Role of the small GTPase RhoA in the hypoxia-induced decrease of plasma membrane Na,K-ATPase in A549 cells

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Accepted 3 May 2007
Journal of Cell Science 120, 2214-2222 Published by The Company of Biologists 2007
doi:10.1242/jcs.003038

Summary

Hypoxia impairs alveolar fluid reabsorption by promoting Na,K-ATPase endocytosis, from the plasma membrane of alveolar epithelial cells. The present study was designed to determine whether hypoxia induces Na,K-ATPase endocytosis via reactive oxygen species (ROS)-mediated RhoA activation. In A549 cells, RhoA activation occurred within 15 minutes of cells exposure to hypoxia. This activation was inhibited in cells infected with adenovirus coding for gluthatione peroxidase (an H_2O_2 scavenger), in mitochondria depleted (ρ^0) cells or cells expressing decreased levels of the Rieske iron-sulfur protein (inhibitor of mitochondrial complex III), which suggests a role for mitochondrial ROS. Moreover, exogenous H_2O_2 treatment during normoxia mimicked the effects of hypoxia on RhoA, further supporting a role for ROS. Cells expressing dominant negative RhoA failed to endocytose the Na,K-ATPase during hypoxia or after H_2O_2 treatment. Na,K-ATPase endocytosis was also prevented in cells treated with Y-27632, a Rho-associated kinase (ROCK) inhibitor, and in cells expressing dominant negative ROCK. In summary, we provide evidence that in human alveolar epithelial cells exposed to hypoxia, RhoA/ROCK activation is necessary for Na,K-ATPase endocytosis via a mechanism that requires mitochondrial ROS.

Key words: Na,K-ATPase, ROS, Hypoxia, Endocytosis, Alveolar epithelium

Introduction

Pulmonary edema develops when fluid movement into the air spaces exceeds the ability of the lung to clear it (Mutlu and Sznajder, 2005; Staub, 1974). During acute hypoxemic respiratory failure, alveoli flood with edema thus impairing the transfer of oxygen from the airspaces into the pulmonary circulation (Ware and Matthay, 2000). Transport of Na^+ and edema reabsorption from alveolar spaces into interstitial and vascular spaces is crucial for improving hypoxemia and restoring normal lung function (Jain and Sznajder, 2005). Sodium enters the apical domains of alveolar epithelial cells (AECs) through amiloride-sensitive Na^+ channels and is transported out across the basolateral membrane by the Na,K-ATPase (Sznajder et al., 1995; Ware and Matthay, 2000). It has been shown that hypoxia decreases transepithelial fluid clearance from the alveoli of hypoxic rats (Litvan et al., 2006; Vivona et al., 2001) and that, in vitro, hypoxia inhibits active Na^+ transport by impairing the activity of both apical Na^+ channels and the Na,K-ATPase, whereas a more prolonged exposure leads to their degradation and inhibition of gene transcription (Clerici and Matthay, 2000; Comellas et al., 2006; Planes et al., 2002; Planes et al., 1996). We have previously shown that, in AECs, acute exposure to severe hypoxia inhibits Na,K-ATPase by promoting its endocytosis from the cell surface via a clathrin-adaptor protein 2-dependent mechanism (Chen et al., 2006; Dada et al., 2003).

Endocytosis of plasma membrane proteins requires the interaction of different proteins to cluster cargo molecules, invaginate the membrane and release the vesicles. The importance of a dynamic actin cytoskeleton in the endocytic process has been reported in the budding yeast (Ayscough, 2005) and more recently in mammalian cells (Apodaca, 2001; Engqvist-Goldstein and Drubin, 2003; Lamaze et al., 1997; Qualmann et al., 2000; Yarar et al., 2005). The ability of Rho proteins (Rho, Rac and Cdc 42) to regulate actin dynamics has been described (Hall, 1998; Takai et al., 2001). Like other GTPases, Rho is maintained inactive in the cytosol when complexed with the GDP-dissociation inhibitor and active when bound to GTP at the membrane (Matozaki et al., 2000). Activated RhoA has been implicated in the regulation of receptor internalization (Lamaze et al., 1996; Symons and Rusk, 2003). RhoA exerts its biological function through the activation of a number of downstream effectors. The Rho-associated serine/threonine kinase (ROCK) is a major RhoA effector and is involved in the formation of stress fibers and focal adhesions, contractility of smooth muscle, and ICAM internalization in endothelial cells, among other functions (Muro et al., 2003; Wettschureck and Offermanns, 2002). Rho-kinase is activated by the GTP-bound active form of RhoA.

A growing body of evidence suggests that hypoxia increases the generation of reactive oxygen species (ROS) (Chandel and Schumacker, 2000; Duranteau et al., 1998; Waypa and Schumacker, 2005), which have been implicated in modulating cell signaling pathways (Hool, 2006; Schulze-Osthoff et al., 1997). We have previously found that hypoxia causes an increase in ROS generation in AECs (Comellas et

al., 2006; Dada et al., 2003). In the present study, we report that under hypoxic conditions mitochondrial ROS activate RhoA, and that the RhoA/ROCK regulate Na,K-ATPase endocytosis from the plasma membrane into intracellular compartments.

Results

Effect of hypoxia on alveolar epithelial Na,K-ATPase

We have previously described that exposing A549 cells to 1.5% O_2 for 60 minutes resulted in a decrease in plasma membrane Na,K-ATPase α_1 expression by ~50%, while milder hypoxic conditions (3% O_2) induced similar results by 120 minutes (Dada et al., 2003). To characterize the specificity of the endocytic process we assessed the levels of plasma membrane transferrin receptor during hypoxia. The decrease in plasma membrane Na,K-ATPase protein levels appears to be specific since the levels of the transferrin receptor were unchanged as assessed by a biotinylation assay (Fig. 1A). It has been previously demonstrated that dynamin is a mediator of plasma membrane protein internalization (De Camilli et al., 1995). Here, we examined the ability of hypoxia to regulate Na,K-ATPase protein levels in cells transiently transfected with a plasmid coding for a GFP-tagged dominant negative dynamin-2 (DN-GFP-Dyn K44A). As depicted in Fig. 1B, we found hypoxia failed to induce a significant decrease in Na,K-ATPase protein expression at the plasma membrane in cells expressing the DN-dynamin, which suggests a role for dynamin in promoting Na,K-ATPase endocytosis.

Actin disrupting agents prevent hypoxia-induced Na,K-ATPase endocytosis

To determine whether actin organization was required for hypoxia-induced Na,K-ATPase endocytosis, A549 cells were pre-incubated with the actin-filament-disrupting drugs latrunculin B (LB) and cytochalasin D (Cyt D) and the actin-stabilizing agent phalloidin. As shown in Fig. 2, pre-treatment with LB (Fig. 2A), Cyt D (Fig. 2B) or phalloidin (Fig. 2C) blocked the decrease in plasma membrane Na,K-ATPase levels during hypoxia. By contrast, microtubule polymerization was not required for this process, as the microtubule polymerization inhibitor colchicine did not prevent the hypoxia-mediated Na,K-ATPase downregulation (Fig. 2D).

Hypoxia activates RhoA in alveolar epithelial cells

Upon stimulation, RhoA migrates from the cytosol to the membrane, where it is active (Fleming et al., 1996). Thus, to assess whether RhoA was stimulated by hypoxia, we incubated A549 cells at 1.5% O_2 and, as depicted in Fig. 3A, found that hypoxia stimulated RhoA translocation to the 1% Triton X-100-soluble membrane fraction. Previously, we have determined that levels of Na,K-ATPase in the total membrane fraction (plasma membrane plus intracellular membranes) did not change upon exposure to hypoxia for 60 minutes, thus total membrane α1-subunit protein levels were used as a loading control (Fig. 3A lower panel) (Dada et al., 2003). RhoA activation was also assessed by a pull-down assay and, as shown in Fig. 3B (top panel), A549 cells exposed to hypoxia had a time-dependent increased recovery of RhoA bound to GTP (active) as compared with the normoxic control. The total amount of RhoA in the cell lysates was unchanged indicating the specificity of the increase in the pull-down assay (Fig. 3B, bottom panel). Stress fiber formation is a hallmark of RhoA activation (Ridley and Hall, 1992). To further confirm hypoxia-induced RhoA activation, A549 cells were exposed to hypoxia for different periods of time and stress fiber formation was assessed. Cells in normoxic conditions exhibited a normal pattern of actin staining consisting of cortical actin at the plasma membrane and very few if any stress fibers (Fig. 3C, left panel); cells exposed to 1.5% O_2 for 15 minutes exhibited an increase in stress fiber formation (Fig. 3C middle) indicating RhoA activity, which is reduced at 60 minutes (Fig. 3C, right panel).

Mitochondrial ROS mediate hypoxia-induced RhoA activation

The upstream regulators of the RhoA during hypoxia in alveolar epithelial cells have not been established. Hypoxia has been shown to increase the generation of ROS (Budinger et al., 1998; Chandel et al., 1998) and we have previously shown that ROS are generated in A549 cells during hypoxia (Dada et al., 2003). Because the generation of these ROS was attenuated by selective inhibitors of the mitochondrial electron transport, we concluded that mitochondria should be involved in their production (Dada et al., 2003). To demonstrate whether ROS were required for the hypoxic-activation of RhoA, cells were

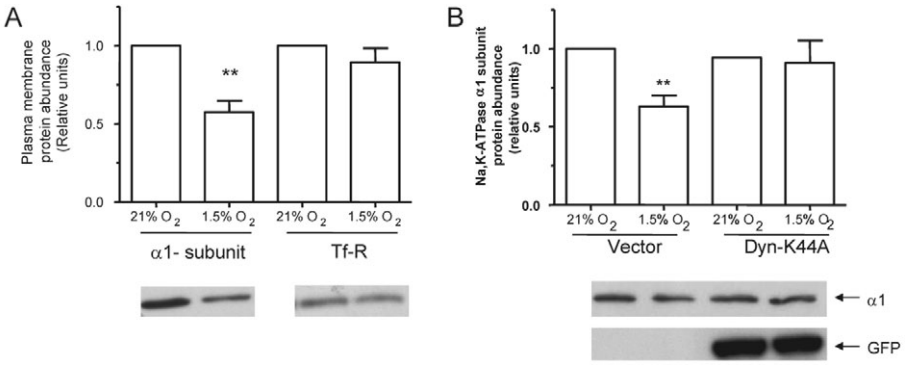

Fig. 1. Effects of hypoxia on Na,K-ATPase endocytosis in A549 cells. (A) Serum-starved A549 cells were exposed to 21 or 1.5% O_2 for 60 minutes and the protein levels of the Na,K-ATPase-α_1 subunit or the transferrin receptor in the plasma membrane were studied by cell surface biotinylation followed by streptavidin pull-down and western blot analysis using specific antibodies. A representative western blot for each protein is shown, mean ± s.e.m. (n=4). $^{**}P$<0.01. (B) COS-7 cells were transiently transfected with vector or GFF-Dyn K44A, serum-starved and exposed to 21 or 1.5% O_2 for 60 minutes. Na,K-ATPase endocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and western blot analysis using a specific anti α_1-subunit antibody. Top: bars represent mean ± s.e.m. (n=3), $^{**}P$<0.01. Bottom: a representative western blot for α_1-Na,K-ATPase endocytosis and GFP-dynamin expression levels.

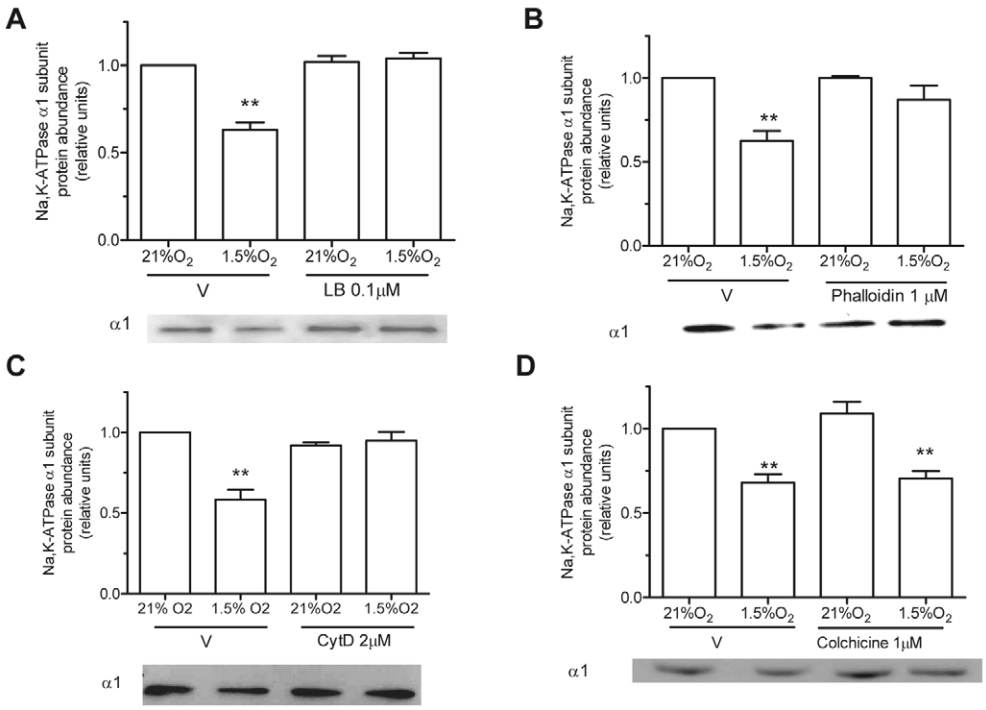

Fig. 2. Effect of cytoskeleton-disrupting agents on hypoxia-induced Na,K-ATPase endocytosis. Serum-starved A549 cells were pretreated in the presence or absence of 0.1 μM LB (15 minutes; A), 2 μM Cyt D (15 minutes; B), 1 μM phalloidin (overnight, C), 1 μM Colchicine (4 hours, D) and then exposed to 21 or 1.5% O_2 for 60 minutes. Na,K-ATPase endocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and western blot analysis using a specific anti α_1-subunit antibody. A representative western blot for each treatment is shown, mean ± s.e.m. (n=3). $^{**}P$<0.01.

infected with an adenovirus encoding for the antioxidant enzyme gluthathione peroxidase 1 (GPX1). Cells overexpressing GPX1 failed to translocate RhoA to the cell membrane during hypoxia (Fig. 4A). To assess the role of mitochondria-generated ROS, we conducted experiments in mitochondria-depleted (ρ^0)-A549 cells, which are not capable of mitochondrial respiration because they lack key components of the electron transfer chain (Chandel et al., 1998; Dada et al., 2003). As shown in Fig. 4B,C, hypoxia failed to translocate to the membrane or to active RhoA in ρ^0-A549 cells. Moreover, it has been suggested that the formation of semi-ubiquinone at complex III is the primary site for mitochondrial superoxide generation (Brunelle et al., 2005). We tested the requirement of a functional complex III in the regulation of RhoA during

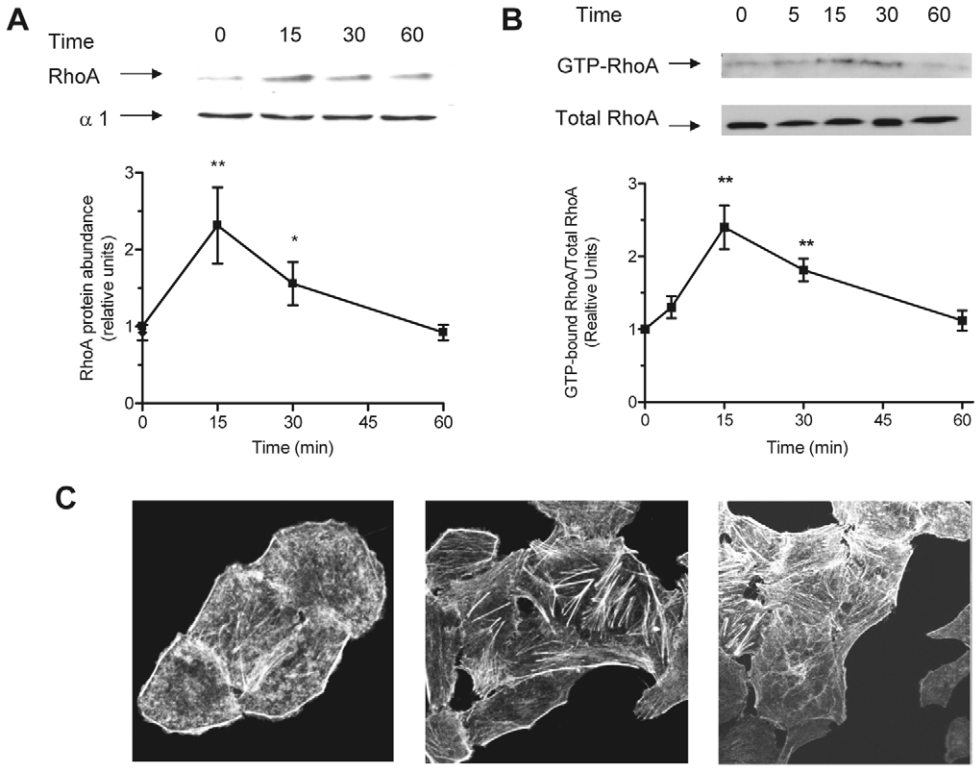

Fig. 3. Hypoxia-induced activation of RhoA in A549 cells. (A) Serum-starved A459 cells were exposed to hypoxia for 15, 30 or 60 minutes, 1% Triton X-100-soluble membrane fractions were obtained and RhoA translocation was evaluated by western blot. Top: a representative western blot for RhoA translocation and total α_1-Na,K-ATPase as loading control; Bottom: points on the curve represent mean ± s.e.m. (n=3). $^{*}P$<0.05; $^{**}P$<0.01. (B) A549 cells were exposed to hypoxia for 5, 15, 30 or 60 minutes and cell lysates were subjected to a pull-down assay with a GST-tagged fusion protein rhotekin Rho binding (RBD) domain. A representative western blot of RhoA bound to GTP (top) and total RhoA (bottom) is shown (n=3). (C) A549 cells were plated onto glass coverslips and exposed to 21 or 1.5% for 15 or 60 minutes. After incubation cells were washed, fixed, stained with rhodamine-phalloidin and evaluated using fluorescence microscopy. Representative images are shown.

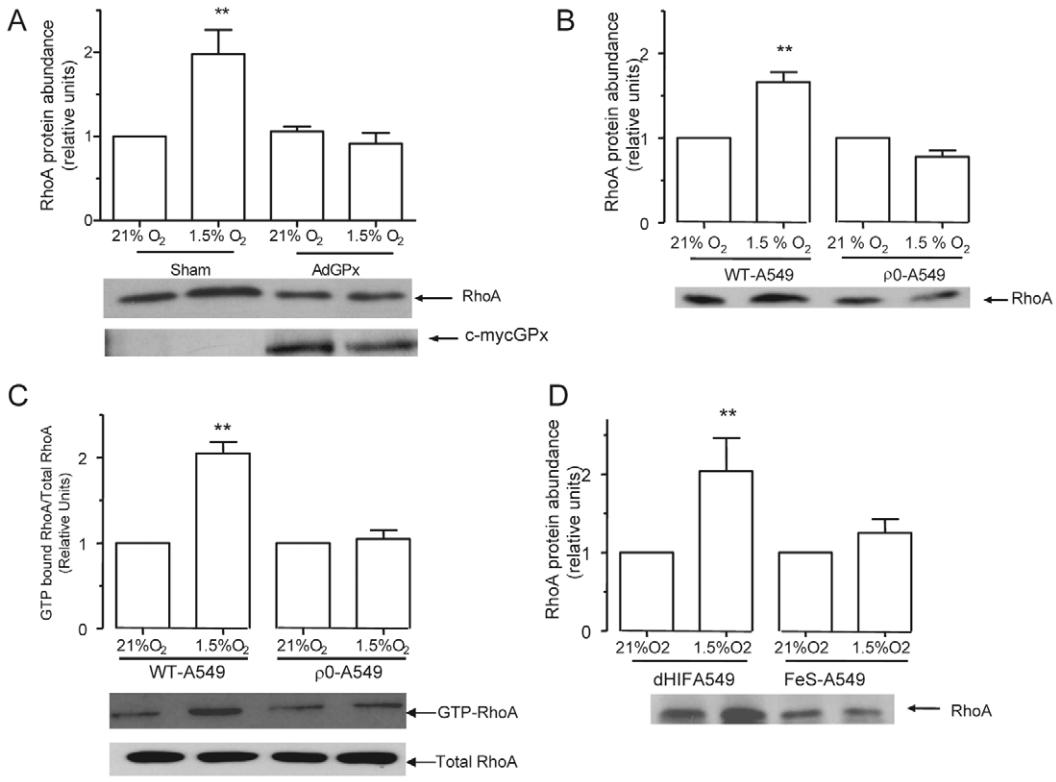

Fig. 4. Effect of mitochondria-generated ROS on RhoA activity and protein levels in A549 cells during hypoxia. (A) A549 cells were infected with null adenovirus (Sham) or adenovirus expressing Myc-tagged GPX1 (AdGPx) and subsequently exposed to 21% O_2 or 1.5% O_2 for 15 minutes. 1% Triton X-100-soluble membrane fractions were obtained and RhoA translocation was evaluated by western blot. Mean ± s.e.m. (n=3), $^{**}P$<0.01. A representative western blot is shown for RhoA and Myc. (B) WT and (ρ^0)-A549 cells were exposed to 21% O_2 or 1.5% O_2 for 15 minutes and then processed as described above. Mean ± s.e.m. (n=3), $^{**}P$<0.01. (C) WT and (ρ^0)-A549 cells were exposed to 21% O_2 or 1.5% O_2 for 15 minutes and cell lysates were subjected to a pull-down assay with a GST-tagged fusion protein RBD and processed as described. Mean ± s.e.m. (n=3), $^{**}P$<0.01. (D) A549 cells stably transfected with RNAi against *Drosophila* HIF (dHIF) or the Rieske iron sulphur protein (FeS) were exposed to 21% O_2 or 1.5% O_2 for 15 minutes and then processed as described above. Mean ± s.e.m. (n=3), $^{**}P$<0.01.

hypoxia by using A549 cells stably transfected with shRNA against a component of complex III, the Rieske iron-sulfur protein (Brunelle et al., 2005). We have previously described that these cells displayed a significant decrease in Fe-S protein levels compared to cells transfected with a control shRNA against *Drosophila* HIF (Comellas et al., 2006). Similarly to the results obtained with (ρ^0)-A549 cells, the cells deficient in the Fe-S protein failed to translocate RhoA to the plasma membrane (Fig. 4D).

To confirm the role of ROS in RhoA activation, we incubated A549 cells with 100 μM t-H_2O_2, a stable analog of H_2O_2, in normoxic conditions. t-H_2O_2 caused a time dependent RhoA translocation to the plasma membrane (Fig. 5A) and stress fiber formation (Fig. 5B).

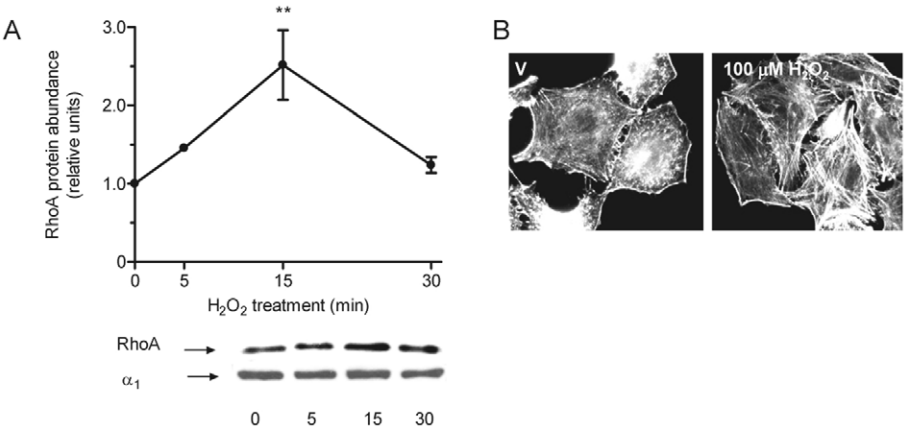

Fig. 5. H_2O_2 induced RhoA activation in A549 cells. (A) Serum-starved A459 cells were treated with 100 μM t-H_2O_2 for 5, 15 and 30 minutes. 1% Triton X-100-soluble membrane fractions were obtained and RhoA translocation was evaluated by western blot. Top: points on the curve represent mean ± s.e.m. (n=3). $^{**}P$<0.01. Bottom: a representative western blot for RhoA translocation and α_1-Na,K-ATPase as loading control. (B) A549 cells were exposed to 100 μM t-H_2O_2 for 15 minutes; stress fiber formation was assessed in cells fixed and stained with rhodamin-palloidin and evaluated by fluorescence microscopy. Representative photomicrographs of control cells (V), and t-H_2O_2 treated cells are shown.

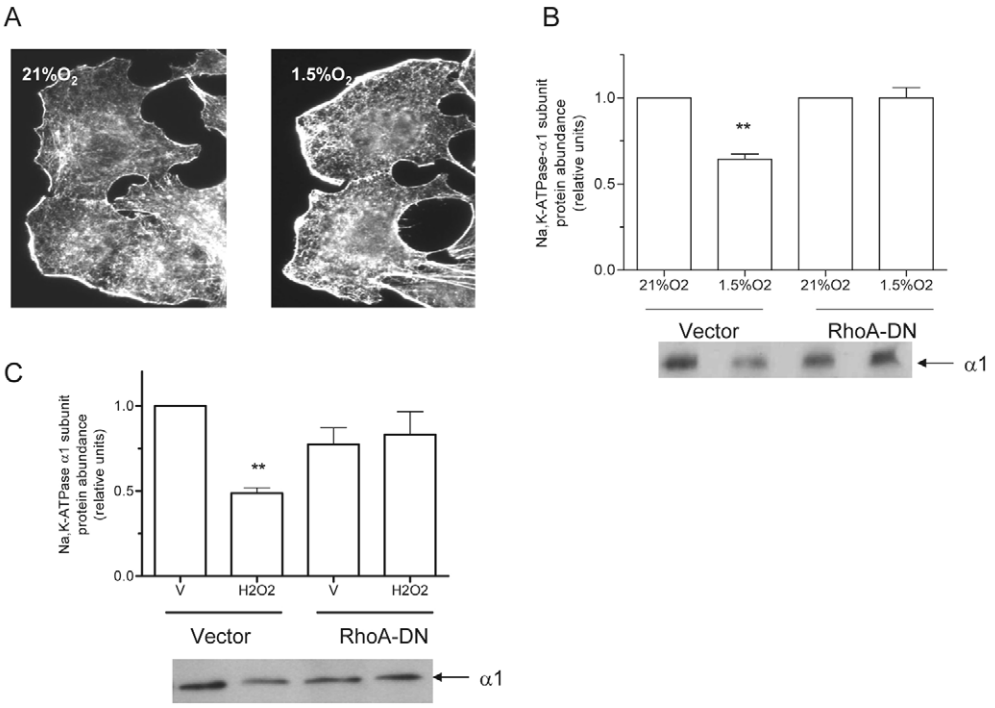

Fig. 6. Dominant negative RhoA prevents hypoxia-induced Na,K-ATPase endocytosis. Permanently transfected A549 cells expressing either vector or dominant negative RhoA (RhoAN19) were grown in the presence of G418. (A) Actin stress fiber formation was assessed in DN-RhoA cells fixed and stained with rhodamin-palloidin and evaluated by using fluorescence microscopy. Representative photomicrographs of control cells (21% O_2), and hypoxia (1.5% O_2)-treated cells are shown. (B) Na,K-ATPase endocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and western blot analysis using a specific antibody after exposure to 1.5% O_2 for 60 minutes. Top: bars represent mean ± s.e.m. (n=3). $^{**}P$<0.01. Bottom: a representative western blot. (C) Na,K-ATPase endocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and western blot analysis using a specific antibody after treatment with 100 μM H_2O_2 for 30 minutes. Top: bars represent mean ± s.e.m. (n=3). $^{**}P$<0.01. Bottom: a representative western blot.

Role of RhoA–ROCK in hypoxia-induced Na,K-ATPase endocytosis

To determine whether RhoA plays a role in hypoxia-mediated Na,K-ATPase endocytosis. We used A549 cells permanently transfected with DN-RhoA (RhoA N19) (Lecuona et al., 2003). As depicted in Fig. 6A, hypoxia did not result in formation of stress fibers in the A549 cells expressing the DN-RhoA and these cells failed to endocytose the plasma membrane Na,K-ATPase when exposed to hypoxia or to exogenous H_2O_2 (Fig. 6B,C).

ROCK inhibits myosin light chain (MLC) phosphatase activity through phosphorylation of its regulatory binding subunit (MYPT), which promotes MLC phopshorylation and its activation (Kimura et al., 1996). To determine whether ROCK was the downstream effector of RhoA, first we examined the MYPT phosphorylation levels during hypoxia in the presence of the ROCK inhibitor Y-27632 in A549 cells. Fig. 7A shows that hypoxia increased MYPT1 phosphorylation at Thr696, which was blocked by pre-treatment of the cells with Y-27632 and thus suggests a role for ROCK in this process. To determine whether Rho kinase was involved in the hypoxia-induced Na,K-ATPase endocytosis, cells were pre-treated with the ROCK inhibitor Y-27632 or transiently transfected with a plasmid coding for a dominant negative form of ROCK (ROCK KD-IA) and then exposed to 1.5% O_2 for 60 minutes. Both strategies prevented hypoxia-induced Na,K-ATPase endocytosis (Fig. 7B,C). Together these results indicate that the hypoxic activation of RhoA/ROCK is necessary for the endocytosis of Na,K-ATPase.

We have previously reported that Na,K-ATPase endocytosis is triggered by the phosphorylation of its α subunit by PKCζ (Dada et al., 2003). Here, we investigated whether PKCζ is a downstream target of RhoA-ROCK. WT and DN-RhoA-A549 cells were incubated under 1.5% O_2 and PKCζ translocation to the plasma membrane was assessed. Fig. 8 shows that PKCζ translocated to the plasma membrane at the same level in WT and DN-RhoA-A549 cells, which suggests that activation of PKCζ is independent of RhoA activation. Total membrane Na,K-ATPase was used as a loading control.

Discussion

Alveolar fluid reabsorption is important for maintaining the airspaces free of edema. This process is accomplished by the active Na^+ transport across the alveolo-capillary barrier via apical Na^+ channels and the basolateral Na,K-ATPase. We have previously reported that in alveolar epithelial cells hypoxia causes Na,K-ATPase endocytosis in a process mediated by mitochondrial ROS (Dada et al., 2003). The decrease in plasma membrane protein levels is specific and not a consequence of general membrane internalization because the levels of transferrin receptor (Fig. 1A) or GLUT-1 (Dada et al., 2003) were not decreased during hypoxia. Here, we also reported that Na,K-ATPase endocytosis is blocked by the dominant negative dynamin mutant, indicating that it is dynamin-dependent

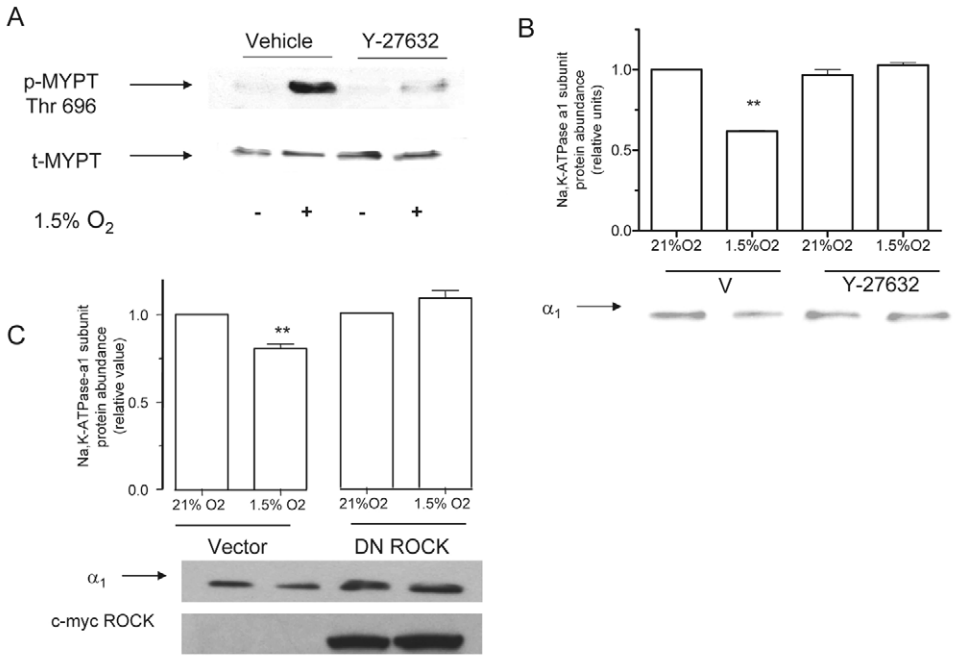

Fig. 7. ROCK activation is necessary in hypoxia-induced Na,K-ATPase endocytosis. (A) A549 cells were exposed to hypoxia in the presence or absence of 10 μM Y-27632 and cell lysates were obtained. Equal amount of proteins were separated by SDS-PAGE and immunoblotted with either phospho-MYPT (Thr696) antibody or pan-MYPT antibody. A representative western blot is shown (n=3). (B) A549 cells were pre-incubated in the presence of vehicle (V) or 10 μM Y-27632 and then exposed to hypoxia for 60 minutes. Na,K-ATPase endocytosis was studied by biotin labeling of surface proteins followed by streptavidin pull-down and western blot analysis using a specific antibody. Top: bars represent mean ± s.e.m. (n=3), $^{**}P$<0.01. Bottom: a representative western blot. (C) COS-7 cells were transiently transfected with vector, DN-ROCK, and Na,K-ATPase endocytosis was studied by biotin labeling of surface proteins. Top: bars represent mean ± s.e.m. (n=3), $^{**}P$<0.01. Bottom: a representative western blot for α_1-Na,K-ATPase endocytosis and Myc-ROCK expression levels.

process. Dynamin is a crucial factor in endocytosis that participates in membrane fission (Roux et al., 2006). Our results showing that dynamin K44A inhibits Na,K-ATPase endocytosis are concordant with a previous study reporting that in renal cells clathrin-dependent Na,K-ATPase endocytosis requires dynamin (Efendiev et al., 2002). Collectively, these results provide evidence that, during hypoxia, changes in Na,K-ATPase protein levels at the plasma membrane are mediated by a defined intracellular signaling mechanism and not as a result of generalized cell damage.

It has been reported that actin assembly may have a role in endocytosis (Fujimoto et al., 2000; Qualmann et al., 2000). We investigated the role of the actin cytoskeleton, the small GTPase RhoA and its downstream effector ROCK in hypoxia-mediated Na,K-ATPase endocytosis in A549 cells. To investigate the role of actin in endocytosis, we used three toxins that perturb actin dynamics: cytochalasin D, latrunculin B and phalloidin. These toxins have been shown to display variable effects on receptor-mediated endocytosis (Lanzetti et al., 2001) and phalloidin has already been used to study Na,K-ATPase exocytosis in alveolar epithelial cells (Bertorello et al., 1999). The decrease in Na,K-ATPase plasma membrane protein during hypoxia, measured by biotin cell surface labeling, was inhibited by pre-treatment of the cells with these toxins, which suggests that the local re-arrangement of the actin cytoskeleton (i.e. a combination of polymerization and de-polymerization) is required. By contrast, de-polymerization of microtubules with colchicine did not prevent the hypoxia-induced internalization of Na,K-ATPase (Fig. 2). In other systems, microtubules have been described to play a role in transport of molecules between early sorting endosomes, late endosomes and the recycling endosomes (Aniento et al., 1993; Gruenberg et al., 1989). Therefore, a role for microtubules in the downstream trafficking of the Na,K-ATPase cannot be ruled out.

Recent evidence suggests that Rho GTPase family members are key regulatory molecules of filamentous actin reorganization and have been implicated in the control of endocytosis (Fernandez-Borja et al., 2005; Hall, 1998; Leung et al., 1999; Symons and Rusk, 2003). Here, by studying translocation to the membrane fraction and pull-down of the GTP-bound RhoA, we found that RhoA is activated within 15 minutes of exposure to hypoxia. This activation was transient as the levels of active RhoA returned to control levels after 60 minutes of exposure to hypoxia. The activation of RhoA by hypoxia in A549 cells was confirmed by actin stress fiber formation, which was prevented in DN-RhoA A549 cells. In addition, in DN-RhoA A549 cells the hypoxia-stimulated endocytosis of Na,K-ATPase was prevented. Taken together, these results suggest an important role for RhoA in the endocytosis of Na,K-ATPase. Lamaze et al. described that activated Rho and Rac inhibited the endocytosis of transferrin and epidermal growth factor receptor in HeLa cells (Lamaze et al., 1996). Here, we observed that the activation of RhoA is necessary for Na,K-ATPase endocytosis during hypoxia. Our results are in agreement with a previous report describing the role of RhoA in constitutive clathrin-independent Na,K-

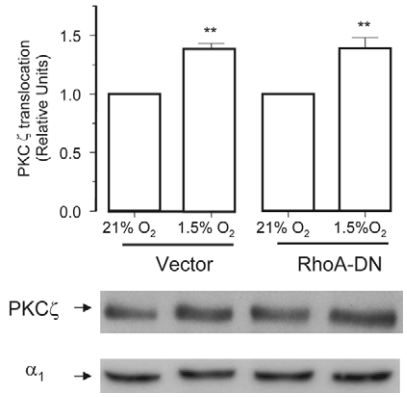

Fig. 8. Hypoxia-mediated translocation of PKCζ is independent of RhoA. Serum-starved WT and DN-RhoA-A549 cells were exposed to 21% O_2 or 1.5% O_2 for 20 minutes. Triton X-100-soluble membrane fractions were obtained and PKCζ translocation was evaluated by western blot. Top: bars represent mean ± s.e.m. (n=3). $^{**}P$<0.01. Bottom: a representative western blot for PKCζ translocation and total α_1-Na,K-ATPase as loading control.

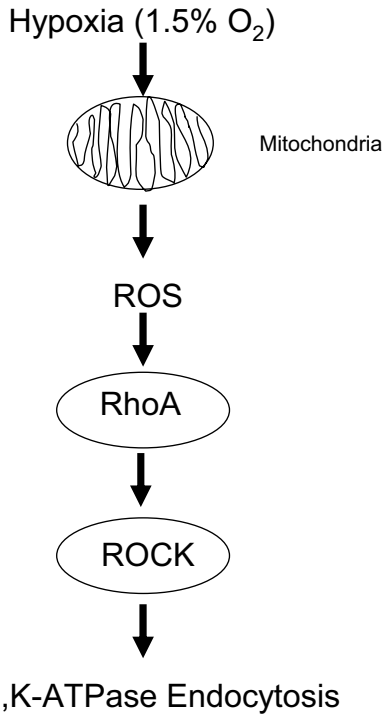

Fig. 9. A signaling model for hypoxic activation of RhoA and Na,K-ATPase regulation in alveolar epithelial cells. Based on our current findings we propose that hypoxia stimulates oxidant production within mitochondria. These oxidants activate the RhoA/ROCK pathway, resulting in stress fiber formation and a decrease in Na,K-ATPase protein levels at the plasma membrane.

ATPase endocytosis (Schmalzing et al., 1995). Moreover, in polarized epithelial cells active RhoA stimulates both the apical and basolateral endocytosis of the polymeric immunoglobulin receptor, whereas RhoAN19 expression decreased the rate of both (Leung et al., 1999). Similar inhibition was observed for the epithelial Na/H exchanger and for thromboxane A2 receptor endocytosis (Laroche et al., 2005; Szaszi et al., 2000). RhoA might regulate Na,K-ATPase endocytosis via its effects on the actin cytoskeleton or by RhoA-dependent phosphorylation of yet undefined targets. Recently it was reported in HEK293 cells that, after receptor stimulation, endocytic vesicles were aligning with actin stress fibers converging towards the endosomal compartment (Laroche et al., 2005). We demonstrated that hypoxia promoted the formation of stress fibers in AECs and induced Na,K-ATPase endocytosis. This was inhibited in RhoA-DN cells that are unable to form stress fibers and so suggests that actin stress fibers play a role directing the trafficking of the Na,K-ATPase from the plasma membrane towards the endosomal compartments. To better understand the mechanisms involved in RhoA regulation of Na,K-ATPase endocytosis, we focused on ROCK as a downstream effector of RhoA. Here, we provide evidence that hypoxia-mediated Na,K-ATPase endocytosis was prevented in A549 cells pretreated with the ROCK inhibitor Y27632 and in cells expressing DN-ROCK, which suggests that the pathway leading to Na,K-ATPase endocytosis requires RhoA/ROCK. We have previously shown that during hypoxia PKCζ phosphorylation of the Na,K-ATPase α_1 subunit triggers its endocytosis in a process mediated by ROS (Dada et al., 2003) and here we have provided evidence that activation of PKCζ during hypoxia is independent of RhoA (Fig. 8). We reasoned that hypoxia-ROS in alveolar epithelial cells activate different signal pathways that, independently from each other, contribute to Na,K-ATPase endocytosis.

The effects of hypoxia on the alveolar epithelia are rapidly reversed by treatment with β2-receptor agonists (Litvan et al., 2006; Planes et al., 2002; Vivona et al., 2001). Increased rates of alveolar fluid reabsorption by β-adrenergic stimulation were probably mediated by the recruitment and translocation of Na,K-ATPase from intracellular pools to the cell plasma membrane in alveolar epithelium (Litvan et al., 2006), but the signal pathways involved in this process and the role of the actin cytoskeleton warrants further studies.

Analysis of upstream RhoA events suggests a crucial role for mitochondrial ROS. Mitochondria have been proposed as oxygen sensors and ROS as signaling molecules (Brunelle et al., 2005; Chandel and Schumacker, 2000; Guzy et al., 2005). Although superoxide ions and H_2O_2 are considered to be toxic in high concentrations, recent studies suggest that low levels of these ROS participate in signal transduction pathways (Chandel et al., 2000; Emerling et al., 2005; Gabbita et al., 2000). It has been suggested that hypoxia partially inhibits mitochondrial electron transport and results in redox changes in the electron carriers that increase the generation of ROS (Chandel and Schumacker, 2000), which act as second messengers in the cytosol. In the present study, we provide evidence that hypoxia-induced RhoA activation is prevented in cells depleted of mitochondrial DNA or cells lacking the Rieske iron-sulfur protein (Fig. 7), which suggests that a functional electron transport chain is required for the hypoxic activation of RhoA. As such, we propose that hypoxia, by generating superoxide at the mitochondrial complex III, initiates a signal transduction pathway and that superoxide is then converted to H_2O_2. In agreement with this reasoning treatment of A549 cells with H_2O_2 in normoxic conditions induced RhoA and stress fiber formation Furthermore, in cells expressing DN-RhoA, H_2O_2

failed to induce Na,K-ATPase endocytosis suggesting that H_2O_2 is sufficient to cause RhoA activation.

As depicted schematically in Fig. 9, we provide evidence that hypoxia-induced Na,K-ATPase endocytosis is dependent on an intact actin cytoskeleton, which can be regulated, via mitochondrial ROS, by the small GTPase RhoA and its downstream effector ROCK. In summary, these data provide a molecular link between hypoxia-induced signal transduction and the endocytosis of Na,K-ATPase. Decreased Na,K-ATPase activity leads to an impairment in fluid reabsorption, which has significant adverse effects on epithelial function.

Materials and Methods

Materials

Latrunculin B, phalloidin, colchicine, Rho-associated kinase inhibitor: (R)-(+)-*trans*-N-(4-Pyridyl)-4-(1-aminoethyl cyclohexane carboxamide-2HCl (Y-27632) were from Calbiochem (La Jolla, CA). Ouabain was purchased from ICN Biomedicals (Aurora, OH). t-butyl-hydroperoxide (t-H_2O_2) was purchased from Sigma-Aldrich (St Louis, MO). Rhodamine-phalloidin was from Molecular Probes (Eugene, OR). The Na,K-ATPase α1 subunit monoclonal antibody (clone 464.6) and antiphospho-myosin phosphatase target subunit (MYPT) (Thr696) polyclonal antibody were from Upstate Biotechnology (Lake Placid, NY). RhoA monoclonal antibody and PKCζ monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Transferrin receptor monoclonal antibody was from Zymed (San Francisco,CA). MYPT polyclonal antibody was from Covance (Berkeley, CA). All other reagents were commercial products of the highest grade available.

Cell culture

A549 cells (ATCC CCL 185, a human adenocarcinoma cell line) or A549 cells expressing the rat Na,K-ATPase α_1 subunit isoform, which were generated as described previously (Dada et al., 2003; Efendiev et al., 2000), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 3 μmol/l ouabain to suppress the endogenous Na,K-ATPase α1 subunit. A549 cells permanently expressing DNRhoA (RhoAN19) (Lecuona et al., 2003) were propagated in complete DMEM supplemented with of 400 μg/ml geneticin (G418; Mediatech, Herndon, VA). A549 cells stably transfected with small hairpin RNA (shRNA) targeted to the Rieske iron-sulfur gene or the control *Drosophila* HIF (d-HIF) gene were obtained from N. Chandel (Northwestern University) and culture as described (Comellas et al., 2006). To generate mitochondria-depleted (ρ^0)-A549 cells, wild-type A549 cells were incubated in medium containing ethidium bromide (50 ng/ml), sodium pyruvate (1 mM) and uridine (50 μg/ml) for 4-6 weeks (King and Attardi, 1996). The ρ^0 status of cells was confirmed by the absence of cytochrome oxidase (Dada et al., 2003). COS-7 cells (ATCC-CRL-1651, monkey kidney fibroblast cell line) were grown in DMEM supplemented as described above. Cells were seeded in 6- or 10-cm plates and grown to confluence.

Cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. Hypoxic conditions 1.5%O_2/93.5%N_2/5%CO_2 were achieved in a humidified variable aerobic workstation (INVIVO O_2, Ruskinn Technologies, Leeds, UK) that continuously monitors the chamber oxygen tension as previously described (Dada et al., 2003).

Biotinylation of cell surface proteins

Cells were exposed to 21% O_2 (normoxia) or 1.5% O_2 (hypoxia) at 37°C, placed on ice, washed twice with ice-cold phosphate-buffered saline (PBS), and surface proteins were labeled for 20 minutes using 1 mg/ml EZ-link NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) as described before (Dada et al., 2003). After labeling, the cells were rinsed three times with PBS containing 100 mM glycine to quench unreacted biotin, and then lysed in modified RIPA buffer (m RIPA: 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40 and 1% sodium deoxycholate and protease inhibitors). 100-200 μg proteins were incubated overnight at 4°C with end-over-end shaking in the presence of Streptavidin beads (Pierce Chemical Co., Rockford, IL). Beads were thoroughly washed, resuspended in 30 μl of Laemmli's sample buffer solution (Laemmli, 1970) and analyzed by western blot.

Western blot analysis

Protein was quantified by Bradford assay (Bradford, 1976) (Bio-Rad, Hercules, CA) and resolved in 10%-15% polyacrylamide gels (SDS-PAGE). Thereafter, proteins were transferred onto nitrocellulose membranes (Optitran, Schleider & Schuell, Keene, NH) using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Incubation with specific antibodies was performed overnight at 4°C. When more than one primary antibody was used in the same membrane, blots were stripped by incubating 1 hour at 55°C in stripping solution (62.5 mM Tris-HCl, 2% SDS, 100 mM 2-mercaptoethanol, pH 6.8). Blots were developed with a chemiluminescence detection kit (PerkinElmer Life Sciences, Boston, MA) used as recommended by the manufacturer. The bands were quantified by densitometric scan (Image 1.29×, National Institutes of Health).

RhoA pull-down assay

Activation of RhoA was determined using the Rho activation assay kit (Upstate Biotechnology, Lake Placid, NY) as recommended by the manufacturer and previously described (Lecuona et al., 2003). In brief, after hypoxia exposure cells were lysed and lysates were incubated for 45 minutes with agarose beads coupled with GST-tagged fusion protein, corresponding to residues 7-89 of the mouse rhotekin Rho binding domain (RBD). After washing, the beads were resuspended in Laemmli loading buffer and analyzed using SDS-PAGE.

1% Triton X-100 soluble membrane fraction

Cells were exposed to 21 or 1.5% O_2 at 37°C, placed on ice and washed twice with ice-cold PBS. Cells were scraped in PBS, centrifuged, resuspended in homogenization buffer (1 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl, pH 7.5, and protease inhibitors) and homogenized. Homogenates were centrifuged at 500 ***g*** to discard nuclei and debris and the supernatant was centrifuged at 100,000 ***g***, for 1 hour at 4°C (TL ultracentrifuge, Beckman, Rotor TLA 100.2). The pellet containing the crude membrane fraction was resuspended in homogenization buffer with 1% Triton X-100 and centrifuged at 100,000 ***g*** for 30 minutes at 4°C. The supernatant was considered to be the 1% Triton X-100 soluble membrane fraction.

Transient transfection

COS-7 cells were plated in 6 cm plates at 5×10^5 cells/plate and transfected with 5 μg of plasmid DNA [dominant negative (ROCK KD-IA) form of ROCK, a gift of S. Narumiya, Kyoto University] (Ishizaki et al., 1997) using jetPEI (polyethyleneimine) reagent (Polyplus-Transfection, France), as indicated by the manufacturer. The dominant negative GFP-dynamin 2 K44A was generously provided by P. De Camilli (Yale University, New Haven, CT) (Ochoa et al., 2000); 6 μg plasmid was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were used 36 hours post-transfection.

Myosin phosphate target subunit determination

After treatment, incubations were terminated by placing the cells on ice, washing them twice with ice-cold PBS, then lysed in modified RIPA buffer. Equal amounts of protein were resolved by 10% SDS-PAGE and analyzed by immunoblotting using phospho-MYPT antibody. Membranes were then stripped with stripping buffer and probed with total MYPT antibody.

Immunofluorescence

A549 cells were grown over glass coverslips (~5×10^5 cells/coverslip), allowed to attach, serum-starved for 4 hours prior to treatments and fixed with 2% formaldehyde in PBS for 7 minutes at room temperature. After permeabilization with 0.1% Triton X-100 and blocking with 1.5% normal goat serum, cells were stained with rhodamine-phalloidin in blocking solution (1:100) for 30 minutes at 37°C to label F-actin and mounted on Gelvatol.

Adenoviral infection

A549 cells (~70% confluent) were infected with 10 μl (10^6/μl pfu) of Myc-tagged gluthathione peroxidase (Li et al., 2001). RhoA translocation was studied 24 hours after infection as described above.

PKC translocation assay

PKC translocation was determined as we previously described (Ridge et al., 2002). In brief, after hypoxia exposure cells were scraped into a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA and protease inhibitors, and homogenized for 2 minutes. Homogenates were then processed as described for RhoA translocation. Membrane fractions (20-50 μg) were then subjected to immunoblotting by using specific anti-PKCζ antibody.

Statistical analysis

Data are reported as mean ± s.e.m. Statistical analysis was carried out using a one-way ANOVA and Dunnett correction or *t*-test. Results were considered significant when $P<0.05$.

We thank Karen Ridge for critical review of the manuscript. This work was supported in part by HL-PO1-71643 National Institutes of Health Grant.

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