Calpains mediate acute renal cell death: role of autolysis and translocation

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Liu, Xiuli, Juanita J. Rainey, Jay F. Harriman, and Rick G. Schnellmann. Calpains mediate acute renal cell death: role of autolysis and translocation. Am J Physiol Renal Physiol 281: F728–F738, 2001.—The goals of this study were to determine 1) the expression of calpain isoforms in rabbit renal proximal tubules (RPT); 2) calpain autolysis and translocation, and calpastatin levels during RPT injury; and 3) the effect of a calpain inhibitor (PD-150606) on calpain levels, mitochondrial function, and ion transport during RPT injury. RT-PCR, immunoblot analysis, and FITC-casein zymography demonstrated the presence of only \( \mu \)- and \( m \)-calpains in rabbit RPT. The mitochondrial inhibitor antimycin A decreased RPT \( \mu \)- and \( m \)-calpain and calpastatin levels in conjunction with cell death and increased plasma membrane permeability. No increases in either \( \mu \)- or \( m \)-calpain were observed in the membrane nor were increases observed in autolytic forms of either \( \mu \)- or \( m \)-calpain in antimycin A-exposed RPT. PD-150606 blocked antimycin A-induced cell death, preserved calpain levels in antimycin A-exposed RPT, and promoted the recovery of mitochondrial function and active \( \mathrm{Na}^+ \) transport in RPT after hypoxia and reoxygenation. The present study suggests that calpains mediate RPT injury without undergoing autolysis or translocation, and ultimately they leak from cells subsequent to RPT injury/death. Furthermore, PD-150606 allows functional recovery after injury.

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subunit (7, 39). The procalpains are predominantly localized in the cytosol, and several cellular events can increase the activity of calpains: a rise in intracellular Ca\(^{2+}\), translocation of calpains to membranes, autolysis of calpains, dissociation of the calpain subunits, decreased levels of calpastatin (the endogenous inhibitor of calpain), and interaction with calpain activator and phospholipids (7, 26, 27). In the autolysis-dissociation model, Ca\(^{2+}\) binding to calpain induces dissociation of the calpain subunits and two successive autolytic events (80 to 78 to 76 kDa); the autolyzed large subunit makes the dissociation irreversible (21, 28, 48). In contrast, there is evidence that calpains are active without undergoing autolysis. For example, Molinari et al. (29) reported that \(\mu\)-calpain is active in the erythrocyte membrane in its nonautolyzed 80-kDa form, and Elce et al. (13) demonstrated that a mutation in the m-calpain large-subunit autolysis site prevented autolysis but had no effect on protease activity. Despite extensive investigation, the role of autolysis during intracellular calpain activation is still debated.

The translocation of calpain from the cytosol to the plasma membrane was proposed to be a critical step in the calpain activation process in platelets and red blood cells (15, 28). A redistribution of calpain from the cytosol to the membrane fraction was detected previously after traumatic rat brain injury using casein zymography (51). However, N-methyl-\(\alpha\)-aspartic acid-induced calpain activation was independent of calpain translocation in primary rat cortical neurons (20). Also, Blomgren et al. (3) reported that calpain immunoreactivity decreased in the cytosolic fraction with no significant changes in the membrane fractions in cortical tissue from neonatal rats subjected to cerebral hypoxia-ischemia. Calpain activation in these models was confirmed by increased spectrin proteolysis.

With respect to calpain activation during renal cell injury, Edelstein et al. (9, 10, 12) observed an increase in calpain activity before membrane damage using a fluorescent calpain substrate and degradation of spectrin in rat RPT subjected to hypoxia. Using a calpain substrate, our laboratory (42, 43) suggested that calpain activity translocated from the cytosol to the membrane fraction during mitochondrial inhibitor-induced RPT cell injury. However, it is unknown which calpain isoform(s) are expressed in rabbit RPT and whether \(\mu\)- and/or m-calpain undergoes autolysis or translocates to the membrane during RPT injury.

The aims of this study were to determine 1) the expression of calpain isoforms in rabbit RPT; 2) calpain autolysis and translocation during RPT cell injury; 3) calpastatin levels during RPT cell injury; and 4) the effect of a calpain inhibitor (PD-150606) on calpain levels, mitochondrial function, and ion transport during RPT cell injury.

**MATERIALS AND METHODS**

**Reagents.** Purified \(\mu\)-calpain (from porcine erythrocyte), purified m-calpain (from porcine kidney), and the calpain inhibitor PD-150606 were purchased from Calbiochem (La Jolla, CA). Antimycin A, DMSO, and casein fluorescein isothiocyanate (FITC-casein) were obtained from Sigma (St. Louis, MO). An enhanced chemiluminescence (ECL) kit and autoradiography film were obtained from Amersham Pharmacia (Arlington Heights, IL). TRizol reagent and QIAquick gel extraction kit were purchased from GIBCO BRL (Grand Island, NY) and from Qiagen (Chatsworth, CA), respectively. Moloney murine leukemia virus reverse transcriptase (MuLV-RT) was obtained from PerkinElmer (Foster City, CA). The sources of the remaining chemicals have been reported previously (17, 33) or were from Sigma. All glassware was silanized and autoclaved before use. All media and buffers were sterilized by filtering before use.

**Isolation of RPT S2 segments, RNA extraction, and RT-PCR amplification.** Female New Zealand White rabbits (Myrtle’s Rabbitry, Thompson Station, TN) were injected with 500 units/kg heparin sulfate and euthanized with an overdose of pentobarbital sodium (50 mg/kg), and kidneys were removed. Rabbit RPT S2 segments were individually isolated using microdissection as described by Zalups and Barfuss (49). Total cellular RNA was isolated from RPT S2 segments using TRIzol reagent following the manufacturer's instructions. The RNA was stored in RNase-free water at ~80°C until it was used. The rabbit \(\mu\)- and m-calpain large-subunit mRNA sequences were identified in the National Center for Biotechnology Information (NCBI) Entrez database (accession no. M13363 and M13797 for \(\mu\)- and m-calpain large subunits, respectively). Two sets of primers were designed for use in PCR amplification studies: \(\mu\)-calpain large subunit, CU5 (sense) 5’-TGGTACCAAGGAGCTTCA-3’, CU6 (antisense) 5’-AGGGACATGAGGAAGCTGG-3’; m-calpain large subunit, CM1 (sense) 5’-ACATGACACATCGGTTCTC-3’, and CM2 (antisense) 5’-GGAGGCAAGAAGGATTGT-GTC-3’. For the m-calpain large subunit, a specific 5’ inner primer, CU3, 5’-CATTGAGTTCTGMGCCATC-3’ also was used for nested PCR amplification. The expected product from first-round PCR was 700 base pairs (bp) for the m-calpain large subunit and 700 bp for the \(\mu\)-calpain large subunit. The expected product from the nested PCR for the \(\mu\)-calpain large subunit was 500 bp.

First-strand cDNA was synthesized from 2 \(\mu\)l of total cellular RNA isolated from RPT S2 segments using MuLV-RT and a downstream antisense primer according to the manufacturer’s instructions. Control experiments were conducted in which neither RNA template nor RT was added to the RT reaction tubes. RT experiments consisted of one cycle of 2 min at 95°C, 5 min at 99°C, and 5 min at 4°C. The products from the RT reactions were amplified by PCR. First-round PCR was carried out for 1 cycle of 2 min at 95°C, 30 cycles consisting of 30 s at 94°C, 30 s at 50°C (for \(\mu\)-calpain large subunit) or 45°C (for m-calpain large subunit), and 60 s at 72°C, and followed by 1 final cycle of 5 min at 72°C. For \(\mu\)-calpain large subunit, 1 \(\mu\)l of the product from the first-round PCR was reamplified using a 5’ inner primer and the same PCR reaction conditions. The PCR products were analyzed by electrophoresis in 1.5% agarose gels containing 0.50 \(\mu\)g/ml ethidium bromide and viewed under ultraviolet (UV) light. A 700-bp DNA band from RPT S2 segments amplified with m-calpain large subunit-specific primers (CMI/CM2) and a 500-bp DNA band from RPT S2 segments amplified with \(\mu\)-calpain large subunit-specific primers (CU3/CU6) were isolated from agarose gels after the RT-PCR or nested PCR amplification using QIAquick according to the manufacturer’s instructions. The 700- and 500-bp fragments were directly sequenced using an automated DNA sequencer (ABI-PRISM, model 377, PerkinElmer).

**Isolation and incubation of rabbit RPT.** RPT were isolated and purified by the method described by Rodeheaver et al.
after the preincubation, the mitochondrial fraction were obtained by centrifugation (14,000 g for 10 min at 37°C). The pellet was resuspended in zymography buffer and lysed with 1% Triton X-100 on ice for 10 min. The cytosol and the membrane-associated fractions were mixed with 2× zymography loading buffer [100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 20% (vol/vol) glycerol, 10 mM 2-mercaptoethanol, and 0.02% bromphenol blue]. Matched samples were taken for protein concentration and determination using the BCA assay.

To measure total activity of μ- and m-calpain in RPT, RPT were centrifuged at 1,000 g for 1 min, resuspended in zymography buffer, and lysed with 1% Triton X-100 for 10 min at 37°C. The lysate was centrifuged (14,000 g for 10 min) and the supernatant was mixed with 2× zymography loading buffer.

Ten-microgram samples (unless otherwise indicated) were loaded onto 10% polyacrylamide gels containing 0.025% FITC-casein and subjected to electrophoresis under non-denaturing conditions with buffer containing (in mM) 25 Tris base, 125 glycine, 1 EDTA, and 10 2-mercaptoethanol, pH 8. Thirty nanograms of purified μ- and m-calpains and a mixture of the two were loaded onto the same gels as positive controls. After electrophoresis, the gels were incubated with a buffer containing (in mM) 50 Tris-HCl, 10 CaCl2, and 10 2-mercaptoethanol, pH 7.6, twice for 30 min at room temperature. The gels were then incubated with the same buffer at 4°C for 16 h. Photographs were taken under UV light and scanned, and the densities for the bands were determined with NIH Image software. Results are expressed as the percentage of control RPT.

Immunoblot analysis of calpastatin. The RPT supernatant used for total calpain activity evaluation by zymography was also used for determination of calpastatin. The supernatant samples were mixed with 2× loading buffer (100 mM Tris, 4% SDS, 20% (vol/vol) glycerol, 10% 2-mercaptoethanol, and 0.04% bromphenol blue) and boiled for 10 min. Matched samples were taken for protein concentrations and determined either by the method described by Lowry et al. (24), or by the BCA assay (Pierce, Rockford, IL), using BSA as standards.

To estimate the total calpain isoforms in RPT, the RPT were centrifuged at 1,000 g for 1 min, resuspended in imidazole buffer, and lysed with 1% Triton X-100 for 30 min at 37°C. The Triton X-100-soluble fraction was obtained by centrifugation (14,000 g for 2 min) and contained more than 95% of the total cellular protein (data not shown).

Thirty-microgram (unless otherwise indicated) or 20-μl samples of incubation buffer were loaded onto 10% SDS-polyacrylamide gels and subjected to electrophoresis, and the proteins were transferred to a nitrocellulose membrane. The membrane was incubated overnight in blocking buffer (2.5% casein, 0.9% NaCl, 5 mM Tris, and 0.25 μM thimerosal, pH 7.6) and then incubated overnight with either the anti-μ-calpain antibody (1:2,500) (19) or the m-calpain antibody (1:2,000) (18). The membrane was washed and incubated with a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase. The membrane was washed and developed using the ECL system following the manufacturer’s instructions. For μ- and m-calpains, densities of the corresponding bands were determined with National Institutes of Health (NIH) Image software. Results were expressed as percentage of controls.

FITC-casein zymography. RPT suspensions were separated into cytosolic and membrane-associated fractions as described previously (42, 43) with the following modifications. RPT were centrifuged at 1,000 g for 1 min, resuspended in zymography buffer [50 mM HEPES, 150 mM NaCl, 10% (vol/vol) glycerol, 5 mM EDTA, 100 μM PMSF, 10 μg/ml leupeptin, and 10 mM 2-mercaptoethanol, pH 7.6], and permeabilized with 100 μM digitonin for 10 min at 37°C. The cytosol and the membrane fraction were obtained by centrifugation (14,000 g for 10 min at 4°C). The pellet was resuspended in zymography buffer and lysed with 1% Triton X-100 on ice for 10 min. The cytosol and the membrane-associated fractions were mixed with 2× zymography loading buffer [100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 20% (vol/vol) glycerol, 10 mM 2-mercaptoethanol, and 0.02% bromphenol blue]. Matched samples were taken for protein concentration and determination using the BCA assay.

To measure total activity of μ- and m-calpain in RPT, RPT were centrifuged at 1,000 g for 1 min, resuspended in zymography buffer, and lysed with 1% Triton X-100 on ice for 10 min. The cytosol and the membrane-associated fractions were mixed with 2× zymography loading buffer [100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 20% (vol/vol) glycerol, 10 mM 2-mercaptoethanol, and 0.02% bromphenol blue]. Matched samples were taken for protein concentration and determination using the BCA assay.

Hypoxia/reoxygenation exposure and QO2 measurement. RPT were subjected to hypoxia (95% N2-5% CO2, 1 h) before reoxygenation (95% air-5% CO2, 1 h), as described previously (31). Immediately after the hypoxic period, aliquots of RPT were removed for determination of LDH release. After reoxygenation, aliquots of RPT were removed for determination of LDH release or oxygen consumption (QO2). QO2 was measured polarographically using a Clark-type electrode as described previously (34). After basal oxygen consumption was measured, an oxygen-insensitive QO2 was measured in the presence of 0.1 mM ouabain, and the oxygen-insensitive QO2 was calculated as the difference between basal and ouabain-insensitive QO2. The calpain inhibitor PD-150606 (100 μM) was added immediately before hypoxia. Protein concentration was determined by the BCA assay.

LDH analysis. The release of LDH into the incubation buffer was measured as a marker of cellular death/lysis, as described previously (30).

Statistics. The data are expressed as means ± SE. RPT suspensions isolated from one rabbit represent a single experiment (n = 1). Data were analyzed by ANOVA; multiple means were compared using Fisher’s protected least signifi-
cance difference test with a level of significance of \( P < 0.05 \). Two means were compared using Student’s \( t \)-test with the same level of significance.

**RESULTS**

Expression of calpain isoforms in rabbit RPT by RT-PCR, immunoblot analysis, and FITC-casein zymography. Freshly isolated rabbit RPT suspensions provide a mixture of S1 and S2 segments of RPT (96–99% purity) with the majority of the contamination being glomeruli and distal tubular segments (17). To avoid contamination of non-RPT cells in determining the expression of calpain isoforms in RPT using RT-PCR, individual RPT S2 segments were micro-dissected and pooled, and total cellular RNA was extracted. RNA was subjected to RT-PCR using either rabbit \( \mu \)- or m-calpain large subunit-specific primers. First round PCR amplified a cDNA of the expected size of 700 bp using the m-calpain large subunit primers (Fig. 1A). A product was not observed after first-round PCR using the \( \mu \)-calpain large subunit primers. However, nested PCR using a 5’ inner primer resulted in the expected 500-bp product (Fig. 1B). No products were observed in reactions lacking the RNA template or RT (data not shown). Sequencing of both PCR products revealed sequences that were identical to \( \mu \)- and m-calpain large subunit mRNA sequences reported in the NCBI Entrez database. Therefore, the RT-PCR data demonstrate that both \( \mu \)- and m-calpain large subunits are expressed in rabbit RPT S2 segments at the mRNA level.

Immunoblot analysis also was employed to demonstrate the presence of both \( \mu \)- and m-calpain large subunit proteins in rabbit RPT. A previously characterized anti-\( \mu \)-calpain large subunit antibody that recognizes the 80-, 78-, and 75-kDa \( \mu \)-calpain forms and a characterized anti-m-calpain large subunit antibody that recognizes brain m-calpain were used (18, 19).

![Fig. 1. Expression of \( \mu \)- and m-calpain large subunits in rabbit renal proximal tubule (RPT) S2 segments. RPT S2 segments were micro-dissected, and RNA were extracted and subjected to RT-PCR under conditions described in MATERIALS AND METHODS using \( \mu \)- or m-calpain large subunit-specific primers. Products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide. A: a 700-bp product amplified from the m-calpain large subunit using m-calpain large subunit-specific primers; B: a 500-bp product amplified from the \( \mu \)-calpain large subunit using \( \mu \)-calpain large subunit-specific primers; in this case, RT-PCR was followed by a nested PCR using a 5’ inner primer. S2, RPT S2 segments; M, DNA molecular weight marker.](http://ajprenal.physiology.org/)

![Fig. 2. Characterization of anti-\( \mu \)- and m-calpain large subunit antibodies by immunoblot analysis using purified \( \mu \)- and m-calpains. A: detection of an 80-kDa immunoreactive band for \( \mu \)-calpain using anti-\( \mu \)-calpain large subunit antibody. No immunoreactivity was detected using purified m-calpain. B: detection of an 80-kDa immunoreactive band for m-calpain using anti-m-calpain large subunit antibody. No immunoreactivity was detected on purified \( \mu \)-calpain. MW, protein molecular weight standards.](http://ajprenal.physiology.org/)

Immunoblot analysis using the anti-\( \mu \)-calpain large subunit antibody and purified \( \mu \)-calpain revealed an 80-kDa band, but no band was detected using purified m-calpain (Fig. 2A). Immunoblot analysis with purified \( \mu \)-calpain incubated in the presence of 1 mM free \( \text{Ca}^{2+} \) confirmed that this antibody was able to recognize all three forms, 80, 78, and 75 kDa (data not shown). Similarly, immunoblot analysis using the anti-m-calpain large subunit antibody and purified m-calpain revealed an 80-kDa band, but no band was detected using purified \( \mu \)-calpain (Fig. 2B). Immunoblot analysis with purified m-calpain incubated with 10 mM free \( \text{Ca}^{2+} \) confirmed that this antibody was able to recognize all three forms, 80, 78, and 76 kDa (data not shown). These results demonstrate the utility and specificity of the antibodies.

RPT cytosolic and membrane proteins were subjected to immunoblot analysis using the same antibodies. Immunoblot analysis using the anti-\( \mu \)-calpain large subunit antibody revealed the presence of 80- and 78-kDa \( \mu \)-calpain in the cytosolic and membrane fractions, respectively (Fig. 3, A and B). The signal for the
80-kDa band in the cytosolic fraction was stronger than that for the 78-kDa band in the membrane fraction. Immunoblot analysis demonstrated the presence of the 80 kDa and the autolytic 76-kDa form of m-calpain large subunit in the cytosol using the anti-m-calpain large subunit antibody (Fig. 3C). Immunoblot analysis using the same antibody on the membrane fraction did not reveal a band for m-calpain large subunit (data not shown).

FITC-casein zymography is a sensitive method for distinguishing μ- and m-calpain activities (1). Samples are run under nondenaturing conditions, and proteins are separated primarily by their net charges. As shown in Fig. 4, FITC-casein zymography of purified calpains revealed one band for μ-calpain (top) and a doublet for m-calpain (bottom), demonstrating the ability of this method to separate μ- from m-calpain activities. The presence of double bands for purified m-calpain is consistent with previous observations in the literature (1). FITC-casein zymography of the RPT cytosol demonstrated the presence of both μ- and m-calpain activities in the cytosol as single bands parallel with purified μ- and m-calpain (Fig. 4A). Compared with the cytosolic fraction, FITC-casein zymography of the membrane fraction revealed the presence of weak bands for μ- and m-calpain (Fig. 4B). No additional calpain isoforms were identified in the cytosolic or membrane fractions using FITC-casein zymography (Fig. 4). These results strongly suggested the presence of only μ- and m-calpain in RPT.

Alterations of μ- and m-calpains in the cytosolic and membrane fractions during mitochondrial inhibitor-induced RPT cell injury. RPT were exposed to the mitochondrial inhibitor antimycin A to induce cell injury/death. Antimycin A treatment resulted in time-dependent cell death, indicated by increased LDH release at 30 min of treatment (Fig. 5A). FITC-casein zymography was performed on the cytosolic and membrane fractions of RPT after different times of antimycin A exposure. As demonstrated in Fig. 5, B and C, cytosolic μ- and m-calpain activities did not change at the early stage of RPT injury produced by antimycin A exposure. However, antimycin A treatment resulted in decreases in cytosolic μ- and m-calpain activities after 30 min. FITC-casein zymography of the membrane fraction obtained from RPT exposed to antimycin A for up to 15 min did not reveal any increases in either μ- or m-calpain isoform (Fig. 5, D and E).

Fig. 4. FITC-casein zymographic assay of the cytosol and membrane fraction from rabbit RPT. A: the zymography results using rabbit RPT cytosolic protein (10 μg). B: the zymography results using the membrane protein from rabbit RPT (50 μg). μ- and m- are used to indicate the positions of purified μ (30 ng)- or m-calpain (30 ng).

However, after 30 min of exposure, both μ- and m-calpain activities in the membrane fractions were decreased, similar to that observed in the cytosol. Therefore, the FITC-casein zymography results demonstrate the lack of μ- or m-calpain translocation during the early phases of cell injury and decreases in μ- and m-calpain activities in both fractions in concert with cell death/lysis produced by antimycin A exposure.

Immunoblot analysis using the anti-μ-calpain large subunit antibody revealed decreases in the cytosolic 80-kDa μ-calpain large subunit protein, and the reductions correlated with increased cell death/lysis after antimycin A exposure (Fig. 6, A and B). The decreases in the cytosolic 80-kDa μ-calpain large subunit were not accompanied by the appearance of either 78- or 75-kDa autolyzed μ-calpain large subunit forms in the cytosol (data not shown). Immunoblot analysis of the membrane fraction revealed no increases in the 78-kDa μ-calpain large subunit or the appearance of any additional autolytic forms of the μ-calpain large subunit (data not shown). The immunoblot analyses confirm the FITC-casein zymography results and suggest that the decrease in cytosolic μ-calpain levels was due to leakage from the cell and not due to translocation to the membrane or autolysis.

Immunoblot analysis with the anti-m-calpain large subunit antibody was performed on the cytosol and the membrane fraction of RPT exposed to antimycin A. Under control conditions, this antibody recognized the 80-kDa and the autolytic 76-kDa form of m-calpain large subunit in the cytosol. Antimycin A exposure for 30 min resulted in a 25% decrease in the 80-kDa m-calpain large subunit and a 90% decrease in the 76-kDa autolytic m-calpain large subunit (data not shown), which is consistent with a decrease in m-calpain activity revealed by FITC-casein zymography. Immunoblot analysis of the membrane fractions of control or antimycin A-exposed RPT did not reveal the presence of m-calpain. These results show that the decrease in cytosolic m-calpain levels was due to leakage from the cell and not due to translocation to the membrane or autolysis.

Leakage of calpain isoforms from RPT during mitochondrial inhibitor-induced cell injury. The possibility that decreases in cytosolic μ- and m-calpains was due to increased cell membrane permeability during RPT cell injury/death was explored further. Total μ- or m-calpain levels were measured in antimycin A-treated RPT and compared with that of controls. Loss of cytosolic protein from antimycin A-treated RPT decreased the ratio of cytosolic-to-membrane protein and would introduce an artificial decrease in the calpain isoforms contained per milligram total protein. Therefore, the same sample volume (20 μl) instead of the same amount of total protein from control and antimycin A-treated RPT was used for FITC-casein zymography or immunoblot analysis. FITC-casein zymography revealed that antimycin A exposure resulted in no changes in both calpain isoforms during the first 15 min of RPT injury (Fig. 7, A and B). However, after
30 min, decreased activities for calpain isoforms were observed (Fig. 7, A and B). Immunoblot analysis using the anti-μ-calpain large subunit antibody on the Triton X-100-soluble fraction revealed decreases in total 80-kDa μ-calpain large subunit after 30 and 60 min of antimycin A exposure (Fig. 8A), further supporting the FITC-casein zymography results. Correlated with the decreases in protein or activities of both calpains were similar increases in cell death, indicated by increased LDH release and the appearance of both μ- and m-calpain large subunits in the incubation buffer (Figs. 5A and 8). These results show that calpains leak from the RPT cells during antimycin A-induced RPT injury/death.

Changes in calpastatin during mitochondrial inhibitor-induced RPT cell injury. The protein level of the endogenous calpain inhibitor calpastatin was examined using immunoblot analysis. The antibody recognized a 120-kDa protein in RPT, which is consistent with previous reports (25). Immunoblot analysis demonstrated that calpastatin is present primarily in the RPT cytosol (data not shown). As shown in Fig. 9, A and B, antimycin A exposure up to 15 min did not result in decreases in calpastatin levels. A decrease in calpastatin protein level was found 30 min after antimycin A exposure (Fig. 9B) and corresponded to the increased LDH release (Fig. 5A).

Effect of PD-150606 on μ-calpain level, mitochondrial function, and active Na+ transport in RPT exposed to antimycin A or subjected to hypoxia/reoxygenation. The present study investigated the effects of PD-150606 on total μ-calpain levels during RPT cell injury/death induced by antimycin A exposure. As illustrated in Fig. 10, the presence of 100 μM PD-150606 prevented the decreases in total 80-kDa μ-calpain large subunit (A) and protected against RPT cell injury/death produced by 30 min of antimycin A exposure (B).

Fig. 5. A: RPT cell death produced by antimycin A exposure. B: FITC-casein zymographic assay of the cytosolic μ-calpain (10 μg) in rabbit RPT subjected to antimycin A exposure. C: FITC-zymographic assay of cytosolic m-calpain (10 μg) in rabbit RPT subjected to antimycin A exposure. D: FITC-casein zymographic assay of μ-calpain in the membrane fraction (50 μg) of RPT subjected to antimycin A. E: FITC-casein zymographic assay of m-calpain in the membrane fraction (50 μg) of RPT subjected to antimycin A. RPT were exposed to DMSO (CON) or antimycin A (AA; 10 μM) for up to 30 min. Bars are means ± SE, n = 6. Bars with different letters are significantly different, P < 0.05.
Whereas the above results and our previous data demonstrated that the calpain inhibitor PD-150606 prevented cell death/lysis produced by multiple insults, the prevention of cell death/lysis may not necessarily reflect true cytoprotection. Therefore, in the present study, basal and ouabain-sensitive QO2 were measured as markers of mitochondrial function and active Na\(^{+}\) transport in rabbit RPT after hypoxia/reoxygenation. One-hour hypoxia resulted in extensive cell death (Fig. 11A). The reoxygenation period did not increase cell death further (Fig. 11A). The addition of the calpain inhibitor PD-150606 at the onset of hypoxia completely blocked LDH release after the hypoxia/reoxygenation period (Fig. 11A). RPT subjected to hypoxia/reoxygenation displayed impaired mitochondrial function and active Na\(^{+}\) transport indicated by decreases in basal QO2 and ouabain-sensitive QO2 (Fig. 11, B and C). The presence of PD-150606 markedly improved basal QO2 and ouabain-sensitive QO2. These results show that calpain inhibition prevented LDH and calpain leakage and promoted the recovery of mitochondrial respiration and active Na\(^{+}\) transport in RPT exposed to antimycin A or subjected to hypoxia/reoxygenation.

**DISCUSSION**

Many hypotheses have been proposed for the underlying mechanisms of oncosis. There is now a significant amount of data demonstrating that increases in intracellular Ca\(^{2+}\) play a critical role in RPT cell injury/death. For example, decreasing the extracellular Ca\(^{2+}\) concentration or chelating intracellular Ca\(^{2+}\) reduced LDH release from rabbit RPT subjected to anoxia, hypoxia, or mitochondrial inhibitor, and rat RPT subjected to hypoxia (12, 40, 42, 45). In addition, the Ca\(^{2+}\) channel blocker nifedipine inhibited Ca\(^{2+}\) uptake and LDH release and promoted the recovery of physiological functions after hypoxia and reoxygenation injury (31, 42). Finally, prior depletion of ER Ca\(^{2+}\) stores prevents the cascade that causes an influx of extracellular Ca\(^{2+}\) and Cl\(^{-}\) and cell injury/death in rabbit RPT subjected to hypoxia or exposed to antimycin A (44).

Calpains are a family of Ca\(^{2+}\)-activated proteases, with \(\mu\)- and \(m\)-calpains being ubiquitously and constitutively expressed in most mammalian cells (7, 39), and were proposed to be a cell injury/death mediator after increased intracellular Ca\(^{2+}\). Edelstein et al. (12) reported the presence of only \(\mu\)-calpain in rat RPT. Therefore, RT-PCR, immunoblot analysis, and FITC-casein zymography were employed in the present study to investigate the expression of calpain isoforms in rabbit RPT, and the results demonstrated that rabbit RPT cells express both \(\mu\)- and \(m\)-calpain.

Immunoblot analysis demonstrated that the majority of \(m\)-calpain large subunits are present in the cytosol as an 80-kDa form whereas a small amount of the 80 kDa \(\mu\)-calpain was present in the cytosol. Immunoblot analysis of the cytosolic 80 kDa \(\mu\)-calpain (30 \(\mu\)g) in rabbit RPT subjected to antimycin A exposure. RPT were exposed to DMSO (CON) or antimycin A (AA, 10 \(\mu\)M) for 30 min. Bars are means ± SE, \(n = 6\). Bars with different letters are significantly different, \(P < 0.05\).

**Fig. 6.** A: RPT cell death produced by antimycin A exposure. B: immunoblot analysis of the cytosolic 80 kDa \(\mu\)-calpain (30 \(\mu\)g) in rabbit RPT subjected to antimycin A exposure. RPT were exposed to DMSO (CON) or antimycin A (AA, 10 \(\mu\)M) for 30 min. Bars are means ± SE, \(n = 6\). Bars with different letters are significantly different, \(P < 0.05\).

**Fig. 7.** A: FITC-casein zymographic assay of \(\mu\)-calpain (A) and \(m\)-calpain (B) in rabbit RPT subjected to antimycin A exposure. RPT were exposed to DMSO (CON) or antimycin A (AA, 10 \(\mu\)M) for up to 30 min. At 5, 10, 15, and 30 min after antimycin A exposure, aliquots of RPT were taken and processed as described in MATERIALS AND METHODS. Twenty-microliter samples were subjected to zymography. Bars are means ± SE, \(n = 5\). Bars with different letters are significantly different, \(P < 0.05\).
78-kDa intermediate autolytic form is associated with the membrane. Localization of μ-calpain mainly in the cytosol is consistent with previous reports in the literature (15) and was supported by the FITC-casein zymography results. The presence of the 80-kDa μ-calpain large subunit and the absence of autolytic forms in the cytosol suggest that cytosolic μ-calpain is an inactive proenzyme or may have activity without undergoing autolysis. It was reported that μ-calpain has a long half-life and was suggested that it is active without autolysis (50). The presence of the autolyzed 78-kDa form only in the membrane fraction confirmed its preferential formation and association with the membrane fraction as described previously (26) and suggests that it may form and act on membrane substrates. The 80-kDa m-calpain and its autolytic 76-kDa form were present only in RPT cytosol. These results are consistent with previous observations in other tissues that m-calpain primarily localizes in the cytosol (51).

The presence of the 78-kDa μ-calpain in the membrane fraction and the 76-kDa m-calpain in the cytosol under control conditions suggests that both calpains have physiological functions in RPT. The fact that they originated from different calpain isoforms, at different autolytic stages, and localized to different cell compartments, suggests that μ- and m-calpains have different physiological functions under normal conditions. For example, selective inhibition of μ-calpain by anti-sense oligonucleotides or a specific inhibitor disrupted cell spreading and adhesion whereas inhibition of m-calpain had no effect (23). Additionally, Schoenwaelder et al. (37) reported that 78- and 76-kDa μ-calpain autolytic products have different substrates and functions during platelet activation.

Calpains have been implicated in ischemic/hypoxic cell/tissue injury. However, how calpain becomes active/activated intracellularly remains unknown. Translocation of cytosolic calpains to the membrane is
thought to be a critical step before calpain activation. In previous studies from our laboratory, the mitochondrial inhibitor antimycin A decreased cytosolic calpain activity and increased calpain activity in the membrane fraction. This alteration was interpreted as translocation of calpain activity from the cytosol to the membrane fraction (42, 43). However, calpain activity translocation does not reflect the calpain isoform involved nor the form (i.e., proenzyme or autolyzed product). FITC-casein zymography and immunoblot analysis of the membrane fraction failed to detect any increases in either \( \mu \)- or \( m \)-calpain after antimycin A exposure, demonstrating no translocation of either calpain isoform during RPT injury. In the previous study, an in vitro calpain assay and a fluorescent substrate were used, and the increased activity in the membrane fraction could be due to enzymes other than calpains or calpain isoforms undetectable using the anti-\( \mu \)- or \( m \)-calpain large subunit antibodies. However, in the present study, FITC-casein zymography did not reveal any additional calpain isoforms in RPT.

Of equal importance, no increases in autolytic forms of \( \mu \)- or \( m \)-calpain large subunits were observed by immunoblot analysis in antimycin A-exposed RPT. Therefore, it is likely that \( \mu \)- or \( m \)-calpain mediate RPT cell injury in their proenzyme form. The reductions in \( \mu \)- and \( m \)-calpain levels observed during the late phase of RPT injury correlated with the appearance of \( \mu \)- and \( m \)-calpain in the incubation buffer and the increased plasma membrane permeability to LDH release. These results demonstrate that calpains leak from RPT during the cell death process.

Calpains coexist with calpastatin, the endogenous inhibitor in the cell (2). Under normal conditions, calpastatin is localized in specific structures rather than distributed in a diffused form in the cytosol (8). Calpastatin and calpain associate in the presence of \( \text{Ca}^{2+} \) (7). Higher and prolonged increases in intracellular

![Fig. 10](http://ajprenal.physiology.org/)

Fig. 10. Effect of PD-150606 on total 80-kDa \( \mu \)-calpain (A) and LDH release (B) in rabbit RPT subjected to antimycin A. RPT were exposed to DMSO (CON) or antimycin A (AA, 10 \( \mu \)M) for 30 min. PD-150606 (PD, 100 \( \mu \)M) was added 30 min before antimycin A addition. Twenty-microliter samples were subjected to immunoblot analysis. Bars are means ± SE, \( n = 5 \). Bars with different letters are significantly different from one another, \( P < 0.05 \).

![Fig. 11](http://ajprenal.physiology.org/)

Fig. 11. Effect of PD-150606 on LDH release (A), basal oxygen consumption (QO₂) (B), and ouabain-sensitive QO₂ in rabbit RPT subjected to hypoxia/reoxygenation (C). RPT were exposed to 95% air-5% \( \text{CO}_2 \) for 2 h (CON) or 95% \( \text{N}_2 \)-5% \( \text{CO}_2 \) (1 h) followed by 1 h 95% air-5% \( \text{CO}_2 \) (HYPO). PD-150606 (PD, 100 \( \mu \)M) was added immediately before hypoxia. 1 Represents either 1 h hypoxia or 1 h normoxia; 2 represents either 1 h hypoxia followed by 1 h normoxia or 2 h normoxia. Bars are means ± SE, \( n = 5 \). Bars with different letters are significantly different from one another, \( P < 0.05 \).

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Ca\textsuperscript{2+} activate calpain, and the activated calpain mediates degradation of calpastatin (8). Downregulation of calpastatin allows calpain to be active (7). In the present study, a 120-kDa calpastatin was identified using a specific anti-calpastatin antibody. Antimycin A treatment did not result in changes in calpastatin levels at the early stage of RPT injury, suggesting the lack of calpastatin degradation during RPT injury. The reductions in calpastatin levels at 30 min of antimycin A exposure may reflect the leakage from cells subsequent to RPT injury/death, similar to that observed with the calpains. Recently, Edelstein et al. (38) reported a decrease in a 41-kDa protein immunoreactive to a calpastatin antibody in rat kidney cortex subjected to ischemia-reperfusion. However, they did not report any increases in or appearance of any small fragments of calpastatin. When the compromised cell membrane integrity during RPT injury and the small molecular weight of calpastatin are considered, the observed decrease could be due to leakage from the cell rather than degradation. It is also possible that the discrepancy is due to the different model.

The present study demonstrates that the calpain inhibitor PD-150606 decreases LDH release and calpain leakage from RPT subjected to antimycin A, consistent with previous reports (36, 44). Furthermore, the present study shows that PD-150606 promotes the recovery of mitochondrial function and active Na\textsuperscript{+} transport in RPT subjected to hypoxia/reoxygenation, suggesting that calpains mediate mitochondrial dysfunction and inhibit ion transport during RPT injury. Alternatively, the mitochondrial dysfunction and inhibition of ion transport may be due to calpain-mediated plasma membrane permeability changes. However, previous reports from our laboratory demonstrated that the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase isolated from RPT subjected to hypoxia/reoxygenation showed no inhibition of activity when measured in vitro, indicating that calpain does not exert a direct effect on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (31). We hypothesize that calpain may modulate the cytoskeleton (such as spectrin and ankyrin) and interfere with Na\textsuperscript{+}-K\textsuperscript{+}-ATPase function or localization.

In summary, the present study demonstrates that rabbit RPT cells express active calpains, and calpains play a critical role during RPT injury/death without undergoing autolysis or translocation. The calpain inhibitor PD-150606 is a true cytoprotectant, allowing the return of physiological functions in RPT subjected to hypoxia/reoxygenation.

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