BDM inhibited PAK2 activity with any of the substrates tested (Table 2). Similar absence of inhibition was found when PAK2 was activated with trypsin (data not shown). The KT5926 and BDM inhibitor concentrations used were sufficient to effectively inhibit MLCK and myosin II ATPase activity in vitro (Fryer et al., 1988; Herrmann et al., 1993; Cramer and Mitchison, 1995) and block the thrombin-stimulated MLCK response (unpublished observation) and myosin II ATPase in cells in vivo (Cramer and Mitchison, 1995).

Having established that staurosporine was an effective inhibitor of PAK2 activity in vitro whereas KT5926 and BDM had no effect on PAK2 activity, we sought to determine the effects of the inhibitors on PAK2 induced cytoskeletal alterations. A 40 minute control incubation in 50 nM staurosporine alone (Fig. 5B) had minimal effect on F-actin distribution compared to buffer control (Fig. 5A). In addition, staurosporine treatment alone had no measurable effect on the cell area (Table 3). BPAE cells were incubated in the presence of 50 nM staurosporine for 5 minutes prior to microinjection with PAK2 and then incubated for an additional 30 minutes following microinjection in the continued presence of the inhibitor. Cells were then fixed and stained for F-actin. Both cell retraction (Table 3) and F-actin cytoskeletal changes (Fig. 5E) were largely prevented by staurosporine. Fig. 5E shows that treatment with staurosporine prevented the loss of F-actin filaments and the development of the large puddles of F-actin seen with PAK2 microinjection alone (Fig. 5C).

We have previously shown that PAK2 phosphorylates myosin II RLC in a Ca$^{2+}$/calmodulin independent manner and initiates cell retraction in permeabilized monolayers implicating PAK2 in myosin II activation. To confirm the role of PAK2 in myosin II activation, butanedione monoxime (BDM), an inhibitor of myosin II ATPase, was utilized to inhibit myosin II function. Cultures were preincubated in 5 mM BDM for 10 minutes then microinjected with PAK2 (in

![Fig. 3. Effect of constitutively active PAK2 on endothelial cell F-actin. BPAE cells were injected with constitutively active PAK2 (1 mg/ml) together with 0.1 mg/ml FITC-labeled dextrans (10x10$^3$ M$_r$). Cells were then fixed at the appropriate time interval with 3% formaldehyde for 1 hour and permeabilized in stabilization buffer containing 0.1% Triton X-100. F-actin was stained with rhodamine phalloidin and cells viewed with a Bio-Rad MRC 1024 confocal microscope. Images are composite Z-series projections illustrating both the F-actin and FITC-dextran distribution. (A-H) F-
the presence of BDM) and the extent of cell retraction determined at 10, 30 and 60 minutes. As shown in Table 3 BDM effectively inhibited PAK2 induced cell retraction.

To determine if MLCK activation of myosin II was involved in PAK2-induced endothelial cell retraction, cells were pretreated with 150 nM KT5926, a specific inhibitor of MLCK (Nakanishi et al., 1990), for 10 minutes then injected with PAK2 (in the presence of KT5926) and the degree of cell retraction assessed at 10, 30 and 60 minutes. Inhibition of MLCK activity with KT5926 had no significant effect on retraction induced by PAK2 (Table 3). We conclude that PAK2 induced cell retraction is dependent on myosin II but independent of MLCK activation.

**MLCK-mediated cell retraction**

Since the activation mechanisms and phosphorylation events for PAK2 and MLCK are evidently distinct, the effects of the two kinases on cell morphology and actin filament distribution were compared. When purified MLCK is proteolytically cleaved under controlled conditions, it retains its kinase activity in the absence of Ca²⁺/calmodulin. This unregulated form of MLCK was used to test the role of microinjected MLCK in actin rearrangements and endothelial cell retraction. Unregulated MLCK was microinjected into preconfluent BPAE cells and the cultures were fixed and stained with rhodamine phalloidin at 10, 30, and 60 minutes post injection. Fig. 6 shows a representative cell exhibiting the effects of microinjected unregulated MLCK on F-actin distribution. Within 10 minutes post injection (Fig. 6A), F-actin stress fibers begin to develop oriented parallel to one another and to the long axis of the cell. By 30 minutes (Fig. 6C), the cell cytoplasm is filled with prominent filament bundles oriented parallel to one another, and a circumferential rim of actin outlines the cell margin. In addition, numerous fingerlike projections containing F-actin protrude from the cell margin. No detectable cell retraction occurred as a result of microinjection of unregulated MLCK in preconfluent cells used in these experiments.

**Co-precipitation of PAK2 with myosin II**

In a final set of experiments we sought to directly determine if PAK2 and BPAE myosin II were associated intracellularly. Fig. 7 is a western blot of immunoprecipitated PAK2 from BPAE cells probed with affinity purified anti-human platelet myosin II heavy chain antibody (Wysolmerski and Lagunoff, 1991) and polyclonal anti-peptide PAK2 antibody. Cultures were lysed in buffer containing 10 mM ATP and PAK2 was immunoprecipitated with a PAK2 mAb as described in Materials and Methods. Fig. 7A is a representative film illustrating that myosin II co-immunoprecipitates with endogenous PAK2. Immunoprecipitated samples
were also probed with a rabbit anti-PAK2 peptide antibody to verify that endogenous PAK2 was precipitated (Fig. 7B). The amount of myosin II heavy chain detected is consistent with a specific interaction between PAK2 and the contractile protein as opposed to cross reactivity of the PAK2 monoclonal antibody with the entire myosin pool. In addition, samples were screened with an anti-actin monoclonal antibody to ensure that myosin II association with PAK2 was specific and not a result of a nonspecific interaction with actin. No actin was detected in immunoprecipitates.

**DISCUSSION**

Endothelial cell retraction occurs in response to a wide variety of physiological events. In these studies the role of PAK2 in endothelial cell retraction was investigated using microinjection of constitutively active enzyme. Reorganization of the cytoskeleton was monitored by video microscopy and confocal microscopy of phalloidin-rhodamine stained actin filaments. The response to microinjected PAK2 was rapid with actin filament reorganization observed within 5 minutes. Cell retraction associated with extensive reorganization of F-actin was clearly evident in 15 minutes and persisted for up to 60 minutes. Microinjection and osmotic loading of constitutively active PAK2 resulted exclusively in monophosphorylation of myosin II RLC. Cytoskeletal reorganization was significantly different from that observed with microinjected unregulated MLCK. The response to microinjected PAK2 was completely abolished by the protein kinase inhibitor staurosporine. BDM, an inhibitor of myosin II ATPase, also prevented PAK2 induced cell retraction and F-actin rearrangements confirming that phosphorylation and activation of myosin II is necessary for endothelial cell retraction. In contrast, KT5926, an inhibitor of MLCK had no effect on PAK2 induced cell retraction or F-actin reorganization.

Activated Rho promotes stress fiber assembly, Rac1 induces membrane ruffling and Cdc42 causes development of filopodia at the cell periphery (Ridley and Hall, 1992; Ridley et al., 1992; Narumiya, 1996; Sells and Chernoff, 1997). These effects are believed to be mediated through activation of effector kinases. Our findings indicate that PAK2, a protein kinase activated by Cdc42, does not directly promote projection of filipodia from the cell periphery but that these peripheral structures form in the course of cell retraction. Our observations agree in part with two recent reports (Sells et al., 1997; Manser et al., 1997) describing the effects of microinjected PAK1 on fibroblasts and HeLa cells. These investigators showed that microinjection of either constitutively active PAK1 protein or plasmids encoding constitutively active PAK1 induced cell retraction, but in both HeLa cells and fibroblasts there was virtually complete loss of stress fibers. In endothelial cells, PAK2 induces cell retraction and reorganization of peripheral actin filaments without loss of central actin stress fibers. Microinjection of constitutively

<table>
<thead>
<tr>
<th>Inhibitor PAK2 activity (% H4 control activity)</th>
<th>Recombinant PAK2</th>
<th>Placenta PAK2</th>
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</thead>
<tbody>
<tr>
<td>H4</td>
<td>MBP H4-Site2</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>Staurosporine (50 nM)</td>
<td>&lt;1 &lt;1</td>
<td></td>
</tr>
<tr>
<td>KT5926 (50 nM)</td>
<td>104</td>
<td>103</td>
</tr>
<tr>
<td>BDM (5 nM)</td>
<td>97</td>
<td>122</td>
</tr>
</tbody>
</table>

Table 2. Inhibition of PAK2 by staurosporine, KT5926 and BDM

Fig. 4. Effects of PAK2 on myosin II RLC phosphorylation. Preconfluent BPAE cells were microinjected (A) or osmotically loaded (B) with constitutively active PAK2 (cPAK2) as described in Materials and Methods. BPAE cells from 5 cloning coverslips were microinjected with buffer only (A, lane 1) or cPAK2 (A, lane 2) and the phosphorylated states of myosin II analyzed by 12% glycerol/urea gel electrophoresis blotted onto nitrocellulose and probed with anti-myosin II RLC antibodies as described in Materials and Methods. RLC phosphorylation was determined 35 minutes after microinjection was initiated. Microinjection of buffer had no effect on RLC phosphorylation whereas cPAK2 induced a shift from unphosphorylated to monophosphorylated RLC. No increase in diphasphorylated RLC was evident. (B) Representative experiment from preconfluent BPAE cells osmotically loaded with buffer only or cPAK2. BPAE were grown in 12-well plates and treated as follows: lane 1, control cells without treatment; lane 2, cells treated with PBS containing delivery reagent for 30 minutes; lane 3, cells incubated with cPAK2 and delivery reagent for 30 minutes. Control treatments had no effect on RLC phosphorylation. Incubation with cPAK induced exclusively monophosphorylation of RLC (lane 3). (C) To confirm that cPAK2 gained entrance to the cytoplasm cells incubated with either buffer alone (lane 1) or cPAK (lane 2) were lysed in SDS sample buffer separated on 10% SDS-PAGE, transferred to PVDF membrane and probed with a mouse monoclonal antibody to PAK2. Endogenous endothelial cell PAK2 and cPAK2 are recognized by the monoclonal antibody. Active PAK2 (cPAK2) was constructed with a His-tag and migrates with a higher molecular mass than the native enzyme (lane 2).
active PAK1, an isoform not endogenously expressed in endothelial cells, induced endothelial cell retraction and morphological changes similar to PAK2 (Table 1). These results suggest that the effects of the PAK family of kinases may be cell specific rather than isoform specific.

Although some of the morphological changes orchestrated by members of the PAK family of kinases have been documented, little is known of the targets that mediate these events. Initial studies of the role of G protein activation and protein kinase activity in actin reorganization demonstrated that retraction induced by activated PAK1 was dependent on PAK catalytic activity (Sells et al., 1997; Manser et al., 1997). Several potential substrates have been identified in genetic and biochemical studies. However, considerable uncertainty remains with respect to the molecules actually phosphorylated by PAKs and the events leading to cell retraction and cytoskeletal alterations. Recently, we have shown that in vitro PAK2 phosphorylates myosin II RLC only at Ser-19 (Chew et al., 1998) in a Ca2+/CaM-independent manner. The additional finding that activated PAK2 added to permeabilized endothelial cell monolayers resulted in a Ca2+/CaM-independent cell retraction (Chew et al., 1998) suggested that the Ca2+/CaM-independent retraction induced

**Fig. 5.** Staurosporine inhibition of PAK2 induced alterations in F-actin. Preconfluent BPAE cells were microinjected with 1 mg/ml constitutively active PAK2 with or without pretreatment with 50 nM staurosporine. Thirty minutes after microinjection of constitutively active PAK2, cells were fixed and stained for F-actin. Staurosporine prevents the PAK2 induced reorganization of F-actin. (A) Control cell without treatment; (B) BPAE cell incubated in 50 nM staurosporine for 30 minutes only; (C) cell injected with constitutively active PAK2 for 30 minutes only; (E) cells pretreated with staurosporine for 5 minutes, microinjected with constitutively active PAK2 (in the presence of staurosporine) for 30 minutes, then fixed and stained for F-actin. (D and F) Fluorescence signal from the co-injected FITC-dextran confirming the microinjected cell.

**Fig. 6.** Effect of unregulated myosin light chain kinase (MLCK) on F-actin distribution. BPAE endothelial cells were microinjected with unregulated MLCK (0.6 μg/μl) and fixed with 3% formaldehyde at 10 (A) and 30 (C) minutes, respectively. F-actin was stained with rhodamine phalloidin and viewed with a Bio-Rad confocal microscope. Unregulated MLCK bundles actin into prominent stress fibers that traverse the long axis of the cell. Minimal cell retraction occurs in cells microinjected with the unregulated MLCK. Effects of unregulated MLCK on F-actin distribution and cell retraction are distinct from those induced by constitutively active PAK2 (cf Fig. 3). (A) Cell microinjected with unregulated MLCK for 10 minutes; (B) fluorescence signal from the co-injected FITC-dextran; (C) cell microinjected with unregulated MLCK for 30 minutes and (D) fluorescence signal from the co-injected FITC-dextran.
by PAK2 could arise from myosin II activation by monophosphorylation of the RLC. In this report we have extended our in vitro studies to show that PAK2 induces an increase in myosin II RLC phosphorylation in vivo. Microinjection of constitutively active PAK2 (Fig. 4) caused an increase in monophosphorylation of myosin II RLC by 85 and 50%, respectively. These results are consistent with those of Sells et al. (1999) data utilizing a RLC, Ser-19 specific antibody to show immunocytochemically that PAK1 induced an increase in myosin light chain phosphorylation. These data provide strong support for our hypothesis that PAK2 effects endothelial cell retraction through activation of myosin II RLC phosphorylation.

Sanders et al. (1999) investigating the effects of PAK1 on cell spreading showed that PAK1 phosphorylated MLCK reducing its catalytic activity. The authors suggest that altering MLCK catalytic activity and thus RLC phosphorylation prevents cell spreading. It is difficult to compare these studies with ours due to the different methods employed to deliver the PAK enzymes. Microinjection allowed us to control the amount of enzyme delivered within the cytoplasm while the Semiliki Forest viral expression system used by Sanders et al. (1999) continuously express large amounts of the enzyme over a period of hours. Since PAK1 in their experiments was expressed in cells for 6 to 8 hours before initiating experiments, it is conceivable that overexpression of PAK1 could modulate other cellular pathways altering cell spreading.

Based on their studies of human microvascular endothelial cells transiently transfected with active or dominant negative PAK1, Kiosses et al. (1999) suggest that one function of PAK in stimulating cell motility is to cause retraction of the trailing portion of the cell. Our results demonstrating the action of PAK2 on monophosphorylation of MLC resulting in cell retraction fits well with their proposal.

The effects of known agents that block contractility were assayed for their ability to inhibit PAK2 catalytic activity in vitro and inhibit PAK2 induced cell retraction. We utilized three inhibitors, staurosporine, a general kinase inhibitor (Watson et al., 1988; Herbert et al., 1990; Godbois et al., 1992), KT5926, an MLCK inhibitor (Nakanishi et al., 1990), and BDM, a myosin II ATPase inhibitor (Fryer et al., 1988; Herrmann et al., 1993; Cramer and Mitchison, 1995).

Staurosporine while a potent inhibitor of PKC is also an inhibitor of several other kinases (Watson et al., 1988; Herbert et al., 1990; Godbois et al., 1992). Recently Kabuchi's laboratory (Amano et al., 1997) has shown that staurosporine prevented stress fiber and focal adhesion formation in 3T3 fibroblasts microinjected with constitutively active Rho-kinase and it was proposed on this basis that staurosporine was an inhibitor of Rho-kinase, although the effectiveness of staurosporine in inhibiting Rho-kinase catalytic activity was not reported. In our studies, staurosporine was able to completely abolish PAK2 phosphorylation of MLC, H4 and peptide S6-23 in vitro (Table 2), and pretreatment of endothelial cells with staurosporine inhibited the PAK2 induced cell retraction and actin rearrangements.

Having established that PAK2 activity induces endothelial cell retraction, we sought to directly test the involvement of myosin II. BDM, a myosin ATPase inhibitor, which functions by slowing the release of phosphate from myosin after ATP hydrolysis (Herrmann et al., 1993; McKillop et al., 1994) has been shown by Cramer and Mitchison (1995) to be an effective inhibitor of nonmuscle myosin II. Because BDM suffers from a confounding effect of depleting cellular ATP (Hebisch et al., 1993) and causes disruption of F-actin, endothelial cells were incubated in 5, 10 and 20 mM BDM for 20 minutes and F-actin structure examined by confocal microscopy. At 10 mM BDM, cells exhibit disorganization of F-actin and 20 mM BDM causes F-actin fragmentation and cells round up and detach from the

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Table 3. Effect of staurosporine, BDM, KT5926 on PAK2 induced endothelial cell retraction

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10 minutes</th>
<th>30 minutes</th>
<th>60 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% retraction</td>
<td>% inhibition</td>
<td>% retraction</td>
</tr>
<tr>
<td>PAK2 alone</td>
<td>14.3±0.9</td>
<td>46.0±1.1</td>
<td>57.8±0.9</td>
</tr>
<tr>
<td>Staurosporine+PAK2</td>
<td>1.4±0.1</td>
<td>2.9±0.2</td>
<td>4.6±0.2</td>
</tr>
<tr>
<td>BDM+PAK2</td>
<td>6.2±0.8</td>
<td>8.6±7.7</td>
<td>13.1±1.0</td>
</tr>
<tr>
<td>KT5926+PAK2</td>
<td>13.9±1.0</td>
<td>48.3±2.0</td>
<td>58.0±1.8</td>
</tr>
</tbody>
</table>

Effect of inhibitors on PAK2 induced retraction. Cells were preincubated in 50 nM staurosporine (general kinase inhibitor), 5 mM BDM (myosin II ATPase inhibitor), or 150 nM KT5926 (MLCK inhibitor) for 5 minutes prior to microinjection of constitutively active PAK2. The effects of PAK2 were followed by videomicroscopy for 1 hour post injection. Percentage retraction for each time point was calculated as described in Materials and Methods. Percentage inhibition was determined by comparing the percent retraction of cells exposed to the inhibitor and microinjected with constitutively active PAK2 with that of control cells injected with constitutively active PAK2 alone. Footprints of 10 cells were measured for each inhibitor. Data presented as means ± s.d. of 3 separate experiments.
culture dish. However, at the concentration of 5 mM used in these studies, F-actin structure as well as cellular ATP levels are unaffected (data not shown), but there is complete inactivation of myosin II ATPase. Neither did 5 mM BDM have any effect on the PKA2 catalyzed phosphorylation of MLC, H4 or S6-23 peptide in vitro. Yet preincubation of endothelial cells in 5 mM BDM resulted in greater than 80% inhibition of retraction (Table 3) and prevented the reorganization of F-actin in cells microinjected with PKA2, indicating that the retraction induced by PKA2 requires the activation of myosin II. This conclusion is further strengthened by the observation that myosin II can be co-immunoprecipitated with PKA2 suggesting that PKA2 may be localized in the cell in a manner that allows efficient activation of myosin II.

Although several kinases have been shown to phosphorylate myosin II in intact cells (Ludowkye et al., 1989; Satterwhite et al., 1992; Goecckeler and Wysolmerski, 1995), it is generally accepted that myosin II activation results principally from phosphorylation of myosin II RLC by MLCK, a Ca^{2+}/CaM dependent enzyme. MLCK phosphorylation of nonmuscle myosin II RLC at Ser-19 and Thr-18 has been shown to regulate myosin II filament formation (Craig et al., 1983; Higashihara et al., 1989), myosin II ATPase activity (Adelstein and Conti, 1975; Ikebe and Hartshorne, 1985) and in vitro motility (Unemoto et al., 1989). In order to demonstrate that myosin II activation by PKA2 was not initiated by PKA2 microinjection, we inhibited MLCK in PKA-injected cells. KT5925 (Nakanishi et al., 1990), a well characterized inhibitor of MLCK with high specificity for this enzyme in vitro at the concentrations used in this study was employed. MLCK activity in endothelial cell extracts is inhibited by greater than 98% by 150 nM KT5926. At this concentration KT5926 had no effect on PKA2 catalytic activity (Table 2) and did not prevent PKA2 induced cell retraction (Table 3). Collectively, the results of the inhibitors studies support the conclusions that (1) PKA2 kinase activity is required to induce retraction, (2) myosin II activation is required for PKA2 evoked cell retraction, and (3) PKA2 mediated cell retraction is independent of MLCK activation of myosin II.

In vitro PKA2 catalyzes only monophosphorylation of myosin II RLC at Ser-19 (Chew et al., 1998), whereas MLCK (Goecckeler and Wysolmerski, 1995; Chew et al., 1998) and Rho-kinase (Amano et al., 1996) diphosphorylate myosin II RLC at Ser-19 and Thr-18. Microinjection of endothelial cells with unregulated MLCK results in the formation of prominent stress fibers which run parallel to one another along the long axis of the cell (Fig. 6). In intact endothelial cell monolayers (Goecckeler and Wysolmerski, 1995), thrombin induces myosin II RLC phosphorylation at Ser-19 and Thr-18 and causes the formation of stress fibers essentially identical to those seen with microinjection of unregulated MLCK. Treatment of preconfluent endothelial cells with thrombin caused stress fiber formation indistinguishable from those formed after microinjection of unregulated MLCK (data not shown). The bundling of stress fibers induced by the unregulated MLCK thus may be the effect of diphosphorylation of myosin II RLC at Ser-19 and Thr-18. Studies microinjecting constitutively active Rho-kinase (Amano et al., 1997) or Rho-kinase activated with LPA (Chrzanowska-Wodnicka and Burridge, 1996) indicate that this kinase, which also diphosphorylates myosin II RLC, induces stress fibers much like those generated on injection of unregulated MLCK. Additionally, microinjection of constitutively active Rho-kinase into endothelial cells causes stress fiber formation identical to unregulated MLCK (unpublished observations). Clearly, actin structure reorganization and cell retraction mediated by PKA2 are distinct from those of either MLCK or Rho-kinase although each of these kinases effects myosin II activation.

Although the physiological significance of each phosphorylation state is currently unknown, we propose that site specific phosphorylation of myosin II RLC may dictate the formation of specific actin structures defining the extent and character of cell retraction. Little is known about the agonists that activate the PKA2 pathway but a reasonable assumption would be that different stimuli activate PKA2, Rho kinase and MLCK pathways leading to enzyme specific modification of myosin II. In turn, the differential phosphorylation of myosin II promotes cell retraction and/or distinct actin redistribution.

The results presented in this report demonstrate that activated PKA2 produces endothelial cell retraction and characteristic actin rearrangements different from those seen with activation of MLCK. These effects are dependent on PKA2 activation of myosin II and are independent of MLCK activity. Based on these findings, we propose that agonists that lead to the activation of PKA2 initiate a pathway for myosin II activation distinct from the pathway in which MLCK or Rho-kinase participate. We further propose that the distinctive effects of the PKA2 pathway arise from monophosphorylation of myosin II at Ser-19.

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