Dissociation of spectrin-ankyrin complex as a basis for loss of Na-K-ATPase polarity after ischemia

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1Division of Pediatric Nephrology, Albert Einstein College of Medicine, New York, New York 10467; 2Division of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520; and 3Department of Nephrology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio 45229-3039

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Woroniecki, Robert, Jean R. Ferdinand, Jon S. Morrow, and Prasad Devarajan. Dissociation of spectrin-ankyrin complex as a basis for loss of Na-K-ATPase polarity after ischemia. Am J Physiol Renal Physiol 284: F358–F364, 2003. First published October 29, 2002; 10.1152/ajprenal.00100.2002.—The polarized distribution of Na-K-ATPase at the basolateral membranes of renal tubule epithelial cells is maintained via a tethering interaction with the underlying spectrin-ankyrin cytoskeleton. In this study, we have explored the mechanism underlying the loss of Na-K-ATPase polarity after ischemic injury in Madin-Darby canine kidney (MDCK) cells, utilizing a novel antibody raised against a recently described kidney-specific isoform of ankyrin. In control MDCK cells, ankyrin was colocalized with Na-K-ATPase at the basolateral membrane. ATP depletion resulted in a duration-dependent mislocation of Na-K-ATPase and ankyrin throughout the cytoplasm. Colocalization studies showed a partial overlap between the distribution of ankyrin and Na-K-ATPase at all periods after ATP depletion. By immunoprecipitation with anti-ankyrin antibody, the mislocated Na-K-ATPase remained bound to ankyrin at all time points after ATP depletion. However, the interaction between ankyrin and spectrin was markedly diminished within 3 h of ATP depletion and was completely lost after 6 h. In solution binding assays using a fusion peptide of glutathione S-transferase with the ankyrin binding domain of Na-K-ATPase, a complex with ankyrin was detected at all time points after ATP depletion, but spectrin was lost from the complex in a duration-dependent manner. The loss of spectrin binding was not attributable to spectrin degradation but was associated with hyperphosphorylation of ankyrin. The results suggest that a dissociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface may contribute to the loss of Na-K-ATPase polarity after ischemic injury and reaffirm a critical adapter role for ankyrin in the normal maintenance of Na-K-ATPase polarity.

phosphorylation; Madin-Darby canine kidney; adenosine 5-triphosphatase depletion

TETHERING INTERACTIONS BETWEEN integral membrane proteins and the underlying spectrin-based cytoskeleton play key roles in several cellular activities, including the establishment and maintenance of ordered membrane domains (10). Ankyrins are a family of conserved proteins that have emerged as adapter molecules mediating such linkages, because they possess binding sites for a variety of integral membrane proteins as well as for spectrin (3–5, 8–10). A particularly well-characterized example is the linkage between ankyrin and α-Na-K-ATPase, mediated primarily by residues within ankyrin’s repeats domain and the second cytoplasmic domain of α-Na-K-ATPase (11, 37, 40). This interaction is especially critical to the cells lining the kidney tubules, which vectorially transport ions and nutrients via mechanisms that are dependent on the polarized basolateral colocalization of Na-K-ATPase, ankyrin, and spectrin (29, 31, 36). One of the major consequences of acute ischemic injury to renal tubule cells is the disruption of polarity, with a coordinate mislocation of Na-K-ATPase, ankyrin, and spectrin to alternate cellular sites (28, 33, 36). This has been demonstrated both in vitro (24–27) and in human biopsy samples (1), with important implications for the abnormal handling of sodium and glucose by the postischemic kidney (21, 24, 35). However, the molecular basis for the loss of Na-K-ATPase polarity after ischemic renal injury remains incompletely understood.

We have recently cloned and characterized a novel renal isoform of ankyrin, termed AnkG190, based on the predicted size of the polypeptide (37). Others and we have shown that AnkG190 is the major isoform associated with the basolateral domain of renal epithelial cells (30, 37). In this study, we have developed an anti-peptide polyclonal antibody directed to the unique NH2-terminal sequences of AnkG190. We demonstrate that AnkG190 interacts with α-Na-K-ATPase at the lateral domain of Madin-Darby canine kidney (MDCK) cells. ATP depletion results in a duration-dependent loss of α-Na-K-ATPase polarity. Using a series of complementary assays, we show that ankyrin remains bound to α-Na-K-ATPase after ATP depletion but that the interaction between ankyrin and spectrin is lost. We suggest that a dissociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface,

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possibly mediated by a posttranslational mechanism involving serine/threonine and tyrosine phosphorylation of ankyrin, may contribute to the loss of Na-K-ATPase polarity that follows ischemic injury.

MATERIALS AND METHODS

Cell culture and ATP depletion. MDCK type II cells, an established polarized renal tubule epithelial cell line obtained from American Type Culture Collection (Rockville, MD), were passaged in complete DMEM with 10% fetal bovine serum (GIBCO, Gaithersburg, MD) and analyzed within 1 day of reaching confluence. Cells were grown on six-well tissue culture-treated polystyrene plates (Costar, Cambridge, MA) except for microscopy, for which they were grown on coverslips placed within the wells. In a separate set of experiments, cells were grown on Transwell filters (Costar) to ensure complete polarization. ATP depletion was used as a well-established model of reversible ischemic injury to MDCK cells (2, 6, 15, 26, 38). Briefly, confluent cells were washed with PBS and incubated for varying time periods in glucose-free DMEM (GIBCO) in the presence of 1 μM antimycin A (Sigma, St. Louis, MO) as an inhibitor of oxidative phosphorylation (15). We have previously shown that MDCK cells subjected to this protocol undergo partial but reversible ATP depletion (15). After ~8 h, a subset of cells thus stressed initiate the process of apoptosis, but the majority remain adherent and viable (15).

Preparation of polyclonal antibodies to AnkG190. Affinity-purified polyclonal antibodies were generated (Quality Controlled Biochemicals, Hopkinton, MA) in two rabbits after injection of a glutaraldehyde-conjugated synthetic peptide containing residues 15–31 of AnkG190. These residues were chosen because they are unique to AnkG190 (37). Antibody titers of hyperimmune sera were monitored by ELISA using the BSA-coupled peptide.

Microscopy. Immunofluorescence microscopy was performed at room temperature as previously described (12, 13, 29). Briefly, 4-μm sections of paraffin-embedded rat kidney or MDCK cells at confluence or after varying periods of ATP depletion were fixed with acetone for 15 min, blocked in goat serum for 30 min, incubated in primary antibody in 2% BSA containing 10% goat serum for 60 min, washed, incubated in secondary antibodies conjugated to Cy2 or Cy3 (Amersham, Arlington Heights, IL), and visualized with a microscope (Olympus AX70, Lake Success, NY) equipped for epillumination. The antibodies used were the polyclonal to AnkG190 at 1:200 dilution and a monoclonal to tubulin at 1:10,000 (Sigma).

Immunoprecipitations. Immunoprecipitations were performed as previously described (13, 29). Briefly, cells in six-well plates were lysed for 20 min at 4°C in 2 ml of IP buffer [10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.5% deoxycholate, 1% Nonidet P-40, and 1× Complete protease inhibitor (Roche Applied Science, Indianapolis, IN)]. In a separate set of experiments, cells were extracted in situ with a low-salt buffer (10 mM PIPES, pH 6.8, 50 mM NaCl, 300 mM sucrose, 0.5% Triton X-100, and 1× Complete protease inhibitor (Roche)) to isolate a cytosolic fraction, as previously described (11–13, 37, 40). The lysates were centrifuged for 1 min at 10,000 g, washed three times with IP buffer, and the pellet was subjected to SDS-PAGE and Western analysis. The antibodies used were the polyclonal to AnkG190 at 1:200 dilution, a monoclonal to α-Na-K-ATPase at 1:5,000 (Upstate Biotechnology), a polyclonal to βII spectrin (10D) at 1:200 dilution, and a monoclonal to tubulin at 1:10,000 (Sigma).

Ankyrin binding assay. Solution binding assays were performed using glutathione S-transferase (GST) fusion peptides as previously described (11, 40). The minimal ankyrin binding (MAB) domain of α-Na-K-ATPase lies within residues 142–166, and the preparation and purification of recombinant peptides representing this region have been previously detailed (11, 40). A construct encoding for MAB was expressed in bacteria as a GST fusion using the pGEX pro-karyotic expression system (Pharmacia, Piscataway, NJ) and purified using glutathione-agarose (11, 40). GST alone was expressed as a control peptide. Proteins were analyzed by SDS-PAGE followed by staining with Coomassie blue and were quantified by a Bradford assay (Bio-Rad, Hercules, CA). Each fusion protein (GST or GST-MAB, 50 μg at 1 mg/ml) was conjugated to 50 μl of a 50% slurry of glutathione-agarose for 1 h at 4°C with gentle rotation and incubated at 4°C overnight with 1 ml of a cytoskeletal fraction (300 μg of total protein) from confluent MDCK cells extracted in situ, using a buffer containing 10 mM PIPES, 500 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 0.5% Triton X-100, and 1× Complete (Roche) protease inhibitors (11, 40). The beads were pelleted and washed twice with PBS, and aliquots were analyzed by SDS-PAGE followed by Western blotting and enhanced chemiluminescence (Amersham) with antibodies as above.

Ankyrin phosphorylation assays. The phosphorylation status of ankyrin was examined using protocols as recommended by the manufacturer (Upstate Biotechnology). Briefly, cells were preincubated for 60 min with protease inhibitor [1× Complete protease inhibitor (Roche)] and a cocktail of phosphatase inhibitors including 1 mM sodium vanadate (Calbiochem), 10 mM okadaic acid (GIBCO), and 1 mM calyculin A (GIBCO). Cells were then extracted in situ with high-salt lysis buffer (500 mM NaCl, 1% Nonidet P-40, 50 mM Tris, pH 8.0), and lysates were centrifuged at 14,000 rpm for 10 min at 4°C. The clear supernates (1 ml) were incubated overnight at 4°C with either 20 μl of preconjugated anti-phosphotyrosine agarose (Upstate Biotechnology) or with a combination of 20 μl of anti-phosphoserine/threonine antibody and 20 μl of IgG-agarose (Upstate Biotechnology). Bound complexes were recovered by centrifugation, washed with 1× PBS, and subjected to Western analysis with antibody to AnkG190 as described above.

RESULTS

Ankyrin is polarized to the basolateral membrane of kidney tubules and MDCK cells. We generated polyclonal antibodies directed to the unique NH2-terminal sequences of AnkG190. The antibody specifically recognized an immunoreactive peptide at 190 kDa in rat kidney lysates and a 210-kDa peptide in MDCK cell lysates, whereas the preimmune serum was devoid of cross-reactivity (Fig. 1). By immunofluorescence microscopy, AnkG190 was localized predominantly to the basolateral domains of kidney tubule cells (not shown) and cultured MDCK cells (Fig. 1), whereas no staining was observed with the preimmune serum (not shown). Double-staining of MDCK cells revealed a colocaliza-
tion of AnkG190 with α-Na-K-ATPase along the basolateral membrane (Fig. 2G).

ATP depletion results in a duration-dependent loss of polarized distribution of Na-K-ATPase and ankyrin. The well-established ATP depletion model was used to study the behavior of ankyrin and Na-K-ATPase after ischemic injury in MDCK cells. Within 3 h of ATP depletion, there was a partial loss of basolateral membrane staining of AnkG190 (Fig. 2B) and α-Na-K-ATPase (Fig. 2E), with both proteins beginning to assume a punctate cytoplasmic distribution characteristic of internalized Na-K-ATPase. By 6 h of ATP depletion, the cytoplasmic mislocation of both AnkG190 (Fig. 2C) and α-Na-K-ATPase (Fig. 2F) was complete, and no staining of either protein was visible at the basolateral membrane. Another uniform observation was that the abundance of immunoreactive ankyrin was increased after 6 h of ATP depletion (Fig. 2, C vs. A). This is consistent with our previous findings of enhanced ankyrin expression in a rat model of renal ischemia (34).

Ankyrin remains bound to Na-K-ATPase after ATP depletion. Because a tethering interaction with ankyrin is crucial to the normal basolateral distribution of Na-K-ATPase, it was of interest to determine the behavior of ankyrin in cells with mislocated Na-K-ATPase after ATP depletion. Double-immunostaining revealed that AnkG190 remained partially colocalized with α-Na-K-ATPase at all time periods after ATP depletion (Fig. 2, H and I).

The finding that ankyrin continues to interact with Na-K-ATPase after ATP depletion was confirmed using two additional complementary assays. First, analysis of the polyclonal AnkG190 antibody immunoprecipitates demonstrated that control cells contained functional complexes of AnkG190, α-Na-K-ATPase, and βII-spectrin (Fig. 3A). Conversely, in cells depleted of ATP, α-Na-K-ATPase continued to immunoprecipitate with ankyrin, whereas spectrin was lost from the complex. The loss of spectrin from the complex was duration dependent (Fig. 3A) and could not be attributed to its degradation, because the 240-kDa immunoreactive spectrin band remained intact in whole cell lysates at all time periods examined (Fig. 3B). In a separate set of experiments, with cells grown on Transwell filters to

Fig. 2. ATP depletion results in a duration-dependent loss of polarized distribution of Na-K-ATPase and ankyrin. MDCK cells, untreated (left) or ATP depleted for 3 (middle) or 6 h (right), were double-stained with antibodies to ankyrin (red, A–C) and α-Na-K-ATPase (green, D–F). G–I: merged images. Results are representative of 3 experiments. Bar = 10 μm.
optimize the generation and maintenance of cell polarity, identical results were obtained (not shown). In another set of experiments, the cells were extracted with a low-salt buffer to isolate a purely cytosolic fraction. Ankyrin in this cytosolic fraction continued to associate with \( \beta_{2} \)-Na-K-ATPase after 6 h of ATP depletion but not with spectrin (not shown).

In the second assay, the in vitro interaction of ankyrin from ATP-depleted cell lysates with a GST fusion protein containing the minimal ankyrin binding domain of \( \alpha \)-Na-K-ATPase (GST-MAB) was evaluated. In control cells, this peptide bound strongly to an ankyrin-spectrin complex (Fig. 4). After ATP depletion, GST-MAB continued to avidly bind ankyrin, but spectrin was progressively lost from the complex as a function of the duration of ATP depletion (Fig. 4). The negative control, GST alone, did not interact with ankyrin or spectrin.

An additional observation gleaned from Figs. 3 and 4 pertains to the enhanced ankyrin expression after ATP depletion. A significant increase in the abundance of immunoreactive ankyrin was noted after 6–12 h of ATP depletion, consistent with the enhanced ankyrin immunofluorescence observed in Fig. 2 and with our previous in vivo observations (34). Densitometric analysis of multiple blots revealed that ankyrin protein abundance increased by 2.5 ± (SD) 0.5-fold at 6 and 12 h of ATP depletion.

**Ankyrin undergoes phosphorylation after ATP depletion.** Because membrane-cytoskeletal interactions in general, and the interactions between spectrin and ankyrin in particular, are often regulated by phosphorylation (7, 10, 22), it was of interest to determine whether the phosphorylation status of ankyrin changed after ATP depletion. This was measured by evaluating the amount of ankyrin that was present in anti-phosphotyrosine or anti-phosphoserine/threonine precipitates. Surprisingly, despite the diminished ATP content of these cells, increases in both tyrosine and serine/threonine phosphorylation on ankyrin were detected, as shown in Fig. 5. Enhanced phosphorylation was detected as early as 3 h after the start of ATP depletion and persisted through 6 h of ATP depletion. These changes correlated with the rate of spectrin loss from the ankyrin-Na-K-ATPase complex.

**Fig. 3.** Ankyrin remains bound to Na-K-ATPase after ATP depletion-immunoprecipitation assays. A: MDCK cells, untreated (Con) or ATP depleted for various periods as shown, were analyzed for complexes with ankyrin. The negative control was beads alone (Beads). Equal aliquots of cell lysates were probed with anti-tubulin antibody before precipitation to verify equal loading of samples. B: Western blot of cell lysates at various time periods as indicated, with anti-spectrin antibody. Left: molecular mass (in kDa). Results are representative of 3 experiments.

**Fig. 4.** Ankyrin remains bound to Na-K-ATPase after ATP depletion-solution binding assays. Top: Coomassie blue-stained gel of the recombinant peptides glutathione S-transferase (GST) alone (negative control (Con)) and GST-minimal ankyrin binding (MAB) construct. Middle and bottom: Western blots with antibodies to ankyrin and spectrin, respectively. Left: molecular mass markers (in kDa). Results are representative of 2 experiments.

**Fig. 5.** Ankyrin is phosphorylated after ATP depletion. Western blot with ankyrin antibody of MDCK cell lysates after various periods of ATP depletion and immunoprecipitation with anti-phosphotyrosine or anti-phosphoserine/threonine. Equal aliquots of cell lysates were probed with anti-tubulin antibody before precipitation to verify equal loading of samples.
DISCUSSION

We have developed a polyclonal antibody directed to the unique NH$_2$-terminal sequences of AnkG190 and demonstrated that AnkG190 interacts with α-Na-K-ATPase at the lateral domain of polarized MDCK cells. On ATP depletion, there is a duration-dependent loss of Na-K-ATPase and ankyrin polarity. Using a series of complementary assays, we have demonstrated that ankyrin remains bound to Na-K-ATPase after ATP depletion but that the interaction between ankyrin and spectrin is lost. We suggest that a dissociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface may contribute to the loss of Na-K-ATPase polarity and contribute to its internalization after ATP depletion.

Ischemic renal injury remains a leading cause of acute renal failure, and despite important technical advances in treatment the associated mortality and morbidity remain dismally high (28, 33, 36). An improved understanding of the pathophysiology from a cellular and molecular standpoint may facilitate the development of novel therapeutic interventions. Although the pathogenesis of acute renal failure is clearly multifactorial, persistent afferent arteriolar vasoconstriction is considered an important factor and has been postulated to occur secondarily to the loss of proximal tubule cell polarity (36). Previous studies have established that ischemic injury modeled by ATP depletion results in a disruption of polarized basolateral membrane distribution of Na-K-ATPase, ankyrin, and spectrin in cultured proximal tubule cells (24–27), leading to impaired sodium reabsorption (24, 35). Loss of Na-K-ATPase polarity and increased delivery of filtered sodium to the macula densa have also been documented in human cadaveric kidneys after ischemia-reperfusion injury (1, 21). It is reasonable to hypothesize that the ensuing activation of tubuloglomerular feedback contributes to the persistent vasoconstriction characteristic of ischemic acute renal failure. However, the molecular basis for the loss of Na-K-ATPase polarity after ischemic renal injury remains incompletely understood.

Several lines of evidence gleaned from the present study suggest that a dissociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface may be responsible for the loss of Na-K-ATPase polarity after ATP depletion. First, the internalized and mislocalized α-Na-K-ATPase remains substantially colocalized with ankyrin. Second, ankyrin remains complexed with α-Na-K-ATPase in immunoprecipitates, whereas spectrin is lost from the complex in proportion to the duration of ATP depletion. Third, the isolated ankyrin binding domain of Na-K-ATPase continues to interact in vitro with ankyrin after ATP depletion whereas spectrin does not. Fourth, βII spectrin remains intact and does not undergo degradation after ATP depletion. Collectively, these results suggest earlier studies demonstrating the lack of association between ankyrin and spectrin after ATP depletion in LLC-PK$_1$ cells (26) and demonstrate the persistence of a crucial ankyrin-Na-K-ATPase linkage. Others have similarly shown that in large measure, both spectrin and ankyrin remain intact after ischemic injury to MDCK cells (14).

The phosphorylation state of ankyrin is an important determinant of its interaction with spectrin (7, 16–19, 22, 23). Unphosphorylated ankyrin preferentially binds to the fully functional tetrameric spectrin unit rather than to dimers with a 10-fold greater affinity; this preferential binding is abolished by phosphorylation. Although the sites and physiological significance of phosphorylation remain unclear, an attractive mechanism by which ATP depletion might influence spectrin-ankyrin interactions would be by altering the phosphorylation status of ankyrin. Our findings indicate that ATP depletion results paradoxically in a marked increase in both tyrosine and serine/threonine phosphorylation of ankyrin, suggesting a possible biochemical mechanism underlying the loss of association between spectrin and ankyrin. While the role of phosphorylation in ankyrin is unknown, it has been shown that hyperphosphorylation of ankyrin on serine/threonine in avian red blood cells by casein kinase II suppresses its ability to bind spectrin (16, 18). Casein kinase II is also known to phosphorylate spectrin (39). Normally, ankyrin and spectrin undergo a futile cycle of rapid phosphorylation and dephosphorylation, reflecting dynamic control of membrane and cytoskeletal organization. During a period of ATP depletion, this cycle is presumably interrupted or unbalanced. The mechanisms that account for changes in kinase/phosphatase activity under these conditions remain unexplored. Another factor that may influence the spectrin-ankyrin interaction after ATP depletion includes changes in the oligomeric status of spectrin per se. In erythrocytes, the affinity of spectrin for ankyrin is cooperatively linked to its oligomeric state, with ankyrin favoring spectrin oligomers and tetramers over spectrin dimers (7, 19). Because spectrin's oligomerization state can be influenced by a variety of factors including phosphorylation, calcium, calmodulin, and proteolysis (20, 23), these factors may also contribute, under conditions of renal tubule cell injury, to the deconstruction of the cortical spectrin-ankyrin skeleton.

Finally, an additional observation offered by this study is the finding that immunodetectable ankyrin is upregulated after ATP depletion in MDCK cells, consistent with our previous findings in a rat model of renal ischemia (34). Although the molecular mechanisms and significance of this change are unclear, it is intriguing to speculate that the overexpressed ankyrin, via its continued ability to interact with Na-K-ATPase, may play a role in the restoration of cell polarity during recovery from ischemia, lending emerging evidence for the notion that ankyrin-spectrin binding is a necessary cofactor for the appropriate membrane delivery and sorting of a subset of proteins that includes Na-K-ATPase (13), anion exchanger (17), CD45 (32), and others (5).
In summary, the present study suggests that a disassociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface may contribute to the loss of Na-K-ATPase surface display and polarity after ATP depletion. It will be important in future studies to examine the phosphorylation status of ankyrin after ischemic injury in animal models to further elucidate the possible mechanisms underlying the selective loss of spectrin binding and preferential retention of the interaction between ankyrin and Na-K-ATPase.

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