Rho GTPase signaling regulates tight junction assembly and protects tight junctions during ATP depletion

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Gopalakrishnan, Shobha, Narayan Raman, Simon J. Atkinson, and James A. Marrs. Rho GTPase signaling regulates tight junction assembly and protects tight junctions during ATP depletion. Am. J. Physiol. 275 (Cell Physiol. 44): C798–C809, 1998.—Tight junctions control paracellular permeability and cell polarity. Rho GTPase regulates tight junction assembly, and ATP depletion of Madin-Darby canine kidney (MDCK) cells (an in vitro model of renal ischemia) disrupts tight junctions. The relationship between Rho GTPase signaling and ATP depletion was examined. Rho inhibition resulted in decreased localization of zonula occludens-1 (ZO-1) and occludin at cell junctions; conversely, constitutive Rho signaling caused an accumulation of ZO-1 and occludin at cell junctions. Inhibiting Rho before ATP depletion resulted in more extensive loss of junctional components between transfected cells than control junctions, whereas cells expressing activated Rho better maintained junctions during ATP depletion than control cells. ATP depletion and Rho signaling altered phosphorylation signaling mechanisms. ZO-1 and occludin exhibited rapid decreases in phosphoamino acid content following ATP depletion, which was restored on recovery. Expression of Rho mutant proteins in MDCK cells also altered levels of occludin serine/threonine phosphorylation, indicating that occludin is a target for Rho signaling. We conclude that Rho GTPase signaling induces posttranslational effects on tight junction components. Our data also demonstrate that activating Rho signaling protects tight junctions from damage during ATP depletion.

junctional complex; signal transduction; ischemia

EPITHELIAL CELLS FORM barriers between biological compartments that regulate the composition of these compartments. The tight junction is a cell-cell junctional complex that forms a belt at the apical-most region of the lateral plasma membrane and is responsible for generating and maintaining a permeability barrier that regulates paracellular solute flow (7, 15, 28). Tight junctions also regulate lateral diffusion between apical and basolateral plasma membrane domains, which maintain plasma membrane protein and lipid polarity (15, 28). Morphologically, tight junctions are close appositions of the lateral plasma membrane and have an associated plaque that interacts with actin filaments. Freeze-fracture analysis of the tight junction shows a series of interlacing strands of intramembranous particles (24).

One integral membrane component of the tight junction has been identified, called occludin (26). Occludin was localized to intramembranous strands, and data suggest that occludin is a major constituent of these intramembranous strands (25). Occludin regulates transepithelial resistance, paracellular permeability, and the lateral diffusion barrier for lipids between the apical and basolateral plasma membrane domains (12, 18, 41, 65). Serine/threonine phosphorylation regulates occludin assembly into the junctional complex (52).

Several other protein components of the tight junction have now been identified, but specific functions for these proteins have not been established (5). Most are thought to be structural components, but these proteins may also have signaling roles. Zonula occludens-1 (ZO-1) was the first component identified of the tight junction. ZO-1, together with the related tight junctional protein ZO-2, is a member of the membrane-associated guanylate kinase (MAGuK) gene superfamily (5). Several MAGuK family members are developmental signaling molecules (33), suggesting that ZO-1 and other MAGuK family members in the tight junction may have intracellular signaling functions (4). MAGuK proteins act to cluster membrane proteins in specialized plasma membrane subdomains (epithelial junctional complexes and neuronal synapses) (55). ZO-1 binds directly to the occludin cytoplasmic domain (27), and ZO-1 clusters occludin in the tight junction (41a).

Paracellular permeability is regulated by intracellular signaling and as a pathophysiological consequence of disease (9, 13, 15, 37, 53). Signaling pathways that affect tight junction function, and how these pathways are affected by pathophysiological events, remain unclear. Both protein tyrosine and serine/threonine phosphorylation mechanisms have been implicated in the regulation of tight junction assembly and paracellular permeability changes. Tyrosine kinase agonists and tyrosine phosphatase inhibitors were shown to affect phosphotyrosine levels in ZO-1 and ZO-2, and this was correlated with altered permeability properties and tight junction component redistribution in epithelial and endothelial cells (56, 59, 63). Protein kinase C activation results in cadherin-independent tight junction assembly and increased barrier function in Madin-Darby canine kidney (MDCK) cell monolayers (10), but protein kinase C activation in established epithelial monolayers increases permeability and inhibits tight junction assembly (19, 45, 37). Epithelial and endothelial cell permeabilities are compromised as a consequence of cell injury. Ischemic events in the kidney...
result in a rapid opening of tubular epithelial cell tight junctions (16, 39, 44). In the brain, injury (for example, stroke or head trauma) that causes cerebral ischemia may lead to disruption of endothelial cell tight junctions (see discussion in Ref. 54). Signaling mechanisms disrupted during ischemia that lead to tight junction dysfunction remain unknown.

Rho is a member of the Ras superfamily of small GTP-binding proteins that switch between GTP-bound (active) and GDP-bound (inactive) conformations (30). Activated (GTP-bound) Rho interacts with a growing list of effector molecules that propagate downstream signaling (3, 34, 50). Switching between GTP- and GDP-bound forms is regulated by GTP hydrolysis and signaling (3, 34, 50). Rho-N19 has preferential affinity for GDP but lacks the capacity to activate downstream targets. Therefore, growing evidence shows that ATP depletion on Rho expression led to accumulation of the tight junction components ZO-1 and occludin in MDCK cell junctional complexes. Analysis of redistribution of tight junction components after ATP depletion on MDCK cells expressing Rho mutant proteins suggests that these treatments affect the same tight junction assembly pathway. To examine the potential mechanisms affected by ATP depletion and Rho signaling that lead to tight junction disassembly, protein phosphorylation of tight junction components was examined. Both ATP depletion and mutant Rho expression in MDCK cells affect phosphorylation of tight junction components. These data suggest that tight junction disassembly in response to ATP depletion results from inactivation of Rho signaling, leading to posttranslational effects on tight junction components and then tight junction disassembly/dysfunction. This model may apply generally to other cellular injury events that lead to cytoskeletal disruption and junctional complex disassembly.

MATERIALS AND METHODS

Cell culture, antibodies, and reagents. 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). Reagents were purchased from Sigma Chemical (St. Louis, MO) or Midwest Scientific (St. Louis, MO) unless otherwise indicated.

Polyclonal antibodies against ZO-1 and occludin were purchased from Zymed (South San Francisco, CA). The ZO-1 hybridoma, R26.4C (6, 57), was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, The University of Iowa (Iowa City, IA) under contract from the National Institute of Child Health and Human Development. Dr. Mark Wagner (Indiana University) provided the hybridoma 9E10 against the Myc epitope. Polyclonal antibodies against ZO-2 were a gift from Drs. Alan Fanning and Jim Anderson (Yale University). Horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibody was purchased by 10.220.33.3 on October 20, 2017 http://ajpcell.physiology.org/ Downloaded from http://ajpcell.physiology.org/
medium. Cells were analyzed at various times, but the experiments shown were analyzed 48 h posttransfection.

ATP depletion. MDCK cells were plated at a density of 2 x 10^4 per 35-mm culture dish. After 72 h, cells were rinsed in prewarmed depletion medium; cells were ATP depleted for different times by incubating cells with depletion medium containing 0.1 μM antimycin A (16). For transfected cells, ATP depletion was performed 48 h posttransfection. ATP levels were assayed as described previously (16).

Immunoprecipitation and immunoblotting. Cells were rinsed with ice-cold PBS (in mM: 2.7 KCl, 1.5 KH₂PO₄, 137 NaCl, 8.1 Na₂HPO₄) and lysed in RIPA buffer (0.1 M NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.05 M Tris, pH 8.0) for 15 min on ice. Cells were scraped, and lysates were collected and cleared by centrifugation in a microcentrifuge at 13,000 rpm for 5 min at 4°C. Primary antibody (anti-ZO-1, Zymed) 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 in lysis buffer. Beads were resuspended in SDS-PAGE sample buffer for analysis and separated on 7.5% SDS polyacrylamide gels.

For immunoblotting total cellular proteins, cells were extracted in hot SDS-containing buffer (1% SDS, 10 mM Tris, pH 7.5, 2 mM EDTA). Cells were scraped, and lysates were collected, heat at 100°C for 5–10 min, and sonicated. Samples were cleared by centrifugation. Protein assays were performed on supernatants using the bichinoninic acid kit (Pierce Chemical, Rockford, IL). Thirty micrograms of samples were separated on 10% SDS polyacrylamide gels.

RESULTS

Rho signaling is required for tight junction assembly in MDCK cells. MDCK cells have been used extensively as a model for a polarized epithelial cell phenotype and to study junctional complex assembly (8, 39, 51) and used to model renal ischemia and recovery in vitro by ATP depletion and repletion (8, 39, 40, 61). We utilized the MDCK cell model system to examine roles of Rho GTPase signaling in tight junction assembly and in ATP depletion-induced tight junction dysfunction. Previous experiments used C3 transferase (a specific inhibitor of Rho GTPase) to show that Rho GTPase regulates tight junction assembly in Caco-2 cells and MDCK cells (46, 58). We also examined the effects of C3 transferase on tight junction assembly in MDCK cells. Bacterially expressed and purified C3 transferase was microinjected into MDCK cells in small colonies, and FITC-conjugated dextran was co-injected for identification of injected cells. Injected cell cultures were incubated for 45 min at 37°C before fixation and processing for immunofluorescence to determine ZO-1 distribution.

ZO-1 staining at junctions, either between pairs of injected cells, between an injected cell and an uninjected cell, or between pairs of uninjected cells, was analyzed by quantitative image analysis (see MATERIALS AND METHODS). In pairs of microinjected cells directly adjacent to one another, C3 transferase results in decreased ZO-1 localization at sites of cell-cell contact to 0.35-fold relative to uninjected cell pairs (differences were significant to P < 0.0001, t-test). In addition, junctions between injected cells and uninjected cells showed decreased ZO-1 localization at sites of cell-cell contact but to a lesser extent than junctions between pairs of injected cells: 0.49-fold relative to uninjected cell pairs (P < 0.0005, t-test). These data are in contrast to
to the conclusions of Takaishi et al. (58), who did not
observe an effect of C3 transferase on tight junctions
between injected cells and uninjected cells. Our data
suggest that Rho family GTPase signalling is required
for normal tight junction maintenance in MDCK cells
and that Rho function is required in both cells that
contribute to the junction.

To further investigate the effects of Rho on tight
junction assembly, transient transfection was per-
formed with plasmids encoding dominant negative Rho
GTPase mutant (Rho-N19) and dominant active Rho
GTPase mutant (Rho-V14) proteins. Both these con-
structs contain a Myc epitope tag engineered at the
amino terminus that allowed detection of the exog-
eous proteins and identification of transfected cells
using a monoclonal antibody (9E10). Subconfluent
MDCK cell cultures were transfected, and mutant Rho
expression was examined at various times following
transfection. Transfected cell cultures achieved conflu-
ence, usually by 24 h posttransfection. Peak expression
was observed at 48 h posttransfection, and this time
point was used for all experiments reported here.
However, effects of mutant Rho GTPases were observed
at earlier and later time points (data not shown).

MDCK cells transfected with mutant Rho proteins
were fixed and processed for double-label immunofluo-
rescence to detect Myc-tagged Rho proteins and ZO-1 or
occludin. Again, a z-series of x-y images through the
entire volume of the cells was collected using a laser
scanning confocal microscope and combined, to avoid
missing fluorescence from other focal planes. Levels of
fluorescence for tight junction components ZO-1 and
occludin were reduced at junctions between Rho-N19-
transfected cells, relative to junctions between untrans-
fected cells (Fig. 1; see also Figs. 2 and 3). As with C3
transferase-mediated Rho inhibition, dominant nega-
tive Rho expression in MDCK cells inhibited tight
junction assembly. Expression of dominant active Rho-
V14 showed an opposite effect on tight junction assem-
bly to that of Rho-N19. Rho-V14 expression resulted in
alterations in tight junction structure. However, with
the consideration that tight junction effects precede
effects on adherens junctions, these data suggest that
Rho signaling also affects tight junctions independently
effects on adherens junctions.

Effect of ATP depletion on tight junction assembly in
MDCK cells expressing Rho mutant proteins. Next, we
tested whether Rho GTPase signaling affected tight
junction assembly in the model system for renal ische-
emia, ATP depletion of MDCK cell monolayers. ATP
depletion was accomplished by incubating cells with
antimycin A, a reversible inhibitor of cytochrome reduc-
tive electron transport, which caused ATP levels to drop
rapidly to <5% of control within 15 min following
antimycin A treatment. Nearly all cells survive and
recover from this injury, and ATP levels recover to
~50% of control levels 60 min after cells are returned
to normal growth medium. The effects of ATP depletion
on cultured epithelial cells strongly resemble the conse-
quences for tubular epithelial cells during renal ische-
mia in vivo, in which ATP levels also decrease due to
restricted perfusion in tissues. Both ATP depletion in

![Fig. 1](http://ajpcell.physiology.org/)

**Fig. 1.** Distribution of tight junction components in
cells expressing mutant Rho GTPase proteins. Madin-Darby canine kidney (MDCK) cells were transiently
transfected with Myc-tagged dominant negative Rho-
N19 or dominant active Rho-V14 (arrowheads). At 48
h posttransfection, cells were fixed and processed for
double-label, indirect immunofluorescence using zonula
occludens-1 (ZO-1) antibodies (A) or occludin antibodies
(B). Mutant Rho GTPase expressing cells were
observed using anti-Myc tag antibodies (Myc).
Each image shown is 106.75 µm². Data shown are
representative of 8 independent experiments.
vitro and renal ischemia in vivo cause the disassembly of tight junctions (16, 23, 39, 44).

To test the effect of Rho GTPase signaling during ATP depletion, subconfluent MDCK cell cultures were transfected with constructs encoding mutant Rho GTPases, and, at 48 h posttransfection, these MDCK cell monolayers were ATP depleted for 60 min or untreated in controls. Cells were then fixed and processed for double-label immunofluorescence to detect Myc-tagged Rho proteins and ZO-1 or occludin. Effects of Rho GTPase signaling and ATP depletion were assayed using confocal microscopy and quantitative image analysis. Fluorescence intensity per unit length was measured using MetaMorph image analysis software. To determine the consequences of expressing Rho mutant proteins, a fluorescence intensity ratio of the fluorescence intensity per unit length in junctions between two transfected cells divided by the fluorescence intensity per unit length in junctions between two untransfected cells was calculated. This ratio expresses the magnitude of decrease or increase in fluorescence intensity per unit length as a consequence of mutant Rho protein expression. This ratio also allows us to distinguish relative changes in fluorescence after ATP depletion because the ratio is internally normalized to untransfected cell-cell junctions.

MDCK cells expressing Rho-N19 that were ATP depleted for 60 min showed a more dramatic decrease in tight junction assembly compared with parallel cultures of MDCK cells expressing Rho-N19 that were not subjected to ATP depletion (Figs. 2 and 3). The ratio of fluorescence intensity for junctions between Rho-N19 transfected cell pairs divided by untransfected cell pairs stained for ZO-1 was reduced from 0.27 ± 0.05 (SD) in control cultures to 0.09 ± 0.02 (SD) in parallel cultures that were ATP depleted for 60 min (Fig. 2B). For occludin, the ratio of fluorescence intensity for junctions between Rho-N19 transfected cell pairs divided by untransfected cell pairs was reduced from 0.40 ± 0.12 (SD) in control cultures to 0.17 ± 0.01 (SD) in parallel cultures that were ATP depleted for 60 min (Fig. 3B), similar to that for ZO-1. Because it has been previously shown that the tight junction structure was disrupted by ATP depletion over a 60-min time course (B), the decreased fluorescence intensity ratio we observed in cells expressing Rho-N19 relative to cells not ATP depleted probably represents acceleration of disassembly mechanisms resulting from Rho signaling inhibition.

MDCK cells expressing dominant active Rho-V14 were ATP depleted for 60 min or untreated in controls, and quantitative image analysis was performed on confocal, extended focus images of double-label immunofluorescence images stained to detect Myc-tagged Rho proteins and tight junction component proteins (Figs. 4A and 5A). Rho-V14 expression in MDCK cells protected tight junctions from disassembly during ATP depletion. The ratio of fluorescence intensity for junctions between Rho-V14-transfected cell pairs divided by untransfected cell pairs stained for ZO-1 was increased from 7.9 ± 1.0 (SD) in control cultures to 15.3 ± 1.8 (SD) in parallel cultures that were ATP depleted for 60 min (Fig. 4B). The ratio of fluorescence intensity for occludin staining in cell-to-cell contacts between Rho-V14-transfected cell pairs divided by untransfected cell pairs was increased from 4.6 ± 1.7 (SD) in control cultures to 16.3 ± 2.2 (SD) in parallel cultures that were ATP depleted for 60 min (Fig. 5B). This fluorescence intensity ratio increase in cells expressing Rho-V14 that were subjected to ATP depletion suggests that
Rho-V14 expression inhibited the injury-induced disassembly mechanisms, thereby protecting cells from injury to tight junctions.

Protein phosphorylation pathways for tight junction components are disrupted by ATP depletion in MDCK cells. Both ATP depletion and Rho GTPase signaling affect tight junction assembly (16, 23, 39, 44, 46, 58), and data presented above suggest that Rho-mediated signaling mechanisms are affected by ATP depletion. Rho effectors are being identified and characterized, and protein phosphorylation mechanisms are common among these pathways (3, 34, 50). ATP depletion down-regulates protein phosphorylation signaling mechanisms (35). We tested whether alterations in phosphorylation states for ZO-1 and occludin accompany changes in tight junction assembly that were observed in re-

Fig. 3. Effect of ATP depletion on distribution of occludin in cells expressing dominant negative Rho GTPase mutant protein. MDCK cells were transiently transfected with Myc-tagged dominant negative Rho [Rho-N19 (DN), arrows]. At 48 h posttransfection, cells were ATP depleted for 60 min or left untreated (control). A: cells were processed for double-label, indirect immunofluorescence using occludin antibodies, and mutant Rho GTPase expressing cells were detected using anti-Myc antibodies. Each image shown is 179.2 µm². B: mean fluorescence intensity was calculated for junctions between pairs of mutant Rho GTPase expressing cells (transfected) and between pairs of untransfected cells from the same experiment. Ratio decreased from 0.40 for Rho-N19 alone (n = 20) to 0.17 for Rho-N19 with 60-min ATP depletion (n = 20) (P = 3.3 × 10⁻⁸, t-test). Dashed line shows mean fluorescence intensity ratio in untransfected junctions for reference. Data shown are representative of 3 independent experiments.

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Fig. 4. Effect of ATP depletion on distribution of ZO-1 in cells expressing dominant active Rho GTPase mutant protein. MDCK cells were transiently transfected with Myc-tagged dominant active Rho [Rho-V14 (DA), arrows]. At 48 h posttransfection, cells were ATP depleted for 60 min or left untreated (control). A: cells were processed for double-label, indirect immunofluorescence using ZO-1 antibodies, and mutant Rho GTPase expressing cells were detected using anti-Myc antibodies. Each image shown is 179.2 µm². B: mean fluorescence intensity was calculated for junctions between pairs of mutant Rho GTPase expressing cells (transfected) and between pairs of untransfected cells from the same experiment. Ratio increased from 7.9 for Rho-V14 alone (n = 25) to 15.3 for Rho-V14 with 60-min ATP depletion (n = 25) (P = 4.1 × 10⁻⁹, t-test). Dashed line shows mean fluorescence intensity ratio in untransfected junctions for reference. Data shown are representative of 4 independent experiments.
sponse to altered Rho signaling and ATP depletion. ZO-1 is reportedly serine/threonine and tyrosine phosphorylated (6, 56), and occludin was shown to be serine/threonine phosphorylated (52). Tyrosine phosphorylation of ZO-1 and serine/threonine phosphorylation of occludin correlate with tight junction assembly and function (52, 56, 59).

Confluent MDCK cell monolayers were ATP depleted and allowed to recover in normal growth medium. At various times, cells were assayed for protein phosphorylation changes. ZO-1 was immunoprecipitated from cell extracts. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted to detect phosphotyrosine or blotted to detect ZO-1 to show that comparable amounts of ZO-1 were immunoprecipitated (Fig. 6). A second protein of ~160 kDa was coimmunoprecipitated with ZO-1 and detected with the anti-phosphotyrosine monoclonal antibody (Fig. 6). Immunoblotting our ZO-1 immunoprecipitates with specific antibodies to ZO-2 (a 160-kDa protein that is structurally related to ZO-1, binds directly to, and coimmunoprecipitates with ZO-1) (32) showed that the coimmunoprecipitating protein was recognized by these antibodies (data not shown). ZO-1 and ZO-2 tyrosine phosphorylation decreased rapidly during ATP depletion. Within 30 min, phosphotyrosine content in ZO-1 and ZO-2 was nearly undetectable (Fig. 6).

We also examined whether phosphotyrosine content in ZO-1 returned during recovery and whether duration of ATP depletion affected the extent of ZO-1 phosphotyrosine recovery. MDCK cells were ATP depleted for 30 or 60 min, and cells subjected to injury for these different times were allowed to recover for 60 min. Both 30 and 60 min of injury were sufficient to reduce ZO-1 and ZO-2 phosphotyrosine levels to undetectable levels (Fig. 6B), as expected from the earlier time course experiment (Fig. 6A). However, the recovery of ZO-1 and ZO-2 phosphotyrosine levels after 60

Fig. 5. Effect of ATP depletion on distribution of occludin in cells expressing dominant active Rho GTPase mutant protein. MDCK cells were transiently transfected with Myc-tagged dominant active Rho (Rho-V14, arrows). At 48 h posttransfection, cells were ATP depleted for 60 min or left untreated (control). A: cells were processed for double-label, indirect immunofluorescence using occludin antibodies, and mutant Rho GTPase expressing cells were detected using anti-Myc tag antibodies. Each image shown is 179.2 µm². B: mean fluorescence intensity was calculated for junctions between pairs of mutant Rho GTPase expressing cells (transfected) and between pairs of untransfected cells from the same experiment. Ratio increased from 4.6 for Rho-V14 alone (n = 25) to 16.3 for Rho-V14 with 60-min ATP depletion (n = 25) (P = 8.1 × 10⁻⁸, t-test). Dashed line shows mean fluorescence intensity ratio in untransfected junctions for reference. Data shown are representative of 3 independent experiments.

Fig. 6. Effect of ATP depletion and recovery on phosphotyrosine content of ZO-1 in MDCK cells. Confluent MDCK cells were ATP depleted for the indicated times, and ZO-1 was immunoprecipitated from cell extracts. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect phosphotyrosine (top). A: short time course of ATP depletion, from 5 min (5') to 25 min (25'). B: MDCK cells were untreated (control) or ATP depleted for 30 min (30') or 60 min (60'), and then cells were allowed to recover from 30- and 60-min injuries for 60 min (30'/60' and 60'/60'). Arrows indicate ZO-1 and arrowheads indicate the coimmunoprecipitating protein identified as ZO-2. Data shown are representative of 3 independent experiments.
We next tested the effect of injury duration on recovery of serine/threonine phosphorylation occludin variants. MDCK cells were ATP depleted for 30 or 60 min and then allowed to recover for 60 min. Again, both 30 and 60 min of injury showed significant reduction in serine/threonine phosphorylated occludin isoforms (Fig. 7B). The extent that serine/threonine-phosphorylated occludin isoforms returned was less for cells subjected to 60 min of ATP depletion compared with cells subjected to 30 min of ATP depletion (Fig. 7B), suggesting that recovery of serine/threonine phosphorylation isoforms of occludin was affected by duration of the initial injury.

Protein phosphorylation pathways for occludin are affected by Rho signaling in MDCK cells. Quantitative image analysis showed that Rho GTPase signaling mechanisms and ATP depletion act together to affect tight junction assembly. ATP depletion also led to rapid reductions in phosphorylation of ZO-1 and occludin. Based on our hypothesis that Rho signaling is inhibited during ATP depletion, we would predict that Rho GTPase inhibition would affect phosphorylation of ZO-1, occludin, or both. To test our prediction, subconfluent MDCK cell cultures were transiently transfected with plasmids encoding Rho mutant proteins. For these experiments, transfection efficiency was optimized to detect the effects of Rho signaling on tight junction components biochemically; we achieved ~50% transfection. Phosphorylation status for tight junction components in transfected cell monolayers was examined at 48 h posttransfection using methods described for Figs. 6 and 7.

ZO-1 tyrosine phosphorylation was not affected in cells expressing mutant Rho proteins (data not shown). At 48 h posttransfection with plasmids encoding Rho-V14, Rho-N19, or vector alone, cell extracts were immunoprecipitated using antibodies specific for ZO-1. These immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect phosphotyrosine and to detect ZO-1. Expressing Rho-V14 or Rho-N19 did not reproducibly change phosphotyrosine levels in ZO-1 relative to vector-transfected cells, suggesting that Rho signaling does not affect ZO-1 tyrosine phosphorylation. It is possible that effects were transient or not sufficiently robust to be detected in our system.

Levels of serine/threonine phosphorylation variants of occludin were affected by expressing mutant Rho protein in MDCK cells. Cells were transfected with vector and Rho-V14 and Rho-N19 expression plasmid constructs. Cell extracts were prepared 48 h posttransfection. Equal amounts of protein from these extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect occludin. Expression of Rho-N19 in MDCK cells caused a reduction (0.42-fold relative to vector-transfected control cells per unit protein) of slower-migrating, more highly phosphorylated occludin isoforms (Fig. 8). This suggests that reductions in occludin phosphorylation during ATP depletion are a result of inhibiting Rho GTPase signaling mechanisms. In addition, Rho-V14 expression in

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**Fig. 7. Effect of ATP depletion and recovery on the presence of phosphoserine/phosphothreonine occludin isoforms in MDCK cells.**

**A.** ATP depletion results in decreased levels of slower-migrating, more highly serine/threonine phosphorylated isoforms of occludin in MDCK cells. Confluent MDCK cells were untreated (control) or ATP depleted for 5 to 30 min (5–30). Thirty micrograms of total protein from control or treated cell extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect occludin. Shown are representative of 3 independent experiments.

**B.** Recovery of serine/threonine phosphorylation of occludin in MDCK cells on ATP repletion. Confluent MDCK cells were untreated (control) or ATP depleted for 30 min (30') or 60 min (60'), and cells were then allowed to recover from 30- and 60-min injuries for 60 min (30'/60' and 60'/60'). Thirty micrograms of total protein from each sample were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect occludin. Arrows indicate the fastest-migrating, underphosphorylated occludin isoform, and brackets indicate slower-migrating, more highly phosphorylated occludin isoforms. Data shown are representative of 3 independent experiments.
signaling activity affects myosin light chain phosphorylation via protein kinases (50). In particular, Rho GTPase signaling affects tight junction assembly via specific occludin phosphorylation. These data indicate that Rho signaling stimulates tight junction assembly and increased levels of tight junction assembly and reduced levels of Rho-mediated tight junction disassembly. We show that inhibiting Rho signaling decreases tight junction assembly. Our results and those of others (46, 58) demonstrated that Rho GTPase signaling during cellular injury and recovery. 

Evidence indicates that Rho signaling regulates tight junction assembly in epithelial cells (14, 46, 58) and Rac also regulates adherens junction assembly (14, 31, 58). In this study, effects of Rho GTPase signaling on tight junction assembly were examined in normal MDCK cells and during ATP depletion processes (ATP depletion, a model of renal ischemia/acute renal failure). Our results suggest that Rho regulates tight junction assembly by affecting occludin protein phosphorylation states and that Rho-mediated tight junction assembly mechanisms are disrupted during ATP depletion. These studies provide novel insights into Rho GTPase actions in cell-cell junctional complex assembly and the role of Rho family GTPase signaling during cellular injury and recovery.

Rho signaling and mechanisms for tight junction regulation. Our results and those of others (46, 58) show that tight junctions are regulated by Rho GTPase signaling. We show that inhibiting Rho signaling decreased tight junction assembly and reduced levels of occludin phosphorylation, and activating Rho signaling stimulates tight junction assembly and increased levels of occludin phosphorylation. These data indicate that Rho signaling affects tight junction assembly via specific protein phosphorylation mechanisms.

Rho GTPase signaling mechanisms are generally mediated by protein kinases (50). In particular, Rho signaling activity affects myosin light chain phosphorylation. The Rho effector, Rho kinase, affects myosin light chain phosphorylation and, activation of Rho increases myosin activity (2, 3, 34). Effects on myosin light chain phosphorylation are well-characterized actions of Rho signaling, but Rho signaling mechanisms affect other kinase substrates (50). How Rho signaling pathways regulate tight junction component phosphorylation is unclear. Further experimentation will be necessary to define Rho signaling pathways that lead to altered tight junction assembly.

An alternative mechanism for Rho effects on tight junction assembly may be indirect, via Rho effects on cadherin-mediated adhesion. Tight junction assembly in epithelial cells requires cadherin function (29, 64). Blocking Rho or Rac function in keratinocytes inhibited cadherin-mediated adherens junction assembly (14, 31, 58). The effects of inhibiting Rho signaling on cadherin function may lead to disassembly of tight junctions in MDCK cells. Activated Rho did not seem to increase adherens junction assembly in keratinocytes (14), and consequences of strengthened cadherin-mediated adhesion for tight junction assembly are not well characterized. Like Madara and colleagues (46), we did not observe redistribution of E-cadherin as a consequence of short-term effects of inhibiting Rho signaling (33, 44). Blocking Rho or Rac function in keratinocytes inhibited cadherin-mediated adhesion. Tight junction assembly may be indirect, via Rho effects on actin cytoskeleton rearrangements.

ATP depletion causes tight junction disassembly, perhaps by inhibiting Rho. Renal ischemia is a consequence of renal injury and numerous renal diseases, resulting in a rapid decrease in cellular ATP levels (60). ATP-depleting epithelial cells in tissue culture are an in vitro model of renal ischemia; ATP levels recover rapidly following removal of the drug, and, with time, cells recover a normal epithelial phenotype (23). This model recapitulates numerous cellular features of renal ischemia in vivo that have been documented in animal models and human patients, including the effects on actin cytoskeleton, cell polarity, and junctional complexes (1, 8, 16, 23, 39, 40, 42–44). With the use of this model system to study the role of Rho GTPase signaling on tight junction disassembly during ATP depletion, our studies support the idea that Rho GTPase signaling was inhibited during ATP depletion. Furthermore, Rho signaling mechanisms protect tight junctions from injury in epithelial cells.

 Renal ischemia in vivo and ATP depletion of cultured epithelial cells result in a rapid breakdown of the tight junction permeability barrier (16, 39, 44). ATP depletion of MDCK cells resulted in paracellular permeability barrier dysfunction within 10 min, but the lateral diffusion barrier function of the tight junction was not compromised until later times (8, 39). Also, with longer times of ATP depletion, ZO-1 distribution and freeze-fracture tight junction ultrastructure were disrupted (8). These data suggest that ATP depletion rapidly...
disrupts signaling processes that result in paracellular barrier dysfunction, before dramatic structural effects on tight junctions, which occur later. Structural changes in tight junctions following ATP depletion are also subsequent to major rearrangements of the actin cytoskeleton, where normal actin structures disassemble and filamentous actin accumulates in perinuclear aggregates (8). Peripheral tight junction components (ZO-1, ZO-2, and cingulin) are redistributed to the cytoplasm and accumulate in large-molecular-weight complexes containing actin and fodrin with extended times of ATP depletion (61). We propose that a key event during the rearrangement of the tight junction is inhibition of Rho GTPase, triggering disassembly and dysfunction.

Tight junction disassembly during ATP depletion was more dramatic in MDCK cells expressing dominant negative Rho mutant proteins. We observed that tight junction components were rapidly dephosphorylated during ATP depletion. Consistent with our hypothesis that Rho signaling is inhibited during ATP depletion, we found that levels of occludin phosphorylation were reduced in MDCK cells expressing dominant negative Rho relative to vector transfected cells. These data suggest that inhibition of Rho during ATP depletion leads to dephosphorylation of occludin and subsequent disassembly and dysfunction of the tight junction. The observation that constitutively active Rho (Rho-V14) partially rescues cells from tight junction disassembly during ATP depletion strongly suggests that Rho signaling provides a specific protective mechanism from cellular injury. Occludin phosphorylation may be the target of this protective signaling pathway.

Rho GTPase signaling may regulate junctional complex assembly in a variety of situations, not limited to cellular injury. Normal physiological responses lead to changes in tight junction permeability (36, 37, 49, 62). Future studies should address whether Rho GTPase signaling regulates tight junction permeability during these physiological events. Protection of tight junctions from disassembly during ATP depletion by Rho GTPase signaling reveals novel signaling events that are disrupted during renal ischemia, leading to pathophysiological consequences in this condition. Rho GTPase activation also protects cellular actin structures from disassembly during ATP depletion (Raman and Atkinson, unpublished observations), suggesting that protective effects of Rho signaling for epithelial cells are more widespread. Other Rho family GTPases may be inactivated during ATP depletion, and their effects will also require study. Analysis of Rho family GTPase signaling will provide a more complete understanding of cellular events that lead to rearrangement of the actin and junctional complexes.

We thank Dwarns, Marc Symons (Onyx Pharmaceuticals) for providing mutant Rho expression plasmids and Alan Hall (MRC, University College, London) for bacteria expressing C3 transferase. Thanks to Drs. James Anderson and Alan Fanning (Yale University) for providing anti-ZO-2 antibodies and Dr. Mark Wagner for the 9E10 hybridoma. We thank Dr. Ken Dunn and Paul Brown (Indiana University, Renal Epithelial Biology Laboratory Imaging Facility) for providing expert assistance with confocal microscopy, image analysis, and statistical analysis. We are grateful to Bill Mokanyk and Matt Muterspaugh for technical assistance.

This work was supported by a fellowship from the American Heart Association, Indiana Affiliate (S. Gopalakrishnan), an award from the Showalter Research Trust Fund (S. J. Atkinson and J. A. Marrs), and National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-54518 (J. A. Marrs).

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Received 24 March 1998; accepted in final form 8 June 1998.

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