Caveolae are a family of cell-cell adhesion molecules that nucleate the assembly of adherens junctions (56). Normal cadherin function requires association with a set of membrane-cytoskeletal proteins that include the catenins. The cadherin-catenin complex links the adherens junction to the actin cytoskeleton. Dynamic regulation of cell-cell adhesion is necessary for normal cellular processes (59). Important regulators of adherens junction assembly are the Rho family GTPases (5, 14, 15, 20, 24, 25, 51).

Rho family GTPases are members of the Ras GTPase superfamily of proteins that switch between GDP-bound active and GDP-bound inactive conformations (31). The Rho family includes Rho, Rac, and Cdc42. These signaling proteins control cytoskeleton and junctional complex assembly in response to various extracellular signals, including growth factors (20, 31). RhoA signaling induces stress fiber and focal adhesion assembly; Rac1 signaling induces actin polymerization at the cell periphery and lamellipodia formation; and Cdc42 signaling induces filopodia formation (20). In epithelial cells, RhoA and Rac1 regulate tight junction and adherens junction assembly and function and Cdc42 regulates adherens junction assembly (5, 14, 15, 20, 24, 25, 37, 51).

Protein phosphorylation pathways that regulate cell adhesion and junctional complex assembly have been studied extensively, but the specific mechanisms remain obscure (12, 62). Loss of cell adhesion and disruption of adherens junction assembly have been correlated with tyrosine phosphorylation of β-catenin and p120^ctn (1, 35, 41–43, 50). However, Tsukita and colleagues (52), using E-cadherin/α-catenin fusion proteins, showed that the transition from a strong cadherin adhesion state to a weak cadherin adhesion state was independent of the presence of β-catenin in the cadherin complex. The role of serine/threonine phosphorylation of cadherin-catenin complex components in the regulation of cell adhesion has been studied less. However, detailed information comes from studies of β-catenin serine phosphorylation and dephosphorylation in the Wnt and adenomatous polyposis coli (APC) tumor suppressor signaling pathways (39).

Renal ischemia is a consequence of numerous disease processes, and it results in significant morbidity and mortality (53). ATP content in epithelial cells is rapidly depleted during renal ischemia. Cellular consequences of renal ischemia have been investigated in human biopsy tissue samples and animal models (30). Hallmark features of ischemic injury include epithelial cell damage in the proximal tubule that alters epithelial cell polarity and actin-associated cellular structures (13, 30). Many of the consequences to renal epithelial cells in vivo are recapitulated in cultured epithelial cells that are subjected to ATP depletion (13). Tight junctions and adherens junctions are among the actin-associated structures that are disrupted in response to ATP depletion in the kidney (7, 28, 29, 36) and in cultured epithelial cell models (28, 32, 33, 57). Mechanistic details of the injury processes are still largely unknown.

We have hypothesized (18) that Rho family GTPase signaling is inhibited during ATP depletion of cultured epithelial cells. We found (18) that activating RhoA...
signaling protects tight junctions from disassembly during ATP depletion and inhibiting RhoA accelerates tight junction disassembly during ATP depletion. Cadherin adhesion (adherens junction) and tight junction assembly are coupled (19, 54, 55). In this study, we examined adherens junction disassembly during ATP depletion. RhoA and Rac1 signaling regulates adherens junction assembly (4, 5, 24, 51). ATP depletion also alters assembly and phosphorylation state of cadherin-catenin complex components. However, RhoA signaling did not alter adherens junction disassembly rates during ATP depletion. This indicates that the protective role of RhoA signaling on tight junctions and actin structures during ATP depletion of epithelial cells is limited and selective (18, 40). In contrast, activation of Rac1 signaling protects adherens junctions from disassembly during ATP depletion, and inhibiting Rac1 accelerates adherens junction disassembly during ATP depletion. These data suggest that Rac1 signaling selectively controls adherens junction assembly state during cellular injury and that Rho family GTPase signaling could provide general protective roles for epithelial cell architecture.

MATERIALS AND METHODS

Cell culture, antibodies, and reagents. Madin-Darby canine kidney (MDCK) type II cells were maintained in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum with penicillin, streptomycin, and glutamine (GIBCO BRL). Chemicals were purchased from Sigma (St. Louis, MO) or Midwest Scientific (St. Louis, MO) unless otherwise indicated.

One monoclonal antibody against E-cadherin (DECMA-1) was purchased from Sigma. Another monoclonal antibody against E-cadherin (rr-1) was purchased from the Developmental Studies Hybridoma Bank (maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract from the National Institute of Child Health and Human Development). The polyclonal antibody against E-cadherin was previously described (34). Anti-phosphotyrosine polyclonal antibody against β-catenin was raised as described previously (7). Monoclonal antibody 9E10 against the myc epitope was purchased from Covance (Berkeley, CA). Horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibody was purchased from Transduction Laboratories (Lexington, KY).

ATP depletion. MDCK cells were plated at a density of 2.0 × 10⁵ cells per 35-mm culture dish. Three- to five-day cultures were rinsed with prewarmed depletion medium and ATP depleted for different times by incubating cells with depletion medium containing 0.1 μM antimycin A (7). For transfected cells, ATP depletion was performed at 30 h after transfection. ATP levels were assayed as described previously (7).  

Immunoblotting. MDCK cells were plated at a density of 2 × 10⁶ cells per 35-mm dish. Three days later cells were ATP depleted for 30 or 60 min or left untreated in control dishes. Cultures were then extracted in SDS-containing buffer (1% SDS, 10 mM Tris-HCl, pH 7.5, and 2 mM EDTA) at 100°C. Cells were scraped, and lysates were collected. Samples were heated at 100°C for 5–10 min and sonicated. Samples were cleared by centrifugation. Protein assays on supernatants were performed with a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical, Rockford, IL). Thirty micrograms of protein were separated on 7.5% SDS polyacrylamide gels. Gels were transferred to nitrocellulose filters (Bio-Rad, Hercules, CA) and blocked in Tris-buffered saline containing Tween 20 (TBST; in mM: 10 Tris-HCl, pH 7.5, 100 NaCl, and 0.1% Tween 20) containing milk (5% nonfat dry milk). Filters were then incubated in primary antibody (E-cadherin 1:10,000 and β-catenin 1:1,000) diluted in blocking solution for 1 h at room temperature, and blots were then washed in TBST for 1 h with several changes. Filters were then incubated for 1 h at room temperature with species-matched HRP-conjugated secondary antibody (Amersham, Arlington Heights, IL) diluted 1:5,000 in blocking solution. Filters were again washed, and signal was detected by enhanced chemiluminescence (ECL kit; Amersham, Arlington Heights, IL) and exposed to film (Kodak Bio-Max ML; Eastman Kodak, Rochester, NY).

Transient transfection. MDCK cells were plated at 2.5 × 10⁵ per 35-mm culture dish. Cells were transfected 24 h later with 1.5–2 μg each of control vector or plasmids with RhoA-V14, RhoA-N19, Rac1-V12, or Rac1-N17 cDNAs expressed from SV40 promoters (generously provided by Dr. Marc Symons, Pfizer Institute for Medical Research, Manhasset, NY) with Lipofectamine plus according to manufacturer's protocol (GIBCO BRL). Cells were incubated with the transfection mixture for 3 h. This mixture was then replaced with normal growth medium. Cells were analyzed at various times, but the experiments shown were analyzed 30 h after transfection.

Immunoprecipitation. Cultures were rinsed with ice-cold phosphate-buffered saline (PBS; in mM: 2.7 KCl, 1.5 KH₂PO₄, 137 NaCl, 8.1 Na₂HPO₄, and 0.45 CaCl₂ with 0.5 M MgCl₂). Cells were extracted with CSK buffer (in mM: 50 NaCl, 300 sucrose, 3 MglCl₂, 1 phenylmethylsulfonyl fluoride (PMSP), 0.5% Triton X-100, and 10 PIPES, pH 6.8) for 15 min on ice. Monolayers were scraped, and lysates were collected. Extracts were cleared by centrifugation in a microtube at 13,000 rpm for 5 min at 4°C. Primary antibody (E-cadherin or β-catenin polyclonal antibody) was added to supernatants, and tubes were rotated at 4°C for 1 h. Immune complexes were collected with protein A-Sepharose beads (Pharmacia, Piscataway, NJ) and washed three times with CSK buffer. Beads were resuspended in SDS-PAGE sample buffer for analysis, and separated on 7.5% SDS polyacrylamide gels. Immunoblotting was performed as described in Immunoblotting except that TBST containing 1% BSA was used for phosphotyrosine blotting.

Confocal microscopy: immunofluorescence, image acquisition, and image analysis. MDCK cells were plated and transfected as described in Transient transfection. At 30 h after transfection, cells were left untreated or ATP depleted as described in ATP depletion. Cells were fixed in PBS containing 3.7% paraformaldehyde for 10 min at room temperature. Cells were washed in PBS and then permeabilized in PBS containing 0.5% Triton X-100 for 10 min at room temperature. Cells were then blocked in PBS containing 0.2% BSA and 2% goat serum for 30 min at room temperature. The first primary antibody diluted in blocking buffer (DECMA-1: 1:100 or β-catenin polyclonal antibody 1:3000) was incubated for 45 min at room temperature. Cells were washed in PBS containing 0.2% BSA and incubated with mouse anti-myc antibody (9E10) at 45 min at room temperature. Coverslips were then rinsed with PBS containing 0.2% BSA and incubated with mouse anti-myc antibody (9E10) for 45 min at room temperature.

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Samples were viewed with a Bio-Rad MRC 1024 confocal microscope. To avoid sampling variability, individual planes through the entire cell volume were collected at 0.5-μm intervals, being careful to avoid saturation of the photomultiplier tube detector. The gain levels were reset between image collections and thus are not always evenly matched in Figs. 1–7. Fluorescein bleed-through into Cy5 channels was avoided by collecting the z-series first with the 647-nm laser line and then collecting the companion image as a single plane with the 488-nm line. A projection image was generated by mathematically summing the z-series images in one 16-bit image for quantification with Metamorph version 4.1.7 software (Universal Imaging, West Chester, PA). This image was background corrected by subtracting from each pixel the median pixel value from the surrounding 32 x 32 pixel neighborhood. Junctional regions were highlighted, and the total fluorescence and length of the junction region were calculated. Average fluorescence per unit length was calculated, and the ratios described in RESULTS were calculated from these numbers. For each experimental condition, ~10 junctions between transfected cells and 10 junctions between nontransfected cells were analyzed.

Two-photon microscopy. MDCK cells (2.5 million) were plated on six-well polycarbonate filter inserts (Corning-Costar, Kennebunk, ME) and cultured for 4 days. Cells were ATP depleted for 30 or 60 min with 0.1 μM antimycin A or left untreated in the control filters. Filters were then fixed with 4% paraformaldehyde for 10 min at room temperature and processed for indirect immunofluorescence with antibodies against E-cadherin and β-catenin. FITC-conjugated secondary antibodies were purchased from Jackson Labs. Samples were mounted in PBS containing DABCO, and two-photon microscopy was performed with the use of a Bio-Rad MRC1024 confocal/two-photon system fitted to a Nikon Eclipse inverted microscope (Melville, NY) with a ×60 water-immersion, NA 1.2 objective. Illumination for the multiphoton fluorescence excitation was provided by a Spectra-Physics (Mountain View, CA) Tsunami Lite titanium-sapphire laser. Data sets were collected as z-series of ~100 images with a spacing of 0.1 μm. Acquired images were first processed for background subtraction by applying a 3 x 3 low-pass filter with Metamorph version 4.1.7 image-processing software. The digital contrast of the stacks was manipulated, and a 256 x 256 subsection from each stack was chosen for further analysis. Data stacks were rendered in three dimensions with volume-visualization software (Voxx). Transparency of the volume was manipulated by varying image opacity coefficients with Voxx. Data collection and image analysis for all control and ATP-depleted cells were manipulated identically.

RESULTS

ATP depletion alters adherens junction assembly. Adherens junctions are disrupted during ATP depletion by unknown disassembly mechanisms (33). To study the effects of ATP depletion on adherens junction component distribution in greater detail, distributions of E-cadherin and β-catenin were examined in three-dimensional reconstructions of immunofluorescence images collected with two-photon microscopy (Fig. 1A). The two-photon microscopy minimizes photobleaching during the acquisition of ~100 images used for three-dimensional data analysis. Data sets were processed with a voxel rendering software program (Voxx) developed in the Indiana University Imaging Facility (11). Please refer to the Supplementary Material1 for this article (published online at the American Journal of Physiology-Cell Physiology web site) to view the movies of rotating volumes for each image in Fig. 1A.

MDCK cell monolayers were ATP depleted with antimycin A, a reversible inhibitor of cytochrome reductive electron transport. As previously described, ATP levels rapidly reduced to <5% of control levels within 15 min (7, 18). ATP depletion of MDCK cells is not lethal. The drug can be easily washed out, and ATP levels recover quickly to ~50% of control levels by 60 min (data not shown).

MDCK cells were stained for immunofluorescence with antibodies specific for E-cadherin or β-catenin. Both proteins were concentrated at the apical region of the lateral membrane in the adherens junction (Fig. 1A). After ATP depletion, adherens junction marker proteins were found in cytoplasmic vesicular structures, suggesting that the cadherin-catenin complex was internalized from the plasma membrane. This confirms findings for E-cadherin distributions made by Mandel and colleagues (33), who also found that the appearance of the cytoplasmic vesicular structures coincided with reduced accessibility of E-cadherin to cell surface biotin labeling. The concentration of adherens junction components in the apical region of the lateral membrane was not observed after ATP depletion, suggesting that the integrity of the adherens junction was disrupted. In addition, E-cadherin and β-catenin staining at the lateral membrane was reduced in intensity and less continuous than in control cells. We also found that β-catenin staining showed an overall reduction in intensity, especially after 60 min of ATP-depletion. However, immunoblotting experiments showed that the amounts of E-cadherin and β-catenin were unchanged in control and ATP-depleted cells (Fig. 1B), similar to previous observations (6, 33), and levels of α-catenin and γ-catenin were also unchanged after ATP depletion (6). Immunoprecipitation experiments showed that the cadherin-catenin complex was intact after ATP depletion (Fig. 1C). Together, these data indicate that β-catenin may become part of a protein complex, in which it is inaccessible to the antibody.

ATP depletion decreased cadherin-catenin complex phosphorylation. Adherens junction disassembly that occurs during ATP depletion (Fig. 1; Ref. 33) may be a consequence of protein phosphorylation changes that regulate protein complex assembly. Protein kinases are generally inactivated during ATP depletion, resulting in a general decrease in protein phosphorylation in epithelial cells after injury (27). Therefore, we examined the consequences of ATP depletion on cadherin-catenin complex phosphorylation.

MDCK cells were ATP depleted for various times. Cells were extracted with Triton X-100-containing buffer, and cadherin-catenin complexes were immunoprecipitated with E-cadherin-specific antibodies. These

1Supplemental material to this article is available online at http://ajpcell.physiology.org/cgi/content/full/283/1/C261/DC1.
immunoprecipitates were separated on SDS polyacrylamide gels, transferred to nitrocellulose, and immuno-blotted to detect phosphotyrosine or phosphoserine/phosphothreonine. Immunoprecipitates were also blotted with E-cadherin antibodies to show that comparable amounts of E-cadherin were immunoprecipitated.

/H9252-Catenin and /H9253-catenin tyrosine phosphorylation was detected in E-cadherin immunoprecipitates of control MDCK cells, and these proteins rapidly lost phosphotyrosine content during a short time course of ATP depletion (Fig. 2A). We consistently observed a transient increase in cadherin-associated β- and γ-catenin phosphotyrosine content after ~5 min of antimycin A treatment (Fig. 2A).

Phosphotyrosine loss was complete by 30 min of ATP depletion. There was no additional loss of phosphotyrosine with 60 min of ATP depletion (Fig. 2B). To examine whether phosphotyrosine content in the cadherin-catenin complex recovers from ATP depletion, MDCK cells were ATP depleted for 30 or 60 min and

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**Fig. 1.** Distribution of adherens junction components in control Madin-Darby canine kidney (MDCK) cells and after ATP depletion. A: MDCK cells were stained for immunofluorescence with antibodies specific for E-cadherin or β-catenin. FITC-conjugated secondary antibodies were used to visualize E-cadherin (top) or β-catenin (bottom) distributions. Stacks of ~100 x-y image planes were collected through the volume of the cell monolayer with a two-photon microscope (see MATERIALS AND METHODS). Left: control, untreated MDCK cells. Middle: MDCK cells that were ATP depleted for 30 min. Right: MDCK cells that were ATP depleted for 60 min. All images were rotated or tilted ~5, 9, and 6 degrees on the x-, y-, and z-axes, respectively. Rotating volumes of these data sets can be viewed as movies online as Supplementary Material to this article. Movie files were generated with Voxel software, converted to a video clip with QuickTime (Apple Computer), and compressed with MPEG encoder (TMPGEnc). Animations of image volumes rendered with Voxel include the following number of image planes: E-cadherin control, 137; E-cadherin 30-min ATP depletion, 97; E-cadherin 60-min ATP depletion, 111; β-catenin control, 105; β-catenin 30-min ATP depletion, 192; β-catenin 60-min ATP depletion, 151. B: control, untreated MDCK cells (C); MDCK cells that were ATP depleted for 30 min, and MDCK cells that were ATP depleted for 60 min were extracted with SDS-containing buffer. Equal amounts of protein were separated by SDS-PAGE and immunoblotted with antibodies against E-cadherin and β-catenin. There was no significant change in the amount of these proteins in MDCK cells after ATP depletion. C: E-cadherin complexes were immunoprecipitated from control, untreated MDCK cells, MDCK cells that were ATP depleted for 30 min, and MDCK cells that were ATP depleted for 60 min and immunoblotted with antibodies specific for E-cadherin and β-catenin. Both immunoblots (IB) show E-cadherin immunoprecipitates (IP). The immunoblot on the left was probed with antibodies specific for E-cadherin, and the immunoblot on the right was probed with antibodies specific for β-catenin.
then allowed to recover in normal growth medium for 60 min. Recovery of phosphotyrosine levels in β-catenin, γ-catenin, and the 180-kDa protein [which may be the epidermal growth factor (EGF) receptor] was less for cells ATP depleted for 60 min than for cells ATP depleted for 30 min (Fig. 2C).

We also determined the effects of ATP depletion on the total cellular β-catenin pool by immunoprecipitating β-catenin from control and ATP-depleted cell extracts and immunoblotting for phosphotyrosine.

Fig. 2. Effect of ATP depletion and recovery on phosphotyrosine and phosphoserine/phosphothreonine content in E-cadherin-catenin complex components. Confluent MDCK cells were ATP depleted for the indicated times, and E-cadherin was immunoprecipitated from cell extracts. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect E-cadherin (bottom), phosphotyrosine (A and B, top), or phosphoserine/phosphothreonine (C, top). A: control (untreated) and short time course of ATP depletion is from 5 (5') to 30 (30') min. B and C: MDCK cells were untreated (control) or ATP depleted for 30 (30') or 60 (60') min, and then cells were allowed to recover from 30- and 60-min injuries for 60 min (30'/60' and 60'/60', respectively). Arrows indicate β-catenin, and arrowheads indicate γ-catenin. Asterisks indicate a 180-kDa protein that may be the epidermal growth factor (EGF) receptor. The data shown are representative of 3 independent experiments.

Phosphoserine/phosphothreonine antibody reactivity in β-catenin was lost rapidly during ATP depletion (Fig. 2C), and serine/threonine phosphorylation levels did not show significant recovery after cells were returned to normal growth medium (Fig. 2C).

Fig. 3. Effect of ATP depletion and recovery on phosphotyrosine and phosphoserine/phosphothreonine content in the total β-catenin cellular pool. Confluent MDCK cells were ATP-depleted for the indicated times, and β-catenin was immunoprecipitated from cell extracts. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect β-catenin (bottom), phosphotyrosine (A–C, top), or phosphoserine/phosphothreonine (D, top). A: control (untreated) and short time course of ATP depletion is from 5 to 30 min. B–D: MDCK cells were untreated (control) or ATP depleted for 30 or 60 min, and cells were then allowed to recover from 30- and 60-min injuries for 60 min. In C, cells were ATP depleted with cyanide (left) and antimycin A (right). The data shown are representative of 3 independent experiments.
β-Catenin tyrosine phosphorylation was detected in control MDCK cells, and β-catenin rapidly loses phosphotyrosine content during ATP depletion (Fig. 3A). We also observed a transient increase in phosphotyrosine content of β-catenin in the total β-catenin pool after ~5 min of antimycin A treatment (Fig. 3A).

Recovery of phosphotyrosine content in β-catenin during ATP repletion was also examined. Recovery of phosphotyrosine levels in β-catenin was less for cells ATP depleted for 60 min than for cells ATP depleted for 30 min (Fig. 3B), as observed for β-catenin associated with E-cadherin.

Schwartz and colleagues (48) found that β-catenin tyrosine phosphorylation showed a sustained increase during cyanide-induced ATP depletion of mouse proximal tubule primary culture cells. Our findings appeared to be contradictory to those of Schwartz et al. (48). Therefore, we tested the effect of cyanide in the MDCK cell experimental system. β-Catenin was immunoprecipitated from MDCK cells that were untreated or treated with cyanide for 30 and 60 min, and these β-catenin immunoprecipitates were immunoblotted to detect phosphotyrosine. β-Catenin phosphorylation was rapidly reduced to nearly undetectable levels by 30 min of cyanide-induced ATP depletion, and this reduction was maintained at 60 min of cyanide-induced ATP depletion (Fig. 3C), similar to antimycin A-induced ATP depletion performed in parallel experiments (Fig. 3C). This indicates that there is a cell type-specific difference in the response of β-catenin tyrosine phosphorylation to ATP depletion between MDCK cells and mouse proximal tubule primary culture cells (48).

β-Catenin phosphorylation was also examined with phosphoserine/phosphothreonine antibodies from β-catenin immunoprecipitates in control MDCK cells (Fig. 3D). β-Catenin phosphoserine/phosphothreonine antibody reactivity was lost rapidly during ATP depletion (Fig. 3D). Recovery of serine/threonine phosphorylation levels in the total pool of β-catenin was significantly less for cells ATP depleted for 60 min than for cells ATP depleted for 30 min (Fig. 3D).

RhoA signaling did not protect adherens junctions from disassembly during ATP depletion. Previous work showed that tight junctions, stress fibers, and the cortical actin cytoskeleton are protected from disassembly during ATP depletion by constitutive RhoA signaling (18, 40). We examined whether RhoA signaling protects adherens junctions. MDCK cells were transfected with plasmids encoding RhoA mutant proteins, and these transiently transfected monolayers were ATP depleted with antimycin A.

Effects of RhoA GTPase signaling and ATP depletion were assayed by confocal microscopy and quantitative image analysis. MDCK monolayers transiently transfected with plasmids encoding either RhoA-N19

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Fig. 4. Effect of ATP depletion (depl) on distribution of E-cadherin and β-catenin in cells expressing dominant-negative RhoA GTPase mutant protein. MDCK cells were transiently transfected with myc-tagged dominant-negative RhoA (RhoA-N19). At 30–36 h after transfection, cells were ATP depleted for 60 min or left untreated (control). A: cells were processed for double-label indirect immunofluorescence with E-cadherin antibodies, and mutant RhoA GTPase-expressing cells were detected with anti-myc antibodies (Myc). Each image shown is 105 μm². B: mean fluorescence intensity ratio was calculated for junctions between pairs of mutant RhoA GTPase-expressing cells (transfected) and between pairs of nontransfected cells from the same experiment. The ratio changed from 0.39 (control; n = 20) to 0.35 (60’ ATP depletion; n = 20) (P = 0.29, t-test). The data shown are representative of 7 independent experiments. C: cells were processed for double-label indirect immunofluorescence with β-catenin antibodies, mutant RhoA GTPase-expressing cells were detected with anti-myc antibodies, and image analysis was performed. Mean fluorescence intensity was calculated for junctions between pairs of mutant RhoA GTPase-expressing cells (transfected) and between pairs of nontransfected cells from the same experiment. The ratio changed from 0.44 (control; n = 25) to 0.49 (60’ ATP depletion; n = 25) (P = 1.19, t-test). The data shown are representative of 5 independent experiments.

AJPCell Physiol • VOL 283 • JULY 2002 • www.ajpcell.org
or RhoA-V14 were fixed and processed for double-label immunofluorescence to detect myc-tagged mutant RhoA proteins and adherens junction components (E-cadherin or β-catenin). A z-series of x-y images through the entire volume of the monolayer was collected with a laser scanning confocal microscope and combined to avoid missing fluorescence from other focal planes. Fluorescence intensity per unit length was measured with Metamorph image analysis software. To determine the consequences of expressing RhoA mutant proteins, a fluorescence intensity ratio of the intensity measurements from junctions between two transfected cells divided by the intensity measurements from junctions between two nontransfected cells was calculated. This ratio expresses the magnitude of decrease or increase in fluorescence intensity per unit length as a consequence of mutant RhoA protein expression. This ratio also allows us to distinguish relative changes in fluorescence after ATP depletion because the ratio is internally normalized to nontransfected cell-cell junctions.

MDCK cell monolayers transfected with dominant-negative RhoA-N19 expression plasmid were examined by immunofluorescence before or after 60 min of ATP depletion. Fluorescence intensity ratios for adherens junction components did not significantly change after ATP depletion (Fig. 4). As previously shown (Ref. 33; Fig. 1), adherens junctions disassemble as a consequence of ATP depletion. Because the fluorescence ratio was not altered during ATP depletion (Fig. 4), inhibiting RhoA activity by RhoA-N19 expression did not accelerate adherens junction disassembly.

MDCK cells expressing dominant-active RhoA-V14 were processed for immunofluorescence before or after being ATP depleted for 60 min. Fluorescence intensity ratios for E-cadherin and β-catenin were not significantly altered during ATP depletion (Fig. 5). Unlike tight junctions and actin cytoskeleton structures (18, 40), RhoA signaling did not protect adherens junctions from disassembly. Instead, adherens junction disassembly was similar in transfected and nontransfected cells (Fig. 5).

Rac1 signaling protects adherens junctions from disassembly during ATP depletion. Because RhoA GTPase signaling did not protect adherens junctions from disassembly during ATP depletion, we sought to determine whether another Rho family member may have a protective role. Rac1 GTPase signaling also regulates adherens junction assembly, and consequently, Rac1 may protect adherens junctions from disassembly during ATP depletion. MDCK monolayers transiently transfected with plasmids encoding either Rac1-N17 or Rac1-V12 were fixed and processed for double-label immunofluorescence to detect myc-tagged mutant Rac1 proteins and adherens junction components (E-cadherin or β-catenin). Fluorescence intensity ratio...
of the intensity measurements from junctions between two transfected cells divided by the intensity measurements from junctions between two nontransfected cells was calculated as in the RhoA experiments.

MDCK cell monolayers were transfected with dominant-negative Rac1-N17 expression plasmid and examined by immunofluorescence before or after 60 min of ATP depletion (Fig. 6A). The ratio of fluorescence intensity for junctions between Rac1-N17-transfected cell pairs divided by fluorescence intensity for junctions between nontransfected cell pairs stained for E-cadherin was reduced from 0.37 ± 0.1 (SD) in control cultures to 0.12 ± 0.08 in parallel cultures that were ATP depleted for 60 min (Fig. 6B). β-Catenin fluorescence intensity ratio of Rac1-N17-transfected cell pairs divided by nontransfected cell pairs was also reduced from 0.48 ± 0.1 in control cultures to 0.2 ± 0.05 in parallel cultures that were ATP depleted for 60 min (Fig. 6C). The decreased fluorescence intensity ratio after ATP depletion indicates that inhibiting Rac1 signaling accelerates disassembly mechanisms that are activated by cell injury.

MDCK cell monolayers expressing dominant-active Rac1-V12 were examined by immunofluorescence and quantitative image analysis before and after 60 min of ATP depletion (Fig. 7A). Fluorescence intensity ratio for junctions between Rac1-V12-transfected cell pairs divided by fluorescence intensity for junctions between nontransfected cell pairs increased from 2.8 ± 0.3 to 4.2 ± 0.4 in control cultures to 6.4 ± 1.4 in parallel cultures that were ATP depleted for 60 min (Fig. 7B). Similarly, the β-catenin fluorescence intensity ratio of Rac1-N17-transfected cell pairs divided by nontransfected cell pairs increased from 2.8 ± 0.34 in control cultures to 6.4 ± 1.4 in parallel cultures that were ATP depleted for 60 min (Fig. 7C). This increase in the adherens junction component fluorescence intensity ratio indicates that Rac1 signaling protects adherens junctions from disassembly during ATP-depletion-induced cell injury.

DISCUSSION

Regulation of junctional complex assembly and function is incompletely understood, but insight comes from the finding that Rho family GTPases regulate epithelial junctional complexes (3, 5, 14, 18, 20, 22, 24, 25, 37, 51). Rho family GTPases regulate intracellular membrane trafficking and epithelial cell polarity (44). Cadherin adhesion helps maintain epithelial monolayer integrity and provides spatial cues that determine epithelial cell polarity (61). Cadherin assembly also controls assembly of the tight junction (which separates membrane domains in epithelial cells) (3, 8, 16, 17, 19, 54, 55, 60).

![Fig. 6. Effect of ATP depletion on distribution of E-cadherin and β-catenin in cells expressing dominant-negative Rac1 GTPase mutant protein. MDCK cells were transiently transfected with myc-tagged dominant-negative Rac1 (Rac1-N17). At 30–36 h after transfection, cells were ATP depleted for 60 min or left untreated (control). A: cells were processed for double-label indirect immunofluorescence with E-cadherin antibodies, and mutant Rac1 GTPase-expressing cells were detected with anti-myc antibodies. Each image shown is 105 μm². B: mean fluorescence intensity ratio was calculated for junctions between pairs of mutant Rac1 GTPase-expressing cells (transfected) and between pairs of nontransfected cells from the same experiment. The ratio changed from 0.37 (control; n = 24) to 0.12 (60’ ATP-depletion; n = 24) (P = 3.25 × 10⁻⁷, t-test). The data shown are representative of 7 independent experiments. C: cells were processed for double-label indirect immunofluorescence with β-catenin antibodies, mutant Rac1 GTPase-expressing cells were detected with anti-myc antibodies, and image analysis was performed. Mean fluorescence intensity was calculated for junctions between pairs of mutant Rac1 GTPase-expressing cells (transfected) and between pairs of nontransfected cells from the same experiment. The ratio changed from 0.48 (control; n = 25) to 0.2 (60’ ATP-depletion; n = 25) (P = 1.65 × 10⁻⁷, t-test). The data shown are representative of 3 independent experiments.

AJP-Cell Physiol • VOL 283 • JULY 2002 • www.ajpcell.org
Adherens junctions are disrupted during ATP depletion by unknown disassembly mechanisms that lead to E-cadherin redistribution (Refs. 6, 33; Fig. 1). We and others (6, 33) found that E-cadherin was internalized during ATP depletion. Appearance of cytoplasmic vesicular structures was coincident with a reduction in E-cadherin biotin labeling on the cell surface (33). These findings suggest that endocytosis mechanisms lead to cadherin-catenin complex internalization during ATP depletion. Endocytosis trafficking of plasma membrane proteins requires ATP. Therefore, direct experiments will be required to determine the specific pathways utilized for E-cadherin internalization during ATP depletion. Reduced cell surface E-cadherin may decrease cell adhesion between epithelial cells, which is consistent with findings that tubular epithelial cells are shed during the ischemic event (30, 53).

We investigated the consequences of ATP depletion on the phosphorylation of cadherin-catenin complexes. Many proteins are dephosphorylated during ATP depletion because ATP levels are reduced below the $K_m$ for protein kinases, yet protein phosphatases remain active (27). Our previous studies (18) and those of other investigators (2, 9, 10, 27, 49, 58) showed that membrane-cytoskeletal and junctional complex components were dephosphorylated during ATP depletion. In this study, we found that cadherin-catenin complex components were rapidly dephosphorylated during cellular injury and that the normal phosphorylation state returned when cells were allowed to recover from ATP depletion. These dephosphorylation events that occur during ATP depletion may contribute directly to junctional complex disassembly and cadherin redistribution.

A transient increase in $\beta$-catenin tyrosine phosphorylation was observed at short times after ATP depletion. Schwartz and colleagues (48) found that $\beta$-catenin tyrosine phosphorylation showed a sustained increase during cyanide-induced ATP depletion of mouse proximal tubule primary culture cells. We ATP-depleted MDCK cells with cyanide, and a sustained increase in $\beta$-catenin tyrosine phosphorylation was not observed. It is unclear whether there is a relationship between the transient increase that we observed in the MDCK cell model and the sustained increase found in the mouse proximal tubule primary culture model (48).

We have examined whether the effects of cellular injury on epithelial junctional complex assembly are regulated by Rho GTPase signaling. Previously, we found (18) that RhoA signaling protects tight junctions from disassembly during ATP depletion. Because RhoA also controls adherens junction assembly and adherens junction assembly controls tight junction assembly, we wanted to determine whether RhoA signaling protection of tight junctions was an indirect consequence of RhoA signaling protection of adherens junction assembly.

Adherens junctions are disrupted during ATP depletion by unknown disassembly mechanisms that lead to E-cadherin redistribution (Refs. 6, 33; Fig. 1). We and others (6, 33) found that E-cadherin was internalized during ATP depletion. Appearance of cytoplasmic vesicular structures was coincident with a reduction in E-cadherin biotin labeling on the cell surface (33). These findings suggest that endocytosis mechanisms lead to cadherin-catenin complex internalization during ATP depletion. Endocytosis trafficking of plasma membrane proteins requires ATP. Therefore, direct experiments will be required to determine the specific pathways utilized for E-cadherin internalization during ATP depletion. Reduced cell surface E-cadherin may decrease cell adhesion between epithelial cells, which is consistent with findings that tubular epithelial cells are shed during the ischemic event (30, 53).

We investigated the consequences of ATP depletion on the phosphorylation of cadherin-catenin complexes. Many proteins are dephosphorylated during ATP depletion because ATP levels are reduced below the $K_m$ for protein kinases, yet protein phosphatases remain active (27). Our previous studies (18) and those of other investigators (2, 9, 10, 27, 49, 58) showed that membrane-cytoskeletal and junctional complex components were dephosphorylated during ATP depletion. In this study, we found that cadherin-catenin complex components were rapidly dephosphorylated during cellular injury and that the normal phosphorylation state returned when cells were allowed to recover from ATP depletion. These dephosphorylation events that occur during ATP depletion may contribute directly to junctional complex disassembly and cadherin redistribution.

A transient increase in $\beta$-catenin tyrosine phosphorylation was observed at short times after ATP depletion. Schwartz and colleagues (48) found that $\beta$-catenin tyrosine phosphorylation showed a sustained increase during cyanide-induced ATP depletion of mouse proximal tubule primary culture cells. We ATP-depleted MDCK cells with cyanide, and a sustained increase in $\beta$-catenin tyrosine phosphorylation was not observed. It is unclear whether there is a relationship between the transient increase that we observed in the MDCK cell model and the sustained increase found in the mouse proximal tubule primary culture model (48).

We have examined whether the effects of cellular injury on epithelial junctional complex assembly are regulated by Rho GTPase signaling. Previously, we found (18) that RhoA signaling protects tight junctions from disassembly during ATP depletion. Because RhoA also controls adherens junction assembly and adherens junction assembly controls tight junction assembly, we wanted to determine whether RhoA signaling protection of tight junctions was an indirect consequence of RhoA signaling protection of adherens junction assembly.
RhoA signaling during cellular injury are selective and specific for a subset of cellular structures and processes. Our finding that RhoA signaling does not protect adherens junctions from disassembly during ATP depletion raised the question of whether another Rho family member may selectively protect adherens junctions from disassembly during cellular injury. Indeed, our current studies show that, like the effect of RhoA on tight junctions, inhibition of Rac1 signaling accelerates ATP-depletion-induced adherens junction disassembly mechanisms and activation of Rac1 signaling protects adherens junctions from disassembly during ATP depletion.

Previous studies described effects of Rac1 signaling on adherens junction development (5, 14, 15, 20, 24, 25, 51). Increased adherens junction assembly is found in cells expressing constitutively active Rac1. Downstream Rac1 signaling effectors that control adherens junction assembly are being studied. For example, IQGAP1 is a target of Rac1 and Cdc42 signaling that binds β-catenin and dissociates α-catenin from the cadherin-catenin complex. Future studies will address how inhibition of Rac1 signaling by ATP depletion may cause cadherin-catenin complex disassembly. In addition, it will be of interest to determine Rac1 effectors that are responsible for protecting adherens junctions during cell injury by ATP depletion.

Actin and associated structures are disrupted by ATP depletion (13, 30). Indeed, the actin-binding proteins vinculin and α-catenin, which are associated with the adherens junction, are also redistributed during ATP depletion (6, 40). RhoA signaling protects actin stress fibers during ATP depletion, but vinculin assembly in focal adhesions was not protected by constitutive RhoA signaling (40). Perhaps differential effects of Rho family GT-Pases signaling on vinculin within the adherens junction during ATP depletion could explain the differential protective effects of RhoA and Rac1. Experiments are under way that examine this issue.

Several Ras superfamily members control adherens junction assembly and cadherin function. Ras itself downregulates cadherin adhesion and increases epithelial cell migration (21, 26, 45, 47). As discussed above, Rho family GT-Pases control cadherin adhesion and adherens junction assembly (5, 14, 15, 20, 24, 25, 51). ARF6, another Ras superfamily of GT-Pase, also regulates cadherin function by controlling intracellular membrane trafficking of cadherin molecules (38). ARF6 signaling also activates Rac1 signaling (46), and this connection may partially explain how ARF6 control adherens junction assembly mechanisms. There are potentially numerous points of control for cadherin function, including cytoskeletal assembly, catenin binding, vesicle trafficking, and protein turnover, that are regulated by small GT-Pases, and many or all these processes may be disrupted by ATP and GTP depletion during ischemia. A more complete understanding of these regulatory and pathophysiological processes will help us determine how epithelial cells are injured during ischemia, which may indicate new therapeutic approaches to reverse this damage.

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