Tight Junction Proteins Form Large Complexes and Associate with the Cytoskeleton in an ATP Depletion Model for Reversible Junction Assembly*

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Tatsuo Tsukamoto‡ and Sanjay K. Nigam§

From the Renal Division, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115

A key feature of the ischemic epithelial cell phenotype is the disruption of tight junctions (TJ). In a Madin-Darby canine kidney cell model for ischemia-reperfusion/hypoxia-reoxygenation injury which employs inhibitors of glycolysis (2-deoxy-D-glucose) and oxidative phosphorylation (antimycin A), transepithelial electrical resistance, a measure of TJ integrity, dropped rapidly, correlating well with declining ATP levels. Although immunocytochemical studies revealed only subtle changes in the distribution of the TJ proteins, zonula occludens (ZO)-1, ZO-2, and cingulin, examination of the Triton X-100 solubilities of these proteins, an indicator of cytoskeletal association, revealed a striking shift of all three TJ proteins into the insoluble pool, consistent with increased cytoskeletal interaction during ATP depletion. In addition, rate-zonal centrifugation analysis of a detergent-soluble fraction showed an increase in the amount of ZO-1 and ZO-2 in high density fractions following ATP depletion, providing further evidence for association of TJ proteins into a large complex possibly involving the cytoskeleton. Analysis of immunoprecipitation data from [35S]methionine-labeled cells revealed that ATP depletion led to the association of a 240-kDa protein with the ZO-1-containing complex. Western blots of this protein immunoprecipitated with anti-ZO-1 antibodies confirmed its identity as fodrin, a protein believed to link membrane and other proteins to the cytoskeleton. Together, our data suggest that in the absence of major immunocytochemical changes, ATP depletion leads TJ proteins to form large insoluble complexes and associate with the cytoskeleton. We propose a model in which a key, potentially regulated, step in the generation of the ischemic epithelial cell phenotype is the interaction between TJ proteins and fodrin and/or other cytoskeletal proteins.

The epithelial intercellular permeability barrier is maintained largely by the tight junction (TJ)‡ (1). The TJ, the most apical of intercellular junctions, consists of a number of proteins, including ZO-1, ZO-2, occludin, cingulin, 7H6, p130, and potentially other proteins (2–9). Considerable indirect evidence suggests that proteins of the TJ are intimately associated with the actin-based cytoskeleton (10–12).

Ischemia and subsequent reperfusion/reoxygenation causes a number of lesions in epithelial cells including mispolarization of at least some membrane proteins, perturbation of the actin cytoskeleton, and disruption of the permeability barrier (13, 14). These lesions have been reproduced in cell culture models for hypoxia-reoxygenation injury using agents that deplete cellular ATP, which has allowed for the analysis of molecular mechanisms underlying ischemic injury (15, 16). Although mechanistic insights into the disruption of the actin-based cytoskeleton are beginning to emerge, little is known about the biochemical basis of the disruption of the TJ after ischemic insult or how the TJ reassembles during recovery of epithelial cells from ischemic injury.

The biochemical basis of the disassembly and reassembly of the TJ has, however, been studied in MDCK cells in a model in which extracellular calcium is manipulated: the “calcium switch” (8, 17–20). When the TJ disassembles in this model under low calcium conditions, transepithelial electrical resistance (TER) is lost, and TJ proteins internalize or are diffusely distributed near the cell surface. In this model, TJ proteins become more extractable with detergent-salt solutions, suggesting a weakening of interactions with the cytoskeleton, and ZO-1, ZO-2, and p130 are found in a complex which sometimes contains other phosphoproteins (8). When extracellular calcium is raised, reassembly of the TJ appears to proceed by classical signaling pathways involving a heterotrimeric G protein, regulated intracellular calcium stores (18–21), and protein kinase C (8). TJ proteins resort to the apico-lateral surface of the plasma membrane, TER develops, and TJ proteins become more resistant to detergent-salt extractions (8, 18, 19).

Superficially, what is known about the behavior of the TJ in MDCK monolayers after ATP depletion and repletion resembles the disassembly of the TJ in low calcium conditions and its reassembly when external calcium is raised. However, in cell culture models for hypoxia-reoxygenation, these issues have not been examined in the same biochemical detail as with low extracellular calcium and the calcium switch. Using physiological, immunocytochemical, and biochemical techniques, we have now explored the behavior of TJ proteins in a MDCK cell model for hypoxia-reoxygenation which employs antimycin A and 2-deoxy-D-glucose (15, 22) and compared this model with dens 1; ZO-2, zonula occludens 2; MDCK, Madin-Darby canine kidney; TER, transepithelial electrical resistance; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; PAGE, polyacrylamide gel electrophoresis.
the calcium switch model. We demonstrate that the biochemical changes observed during TJ disassembly after ATP depletion are very different from those observed when cells are subjected to low calcium conditions. In this model, TJ disassembly is accompanied by the association of TJ proteins into very large complexes, movement of at least three TJ proteins into an insoluble pool, and an increased association of the ZO-1-containing complex with the membrane protein-anchoring cytoskeletal protein, fodrin. Our results provide insights into the molecular pathogenesis of epithelial dysfunction resulting from ischemic insult.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—MDCK cells were purchased from ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Basal TER of confluent monolayer varied between 500 and 800 ohms-cm². Cells were incubated at 37 °C in an air, 5% CO₂ atmosphere and were passaged every week after incubation in phosphate-buffered saline (PBS) and light trypsinization. Cell culture media were obtained from Life Technologies, Inc. Plasticware was from Falcon (Lincoln Park, NJ) except that Transwells were obtained from Costar (Cambridge, MA), and 0.4% low molecular weight (0.15 mol/l NaCl, MA). R40.76 ZO-1 hybridoma and anti-ZO-2 antibody were kindly provided by D. Goodenough (Harvard University), anti-cingulin antibody was from S. Citi (Cornell University Medical College), anti-Na⁺-K⁺-ATPase antibody was from J. Lytton (University of Calgary), and anti-fodrin antibody was from J. H. Hartwig (Harvard). All other reagents used in these experiments were of analytical grade.

ATP Depletion—Depletion of ATP was achieved rapidly in MDCK cells by using a combination of glycolytic (2-deoxy-o-glucose; Sigma) and oxidative (antimycin A; Sigma) inhibitors, as described previously (15, 22, 23). In brief, confluent monolayers were washed with PBS three times, then exposed to Dulbecco’s PBS containing 1.5 mM CaCl₂, 2 mM MgCl₂, 2 mM deoxy-o-glucose, and 10 μM antimycin A for various times. Samples designated as control were obtained from cultures grown in standard growth medium. Experiments were also performed with 1 μM antimycin A and 2 mM deoxy-o-glucose; the results were similar but had greater variability.

TER—MDCK cells were plated at confluent density (2 × 10⁵ cells/cm²) on polycarbonate filters (Transwells) and allowed to establish tight monolayers over 48 h before ATP depletion with metabolic inhibitors. TER measurements were performed by light microscopy (19) for light microscopy (19).

ATP Measurement—ATP measurements were performed using a luciferase-based ATP determination kit (Sigma). Briefly, after rinsing three times with PBS, filters were excised, and the cells were solubilized in 300 μl of buffer (sonic cell ATP-releasing agent) from Sigma. Samples were cleared of insoluble material by spinning for 5 min in a microcentrifuge. Determination of ATP levels was accomplished by combining equal volumes of supernatant with an ATP assay mix (Sigma) and measuring the level of chemiluminescence. Measurements are expressed as a percent of the initial value after subtraction of background readings.

Immunocytochemistry—Confluent monolayers of MDCK cells on glass coverslips were washed three times in PBS and incubated in the ATP depletion buffer. Cells were then rinsed twice in PBS, fixed in −80 °C methanol (Z0-1, Z0-2, cingulin, and Na⁺/K⁺-ATPase), or 2% paraformaldehyde (fodrin and phallolidin), and permeabilized in PBS, 0.05% saponin (PBS-S). Cells were then incubated for 1 h with antibodies. After washing, the coverslip were incubated in tetramethylrhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate-conjugated secondary antibody and mounted in gelvatol (16% polyvinyl alcohol, 40 mg/ml Tris-HCl, pH 8.0, in 60% v/v glycerol). Phallolidin staining of actin filaments was accomplished by incubation of separate 2% paraformaldehyde-fixed coverslips in 0.01 mg/ml TRITC-phallolidin in PBS-S for 30 min. Coverslips were viewed with a laser scanning confocal system (Bio-Rad MRC 600) coupled to a Zeiss Axioskop microscope through a 100 × oil immersion objective. Images were processed using Photoshop software (Adobe, CA) and photographed from the monitor screen (8, 18, 19).

Light Microscopy of Transverse Sections—MDCK cells plated onto 12-mm Transwells at confluence were treated with ATP depletion buffer, as described above. At the end of the treatment, the cells at

Western Immunoblot Analysis—For Western immunoblot analysis, electrophoresed proteins were transferred to nitrocellulose filters (MSI, Westboro, MA) by electroblotting. Membranes were incubated with primary antibodies for 2 h at room temperature. After washing, immunoblots were developed using the SuperSignal™ CL-horseradish peroxidase substrate system (Pierce) with horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratories, West Grove, PA) (19, 24).

ATP Depletion Leads to a Reversible Decrease of TER in MDCK Cell Monolayers—The combination of antimycin A and 2-deoxy-o-glucose has been widely employed as a method for inducing rapid ATP depletion by inhibition of both oxidative phosphorylation and glycolysis. Although this method may also alter the cellular redox state because of increased H₂O₂ production (26), it remains nonetheless an excellent model for hypoxia-reoxygenation injury, where both ATP depletion and H₂O₂ production appear to play key roles. In our studies, treat-
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Fig. 1. Panel A, effect of metabolic inhibition (2 mM 2-deoxy-D-glucose and 10 μM antimycin A) on TER in MDCK monolayers grown on polycarbonate filters (Transwell). Inset, closed circles and solid line indicate values during metabolic inhibition; and open circles and dashed line indicate values during recovery in normal growth media after 1 or 3 h of ATP depletion. Panel B, intracellular ATP values during metabolic inhibition with 2 mM 2-deoxy-D-glucose and 10 μM antimycin A. After TER measurements, the cells on filters were solubilized in an ATP-releasing buffer (Sigma). Samples were evaluated with an ATP assay kit (Sigma) followed by scintillation counting of their chemical luminescences. Inset, closed circles and solid line indicate values after metabolic inhibition; and open circles and dashed line indicate values after recovery. Measurements are expressed (mean ± S.D.) as a percent of initial values after subtraction of backgrounds.

Several TJ Proteins Remain Colocalized at Junctional Sites after ATP Depletion, although Subtle Immunocytochemical Alterations Are Present—To date, immunocytochemical analysis of specific TJ proteins during ischemia has been limited to ZO-1. Therefore, the effects of treatment with metabolic inhibitors on the immunolocalization of two other TJ proteins, ZO-2 and cingulin, in addition to ZO-1, were studied. Immunocytochemical analyses revealed that ZO-1, ZO-2, and cingulin were still colocalized to the lateral surface of the plasma membrane in MDCK monolayers even after 3 h of ATP depletion (Fig. 2). However, slight alterations in the staining pattern of each protein were evident; most impressive was the loss of diffuse intracellular staining of ZO-2 and cingulin (Fig. 2, E, F, H, and I) (5). Moreover, after 6 h of ATP depletion, a time at which recovery is apparently impossible (data not shown), the immunocytochemical changes were quite dramatic. In particular, a striking loss of cell-cell contact was observed, evidenced by the retraction of cells from each other with an apparent loss of junctional contacts (Fig. 2, C, F, and I). Occasional discontinuities in the linear staining pattern of ZO-1 near the cell surface were also observed after 3 h of ATP, although they were not as pronounced as reported previously, probably because of differences in the method of ATP depletion (27). In addition, microscopic examination of intact monolayers as well as transverse sections of MDCK cells grown on filters after 3 h of ATP depletion revealed some alterations in cell shape consistent with disruption of the actin-based cytoskeleton, but cell contacts appeared to be intact throughout (Fig. 3). Together, these data suggest subtle alterations in the distribution of ZO-1 and two TJ proteins heretofore unexamined in the context of ischemia-reoxygenation, ZO-2 and cingulin. In contrast, immunofluorescent localization of Na+,K+-ATPase after ATP depletion revealed a significant increase in non-basolateral staining (Fig. 4, A–C), as reported previously (28, 29).

Since TJ proteins are believed to be closely associated with the actin-cytoskeleton, which is known to be disrupted rapidly by ATP depletion, we examined the effects of ATP depletion on this structure by TRITC-phalloidin. This histocytochemical marker of the actin-cytoskeleton revealed subtle changes of the cortical actin-ring in cells and some nuclear staining of actin after 3 h of ATP depletion; however, stress fibers were markedly disrupted as has been reported (Fig. 4, D–I) (27, 30). After 6 h of ATP depletion, a retraction of cortical actin at the lateral membrane was observed (Fig. 4F).

ATP Depletion Causes TJ Proteins to Fractionate into an Insoluble Pool—Based on the premise that a drop in TER and qualitative changes in TJ protein staining patterns are likely to represent important alterations in the association among TJ proteins and the actin-based cytoskeleton after ATP depletion, we next sought to address this question biochemically. Since detergent extractability is an established biochemical means for analyzing protein-cytoskeletal interactions (8, 18, 19, 31), we examined the Triton X-100 solubility properties of TJ proteins (ZO-1, ZO-2, and cingulin) after metabolic inhibitor treatment. As shown in Fig. 5A, ZO-1 as well as ZO-2 and cingulin became more insoluble after ATP depletion in a time-depend-
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ent manner. Densitometric analyses of the blots indicated that TJ proteins appeared to move independently into insoluble fractions after ATP depletion (Fig. 5B). To examine the effect of ionic strength on extractabilities of three TJ proteins, we varied NaCl in the CSK-1 buffer (see “Experimental Procedures”) from zero to 1 M and compared extractabilities of TJ proteins after ATP depletion. As shown in Fig. 6, after ATP depletion the three TJ proteins could not be completely solubilized even in CSK buffer containing high concentrations of NaCl (~40% of ZO-1 and ~20% of ZO-2 and cingulin remaining in the pellet), whereas the three TJ proteins were almost completely extractable in control (untreated) MDCK cells. Furthermore, when we used a detergent-rich buffer containing 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS to extract TJ proteins, ZO-1 as well as ZO-2 and cingulin remained partly insoluble (~50% of ZO-1, and ~30% of ZO-2 and cingulin) after 3 h of ATP depletion, although these TJ proteins were almost

FIG. 3. Assessment of cell-cell contact in MDCK cell monolayers after 3 h of ATP depletion (when full recovery of TER is still possible). Panel A, phase-contrast microscopy of MDCK monolayers in normal growth medium (a) and after 3 h of ATP depletion (b). Panel B, light microscopy of thin sections of MDCK monolayers in growth media (a) and after 3 h of ATP depletion (b). Note that despite some morphological changes, cells after 3 h of ATP depletion retain extensive cell-cell contact.

FIG. 4. Effect of ATP depletion on the actin-cytoskeleton and Na⁺,K⁺-ATPase. Cells were fixed and processed for immunofluorescence using TRITC-phalloidin (panels A–C, actin-ring; panels D–F, actin stress fibers) or specific antibody (panels G–I, Na⁺,K⁺-ATPase). Data are indicated for control monolayers (left panels) as well as monolayers after 3 h of ATP depletion (middle panels) and 6 h of ATP depletion (right panels). Arrowhead indicates a disruption of the actin-ring.

FIG. 5. Time course study of Triton X-100 extractabilities on TJ proteins after 15 min to 6 h of ATP depletion. Panel A, MDCK monolayers were extracted at indicated times after ATP depletion with CSK-1 buffer (0.5% Triton X-100) as defined under “Experimental Procedures.” Both E (extractable) and R (residue) fractions were separated by 6% SDS-PAGE and transferred to a nitrocellulose filter. Immunoblots were probed with indicated antibodies. Panel B, blots were analyzed and quantified with NIH Image software. Measurements (R/E + R) were expressed as percentage of total density of both fractions (E + R). ●, ZO-1; □, ZO-2; △, cingulin.

FIG. 6. Effect of salt concentration on extractions of TJ proteins after ATP depletion. MDCK monolayers were extracted after ATP depletion with CSK buffer containing various concentrations of NaCl (0–1 M) and analyzed as in Fig. 5. ○, control; ●, ATP depletion for 1 h; ■, ATP depletion for 3 h. Note that solubilities of TJ proteins after ATP depletion were increased significantly with a high salt CSK buffer.
completely (>90%) soluble in this buffer in control MDCK monolayers (Fig. 7).

We next examined the reversibility of ATP depletion-induced TJ protein insolubility after washing out metabolic inhibitors and changing to normal growth medium (ATP repletion). As with TER recovery (Fig. 1A), the effect of metabolic inhibitors on the solubilities of the three TJ proteins was found to be largely reversible even after 3 h of ATP depletion (Fig. 8).

After ATP Depletion, TJ Proteins Fractionate into a Large Macromolecular Complex—The marked insolubility of TJ proteins after ATP depletion suggested that they might associate completely (>90%) soluble in this buffer in control MDCK monolayers (Fig. 7).

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After ATP Depletion, TJ Proteins Fractionate into a Large Macromolecular Complex—The marked insolubility of TJ proteins after ATP depletion suggested that they might associate

with other proteins (possibly cytoskeletal), leading us to examine whether large complexes containing TJ proteins could be identified (23, 25). When analyzed by rate zonal centrifugation through 5–20% sucrose gradients, after ATP depletion, the amount of detergent (1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS) extractable ZO-1 as well as ZO-2 present in the high density fractions (fractions 13–18) increased 1.5–2-fold by densitometric analysis (Fig. 9), although the movement of cingulin into the high density fractions after ATP depletion was somewhat less impressive. Thus, not only did the TJ proteins associate with the CSK-1 buffer-insoluble cytoskeletal fraction after ATP depletion (Figs. 5, 6, and 8), but the ZO-1 and ZO-2 that could be solubilized in a different detergent-rich buffer was found in a high molecular mass complex (Fig. 9).

**ATP Depletion Leads to an Increased Association of the ZO-1-containing Complex of TJ Proteins with Fodrin, a Component of Actin-based Cytoskeleton**—The aforementioned studies did not, however, identify specific proteins with which the TJ proteins might associate in the MDCK cells after ATP depletion. The antibody against ZO-1 is known to coimmunoprecipitate efficiently many proteins of the TJ, including ZO-1, ZO-2, p130, and possibly other phosphoproteins (5, 6, 8, 12, 20). Nevertheless, ATP depletion did not significantly alter the association of ZO-1 with ZO-2 by coimmunoprecipitation (Fig. 10).

To clarify protein-protein interactions after ATP depletion in MDCK cells further, we extracted 35S-labeled MDCK cell monolayers with CSK-1 buffer after ATP depletion, followed by another extraction with CSK-2 buffer containing 1 M NaCl. As expected, in CSK-1 extracts we could not detect much ZO-1 after ATP depletion (Figs. 5 and 11A); however, a significant fraction of ZO-1 could be solubilized in CSK-2 extracts after ATP depletion (Fig. 11A). After immunoprecipitation from this latter fraction with anti-ZO-1 hybridoma supernatant, we detected a ~240-kDa protein (Fig. 11B). Based on its molecular
Fig. 10. Association of ZO-2 with ZO-1 after ATP depletion in MDCK monolayers. Cells were solubilized with a buffer containing 1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS, as described under "Experimental Procedures," and immunoprecipitated with anti-ZO-1 antibody or rat nonimmune serum (NIS) overnight at 4 °C. Immunoprecipitates were then collected with anti-rat IgG-coated beads, washed extensively, solubilized in sample buffer, and separated on 6% SDS-PAGE. Shown are immunoblots with anti-ZO-1 antibody (upper) and anti-ZO-2 antibody (lower). The results of several such experiments did not show major changes in the amount of ZO-2 coimmunoprecipitating with ZO-1.

Fig. 11. Association of ZO-1 with cytoskeletal protein, fodrin, in high salt (CSK-2) extracts. Confluent MDCK monolayers were metabolically labeled overnight in the growth medium in the presence of [35S]methionine/cysteine (200 μCi/ml, Expre35S35S labeling mix, DuPont NEN). Control and MDCK monolayers after ATP depletion were first extracted in CSK-1 buffer (100 mM NaCl). The resulting pellet was then extracted again with CSK-2 buffer (1 mM NaCl). This high salt extract was diluted with a NaCl-free CSK buffer at a final concentration of 100 mM NaCl. After preclearing, the CSK-2 extract (in which ZO-1 was solubilized) was incubated with anti-ZO-1 hybridoma supernatant or rat nonimmune serum (NIS) overnight, followed by another incubation with anti-rat IgG-coated beads for 30 min at 4 °C. Samples were separated by a 5–12% gradient gel and then transferred to nitrocellulose filter. Panel A, immunoblots of both CSK-1 and CSK-2 extracts (before immunoprecipitation) after ATP depletion with anti-fodrin antibody (upper) or anti-ZO-1 antibody (lower). Panel B, autoradiogram of immunoprecipitated (IP) proteins in CSK-2 extracts with anti-ZO-1 hybridoma supernatant. The arrowhead indicates identical signal of ZO-1, and the arrow indicates increased signal at ~240 kDa. Molecular masses are shown on the left. Panel C, immunoblots shown with anti-fodrin antibody (upper) and anti-ZO-1 antibody (lower) on the same filter. Panel D, densitometric analysis of relative association between ZO-1 and fodrin. Gray bars show the ratio of fodrin to ZO-1 at each time point.

Mass and the data already discussed indicating an increased association of ZO-1 with the 0.5% Triton X-100-insoluble (cytoskeletonally enriched) fraction, we sought to determine whether the 240-kDa protein might be fodrin, a protein of the actin-based cytoskeleton believed to be important for the localization of plasma membrane proteins to various subdomains in epithelial cells (11, 32). Immunoblots of the ZO-1-containing immunoprecipitates confirmed that the 240-kDa protein was indeed fodrin (Fig. 11C). By densitometric analysis, the ratio of fodrin to ZO-1 increased 8-fold after 3 h of ATP (Fig. 11D). Under these conditions, we did not detect any degraded fodrin fragment in cell lysates or in the ZO-1 immunoprecipitates (33, 34).

Fodrin Accumulates Near the TJ in MDCK Monolayers after ATP Depletion—Given this result, we examined the localization of fodrin by confocal microscopy in MDCK cell monolayers after ATP depletion. In control cells, fodrin was observed diffusely along the subplasmalemmal region and cytosol in all confocal sections, with small amounts colocalizing with ZO-1 at the level of the TJ (Fig. 12, A and B). After ATP depletion, the colocalization of fodrin at TJ level was increased significantly (Fig. 12, C and D) (35).

DISCUSSION

Previous studies of ischemia in vivo and ATP depletion in vitro have demonstrated a disruption of the actin-based cytoskeleton (14, 27, 30, 36, 37). This conceivably could lead to altered polarity of membrane proteins (e.g. Na+,K+-ATPase, Figs. 4 and 8) and loss of the permeability barrier in epithelial cells (13, 14, 16). As the TJ closely associates with the cytoskeleton, the functional loss of TER after ATP depletion seen by us and others could also be accounted for by cytoskeletal alterations (1, 11, 12). To clarify the mechanisms of TJ disassembly underlying ATP depletion in epithelial cells, we examined an interaction between TJ proteins and cytoskeleton biochemically and morphologically. In these studies, we have shown that after the decline in TER which occurs concomitantly with a decline in cellular ATP levels (Fig. 1), three TJ proteins (ZO-1, ZO-2, and cingulin) become extremely insoluble (Figs. 5, 6, and 8), and those that can be solubilized are found in very large molecular mass complexes (Fig. 9) even though all three TJ proteins remain largely localized to the lateral surface of the plasma membrane (Figs. 2–4). ATP depletion leads the ZO-1-containing complex of TJ proteins to associate, either directly or indirectly, with the actin-associated cytoskeletal protein, fodrin (Figs. 11 and 12). Most of these changes appear to be largely reversible even after 3 h of ATP depletion (Figs. 1 and 8).

Recent reports suggest that alterations of the actin-based cytoskeleton after ischemic insult and ATP depletion might be induced via intracellular calcium changes (37–40). It is possible that these changes in intracellular calcium also contribute to the changes in biochemical behavior of TJ proteins and their cytoskeletal association which we have observed after ATP depletion. However, a high intracellular calcium alone is insufficient to explain such an association, at least not under conditions in which the TJ is neither being assembled nor disassembled. For example, when intracellular calcium is manipulated with cell-permeant chelators or calcium ionophores in the absence of ATP depletion, there is no increase in the extractability of TJ proteins (data not shown). Still,
changes in intracellular calcium might play an important role in stabilizing TJ protein-cytoskeletal interactions after TJ disassembly in the face of ATP depletion; likewise, the ion could play a regulatory role in the weakening of these interactions during reassembly after ATP repletion. There is precedence for such a view; in the calcium switch, changes in intracellular calcium concentration have been linked, at least indirectly, to the solubility properties of TJ proteins (19). In this latter model for TJ disassembly and reassembly, the reassembly of the TJ involves a heterotrimetric G protein (20), regulated intracellular calcium stores (18, 19), and protein kinase C activation (8). It will therefore be interesting to determine in future studies whether the reassembly of the TJ after ATP depletion-repletion is likewise dependent on classical signaling mechanisms.

Fodrin, a non-erythroid spectrin, serves to link a number of membrane proteins to the cytoskeleton network (41–43). Spectrin, as well as ankyrin, has been reported to co-localize with junctional proteins in polarized epithelial cells (32, 44). Our data indicate that after ATP depletion, the relative fraction of fodrin near the TJ increases. Although we were able to co-immunoprecipitate fodrin with the ZO-1-containing complex, it is likewise dependent on classical signaling mechanisms.

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