Calpain Mediates Progressive Plasma Membrane Permeability and Proteolysis of Cytoskeleton-Associated Paxillin, Talin, and Vinculin during Renal Cell Death

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ABSTRACT

The goal of the present study was to determine the role of calpain in changes in plasma membrane permeability and cytoskeleton-associated paxillin, vinculin, talin, and α-actinin levels during acute renal cell death. The mitochondrial inhibitor antimycin A or hypoxia produced graded plasma membrane permeability in renal proximal tubules (RPTs), first allowing propidium iodide (PI, molecular mass 668 Da) influx and then lactate dehydrogenase (LDH, molecular mass 130 kDa) release. Cytoskeleton-associated paxillin levels decreased concomitantly with PI influx and before LDH release, whereas cytoskeleton-associated talin and vinculin levels decreased concomitantly with LDH release. Cytoskeleton-associated α-actinin levels did not change during antimycin A exposure or hypoxia. Purified μ-calpain cleaved paxillin, talin, vinculin, but not α-actinin. The dissimilar calpain inhibitors 3-(4-iodophenyl)-2-mercapto-[Z]-2-propenoic acid (PD150606) or chloroacetic acid N'-(6,7-dichloro-4-phenyl)-3-oxo-3,4-dihydroquinazolin-2-yl hydrazide (SJA7029) preserved cytoskeleton-associated paxillin, talin, and vinculin levels and prevented PI influx and LDH release in antimycin A-exposed or hypoxic RPTs. These results suggest that calpain mediates increased plasma membrane permeability and hydrolysis of cytoskeleton-associated paxillin, vinculin, and talin during renal cell death.

Under physiological conditions, the plasma membrane maintains homeostasis of the intracellular environment by retaining cytosolic contents and ionic gradients. When cells are subjected to harmful insults, the plasma membrane undergoes substantial changes with increased permeability and altered morphology (Lemasters et al., 1987; Garza-Quintero et al., 1993; Zahrebelski et al., 1995; Dong et al., 1998; Chen et al., 2001; Nishimura and Lemasters, 2001). A consequence of increased plasma membrane permeability is loss of cytosolic enzymes and metabolites and collapse of the electrochemical gradients of ions across the plasma membrane. Such events are incompatible with cell viability.

Plasma membrane disruption during oncosis is not a singular or all-or-none event; rather, it is a progressive process with a series of altered permeability phases. For example, Chen et al. (2001) demonstrated three different permeability phases in freshly isolated rabbit RPTs subjected to anoxia. The first phase allowed the entry of the cell-impermeable DNA dye propidium iodide (PI, molecular mass 668 Da). The second phase allowed the entry of dextrans up to 3 kDa, and the last phase allowed the entry of 70-kDa dextrans and the release of cytosolic enzymes such as LDH (molecular mass 130 kDa). Similar changes in plasma membrane permeability were observed in Madin-Darby canine kidney (MDCK) cells exposed to chemical hypoxia (Dong et al., 1998) and in hepatic sinusoidal endothelial cells subjected to chemical hypoxia (Nishimura and Lemasters, 2001).

The mechanism underlying increased plasma membrane permeability during cell injury/death remains poorly understood. However, growing evidence indicates that calpains play a critical role in this process. For example, using freshly isolated rabbit RPT and LDH release as a marker of increased plasma membrane permeability, our laboratory showed that multiple calpain inhibitors, including 3-(4-iodophenyl)-2-mercapto-[Z]-2-propenoic acid (PD150606) and chloroacetic acid N’-(6,7-dichloro-4-phenyl)-3-oxo-3,4-dihydroquinazolin-2-yl)hydrazide (SJA7029) protected against...
increased plasma membrane permeability in the process of acute renal cell injury/death produced by diverse toxicants (Schnellmann et al., 1994; Waters et al., 1997; Schnellmann and Williams, 1998; Harriman et al., 2000; Liu et al., 2001). These included a mitochondrial inhibitor (antimycin A), an alkylating quinone (bromohydroquinone), an oxidant (t-butylhydroperoxide), and a toxicant that forms a reactive electrophile (tetrfluoroethyl-L-cysteine). These observations suggest a key role for calpain in plasma membrane permeability increases during acute renal cell death.

Under physiological conditions, the cytoskeleton network supports the plasma membrane. The significance of this supporting cytoskeleton network in maintaining plasma membrane integrity has been demonstrated in erythrocytes in which deficiencies or defects in the cytoskeletal proteins spectrin or spectrin-associated proteins are associated with increased fragility and lysis of the plasma membrane (Palek and Sahr, 1992). Changes in the cytoskeletal network beneath the plasma membrane may contribute to increases in membrane permeability during acute renal cell injury/death.

Renal epithelia are highly differentiated cells with two distinct membrane domains, apical and basal. The apical and basal membrane domains have different biochemical properties and functions and are supported by different cytoskeletal networks. The membrane-cytoskeleton linkages at the basal membrane are chiefly through focal adhesion complexes. The cytoskeleton-associated proteins paxillin, vinculin, talin, and α-actin locate within the focal adhesion complex and mediate the anchorage of the plasma membrane to the cytoskeleton (Luna and Hitt, 1992; Miyoshi et al., 1996; Iyer and Kusner, 1999; Turner, 2000). These cytoskeleton-associated proteins are regulated by calpain under physiological conditions. For example, talin is associated with calpain in focal adhesion complexes, and calpain cleavage of talin regulates focal adhesion structure and function in several cell types (Beckerle et al., 1987). We hypothesized that calpain-mediated alterations in these cytoskeleton-associated proteins may disrupt the membrane-cytoskeleton linkage of the basal plasma membrane, leading to increased permeability during acute renal cell injury/death.

The goals of the present study were to determine 1) temporal plasma membrane permeability in antimycin A-exposed and hypoxic RPTs; 2) temporal levels of cytoskeleton-associated paxillin, talin, vinculin, and α-actin in antimycin A-exposed and hypoxic RPTs; and 3) the effects of two dissimilar calpain inhibitors (PD150606 and SJA7029) on plasma membrane permeability and levels of cytoskeleton-associated paxillin, talin, vinculin, and α-actin in antimycin A-exposed and hypoxic RPTs.

Materials and Methods

Reagents. Purified μ-calpain (from porcine erythrocyte) and the calpain inhibitor PD150606 were purchased from Calbiochem (La Jolla, CA). Calpain inhibitor SJA7029 was a gift from Dr. Jun Inoue (Senju Pharmaceutical Co., Kobe, Japan). Antimycin A and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Propidium iodide and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes (Eugene, OR). Enhance chemiluminescence kit and autoradiography films were obtained from Amersham Biosciences (Piscataway, NJ). The sources of the remaining chemicals have been reported previously (Rodeheaver et al., 1990; Groves and Schnellmann, 1996) or were from Sigma-Aldrich. All glassware was silanized and autoclaved before use. All media and buffers were sterilized by filtering before use. All chemicals used were of reagent grade.
Carlsbad, CA), subjected to electrophoresis, and the proteins transferred to nitrocellulose membranes. Membranes were incubated overnight in blocking buffer (2.5% casein, 0.9% NaCl, 5 mM Tris, and 0.25% threo-digalactosylerythritol, pH 7.6) and then incubated overnight with anti-paxillin (1:2000; Upstate Biotechnology, Lake Placid, NY), antitalin (1:2000; Sigma-Aldrich), anti-vinculin (1:2000; Sigma-Aldrich), or the anti-α-actinin antibody (1:2000; Sigma-Aldrich). Membranes were washed and incubated with a rabbit anti-mouse secondary antibody conjugated to horseradish peroxidase. Membranes were washed and developed using the enhanced chemiluminescence system following the manufacturer’s instructions. For paxillin, vinculin, talin, and α-actinin, densities of the corresponding bands were determined with NIH image software. Results are expressed as percentage of controls.

**Treatment of RPT Cell Lysates with Purified μ-Calpain.** Aliquots of RPTs were removed and subjected to centrifugation (1000g for 1 min at 4°C). The RPT pellet was resuspended in lysis buffer (100 mM Tris, 150 mM NaCl, and 10 mM EGTA, pH 7.4) and lysed with 1% Triton X-100 for 10 min at 4°C. RPT cell lysates (0.2 mg of protein) were incubated with 10 μg of purified μ-calpain in the presence of 10 mM Ca2+ or 10 mM EGTA at room temperature for 30 min and stopped by adding 2× loading buffer and boiled for 5 min. Proteins (10 μg) from each reaction were subjected to immunoblot analysis using the antibodies described above.

**LDH Analysis.** LDH release was measured as described previously (Moran and Schnellmann, 1996).

**Statistics.** The data were expressed as means ± S.E. RPT suspensions isolated from one rabbit represent a single experiment (n = 1). Data were analyzed by analysis of variance; multiple means were compared with Fisher’s protected least significance difference test using a level of significance of p < 0.05. Two means were compared with the Student’s t test at the same level of significance.

**Results**

**Progressive Plasma Membrane Permeability in Antimycin A-Exposed or Hypoxic RPTs.** Antimycin A exposure produced a time-dependent increase in PI staining (Fig. 1A). In contrast, antimycin A did not increase LDH release at 15 min but did increase LDH release after 30 min of exposure. Similar results were obtained in RPTs subjected to hypoxia (Fig. 1B). These results show that the plasma membrane of RPTs undergoes progressive permeability changes, and that at least two phases can be differentiated. These data support findings from previous studies that used an anoxic RPT model (Chen et al., 2001) or MDCK cells exposed to an uncoupler of mitochondrial phosphorylation (Dong et al., 1998). In the early phase, the plasma membrane is permeable to small molecules and allows PI (molecular mass 668 Da) influx. In the late phase, the plasma membrane is permeable to large molecules and allows the release of the cytoplasmic protein LDH (molecular mass 130 kDa).

**Cytoskeleton-Associated Paxillin, Vinculin, Talin, and α-Actinin Levels in Antimycin A-Treated or Hypoxic RPTs.** Paxillin, vinculin, talin, and α-actinin are key components of plasma membrane-cytoskeleton linkages in the basal membrane and contribute to the maintenance of membrane integrity (Gailit et al., 1993; Muguruma et al., 1995; Miyoshi et al., 1996; van de Water et al., 1998). Therefore, the levels of these proteins were determined in the cytoskeleton fraction obtained from antimycin A-exposed or hypoxic RPTs using immunoblot analysis. RPTs express 65-kDa paxillin, 230-kDa talin, 117-kDa vinculin, and 100-kDa α-actinin (Fig. 7; data not shown). Antimycin A exposure decreased cytoskeleton-associated paxillin, talin, and vinculin levels (Fig. 2). Although the decrease in paxillin was time-dependent and occurred concomitantly with PI staining and before LDH release, the decreases in talin and vinculin occurred concomitantly to LDH release (Fig. 1A). In contrast to paxillin, talin, and vinculin, the levels of cytoskeleton-associated α-actinin did not change during antimycin A exposure (Fig. 2).

Hypoxia also produced decreases in cytoskeleton-associated paxillin (Fig. 3), corresponding to PI staining and LDH release (Fig. 1B). Similar to antimycin A-exposed RPTs, cytoskeleton-associated α-actinin levels did not change during hypoxia (Fig. 3). These results demonstrate that decreases in cytoskeleton-associated paxillin, talin, and vinculin are closely correlated to plasma membrane permeability increases during renal cell death.

**Calpain Inhibitors PD150606 and SJ7029 Block Progressive Plasma Membrane Permeability during RPT Injury.** Multiple calpain inhibitors of dissimilar structures and different inhibitory actions prevented LDH release in RPTs subjected to hypoxia or exposed to a variety of toxicants (Edelstein et al., 1995, 1996, 1997; Waters et al., 1997; Schnellmann and Williams, 1998; Harriman et al., 2000; Liu et al., 2001). However, it is not known whether calpain mediates the early phase of membrane permeability increase (PI influx) during renal cell death. The calpain inhibitors PD150606 or SJ7029 blocked PI staining and LDH release in antimycin A-exposed RPTs (Fig. 4). To test whether calpain-mediated plasma membrane permeability to PI represents a common pathway during acute RPT injury/death, RPTs were subjected to hypoxia or exposed to the model oxidant t-butylhydroperoxide. Similar results were observed in RPTs subjected to hypoxia (Fig. 5) and t-butylhydroperoxide (PD150606 decreased t-butylhydroperoxide-induced PI staining from 53 ± 3 to 20 ± 2% and LDH release from 28 ± 4 to 7 ± 1%). These results strongly suggest that calpain mediates the early phase of plasma membrane permeability increase and that calpain-mediated early membrane permeability to PI represents a common pathway during renal cell death produced by diverse insults.

**Calpain Inhibitors PD150606 and SJ7029 Preserve Cytoskeleton-Associated Paxillin, Talin, and Vinculin during RPT Injury.** Experiments were designed to determine whether calpain inhibitors block the loss of cytoskeleton-associated paxillin, talin, and vinculin during RPT injury. The presence of PD150606 or SJ7029 preserved cytoskeleton-associated paxillin, talin, and vinculin levels in antimycin A-exposed RPTs (Fig. 6). Hypoxia-induced decreases in paxillin (67 ± 5% of control; Fig. 3) were also blocked by PD150606 (187 ± 43% of control) and SJ7029 (105 ± 9% of control). These results strongly suggest that the decreases in cytoskeleton-associated paxillin, talin, and vinculin levels during RPT cell injury are due to the action of calpain.

**Paxillin, Talin, and Vinculin Are Substrates of Purified μ-Calpain.** Experiments were performed to confirm that paxillin, talin, and vinculin are calpain substrates. Because calpain depends on the presence of Ca2+ for its proteolytic activity, RPT lysates were treated with purified μ-calpain in the presence of 10 mM Ca2+ or in the presence of 10 mM EGTA, and the hydrolysis of paxillin, talin, and vinculin was compared. Treatment of RPT lysates with purified μ-calpain in the presence of 10 mM Ca2+ resulted in decreases in
68-kDa paxillin and 117-kDa vinculin and the loss of 230-kDa talin (Fig. 7). The loss of 230-kDa talin and the decrease in 117-kDa vinculin were accompanied by increases in a 190-kDa talin fragment and a 91-kDa vinculin fragment (Fig. 7). The hydrolysis of these proteins by purified μ-calpain was prevented by 10 mM EGTA. These results demonstrate that paxillin, talin, and vinculin are calpain substrates.

Discussion

Cellular swelling and extensive changes in the plasma membrane with increased permeability and altered morphology have been considered landmarks of oncosis (Lemasters et al., 1987; Elliget et al., 1991, 1994; Garza-Quintero et al., 1993; Zahrebelski et al., 1995; Dong et al., 1998; Chen et al.,...
2001; Nishimura and Lemasters, 2001). The increased plasma membrane permeability leads to loss of cytosolic enzymes and metabolites and collapse of electrochemical gradients of ions across the plasma membrane and is incompatible with cell viability.

The plasma membrane disruption during oncosis is not a singular event; rather, current evidence indicates that it is a progressive process with gradually increased permeability. A series of phases were differentiated during renal cell death. In anoxic rabbit RPTs and ATP-depleted MDCK cells, at least three distinctive phases of membrane disruption were differentiated. In phases 1, 2, and 3, the plasma membrane became permeable to PI (668 Da), 3-kDa dextrans, and 70-kDa a-actinin levels or 130-kDa LDH, respectively (Dong et al., 1998; Chen et al., 2001). The present study demonstrates similar progressive membrane permeability increases in RPTs subjected to the mitochondrial inhibitor antimycin A or hypoxia and confirms that the early phase of membrane permeability increase (PI influx) is distinguishable from the late phase of membrane permeability increase (LDH release).

Previous studies have suggested that calpain plays a critical role in the late phase of membrane permeability increase during oncocytic renal cell death. For example, multiple dissimilar calpain inhibitors, including PD150606, SJA7029, and others blocked LDH release during oncosis in rabbit RPTs exposed to a variety of toxic agents and hypoxia and in rat RPTs subjected to hypoxia (Edelstein et al., 1996; Waters et al., 1997; Schnellmann and Williams, 1998; Harriman et al., 2000; Liu et al., 2001, 2002). The present study demonstrates that these two dissimilar calpain inhibitors also prevented PI influx in antimycin A-exposed, t-butylhydroperoxide-exposed, and hypoxic rabbit RPTs. The results provide strong evidence that calpain mediates the early phase of plasma membrane permeability increase and that calpain-mediated membrane permeability increases represent a com-
mon pathway during renal cell death produced by diverse insults.

How calpain mediates plasma membrane permeability increases in the process of oncotic renal cell death remains unknown. In the present study, we show that decreases in cytoskeleton-associated paxillin, talin, and vinculin levels were closely associated with progressive plasma membrane permeability increases. Specifically, paxillin, talin, and vinculin levels decreased at different rates with the decrease in paxillin concomitant with PI uptake and before LDH release and the decreases in talin and vinculin concomitant with LDH release. Paxillin is the most susceptible of these three proteins, but the reason for this susceptibility is not known. Two dissimilar calpain inhibitors preserved cytoskeleton-associated paxillin, talin, and vinculin levels and prevented progressive plasma membrane permeability increases. Treatment of RPT lysate with purified μ-calpain confirmed that paxillin, talin, and vinculin were calpain substrates. These results strongly suggest that calpain cleaves cytoskeleton-associated paxillin, talin, and vinculin during acute renal cell injury and that hydrolysis of these proteins is associated with increased plasma membrane permeability.

Renal epithelia are highly differentiated cells with two distinct membrane domains, apical and basal. Cytoskeleton-associated paxillin, vinculin, and talin function as the membrane-cytoskeleton linkage within focal adhesion complexes in the basal membrane of renal epithelial cells. Hydrolysis of these proteins could break the membrane-cytoskeleton linkage and decrease the physical support to the basal plasma membrane, leading to bleb formation. Doctor et al. (1997) reported that the linking force between the plasma mem-

Fig. 5. Effects of the calpain inhibitor PD150606 on hypoxia-induced PI entry (A) and LDH release (B). RPTs were subjected to 95% air, 5% CO₂ (CON) or hypoxia (95% N₂, 5% CO₂, HYP) for 30 min. The calpain inhibitor PD150606 (+PD, 100 μM) was added immediately before hypoxia. After 30 min of hypoxia, aliquots of RPT were removed for PI staining and LDH release determination. Columns are means ± S.E., n = 3 to 6. Columns with different letters are significantly different, p < 0.05.

Fig. 6. Effects of the calpain inhibitors PD150606 and SJA7029 on cytoskeleton-associated paxillin, talin, and vinculin levels in antimycin A-exposed RPTs. RPTs were exposed to DMSO (CON) or antimycin A (AA, 10 μM). PD150606 (+PD, 100 μM) or SJA7029 (+SJA, 100 μM) was added immediately before antimycin A exposure. At 30 min of antimycin A exposure, RPT aliquots were removed for determination of cytoskeleton-associated paxillin, talin, and vinculin levels. Columns are means ± S.E., n = 5 to 11. Bars with different letters are significantly different, p < 0.05.
brane and the underlying cellular matrix dropped by 95% after 30 min of ATP depletion and was followed by bleb formation at the basal membrane. Two calpain inhibitors blocked plasma membrane bleb formation in cultured rat kidney proximal tubular epithelial cells exposed to HgCl$_2$ (Elliget et al., 1994) and a calpain inhibitor preserved intra-cellular talin and 2-actinin levels and blocked membrane bleb formation and plasma membrane disruption in hepatocytes exposed to t-butylhydroperoxide (Miyoshi et al., 1996). In summary, the above-mentioned data from several models suggest that calpain mediates bleb formation during cell death through hydrolysis of paxillin, talin, and vinculin. Consequently, calpain may increase plasma membrane permeability by causing bleb formation. Indeed, plasma membrane bleb formation is closely related to increased permeability. For example, in ATP-depleted RPTs, basal membrane blebs permeable to dextrans up to 3-kDa were observed (Chen and Wagner, 2001). Although calpain-mediated hydrolysis of paxillin, talin, and vinculin results in bleb formation, it is not known whether bleb formation directly results in increased membrane permeability. An additional possibility is that calpain increases membrane permeability by acting directly on plasma membrane proteins.

Previous studies have demonstrated that Cl$^-$ and water influx play an important role in the terminal events of oncosis cell death (Miller and Schnellmann, 1993, 1995). CI$^-$ influx occurs after 15 min of antimycin A exposure and inhibition of Cl$^-$ influx blocks terminal cell swelling and LDH release (Miller and Schnellmann, 1993, 1995). Furthermore, calpain inhibitors were shown to block this Cl$^-$ influx (Waters et al., 1997). These results suggest that calpain increases plasma membrane permeability and contributes to oncosis through two mechanisms: 1) cytoskeleton disruption with bleb formation, and 2) increased Cl$^-$ influx followed by water influx and cellular swelling.

In summary, the present study demonstrates that calpain mediates the early phase of plasma membrane permeability increase (PI influx) and this increase represents a common pathway during renal cell death produced by diverse insults. Calpain-mediated hydrolysis of the cytoskeleton-associated paxillin, talin, and vinculin may indirectly contribute to the increased plasma membrane permeability. These results support the idea that loss of the plasma membrane permeability barrier during oncosis requires the breakdown of the cytoskeleton-membrane interaction in conjunction with the Cl$^-$ and water influx and the resulting increased osmotic force.

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References


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