Altered membrane-cytoskeleton linkage and membrane blebbing in energy-depleted renal proximal tubular cells

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Chen, Jing, and Mark C. Wagner. Altered membrane-cytoskeleton linkage and membrane blebbing in energy-depleted renal proximal tubular cells. Am J Physiol Renal Physiol 280: F619–F627, 2001.—The effects of energy depletion on two membrane-cytoskeletal linker proteins (ezrin and myosin-1) and membrane bleb formation were studied in isolated rabbit proximal tubule cells. Measurements of cytoskeletal-membrane interactions by using the laser optic trap method revealed a stronger association of control tubule membrane with the apical cytoskeleton compared with the basal cytoskeleton. Energy depletion weakened the apical membrane-cytoskeleton interactions to a greater degree. Biochemical studies demonstrated that energy depletion altered both ezrin and myosin-1β. The salt-insensitive ezrin fraction dissociated from the cytoskeleton; myosin-1β redistributed from the peripheral cytoskeleton to a perinuclear/nuclear complex. These changes in ezrin and myosin-1β and the weakening of the membrane-cytoskeleton interactions correlated with the release of brush-border membrane blebs observed by differential interference contrast microscopy. Permeability of membrane blebs was also evaluated during energy depletion and indicated an increased permeabilization of basal blebs to 3-kDa dextrans. These results support the hypothesis that alterations in membrane-cytoskeleton linkers facilitate the formation and detachment of blebs by weakening membrane-cytoskeleton interactions.

Ezrin; myosin-1; microvilli; adenosine 5′-triphosphate depletion; ischemia

BLEBS OCCUR ON PLASMA MEMBRANES of renal proximal tubular cells during ischemic or anoxic energy depletion (7, 15, 21, 29). Although the mechanisms and pathological consequences of bleb formation remain largely unknown, bleb formation is likely initiated by dysfunction of membrane-cytoskeleton linkers (7, 11, 15, 25). Normally, linker proteins stabilize the plasma membranes by anchoring them to the cytoskeleton (3, 24). The linker proteins at the apical side of renal proximal tubular cells include ezrin and myosin-1 (1–6, 9, 10, 17, 22, 30, 31).

In our previous studies, energy depletion caused ezrin to dissociate from the microvillar actin cytoskeleton (4–6). Ezrin dissociation was further correlated with microvillar disruption, suggesting a possible role for ezrin in destabilizing the microvillar cytoskeleton during energy depletion (4). Although an apical membrane-cytoskeleton link was expected to break with ezrin dissociation, no quantitative assessment of these changes has been conducted. We previously used the optic trap technique to quantify the membrane-cytoskeleton link at the basal side of proximal tubules. This method can measure the apical link as well. Our recent studies suggest that the cytoskeleton-associated ezrin in proximal tubular cells can be divided into two biochemical subgroups (J. Chen, unpublished observations). One subgroup of ezrin is Triton-insoluble in either the presence or absence of salts; the other is Triton-insoluble only in the absence of salts. The two ezrin subgroups may have distinct functions and respond to energy depletion differently. Because the previously reported ezrin dissociation was revealed by Triton extraction without salts, it is unknown whether anoxia can cause both ezrin types to dissociate from the cytoskeleton.

The other apical membrane-cytoskeleton linker, myosin-1, was recently studied by Wagner and Molitoris (31). These studies indicated that ATP depletion altered the Triton solubility of myosin-1β in a cultured renal proximal tubule cell line, LLC-PK₁ (31), suggesting an increase in association of myosin-1β with the actin cytoskeleton. Association of myosin-1β with specific cytoskeleton subdomains was not addressed. Because cultured cell lines often behave differently from primary cells, it was important to determine whether the membrane-cytoskeleton linker function of myosin-1 was altered in renal proximal tubular cells after energy depletion.

The membrane-cytoskeleton link at the apical and basal sides may differ in composition and mechanical strength. At the apical domains, the membrane is linked to the microvillar cytoskeleton by ezrin and myosin-1 (1–6, 9, 10, 17, 22, 30, 31); the extracellular side of the apical membranes is exposed to the lumen of the proximal tubule without external mechanical support. At the basal domains, the cytoplasmic side of the...
plasma membrane is anchored to the cytoskeleton via different proteins such as ankrin and integrins (13, 14, 16); the extracellular side of the basal membrane is supported by extracellular matrix. Due to these structural differences, the mechanical properties of the apical membrane-cytoskeleton links are likely to differ from those on the basal side. These differences may determine where bleb formation occurs during energy depletion.

The apical blebs may affect renal functions in two ways: 1) obstructing tubular flow if apical blebs are detached, and 2) compromising the barrier functions of plasma membranes. The release of apical blebs into the tubular lumen has been suggested by the presence of membrane vesicles in the lumen (20, 28). These vesicles may originate from detached blebs that are released from microvillar tips early after energy depletion, or they may arise from the apical side of necrotic and/or apoptotic cells later. So far, direct evidence for the release of apical membrane blebs from microvillar tips is lacking. The effect of blebbing on the barrier function of plasma membranes also remains unclear. Studies in cultured rat hepatocytes by Zahrebelski et al. (33) suggested that bleb rupture caused leakage of the plasma membrane during energy depletion. In our previous study, blebs on the basal side of rabbit renal proximal tubules remained intact and impermeable to 70-kDa dextrans during 60 min of energy depletion (7). Because recent studies by Dong et al. and us (8, 18) reported a progressive increase in permeability of plasma membranes during energy depletion, we wanted to determine whether blebs on renal proximal tubular cells were permeable to molecules smaller than 70 kDa.

These studies address membrane-cytoskeleton linkage and membrane blebbing by using freshly isolated renal proximal tubules. We compared the responses of two ezrin subgroups to energy depletion, analyzed the effect of energy depletion on myosin-1β, quantified membrane-cytoskeleton binding forces at apical and basal domains under control and energy-depleted conditions, monitored the formation and release of apical membrane blebs, and determined permeability of blebs to different probes.

**MATERIALS AND METHODS**

**Preparation of Rabbit Proximal Tubular Cells**

Proximal tubules (PT) were isolated and purified as previously described (4). Briefly, female New Zealand White rabbits (1–2 kg, Harlan, Indianapolis, IN) were injected with heparin and euthanized by using pentobarbital sodium. The cortices were trimmed from the excised kidneys, minced, and digested for 60 min at 37°C in DMEM containing 150 U/ml collagenase (Worthington Biochemical, Freehold, NJ) and 1 U/ml DNase (Sigma, St. Louis, MO). The digested cortical tissue suspensions were washed free of collagenase, and PTs were isolated from blood vessels, distal tubules, and injured cells by centrifugation on a self-generating 50% Percoll gradient for 30 min at 36,000 g. The collected PT bands were washed 3 times in DMEM, and the final tubule pellets were resuspended in DMEM at a concentration of 2 mg protein/ml.

The final PT suspension contained both intact tubules and clusters of normal tubular cells that dissociated from tubules due to collagenase digestion. The isolated intact tubules and tubular cells were preincubated in DMEM containing 2 mM glycine and gassed with 100% oxygen for 20 min before any treatments.

**Energy Depletion in PT Cells**

Energy depletion was induced with one of the following two methods. For the measurement of Triton solubility of salt-sensitive and -insensitive ezrin, fractionation of myosin-1β, electron microscopy, and determination of permeability of blebbed membranes to 3- and 70-kDa dextrans, energy depletion was induced by gassing PT suspensions with 100% nitrogen for 30 min. For the measurement of membrane-cytoskeleton binding force and observation of microvillar membrane bleb release, energy depletion was induced by incubating PT with 10 μM antimycin A, a site II mitochondrial inhibitor, for 30 min. No differences were observed between these two previously published methods of energy depletion.

**Measurement of Membrane-Cytoskeleton Binding Force**

The membrane-cytoskeleton binding force was measured by using the laser tweezer technique (12, 23, 27). ConA-coated latex beads were attached to membrane glycoproteins. The bead and the plasma membrane were pulled off the cytoskeleton by a laser tweezer to form a tether (Fig. 1). The minimum force (F₀) required to separate the membrane from the cytoskeleton was used to estimate the membrane-cytoskeleton binding force.
General principles of the laser tweezer technique. A spherical bead can be trapped in the center of a laser beam, as described by Kuo and Sheetz (23). When the laser trap is moved, the bead can be dragged around. If there is no resistance when the bead is dragged, the bead center overlaps the trap center (Fig. 1, left). If resistance is faced during the drag, the bead center will deviate from the center of the laser beam. The distance between the two centers is defined as displacement, which is proportional to the resistance strength. A linear relationship was established between the displacement and the resistance force on the bead, which was calculated by Stokes’s law (12).

Calibration of the laser trap. The laser optical trap was calibrated by viscous drag through the aqueous medium in the microscope focal plane (12). The viscous force was generated by oscillatory motion of the specimen by a piezoceramic-driven stage (Wye Creek Instruments, Frederick, MD) at a constant velocity. The position of the bead in the trap was tracked by using a nanometer-level tracking program to analyze video records of the experiments. Positional variation of the bead in the trap with 60 mW was 11 (±1.7) nm. The calibration shows a linear force-distance relationship for the optical tweezers. This calibration was used to calculate the minimum force that was required to separate the membrane from the cytoskeleton. All of these experiments were performed 3–4 μm above the coverslip surface to minimize viscous coupling to the glass surface, and the laser power was simultaneously monitored.

Bead preparation. For latex bead preparations, ConA (Sigma, St. Louis, MO) was prepared at a concentration of 1.0 mg/ml in 50 μl of PBS. Twenty-five milliliters of 0.5-μm Covaspheres MX reagent (Duke Scientific, Palo Alto, CA) was added to the ConA solution and allowed to incubate for 20 h at 4°C. The beads were washed 3 times by using 0.5% BSA in PBS and centrifugation at 10,000 rpm (4°C) for 10 min in PBS. Twenty-five milliliters of 0.5% BSA was added to the ConA solution and allowed to incubate for 20 h at 4°C. The beads were washed 3 times by using 0.5% BSA in PBS and centrifugation at 10,000 rpm (4°C) for 10 min in a microcentrifuge (Beckman Instruments). The final bead pellet was resuspended in 75 μl of 0.5% BSA. Each bead solution was diluted 3:1000 in DMEM-F-12 culture medium, and sonicated.

Laser optical trap manipulations. After preincubation with oxygen, PT were mounted on a coverslip coated with Cell-Tak (Collaborative Biomedical Products, Bedford, MA). Then, a second coverslip was mounted on top to form a flow chamber that was filled with DMEM with/without 10 μM antimycin A. ConA-covered latex beads were added to the flow chamber. The beads attached to microvillar tips and basal membranes of tubular cells. Manipulation of the attached beads by using laser tweezers allowed measurements of apical and basal F0. The samples were viewed with a video-enhanced differential interference contrast (DIC) microscope (IM-35 microscope; Zeiss, Oberkochen, Germany) and an attached fiber optic illuminator. The stage was maintained at 37°C by using an air current incubator. The laser trap consisted of a polarized beam from an 11-W TEM00-mode near-infrared (1,064 nm) laser (model C-95; CVI, Albuquerque, NM) that was expanded by a 3× beam expander (CVI) and then focused through an 80-mm focal length achromatic lens (Melles Griot, Irvine, CA) into the epifluorescence port of the Zeiss IM-35 microscope.

The minimum force F0 needed to pull the membrane off the cytoskeleton, as indicated by the formation of a membrane tether in Fig. 1, was measured as the force of the tether at zero velocity. The bead position in the trap during tether formation was tracked using the nanometer-scale tracking program developed previously, and the F0 of the tether on the bead was calculated from the laser trap calibration.

Observation of Microvillar Membrane Bleb Release

Freshly isolated rabbit PT suspension was preincubated with oxygen, placed in a microscopic perfusion chamber filled with DMEM containing 10 μM antimycin A, and maintained at 37°C by using an air current incubator. Attachment of PT cells to the bottom of the chamber was strengthened with Cell-Tak (Becton-Dickinson Labware, Bedford, MA). Microvilli were examined in single tubular cells and cells located at the ends of intact tubules, because the brush border lining the lumen of intact tubules was not visible from the outside. Microvilli at these two places were monitored and videotaped using video-enhanced DIC microscopy (Zeiss IM-35 microscope).

Measurement of Triton Solubility of Salt-Sensitive and -Insensitive Ezrin

The association of ezrin with the cytoskeleton was determined after Triton X-100 treatment in the presence or absence of salts. After incubation under control conditions or energy depletion induced by gassing the PT with 100% nitrogen for 30 min, the PT suspension-containing tubules and clusters of tubular cells were pelleted at 100 g for 2 min.

Table 1. List of buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
<th>Applications</th>
</tr>
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<tbody>
<tr>
<td>Digitonin extraction buffer</td>
<td>0.012% digitonin, 280 mM sucrose, 200 mM KCl, 20 mM HEPES, 2 mM EGTA, 200 μM PMSF, 10 μM leupeptin, and 10 μM pepstatin; pH 7.4</td>
<td>Extracts cytosolic myosin-1β</td>
</tr>
<tr>
<td>Triton + salt extraction buffer</td>
<td>0.5% Triton X-100, 150 mM KCl, 280 mM sucrose, 5 mM Tris·HCl, 2 mM EGTA, 200 μM PMSF, 10 μM leupeptin, and 10 μM pepstatin; pH 7.4</td>
<td>Extracts cytosolic, membrane-associated, and salt-sensitive cytoskeleton-associated ezrin, or extracts membrane-associated myosin-1β</td>
</tr>
<tr>
<td>Triton – salt extraction buffer</td>
<td>0.5% Triton X-100, 280 mM sucrose, 5 mM Tris·HCl, 2 mM EGTA, 200 μM PMSF, 10 μM leupeptin, and 10 μM Pepstatin; pH 7.4</td>
<td>Extracts cytosolic and membrane-associated ezrin</td>
</tr>
<tr>
<td>Calcium extraction buffer</td>
<td>6 mM CaCl2, 10% Triton X-100, 280 mM sucrose, 150 mM KCl, 20 mM HEPES, 2 mM EGTA, 200 μM PMSF, 10 μM leupeptin, and 10 μM pepstatin A, pH 7.4</td>
<td>Extracts myosin-1β in peripheral cytoskeleton including microvilli</td>
</tr>
<tr>
<td>ATP extraction buffer</td>
<td>3 mM ATP, 280 mM sucrose, 200 mM KCl, 20 mM HEPES, 2 mM EGTA; pH 7.4</td>
<td>Disrupts interactions between myosin-1β and other proteins including actin in the perinuclear complex</td>
</tr>
<tr>
<td>2× PAGE buffer</td>
<td>5% SDS, 25% sucrose, 5 mM Tris·HCl, and 5 mM EDTA; pH 7.4</td>
<td>Solubilizes proteins for electrophoresis</td>
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PMSF, phenylmethylsulfonyl fluoride.
pellets were resuspended in 0.9 ml of ice-cold Triton + salt extraction buffer containing 150 mM KCl (Table 1), or Triton – salt extraction buffer containing no KCl (Table 1) for 10 min. The extracted suspension was then centrifuged at 4,000 g for 10 min. The Triton-soluble proteins (TSP) were precipitated from the supernatant with 3.5× vol of 100% methanol and pelleted at 4,000 g for 10 min. TSP and the Triton-insoluble proteins (TIP) were resuspended in equal volumes of 2× PAGE buffer (Table 1). The total protein in TIP and TSP fractions of proximal tubules was measured by using the bicinchoninic assay (Pierce Biochem). For quantitation of ezrin in TIP and TSP fractions, equal volumes of each fraction were run on an 8% polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blotting.

Fractionation of Myosin-Iβ

Sequential extraction of PT cells with three buffers was used to separate cytosolic, membrane-bound, peripheral cytoskeletal (including microvillar), and perinuclear/nuclear fractions and to determine any possible redistribution of myosin-Iβ in these fractions during energy depletion. After incubation in the medium gassed with 100% oxygen (control) or 100% nitrogen (energy depletion), equal amounts (40 mg protein) of control or energy-depleted proximal tubular cells (including intact tubules and clusters of tubular cells) were first permeabilized with 1 ml of digitonin extraction buffer (Table 1) on ice with agitation for 10 min to extract cytosolic proteins. The digitonin extraction protocol was modified from published methods (19, 26). After digitonin permeabilization, the whole suspension was centrifuged at 4,000 g for 3 min to separate cytosolic proteins (supernatant) from the digitonin-insoluble fraction. Second, the digitonin-insoluble fraction of control and energy-depleted proximal tubular cells was extracted in 1 ml Triton + salt extraction buffer (Table 1) on ice with agitation for 10 min, resulting in a Triton-soluble (membrane-bound protein) fraction and Triton-insoluble (cytoskeleton + nuclei) pellet. Finally, the Triton-insoluble pellet was extracted in 1 ml calcium extraction buffer (Table 1) to extract peripheral (including microvilli) cytoskeletal proteins. The calcium-soluble and calcium-insoluble fractions were separated by centrifugation (4,000 g, 3 min). In some experiments, the calcium-insoluble pellet was further extracted with an ATP extraction buffer (Table 1) to test the possibility of association of myosin-Iβ with actin. After each sequential extraction, the soluble fraction was mixed with an equal volume of 2× PAGE buffer (Table 1), and the final pellet was resuspended in two volumes of 1× PAGE buffer. Thus volumes of all fractions were equalized. The content of myosin-Iβ in each fraction was measured by Western blotting.

In parallel experiments, the pellets after each extraction were fixed in 2% glutaraldehyde and examined with DIC microscopy and electron microscopy.

Western Blotting

Samples were electrophoresed in 8% polyacrylamide gel and transferred to NitroPure membrane (MSI, Westboro, MA). The blotted proteins were then probed with primary antibodies [monoclonal anti-myosin-Iβ (clone M2; Ref. 30), monoclonal anti-actin and anti-villin from Chemicon, Temecula, CA, monoclonal anti-ezrin from Immusine, Hayward, CA] and peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab, West Grove, PA). The presence of the antigens was indirectly detected by using enhanced chemiluminescence (Amersham) and quantitated by scanning densitometry within linear range by series dilution.

Electron Microscopy

In preparation for electron microscopy, PT were fixed with 2% glutaraldehyde in 100 mM NaH2PO4 overnight at 4°C and then dehydrated in ethanol and embedded in epon 812. Sections were double stained with uranyl acetate and lead citrate and viewed with a Phillips 312 electron microscope.

Determination of Permeability of Blebbed Membranes to 3- and 70-kDa Dextrans

At the end of 30 min of energy depletion induced by gassing the PT with 100% nitrogen, 0.1% 3-kDa tetramethylrhodamine dextran and 0.1% 70-kDa fluorescein dextrans were added into the extracellular medium. The 3-kDa tetramethylrhodamine dextrans and the 70-kDa fluorescein dextrans were immediately visualized with confocal microscopy at the excitation/emission wavelengths of 568/590 and 488/515 nm, respectively. The permeability of blebs to the dextrans was determined by the presence or absence of the dextrans in the lumen of the blebs.

Data Analysis

Data are from at least three independent experiments and are shown as means ± SE. Unless noted in the figure legend, ANOVA was used to compare the means of various experimental groups tested for significance with Fisher’s protected least significance difference test and P < 0.05.

RESULTS

Mechanical Properties of Apical and Basal Membrane-Cytoskeleton Links

Under control conditions, the minimum force required to pull the apical membrane off the cytoskeleton (F0 = 22.2 ± 1.3 pN) was much greater (P < 0.05, n = 5) than the force required to separate the basal membrane from the cytoskeleton (F0 = 13.9 ± 0.8 pN) (Fig. 2). This result indicates that the membrane-cytoskeleton link is stronger at the apical side than at the basal side. After 30 min of energy depletion induced by 10 μM antimycin A, F0 at both the apical and basal sides significantly (P < 0.05, n = 5) decreased to a similar level (7.6 ± 0.9 and 7.9 ± 0.7, respectively).

Fig. 2. F0 on apical and basal sides of control and energy-depleted proximal tubules. Under control conditions, a greater force (F0) was required to pull the apical membrane off the cytoskeleton than the basal membrane (#P < 0.05, n = 5). During antimycin A-induced energy depletion, F0 at both the apical and basal sides decreased significantly (*P < 0.05, n = 5).
n.5% Triton extraction buffer significantly (P < 0.05, n = 5), and from 65 ± 6 to 35 ± 4% (P < 0.05, n = 5), respectively (Fig. 3B). The salt-sensitive cytoskeleton-associated ezrin was insignificantly altered by energy depletion from 25 ± 5 to 21 ± 4% (P > 0.05, n = 5).

Fractionation of Cytosolic Proteins, Peripheral Cytoskeleton, and Perinuclear/Nuclear Complex

The sequential extraction with digitonin, Triton, and calcium buffers (see MATERIALS AND METHODS) fractionated the cellular content of normal cells into several portions. After digitonin permeabilization and Triton extraction, cytosolic and membrane-bound proteins were removed, leaving the entire cytoskeleton intact (Fig. 4A). The subsequent extraction of the Triton-insoluble fraction with calcium (see MATERIALS AND METHODS) completely solubilized the peripheral cytoskeleton, as indicated by both DIC and electron micrographs of the pellet (Fig. 4A). This notion was further supported by a complete or near-complete disappearance of two microvillar cytoskeleton marker proteins.

Dissociation of Salt-Insensitive Ezrin During Anoxia

In normal proximal tubular cells, 90 ± 4% of total ezrin was insoluble in 0.5% Triton extraction buffer without salts; this was defined as the total cytoskeleton-associated ezrin (Fig. 3). This value is consistent with earlier reports (4). Addition of 150 mM KCl into 0.5% Triton extraction buffer significantly (P < 0.05, n = 5) decreased the insoluble ezrin from 90 ± 4 to 65 ± 6% (P < 0.05, n = 5) (Fig. 3). Similar effects on the Triton solubility of ezrin were induced by 150 mM NaCl, indicating the nonspecific effect of salts (data not shown). Based on the salt effects, we arbitrarily differentiated the total cytoskeleton-associated ezrin into two subgroups, the salt-sensitive and the salt-insensitive. The salt-sensitive cytoskeleton-associated ezrin can be solubilized by salts and is defined as the difference of total cytoskeleton-associated ezrin and salt-insensitive ezrin. The salt-sensitive ezrin represents 25 ± 5% of total ezrin (Fig. 3B). The salt-insensitive cytoskeleton-associated ezrin cannot be solubilized by salts and represents 65 ± 6% of total ezrin (Fig. 3B). Energy depletion induced by gassing PT with 100% nitrogen for 30 min did not alter total (Triton-soluble + insoluble) cellular ezrin contents but reduced both total cytoskeleton-associated and the salt-insensitive cytoskeleton-associated ezrin from 90 ± 4 to 56 ± 7% (P < 0.05, n = 5), and from 65 ± 6 to 35 ± 4% (P < 0.05, n = 5), respectively (Fig. 3B). The salt-sensitive cytoskeleton-associated ezrin was insignificantly altered by energy depletion from 25 ± 5 to 21 ± 4% (P > 0.05, n = 5).

Fig. 3. Salt-sensitive and -insensitive Triton solubility of ezrin in control and energy-depleted proximal tubules. A: a representative Western blot of ezrin in Triton-soluble (S) and -insoluble (I) fractions of control and energy-depleted proximal tubular cells under different extraction conditions. −Salt, extracted with Triton−salt extraction buffer containing no salt; +Salt, extracted with Triton+salt extraction buffer containing 150 mM KCl. B: statistical analysis of salt-sensitive, and -insensitive cytoskeleton-associated ezrin in control and energy-depleted proximal tubular cells. The amount of ezrin in each fraction is expressed as % total (sum of ezrin in all subcellular fractions). Energy depletion caused a significant decrease in salt-insensitive cytoskeleton-associated ezrin. (*P < 0.05 compared with the sum of salt-sensitive and -insensitive cytoskeleton-associated ezrin under control conditions and energy depletion, or between salt-insensitive cytoskeleton-associated ezrin under control and energy depletion, n = 5).

Fig. 4. Separation of peripheral (including microvillar) cytoskeleton from the perinuclear cytoskeleton nuclear complex. A, left: differential interference contrast (DIC) image of a proximal tubular cell after extraction with Triton+salt extraction buffer indicates the Triton-insoluble pellet as intact cytoskeleton-containing microvilli. Right: DIC and electron micrographs (EM) of proximal tubular cell after sequential extraction with Triton+salt and calcium extraction buffer show the calcium-insoluble (Ca-P) pellet as a nucleus with remnant perinuclear cytoskeleton. The microvilli and peripheral cytoskeleton have been extracted. B: representative Western blot of villin, ezrin, and actin in total (Total) lysate and Ca-P of proximal tubular cells. No ezrin and little villin, which are both microvillar marker proteins, were found in the Ca-P.
proteins, ezrin and villin, from the calcium-insoluble fraction (Fig. 4B). Thus the calcium-soluble fraction represents proteins in the peripheral cytoskeleton including microvilli, whereas the calcium-insoluble pellet contained remnant perinuclear cytoskeleton and the nuclei.

**Redistribution of Myosin-1β From Calcium-Soluble to Insoluble Fraction During Energy Depletion**

Under control conditions, myosin-1β was distributed in all the fractions: 3 ± 2% in the digitonin-soluble fraction, 14 ± 4% in the Triton-soluble fraction, 44 ± 4% in the calcium-soluble fraction, and 39 ± 3.6% in the calcium-insoluble fraction (Fig. 5A, control). Energy depletion induced by gassing PT with 100% nitrogen for 30 min did not significantly alter the total amount of myosin-1β in proximal tubular cells, in the digitonin-soluble fractions or in the Triton-soluble fractions, but shifted a significant amount of myosin-1β (14 ± 3% of total myosin-1β, P < 0.05, n = 3, paired t-test) from the calcium-soluble fraction (peripheral cytoskeleton) to the calcium-insoluble pellet (perinuclear cytoskeleton/nuclei complex) (Fig. 5A). Figure 5B illustrated the increase in calcium-insoluble myosin-1β in three separate energy depletion experiments. Statistical analysis by using the paired t-test revealed a significant difference (P < 0.05, n = 3) between calcium-insoluble myosin-1β under control conditions (39 ± 3.6% of total cellular myosin-1β) and energy depletion (53 ± 5.0%).

Because myosin-1 interacts with actin in an ATP-dependent manner, the calcium-insoluble pellet was extracted with ATP (see MATERIALS AND METHODS) to initially determine whether myosin-1β would be released from the calcium-insoluble pellet. Almost all of the myosin-1β in the pellet of control proximal tubular cells and the majority of the myosin-1β in the pellet of energy-depleted proximal tubular cells were extracted by ATP (Fig. 6).

**Release of Apical Membrane Bleb During Energy Depletion**

Electron microscopy revealed two types of apical membrane blebs on energy-depleted proximal tubular cells: one at the microvillar tips and one at the apical side of damaged cells that had lost their microvilli (Fig. 7). The blebs on microvillar tips were observed early after energy depletion, whereas the blebs at the apical side of the damaged cells appeared later. DIC microscopy captured bleb release from the brush border during energy depletion induced by antimycin A (Fig. 8).

**Permeabilization of Blebbed Membranes to 3-kDa Dextrans**

Blebs occurred on both apical and basal sides of the PTs after 30 min of energy depletion with 100% nitrogen. Fluorescent probes were used to determine bleb permeability. The determination of apical bleb permeability was unfortunately impossible due to the densely packed microvilli, which prohibited us from distinguishing the intramicrovillus (intracellular) space from the intermicrovillus (extracellular) space. Our present technique, however, enabled us to determine the permeability of basal blebs. None of the basal blebs

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**Fig. 5.** Energy depletion-induced redistribution of myosin-1β in various fractions of proximal tubular cells. A: a representative Western blot of myosin-1β in digitonin-soluble (Digit-S), Triton-soluble (Trition-S), calcium-soluble (Ca-S), and Ca-P fractions in control and energy-depleted proximal tubular cells after the sequential extraction with Triton and calcium (see MATERIALS AND METHODS). B: energy depletion induced an increase of Ca-P myosin-1β in all subcellular fractions. Statistical analysis using the paired t-test revealed a significant difference (P < 0.05, n = 3) between the mean Ca-P myosin-1β under control conditions (39 ± 3.6%) and energy depletion (53 ± 5.0%).

**Fig. 6.** ATP releases myosin-1β from the Ca-P pellet. The representative Western blot displays solubility of Ca-P myosin-1β in ATP extraction buffer (see MATERIALS AND METHODS). The majority of the calcium-insoluble myosin-1β in both control (top) and energy-depleted proximal tubular cells (bottom) was solubilized by ATP extraction buffer. The result supports a myosin-1β association with actin in the Ca-P pellet.
was permeable to 70-kDa dextrans (Fig. 9). Interestingly, only large basal blebs were permeable to 3-kDa dextrans, suggesting that permeability of the blebs might depend on bleb size (Fig. 9).

**DISCUSSION**

This study presents valuable information on the mechanisms and consequences of membrane blebbing during energy depletion. It analyzes membrane-cytoskeleton binding forces and reveals changes to two apical membrane-cytoskeleton linker proteins (ezrin and myosin-1β) during energy depletion.

Optic trap experiments under control conditions revealed a stronger membrane-cytoskeleton interaction at the apical side vs. the basal side of renal proximal tubules (Fig. 2). The greater apical binding force strengthens apical membranes and may compensate for the lack of extracellular support that is only available to basolateral membranes. The stronger apical membrane-cytoskeleton connection is likely attributed to the abundance of membrane-cytoskeleton linker proteins in the microvilli. The difference between apical and basal binding forces was eliminated by energy depletion, as indicated by the decrease of both binding forces to a similar level during energy depletion, as suggested by the same F₀ (Fig. 2), apical membranes are expected to be less stable than basal membranes. We further hypothesize that this decrease in the apical binding force is caused by anoxia-induced alterations of ezrin and myosin-1β.

Biochemical studies demonstrated that both ezrin and myosin-1β were altered by energy depletion. Analysis of the total cytoskeleton-associated ezrin confirmed previous findings of ezrin dissociation from the cytoskeleton (Fig. 3A and Ref. 4). The present studies further revealed that energy depletion primarily affected the salt-insensitive cytoskeleton-associated ezrin (Fig. 3B). The mechanism for the differential effects of energy depletion remains to be investigated. Energy depletion did not affect the total amount of Triton-insoluble myosin-1β that was associated with the cytoskeleton in renal proximal tubules, but it...
caused a redistribution of myosin-1β inside the cytoskeletal fraction (Fig. 5). Approximately 14% of total myosin-1β shifted from the peripheral cytoskeleton to a perinuclear/nuclear region during energy depletion (Fig. 5). The shift of myosin-1β to the nuclear complex coincided with our recent finding that energy depletion induced perinuclear sequestration of actin (32). Thus myosin-1β’s redistribution could result from energy depletion-induced translocation of microvillin actin fragments to the perinuclear/nuclear region. Because the myosin-actin interaction is strengthened in the absence of ATP, the association between myosin-1β and actin in the perinuclear/nuclear complex was tested by using ATP extraction. The results (Fig. 6) are consistent with an actin-myosin-1β interaction, but more direct evidence is needed to clarify this binding. The decrease of peripheral cytoskeleton-associated myosin-1β during energy depletion, along with dissociation of ezrin, will further weaken the apical membrane-cytoskeleton link.

Coincidentally with the decreased membrane-cytoskeleton binding force and the alterations of the linker proteins, apical membrane blebbing was recorded by both electron microscopy (Fig. 7) and video-enhanced DIC microscopy (Fig. 8). The apical blebs were formed on both the microvillar tips and the apical surface of the more severely damaged cells that had lost all microvilli (Fig. 7). The microvillar tip blebs were observed early after energy depletion, whereas the apical surface blebs were found at later stages of energy depletion. The release of blebs from the brush border was documented for the first time (Fig. 8). The detached microvillar blebs, combined with a narrowed tubular lumen caused by swollen tubular cells, could significantly reduce tubular flow during energy depletion.

In addition to obstructing tubular flow, membrane blebs might have another adverse effect, i.e., compromising plasma membrane integrity. Studies in cultured rat hepatocytes by Zahrebelski et al. (33) suggested that bleb rupture caused leakage of the plasma membrane during energy depletion. However, our previous studies on renal proximal tubular cells demonstrated that loss of cytoskeletal support to the plasma membrane was not sufficient by itself to cause membrane disruption or permit entry of 70-kDa dextrans into basal blebs (7). The present studies further demonstrate that basal blebs are impermeable to both 70- and 3-kDa dextrans when the blebs are small (Fig. 9). In contrast, 3-kDa dextrans entered large blebs (Fig. 9). These novel findings suggest that the barrier function of blebs was partially lost during energy depletion. However, the possibility of 3-kDa dextrans entering the lumen of large blebs through membrane domains other than the blebs cannot be excluded at this time. The impact of this membrane damage will require further investigation. It will be interesting to determine whether other molecules are differentially handled by these blebs. For instance, extracellular calcium may enter cell bodies through large blebs, resulting in severe cell damage.

In summary, energy depletion caused a greater decrease in the membrane-cytoskeleton binding force on the apical side compared with the basal side. The compromised apical membrane-cytoskeleton linkage was associated with alterations in the linker proteins ezrin and myosin-1β. Apical membrane blebs were formed and released from the microvillar tips. Blebs were also formed on the basal side of PT. Large basal blebs were permeable to 3-kDa dextrans but not to 70-kDa dextrans. These results support our hypothesis that energy depletion induces a chain reaction: alterations of the membrane-cytoskeletal linker proteins initiate membrane-cytoskeleton dissociation, which in turn facilitates membrane blebbing and detachment of the blebs.
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REFERENCES


