Rho GTPases show differential sensitivity to nucleotide triphosphate depletion in a model of ischemic cell injury

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Hallett, Mark A., Pierre C. Dagher, and Simon J. Atkinson. Rho GTPases show differential sensitivity to nucleotide triphosphate depletion in a model of ischemic cell injury. Am J Physiol Cell Physiol 285: C129–C138, 2003.—Rho GTPases are critical for actin cytoskeletal regulation, and alterations in their activity may contribute to altered cytoskeletal organization that characterizes many pathological conditions, including ischemia. G protein activity is a function of the ratio of GTP-bound (active) to GDP-bound (inactive) protein, but the effect of altered energy metabolism on Rho protein activity has not been determined. We used antimycin A and substrate depletion to induce depletion of intracellular ATP and GTP in the kidney proximal tubule cell line LLC-PK1, and measured the activity of RhoA, Rac1, and Cdc42 with GAPse effector binding domains fused to glutathione S-transferase. RhoA activity decreased in parallel with the concentration of ATP and GTP during depletion, so that by 60 min there was no detectable RhoA-GTP, and recovered rapidly when cells were returned to normal culture conditions. Dissociation of the membrane-actin linker ezrin, a target of RhoA signaling, from the cytoskeletal fraction paralleled the decrease in RhoA activity and was augmented by treatment with the Rho kinase inhibitor Y27632. The activity of Cdc42 did not decrease significantly during depletion or recovery. Rac1 activity decreased moderately to a minimum at 30 min of depletion but then increased from 30 to 90 min of depletion, even as ATP and GTP levels continued to fall. Our data are consistent with a principal role for RhoA in cytoskeletal reorganization during ischemia and demonstrate that the activity of Rho GTPases can be maintained even at low GTP concentrations.

Rac; Cdc42; actin; ezrin; adenosine 5′-triphosphate; guanosine 5′-triphosphate

RHO FAMILY GTPASES ARE MEMBERS of the Ras superfamily of p21 G proteins (25). They play a central role in regulating the organization and dynamics of the cytoskeleton and thus integrate cell morphology and behavior with environmental signals and cellular physiology (6). The biological activities of small GTPases are regulated by their guanine nucleotide binding state (12). They interact with and activate their effectors when in the GTP-bound state and are inactive when the bound nucleotide is GDP, and in this way they act as protein molecular switches. The position of the switch is set by the activity of antagonistic regulatory proteins, because the intrinsic GTPase rates and nucleotide dissociation rates for these proteins are slow. GTPase-activating proteins (GAPs) stimulate nucleotide hydrolysis, thereby turning off the signal, whereas guanine nucleotide exchange factors (GEFs) stimulate GDP release and its replacement with GTP (the latter normally being the predominant guanine nucleotide in the cytoplasm), thus turning on signaling (7). However, these mechanisms, as they are commonly presumed to function, presuppose the nucleotide concentrations that prevail in normal healthy cells, but data are currently lacking on the effect of altered guanine nucleotide concentrations on p21 GTPase activity.

Ischemia is a pathophysiological state that is characterized in part by poor tissue perfusion and subsequent anoxia with inhibition of oxidative phosphorylation caused by substrate depletion (13). Although ischemia is a complex multifactorial process, many of its consequences result from, and can be recapitulated by, depletion of the normal intracellular concentration of ATP (3). Ischemic injury to the epithelial cells of the kidney proximal tubule is a factor in the initiation and progression of acute renal failure and typifies many of the features of ischemic injury in other cell types and organs. The normal architecture of the actin cytoskeleton is an early casualty of ischemic injury and ATP depletion, and this disruption directly results in impaired cell-cell and cell-substrate adhesion, loss of tight junction barrier function (3, 17), and mixing of apical and basolateral transporters (30), with resultant impaired organ physiology and structure. There is a loss of actin from the apical microvilli, terminal web, cortical junctional complexes, and stress fibers. This results in membrane blebbing, internalization, and loss of apical microvilli (15, 40). The loss of actin and villin from microvilli is coincident with abnormal perinuclear accumulation of F-actin (42) and an increase in the ratio of F- to G-actin in the cell.

The complexity and pervasiveness of the cytoskeletal alterations that are precipitated by ischemic injury indicate that they are probably the result of the combined effect of many different pathways. Perturbations of normal signaling pathways are probably a major factor in this aspect of injury, and in view of their...
critical role in cytoskeletal regulation, Rho GTPases are obvious candidate molecules. Evidence for the role of RhoA during ATP depletion in kidney epithelium comes from experiments using the constitutively active Rho mutant RhoV14. When RhoV14 is microinjected into tissue culture cells derived from renal proximal tubule epithelium before ATP depletion, cortical actin and stress fibers are maintained during subsequent depletion (33). Conversely, when the bacterial toxin C3 transferase, which specifically inactivates Rho, is injected, stress fibers are not reassembled during recovery from ATP depletion. RhoV14 also protects tight junctions against disassembly during ATP depletion (17), whereas the corresponding constitutively active mutant of Rac, RacV12, protects adherens junctions (16).

Additional evidence for altered activity of Rho GTPases during ischemia or nucleotide triphosphate depletion is furnished by altered regulation of cytoskeletal proteins known to be downstream targets of Rho GTPase signaling. For example, ezrin is a protein link between the plasma membrane and the actin cytoskeleton (1) that is regulated by RhoA. In normal proximal tubule epithelial cells ezrin is localized in microvilli and at sites of cell-cell contact (19, 35). Ezrin binds to the cytoplasmic domain of the cell surface glycoprotein CD44 (36), and the carboxy terminus of ezrin binds to actin (37). The interaction of ezrin and CD44 is of high affinity only in the presence of phosphatidylinositol 4,5-bisphosphate (21). RhoA is thought to regulate this interaction through its effector, phosphatidylinositol 4-phosphate 5-kinase (29). Another effector of RhoA, Rho kinase (ROK), phosphorylates ezrin in vitro (28). The dephosphorylation of ezrin is an early event during ischemia in renal epithelium (8). As a result of dephosphorylation, ezrin dissociates from the cytoskeleton and can be extracted with nonionic detergents.

The intracellular concentration of GTP is tightly coupled to that of ATP, and the two decrease in tandem with ischemia or chemical anoxia (10). Given the evidence for involvement of Rho GTPase signaling and the paucity of data concerning the effect of intracellular GTP levels on Rho protein signaling mechanisms in vivo, we used the ability of the GTPase binding domains from Rho effectors to discriminate between the GTP- and GDP-bound forms of the GTPase (24) to measure the relative amount of active and inactive RhoA, Rac1, and Cdc42 during ATP depletion induced by treatment with the mitochondrial poison antimycin A combined with substrate depletion. We measured ATP and GTP levels in parallel with these assays to establish the degree of correlation between the activation state of the GTPase and nucleotide levels in the cell. We also followed the localization of actin stress fibers and the detergent solubility of ezrin during nucleotide triphosphate depletion and recovery. Finally, we measured the activity of RhoA, Rac1, and Cdc42 during nucleotide depletion and recovery in the presence of constitutively active RhoAL63.

**Materials and Methods**

**Chemicals.** All chemicals are from Sigma Chemical (St. Louis, MO) unless otherwise stated.

**Cell culture.** LLC-PK1 porcine proximal tubule cells were grown with KP medium (DMEM-Ham’s F-12 medium (1:1) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 14 mM NaHCO3, and 12.5 mM HEPESS) with 10% fetal bovine serum in a humidified chamber (95% air-5% CO2) at 37°C. Cells were grown in collagen coated T-25 flasks (Techno Plastic Products, Trasadingen, Switzerland). Cells were grown for 2 days after confluence and rinsed with warm PBS before use. To model ischemia, cells were incubated with depleted DMEM (medium without amino acids, glucose, serum, and antibiotics) and 100 nM antimycin A. If recovery of these cells was required, they were rinsed with warm PBS and incubated with supplemented KP medium. For some experiments cells were rinsed twice and incubated with serum-free normal growth medium to control for the effect of removing growth factors. We analyzed the effect of ATP depletion in quiescent cells by incubating cells in serum-free medium for 24 h before the experiment.

**Nucleotide triphosphate assay.** Cell cultures were rinsed three times with ice-cold PBS and scraped with 300 μl of ice-cold acetonitrile followed by 700 μl of ice-cold water (2). The extract was centrifuged at 16,000 g for 10 min at 4°C. The supernatant was gassed with N2 while on ice for 30 min to evaporate the acetonitrile. The residue was solubilized with 1 N NaOH, and the protein concentration was determined by Coomassie blue assay (Pierce Chemical, Rockford, IL). To separate nucleotide triphosphates, samples were diluted 1:1 and 100 μl was injected onto a 4-μm Nova-Pak C18 HPLC cartridge (100 × 8 mm ID) equipped with a radial compression chamber (Waters, Milford, MA). The elution buffer consisted of 20% acetonitrile, 10 mM ammonium phosphate, and 2 mM PIC Reagent A ion-pairing reagent (Waters). The column was run isocratically at 2 ml/min (18) on a Hewlett-Packard Chemstation model 1100 with ultraviolet detection at 254 nm (Hewlett-Packard, Wilmington, DE). Internal standards showed that nucleotide recovery was >90%.

**Visualization of stress fibers.** Cells were plated on collagen-coated coverslips in 35-mm petri dishes. Cells were treated with antimycin A as described in Cell culture. After the depletion time course, coverslips were fixed in PBS-5% formaldehyde, permeabilized with PBS plus 0.2% Triton X-100, and incubated with 0.66 μM rhodamine phalloidin (Molecular Probes, Eugene, OR). Confocal images were collected with a Zeiss Axiovert LSM510.

**GTPase activity assay.** The ROK Rho binding domain (ROK-BD, amino acids 941–1,075) and the p-21 associated kinase (Pak) binding domain (Pak-BD, amino acids 67–150) cloned into pGEX vectors were transformed into Escherichia coli strain BL21. pGEX-Rok-BD was a kind gift from Dr. Kozo Kaibuchi (Nagoya University, Nagoya, Japan; Ref. 27), and pGEX-Pak-BD was a kind gift from Dr. Gary Bokoch (Scripps Research Institute, La Jolla, CA; 4). Production of the fusion protein was initiated with isopropyl β-D-thiogalactopyranoside (IPTG). The fusion protein was isolated by binding to S-hexylglutathione-agarose as described in the manufacturer’s instructions. The glutathione S-transferase (GST) fusion proteins bound to glutathione agarose (GST-Rok-BD and GST-Pak-BD) were stored in aliquots in liquid nitrogen for no more than 1 mo. The amount of fusion protein required to capture all of the available GTPase was determined empirically.

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Affinity isolation was carried out as described previously (5) with modifications. After treatment with antizymatin A cells were rinsed with warm PBS. Three hundred fifty microliters of "buffer A" (25 mM Tris (pH 7.5 at 4°C), 150 mM K acetate, 5 mM EDTA, 5 mM EGTA, 10 mM dithiothreitol (DTT), 1% Triton X-100, 60 mM n-octyl-β-D-glucopyranoside, 50 μM butylated hydroxytoluene (BHT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 2 μg/ml aproatinin (Roche Molecular Biochemicals, Indianapolis, IN), 1 μg/ml pepstatin A (Roche Molecular Biochemicals), 50 μg/ml leupeptin (Peptides International, Osaka, Japan), and 40 μg/ml bestatin (Roche Molecular Biochemicals)] were added to the T-25 flasks and incubated on ice for 10 min with periodic manual rocking to evenly distribute the extraction buffer. The buffer was removed and centrifuged briefly at 15,000 g. Twenty-five microliters of detergent extract were mixed with thirty microliters of GST-ROK-BD to assay for RhoA activity or fifteen microliters of GST-Pak-BD to assay for Rac1 or Cdc42 activity and incubated at 4°C for 1 h on a rotator. The GST-binding domain was rinsed three times with 500 μl of "buffer A" without detergent or protease inhibi-

To ensure the validity of using a ratio of bound to unbound GTPase, twenty microliters of detergent extract were mixed with thirty microliters of GST-ROKBD to assay for RhoA activity or fifteen microliters of GST-Pak-BD to assay for Rac1 or Cdc42 activity and incubated at 4°C for 1 h on a rotator. The GST-binding domain was rinsed three times with 500 μl of "buffer A" without detergent or protease inhibi-
tors. This unbound fraction was precipitated with 10% trichloroacetic acid, rinsed with 70% ethanol-acetone, dried, and dissolved in SDS sample buffer [50 mM Tris 7.5, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 6 M urea, bromophenol blue]. The bound fraction was dried in a vacuum and solubilized in SDS sample buffer. Bound and unbound fractions for each time point were run on a 15% SDS-PAGE polyacrylamide gel (BioWhittaker Molecular Applications, Rockland, ME). This was followed by Western blotting and immunostaining with 1 μg/ml anti-RhoA antibody (26C4; Santa Cruz Biotechnology, Santa Cruz, CA), 2 μg/ml anti-Rac1 antibody (Santa Cruz Biotechnology), or 2 μg/ml anti-Cdc42 antibody (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated anti-mouse secondary an-
tibody (Jackson ImmunoResearch, West Grove, PA). Blots were visualized with enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) and Biomax ML film (Kodak, Rochester, NY). To determine the ratio of bound to unbound GTPase, film images were digitized and the optical density of each band was quantified with a Fluor-S Multimager with Quantity One 4.1 software (Bio-Rad Laboratories, Hercules, CA).

For assay validation experiments the detergent lysate was incubated with 1 mM GDP or 1 mM GTPγS or without additional nucleotide for 10 min at 30°C. As a control for nonspecific binding to GST a sample of lysate was incubated with glutathione-agarose-GST.

To ensure the validity of using a ratio of bound to unbound GTPase to express activity, the levels of the GTPases were measured by densitometry of immunoblots of whole cell extracts and normalized to the total amount of protein in LLC-PK10 cell lysates. When expressed as GTPase per mi-

Ezrin solubility assay. The solubility of ezrin during deple-
tion and recovery was determined with the lysates from the RhoA activity assay. The amount of ezrin in the detergent-insoluble fraction left after the lysate was made was determined by adding SDS sample buffer to the T flask containing the detergent-insoluble adherent cell layer and heating it to 80°C for 2 min. The viscous solution was scrape-
ted to reduce viscosity, and run with the detergent-soluble fractions on a 12% PAGE gel. Immunoblotting was per-
formed with an anti-ezrin polyclonal antibody (kind gift of Jing Chen, Indiana State University, Terre Haute, IN; Ref. 9). Visualization and quantitation were as described in GTPase activity assay.

To demonstrate the effect of the RhoA signaling pathway on ezrin detergent solubility, 10 μM of the ROK inhibitor Y27632 (Upstate Biotechnology, Lake Placid, NY; Ref. 11) was incubated with cells during recovery from depletion (as above) and the ezrin detergent solubility was determined as described above.

RESULTS

Nucleotide changes during ATP depletion and recovery. Glucose-depleted medium plus 100 nM antimycin A was used to induce chemical anoxia in LLC-PK10 porcine proximal tubule epithelial cells as a model of ischemic ATP depletion. The relative amounts of ATP and GTP were determined by HPLC over the time course for which Rho GTPase activity was also measured (10). The reduction of ATP was rapid (Fig. 1A). Within 5 min ATP was almost reduced in half, to 52 ± 17% of the level in control cells. After 10 min of depletion, ATP was reduced to 28 ± 10.3% and reached a minimum of 1 ± 0.4% of the control value after 60 min. The change in GTP followed similar kinetics (Fig. 1B).

Within 5 min GTP was reduced to less than half, i.e., 62.6 ± 20.3%, of the control value. After 10 min of

![Fig. 1. Nucleotide triphosphate levels during depletion and recovery measured by HPLC. Values are means ± SD; n = 3. Cells were treated with antimycin A (0.1 μM) and substrate-depleted medium for the indicated times (5, 10, 30, 60, and 90 min) to induce depletion or depleted for 90 min and returned to normal medium for 30 or 60 min (90/30, 90/60). A: change in ATP level as % of control (0 min). B: corresponding values for GTP.](http://ajpcell.physiology.org/ by 10:20:33.6 on March 31, 2017)
depletion, GTP was 42.4 ± 10.8% and reached a minimum of only 5.8 ± 3.4% of the control value after 60 min. In addition to a higher relative minimum amount of GTP during ATP depletion conditions, GTP levels recovered more quickly than ATP when the cells were returned to normal growth conditions. GTP levels reached 86.2 ± 9.4% of the control value after 60 min of recovery in normal medium, whereas ATP reached only 42.1 ± 9.1% of the control level after 60 min of recovery.

The actin cytoskeleton is remodeled during ATP depletion (16, 17, 30). Actin stress fibers are regulated by RhoA and found near the basal membrane in LLC-PK10 cells (33). During the time course over which we measured ATP and GTP levels, we followed the integrity of stress fibers by labeling F-actin with rhodamine phalloidin (Fig. 2). Normal stress fibers (Fig. 2; 0 min) began to break down within 5 min after antimycin A treatment started and became progressively more punctate (Fig. 2; 5–90 min). After repletion of ATP with incubation in normal medium, stress fibers progressively reorganized (Fig. 2; 90/30 and 90/60 min of depletion/repletion).

Measurement of RhoA activity. A fusion protein (GST-ROK-BD) consisting of GST and the effector-binding domain of human ROK was used to selectively capture the GTP-bound form of RhoA with glutathione-agarose resin (24). Because only the GTP-bound form of RhoA binds to the GST-ROK-BD, the active and the inactive proteins could be separated and detected by immunoblotting with anti-RhoA antibody (Fig. 3). We compared the active and inactive RhoA fractions in a control cell detergent lysate (Fig. 3A, lanes 1 and 2) and after 90 min of nucleotide triphosphate depletion (Fig. 3A, lanes 7 and 8). After 90 min of depletion, the amount of inactive RhoA increased and the amount of active RhoA decreased. The level of GTP-bound RhoA decreased substantially when the cell lysate was incubated with a large excess of exogenous GDP (Fig. 3A, lanes 3 and 4). Glutathione-agarose was incubated with GST as an additional control. Lanes 5 and 6 in Fig. 3A show that RhoA did not bind to GST-glutathione-agarose nonspecifically. Additionally, after 90 min of nucleotide depletion, excess exogenous GTP (Fig. 3A, lanes 9 and 10), but not GDP (Fig. 3A, lanes 11 and 12), shifted the balance back toward the active form of RhoA.

Because removal of the growth factors present in the normal growth medium could affect the activity of RhoA independently of ATP depletion, we tested the effect of removing serum on RhoA activity in control cells not subjected to ATP depletion and the effect of the presence or absence of serum during the repletion phase. As shown in Fig. 3B, the presence or absence of serum during the assay did not affect RhoA activity significantly, although in the absence of serum RhoA activity levels during recovery were slightly lower than those observed with serum present. Serum starvation for 24 h (Fig. 3C) did not significantly alter RhoA activity in these cells, and it did not alter the affect of nucleotide triphosphate depletion.

RhoA activity during nucleotide triphosphate depletion and repletion with normal medium following the time course of stress fiber disruption seen in Fig. 2 is illustrated in Fig. 4A. Control cells (Fig. 4A, lanes 1

![Fig. 2. Effect of nucleotide triphosphate depletion on stress fiber morphology. Confocal fluorescence micrographs show LLC-PK10 cells stained with rhodamine phalloidin to label filamentous actin. Cells were treated with antimycin A and substrate-depleted medium for 0, 5, 10, 30, 60, and 90 min or treated for 90 min and returned to normal medium (90/30, 90/60). Magnification, ×100.](image-url)
Rho signaling, we followed the detergent solubility of the ERM (ezrin, radixin, moesin) protein ezrin as an indication of its association with the actin cytoskeleton during the time course of nucleotide depletion and repletion (Fig. 5A). The control detergent fractionation of ezrin (0 min) shows that under our conditions much of the protein was recovered in the detergent-soluble fraction but a significant fraction remained in the detergent-insoluble fraction (Fig. 5A, lanes 1 and 2). With nucleotide triphosphate depletion (Fig. 5A, lanes 3–12) the amount of ezrin in the detergent-insoluble fraction decreased, suggesting that ezrin had dissociated from the cytoskeleton. As the cells recovered from depletion (Fig. 5A, lanes 13–16), the amount of detergent-insoluble ezrin increased. An average detergent solubility for ezrin during the time course (n = 3) was expressed as the ratio of detergent insoluble to soluble ezrin. The detergent insolubility of ezrin decreased 60% during nucleotide triphosphate depletion and increased to control level after 30 min of repletion with normal medium (Fig. 5B) and showed an overall time course similar to that of RhoA activity with depletion and repletion (Fig. 4B).

The serine/threonine kinase Rho kinase (or ROCK) is activated by binding to RhoA GTP and phosphorylates ezrin among other targets (28). To demonstrate the effect of RhoA signaling on ezrin detergent solubility, we incubated the ROK inhibitor Y27632 (11) with LLC-PK10 cells during nucleotide triphosphate depletion and repletion. Figure 5C shows a Western blot of a

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**Fig. 3.** Western blot showing assay for RhoA activity. A: LLC-PK10 cells were incubated for 0 or 90 min with antimycin A and substrate-depleted medium. GDP or GTP-S were added to some lysates, and all lysates were then incubated with glutathione-S-transferase (GST)-Rho kinase binding domain (ROK-BD) or GST alone (indicated) and separated into bound fraction (B; containing RhoA-GTP) or unbound fraction (U; containing RhoA-GDP) by incubation and centrifugation with glutathione-agarose resin. Each fraction was analyzed by Western blotting with an anti-RhoA antibody. B: comparison of RhoA activity with serum present in the control (time 0, lanes 1 and 2) or with serum removed for 90 min (lanes 3 and 4) and effect of ATP repletion in the presence (lanes 7 and 8) and absence (lanes 9 and 10) of serum. C: cells were serum starved for 24 h before ATP depletion (serum starvation) or fed with fresh serum-containing medium 24 h before the experiment (no starvation).

and 2) showed that a large fraction of RhoA was active (bound) in a confluent LLC-PK10 cell culture. During the time course of nucleotide triphosphate depletion (Fig. 4A, lanes 3–12) the amount of active RhoA decreased. On repletion of ATP levels with normal medium the level of active RhoA increased (Fig. 4A, lanes 13–16). The average of three experiments expressed as a ratio of bound protein (active) to unbound protein (inactive) is shown in Fig. 4B. A significant loss of RhoA activity was detected after as little as 5 min of nucleotide triphosphate depletion, by which time the activity ratio dropped to 46.2 ± 15.1% of the control value. After 10 min of nucleotide triphosphate depletion the activity ratio dropped to 11.4 ± 8.2% of the control value. No measurable RhoA activity was detected at 60 min of nucleotide triphosphate depletion. On repletion with normal medium, the ratio recovered to 83.9 ± 78.9% of the control value by 30 min.

**Measurement of ezrin detergent solubility.** To determine whether alterations in the amount of activated RhoA during ATP depletion and recovery were reflected in the behavior of a known cytoskeletal target of ezrin detergent solubility. A: LLC-PK10 cells were incubated with antimycin A for various lengths of time or incubated with antimycin A for 90 min followed by recovery in normal medium for 30 or 60 min. Detergent lysates were then incubated with GST-ROK-BD and separated into bound (B; containing RhoA-GTP) or unbound (U; containing RhoA-GDP) fractions by incubation and centrifugation with glutathione agarose. B: graph of 3 experiments as represented in A. The amount of RhoA in each fraction was quantified by densitometry of Western blots and expressed as the mean ± SD ratio of B to U as % of the control (t = 0) value.

Fig. 4. Effect of depletion and recovery on RhoA activity. A: LLC-PK10 cells were incubated with antimycin A for various lengths of time or incubated with antimycin A for 90 min followed by recovery in normal medium for 30 or 60 min. Detergent lysates were then incubated with GST-ROK-BD and separated into bound (B; containing RhoA-GTP) or unbound (U; containing RhoA-GDP) fractions by incubation and centrifugation with glutathione agarose. B: graph of 3 experiments as represented in A. The amount of RhoA in each fraction was quantified by densitometry of Western blots and expressed as the mean ± SD ratio of B to U as % of the control (t = 0) value.
typical experiment. In control cells a substantial amount of ezrin was associated with the cytoskeleton in the detergent-insoluble fraction (Fig. 5C, lanes 1 and 2). After 90 min of depletion, ezrin dissociated from the cytoskeleton and a decreased amount was found in the detergent-insoluble fraction (Fig. 5C, lanes 3 and 4). However, with 60 min of repletion in normal medium (Fig. 5C, lanes 5 and 6), ezrin was again associated with the cytoskeleton in the insoluble fraction. When Y27632 was included in the incubation medium, ezrin failed to reassociate with the cytoskeleton after 60 min of repletion, suggesting that ROK, and therefore RhoA signaling, is important for the stable association of ezrin with the cytoskeleton.

Activity measurement of Cdc42 and Rac1. The depletion of nucleotide triphosphates likely affects the activity of the other Rho family GTPases as well as RhoA itself. We measured the activity of Cdc42 and Rac1 with a similar pull-down assay using a fusion of GST to the effector domain of Pak (containing the CRIB domain) to selectively bind GTP-bound Rac1 and Cdc42. To validate the assay, nucleotides were added to detergent extracts of LLC-PK10 cells to change the activity of the GTPases (Fig. 6A). Without exogenously added nucleotides (Fig. 6A, lanes 1 and 2) most of Rac1 and nearly all of Cdc42 was found in the bound (active) lane. The addition of GDP increased the amount of GTPase in the unbound (inactive) fraction for both Cdc42 and Rac1. GST alone (Fig. 6A, lanes 5 and 6) did not pull down any GTPase. When the cells were first depleted for 90 min with antimycin A, Cdc42 did not show any significant decrease in activity. The level of active Rac1 was decreased after 90 min of depletion, but a significant fraction of GTP-bound Rac1 remained (Fig. 6A, lanes 7 and 8). Incubation with GTPγS increased the amount of GTPase in the active fraction for Cdc42 and Rac1.
both proteins (Fig. 6A, lanes 9 and 10). Addition of GDP decreased the total amount of Cdc42 that was recovered, making it impossible to determine the effect of GDP on the activity of Cdc42 in this in vitro assay. GDP decreased the amount of active Rac1 (Fig. 6A, lanes 11 and 12). We tested whether serum deprivation contributed to the effects we observed. Substituting serum-free medium for normal growth medium, without induction of ATP depletion (Fig. 6B, lanes 1–4), did not affect Rac1 or Cdc42 activity, and it did not alter the effect of ATP depletion (Fig. 6B, lanes 5 and 6). Serum starvation (24 h) had no appreciable effect on Rac1 activity but did result in a decrease in Cdc42 activity (Fig. 6C, lanes 1 and 2). However, Cdc42 and Rac1 maintained high levels of activity after 90 min of ATP depletion, even with prior serum starvation (Fig. 6C, lanes 9 and 10).

Because, in contrast to our results with RhoA (Fig. 3, lanes 7 and 8, and Fig. 4), there was no significant decrease in the amount of active Cdc42 and Rac1 with depletion, we sought to demonstrate that the GTPase assay was capable of detecting physiological changes in the activity of Rac1 and Cdc42 in LLC-PK10 cells. Because cadherin-mediated cell-cell adhesion was shown to result in alterations in Rho GTPase activity (32), the activities of RhoA, Rac1, and Cdc42 were assayed with the pull-down assay 24 h after LLC-PK10 cells were seeded at a range of different densities. A representative Western blot is shown in Fig. 7A. RhoA activity increased >36-fold from the lowest to the highest initial cell density (graphed in Fig. 7B), whereas Rac1 and Cdc42 activity decreased by 91.1 ± 6.5% and 85.2 ± 14.7%, respectively. These experiments showed that our assay conditions were sufficiently sensitive to have detected physiological changes in the activity of all three GTPases during ATP depletion and recovery.

A comparison of the changes in the activity of RhoA, Rac1, and Cdc42 during 90 min of nucleotide triphosphate depletion and depletion, followed by 60 min of repletion in normal medium, is shown in Fig. 8A. Under normal culture conditions (t = 0) in postconfluent cultures all three GTPases were active (bound fraction). Whereas Rac1 was completely inactive after 90 min of depletion (unbound fraction), Rac1 was still active and Cdc42 activity had changed negligibly. A more complete time course of nucleotide triphosphate depletion and repletion for Rac1 activity revealed that Rac1 activity dropped after initiation of depletion to a minimum at 30 min but rebounded to higher levels after 60 and 90 min (Fig. 8B). With repletion Rac1 activity increased to control levels after 30 min.

The increase in Rac1 activity after an initial decrease during depletion raised the possibility that Rac1, RhoA, or Cdc42 activity may fluctuate over a longer time course. However, a time course extended to 240 min of nucleotide triphosphate depletion showed that the activity of each of the three GTPases was relatively constant over longer time periods. RhoA activity remained near zero, Rac1 activity remained steady during 90–240 min of depletion, and Cdc42 remained almost entirely in the active state (Fig. 9).

Effect of activated RhoA during depletion. Considerable evidence exists for cross talk between the activities of different Rho family GTPases (22). Because RhoA activity decreases so rapidly with nucleotide depletion, we considered that this might affect the activity of Rac1 or Cdc42. To test this possibility, the constitutively active RhoA mutant RhoAL63 was transfected into LLC-PK10 cells. Subsequently, nucleotide triphosphate depletion was induced with antimony A and the activities of RhoA, Rac1, and Cdc42 were measured after 90 min of depletion and after depletion followed by 60 min of repletion. No effect was found on the activity of endogenous RhoA or Cdc42 (data not shown). However, the ratio of active to inactive Rac1 decreased 32.1% during repletion in the presence of RhoAL63 compared with nontransfected controls. However, this change did not reach statistical significance (P = 0.189; n = 3).

DISCUSSION

We demonstrate in this report that chemically induced anoxia in a cultured cell line derived from renal proximal tubule results in rapid conversion of RhoA-GTP to RhoA-GDP, with a time course that parallels the decrease in ATP and GTP levels. Repletion of ATP and GTP resulted in corresponding reversal of this effect and increasing levels of RhoA-GTP. The kinetics of RhoA inactivation and reactivation during ATP de-
pleation and repletion were consistent with the cytoskeletal association of the known RhoA effector ezrin through depletion and repletion, which we show here and which is consistent with previous reports (8, 9). We used the ROK inhibitor Y27632 to show the importance of RhoA signaling and the specific effect of ROK inhibition on ezrin cytoskeletal association. In these experiments, Y27632 prevented the reassociation of ezrin with the cytoskeleton during ATP repletion. Myosin II regulatory light chain phosphorylation, another downstream target of RhoA signaling, also changes during ATP depletion, with kinetics consistent with the changes we observe in RhoA activity (34). This pattern of RhoA inactivation in ischemia is consistent with the finding that constitutively active RhoA mutants are able to protect components of the actin cytoskeleton and associated structures in the basal and lateral domains of the cell cortex against disruption during ATP depletion (17, 33) and confirms an important role for RhoA in the pathophysiology of ischemic injury in the kidney.

In contrast to the effect of ATP depletion on RhoA, the activation state of Rac1 and Cdc42 did not directly correlate with the level of ATP or GTP in the cell. We confirmed that our assay was sensitive to physiological changes in Rac1 and Cdc42 activity by measuring large decreases in activity as cell density increased to confluence. It is interesting to note that the changes we observed in RhoA, Rac1, and Cdc42 activity were in the opposite direction from those observed in similar experiments on cell density in another renal epithelial cell line, Madin-Darby canine kidney (MDCK) cells (32). This discrepancy is not necessarily surprising, because other work has shown that signaling pathways involving Rho GTpases differ between MDCK and LLC-PK cells (16a). It should be noted that the assays we used here are valid as an indication of relative changes in GTPase activity that result from the manipulations we used to alter ATP levels or cadherin engagement but cannot be used as an indication of whether the basal level of GTPase activity in these cells is high or low compared with studies in other cells by other investigators, because of the sensitivity of the assay to buffer conditions and other variables in the protocol used.

Our assay showed that Cdc42 activity did not decrease during ATP depletion, whereas the level of active Rac1 initially declined but later recovered somewhat, even as ATP and GTP levels were still falling. Disruption of actin cytoskeletal organization is not, therefore, attributable to a net decrease in the cellular level of Cdc42 activity and cannot be related in a simple way to the level of Rac1 activity. It may be that the activity of Cdc42 and Rac1 is still an important factor in cytoskeletal disruption, but the disruption results instead from an imbalance in the level of activity of these Rho GTpases. In the absence of detectable RhoA activity, continued Cdc42 and Rac1 activity might indeed be one of the causes of aberrant actin polymerization into F-actin aggregates in the center of the cell that characterizes ATP depletion in renal proximal tubule cells (31), as well as in other cell types such as fibroblasts and endothelial cells (14, 20). Actin

![Extended time course of GTPase activity](https://example.com/figure9.png)

**Fig. 8.** A: comparison of RhoA, Rac1, and Cdc42 activity assays. LLC-PK10 cells were treated with antimycin A and substrate-depleted medium for 0 or 90 min or for 90 min with antimycin A followed by recovery in normal medium for 60 min. B: effect of depletion and recovery on Rac1 activity. LLC-PK10 cells were incubated with antimycin A and substrate-depleted medium for various lengths of time or incubated with antimycin A for 90 min followed by recovery in normal medium for 30 or 60 min. Detergent lysates were then incubated with GST-Pak-BD and separated into bound (B; containing GTP-Rac1) or unbound (U; containing GDP-Rac1) fractions by incubation and centrifugation with glutathione agarose. C: graph of 4 experiments as represented in B. The amount of Rac1 in each fraction was quantified by densitometry of Western blots and expressed as the mean ± SD ratio of bound to unbound as % of the control (t = 0) value.

![Extended time course of GTPase activity](https://example.com/figure9.png)

**Fig. 9.** Extended time course of GTPase activity. LLC-PK10 cells were treated with antimycin A for 0, 90, 180, or 240 min. The cells were lysed, and GTPase activities were measured as described in text.
monomers released by the destruction of normal cytoskeletal assemblies after RhoA inactivation, and from the thymosin-sequestered pool, could be induced to polymerize at nuclei provided by the activity of Rac1 and Cdc42 effectors such as cortactin (38, 41).

The partial recovery of Rac1 activity at later times during ATP depletion, when ATP and GTP levels are still falling, is surprising. Because in many cell types RhoA activity antagonizes that of Rac1 (23, 39), we considered that the nearly complete inactivation of RhoA could explain the partial recovery of Rac1. However, the constitutively active RhoA mutant RhoAL63 had no effect in Rac1 activity under ATP-depleted conditions, so the signaling relays that normally operate between the two GTPases appear to be disrupted by ATP depletion but are again operable during repletion.

Under the conditions used here for ATP depletion, cellular ATP levels decrease to ~1% of the level in control cells, whereas the levels of GTP decrease to a minimum of 5.8% of control levels. Assuming that the control cells, whereas the levels of GTP decrease to a cause the relative affinities for GTP are comparable (~0.2 μM), as are their slightly lower affinities for GDP (~0.5–0.6 μM) (43). Exchange factors (GEFs) act by increasing the dissociation rate constant for bound nucleotide, so that in normal cells the cytoplasmic excess of GTP over GDP ensures that when GEFs bind the result is formation of the GTP-bound form of the protein. Under depleted conditions, however, GDP is in excess over GTP (at least over the short time scales used here) and the activity of exchange factors could be expected to result in inactivation of Rho proteins, because the relative affinities for GTP and GDP do not bias the exchange reaction sufficiently to prevent this. This may well be what happens to RhoA because the rapid kinetics of RhoA inactivation that we observe would be hard to account for by the intrinsic dissociation or hydrolysis rates and must therefore result from the action of GEFs or GAPs. A role for regulatory proteins also seems to be required in view of the very different behavior of RhoA, Rac1, and Cdc42. If alterations in Rho protein activity were simply the result of their equilibration to the ambient GTP-to-GDP ratio in the cytoplasm one would expect to see the same effect of ATP depletion on all three GTPases, because their nucleotide affinities, dissociation rates, and hydrolysis rates are comparable. The fact that the effect of ATP depletion is so different for each protein argues that depletion selectively impacts the regulation of upstream pathways that control the activities of the GEF and GAP proteins. These upstream pathways may still directly sense the GTP-to-GDP or ATP-to-ADP ratio but could transduce the effects of alterations in these ratios differentially to each Rho family member. Because it is generally accepted that GAP proteins are more promiscuous and GEF proteins more specific, it is reasonable to suppose that the differences in the effects of ATP depletion on RhoA, Rac1, and Cdc42 result from different effects on specific GEF proteins.

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