Calpains Mediate Calcium and Chloride Influx During the Late Phase of Cell Injury

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ABSTRACT
The role of Ca\(^{2+}\) in cell death is controversial. Extracellular Ca\(^{2+}\) influx and calpain activation occurred during the late phase of renal proximal tubule cell injury produced by the mitochondrial inhibitor antimycin A. Chelation of intracellular Ca\(^{2+}\), extracellular Ca\(^{2+}\), the calcium channel blocker nifedipine, calpain inhibitor 1 and the dissimilar calpain inhibitor PD150606 blocked antimycin A-induced influx of extracellular Ca\(^{2+}\) and cell death. The calcium channel blocker verapamil was ineffective. Calpain inhibitor 1 and PD150606 were cytoprotective also against tetrafluoroethyl-L-cysteine-, bromohy-

Cell death is generally thought to occur through one of two pathways: necrosis (oncosis) or apoptosis (Majno and Joris, 1995). Necrosis, oncosis or necrotic cell death is the form of cell death normally associated with inflammation and organ failure. In necrotic cell death, the organelles swell, cell volume increases and the cell ruptures/lyses, releasing its contents and triggering inflammation. In general, most toxicants that produce organ dysfunction are thought to produce cell death through necrosis.

The role of Ca\(^{2+}\) in oncosis has received much attention over the past three decades and remains controversial (Choi, 1995; Harman and Maxwell, 1995; Trump and Berezesky, 1995). In a 1979 landmark report, Schanne et al. suggested that an increase in cytosolic free Ca\(^{2+}\) (Ca\(^{2+}\)) represented the final common pathway in cell death/lysis. However, a number of investigators have questioned this hypothesis. For example, Weinberg et al. (1991) and Jacobs et al. (1991) used rabbit RPT subjected to anoxia or exposed to mitochondrial inhibitors and the Ca\(^{2+}\)-sensitive fluorescent dye fura 2 and observed an increase in Ca\(^{2+}\) immediately before cell death/lysis. Similar results were reported by Lemasters et al. (1987) using hepatocytes and chemical hypoxia. These authors concluded that an increase in Ca\(^{2+}\) occurred in concert with the loss of cell viability and thus was not an obligatory step in cell death. In contrast, Kribben et al. (1994), using fura 2 and rat RPT subjected to hypoxia, demonstrated that extracellular Ca\(^{2+}\) levels increased significantly before cell death. In addition, Takano et al. (1987), Wetzels et al. (1993) and Rose et al. (1994) showed that decreasing the extracellular Ca\(^{2+}\) concentration reduced the release of LDH from rabbit RPT subjected to anoxia and rat RPT subjected to hypoxia. Therefore, the exact role that Ca\(^{2+}\) plays during cell injury and death is still not clear.

In support of the hypothesis that Ca\(^{2+}\) plays an important role in cell injury, calcium channel blockers have been re-

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ABBREVIATIONS: RPT, renal proximal tubules; LDH, lactate dehydrogenase; PD150606, 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid; SLLVY-AMC, N-succinyl-Leu-Leu-Val-Tyr-AMC; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate; DPC, diphenylamine-2-carboxylate; IAA-94, indanyloxycetic acid.

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ported to be protective against various forms of cell injury. For example, verapamil or nifedipine decreased cell death in rat RPT subjected to hypoxia and anoxia and rabbit RPT subjected to anoxia (Almeida et al., 1992; Rose et al., 1993; Wetzel et al., 1992). Furthermore, McCarty and O’Neil (1991) reported that rabbit RPT contain both a “base-line” verapamil-sensitive Ca\(^{2+}\) entry pathway and a nifedipine-sensitive Ca\(^{2+}\) entry pathway that is activated during regulatory volume decreases. These studies provide additional evidence that extracellular Ca\(^{2+}\) influx may play a role in renal cell injury.

It is generally hypothesized that if Ca\(^{2+}\) plays a role in cell death, it is the consequence of a supraphysiological and/or prolonged increase in Ca\(^{2+}\), that activates degradative enzymes, including proteases and phospholipases (Choi, 1995; Harman and Maxwell, 1995; Trump and Berezovsky, 1995). Investigators have suggested that nonlysosomal calcium-activated cysteine proteases, calpains (E.C., 3.4.22.17), are activated and contribute to anoxia- or toxicant-induced cell death (Bronk and Gores, 1993; Croall and Demartino, 1991; Edelstein et al., 1995; Nicotera et al., 1986; Saito et al., 1994; Wang and Yuen, 1994). These results are primarily based on the inhibition of cell death by calpain inhibitors and to a limited extent on the measurement of calpain activity or calpain-mediated protein degradation. For example, Edelstein et al. (1996) reported an increase in calpain activity in rat RPT subjected to hypoxia, and Bronk and Gores (1993) demonstrated an increase in calpain-like protease activity in rat hepatocytes subjected to anoxia. Both studies showed that inhibition of calpains resulted in cytoprotection. We demonstrated that calpain inhibitor 2 was cytoprotective to RPT exposed to anoxia and a diverse group of toxicants that included an alkylating quinone (bromohydroquinone), an oxidant (t-butylhydroperoxide) and a toxicant that forms a reactive electrophile (tetrafluoroethyl-L-cysteine) (fig. 1; Schnellmann et al., 1994). These results suggest that calpains play a critical role in diverse forms of cell injury; however, progress in this area has been limited due to difficulties in measuring calpain activity, the lack of specific calpain substrates and inhibitors and the identification of endogenous intracellular substrates (Saito et al., 1993; Sasaki et al., 1984; Wang and Yuen, 1994).

The majority of calpain inhibitors, including calpain inhibitor 1 and calpain inhibitor 2, are modified peptides that bind to the active site of calpain (Wang and Yuen, 1994). The disadvantage of these compounds is diminished selectivity, a direct result of the similarity of the active site among the different classes of cysteine proteases (Wang and Yuen, 1994). Recently, Wang et al. (1996a) identified a novel class of calpain inhibitors, including the compound PD150606. As opposed to binding to the active site of the protease, PD150606 inhibits calpains by binding to the calcium-binding domain of the enzyme. Because calcium-binding domains are not located in other proteases, PD150606 selectively inhibits the calpain enzyme.

The early events in anoxia- and toxicant-induced cell death have been well characterized in numerous models and include inhibition of cellular respiration followed by the loss of intracellular ATP, K\(^+\) efflux and Na\(^-\) influx. Those events that occur during the late phase of cell injury have not been completely elucidated; however, we have shown that Cl\(^-\) influx does not occur passively with the initial Na\(^+\) influx after antimycin A exposure and that Cl\(^-\) influx occurs after a lag period during the late phase of cell injury through a nifluclidamic acid-, 5-nitro-2-(3-phenylpropylamino)benzoic acid-, diphenyl-2-carboxylate- and indanyloxyacetic acid-sensitive channel (Miller and Schnellmann, 1993, 1995; Waters and Schnellmann, 1996). The goal of this study was to obtain a more complete understanding of the events that occur during the late phase of cell death by exploring the roles and interactions of calpains, Ca\(^{2+}\) influx and Cl\(^-\) influx using rabbit RPT suspensions as a model. Specifically, we addressed (1) whether Ca\(^{2+}\) influx occurs and plays a role in cell death, (2) the mechanism and pathway by which Ca\(^{2+}\) influx occurs, (3) whether calpains are activated and play a role in cell death, (4) the subcellular localization of calpain activity during cell injury and (5) whether calpains play a role in the extracellular Cl\(^-\) influx that occurs during the late phase of cell injury.

### Materials and Methods

**Reagents.** Tetrafluoroethyl-L-cysteine was a gift from Dr. Edward A. Lock (Zeneca, Cheshire, UK). SLLVY-AMC was purchased from Bachem Bioscience (Philadelphia, PA). Ionomycin and EGTA-AM were obtained from Calbiochem (San Diego, CA). Calpain inhibitors 1 and 2 were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Bromohydroquinone was purchased from ICN Pharmaceuticals (Plainview, NY). \(^{36}\)Cl\(^-\) (Na\(^+\)), \(^{45}\)Ca\(^{2+}\) (Cl\(^-\)) and \(^{14}\)C\(\text{dextran was obtained from Dupont NEN (Boston, MA). NPPB and IAA-94 were obtained from Research Biochemicals (Natick, MA). Antimycin A, 3,4-dichloroisoucoumarin, dim-
ethylsulfoxide, t-butyl hydroperoxide, niflumic acid, DPC, N-[N(t-3-
trans-ethoxycarboxyl-oxirane-2-carbonyl)-L-leucyl]-3-methyl-
butylamine and N-p-tosyl-l-lysine chloromethyl ketone were purchased from Sigma Chemical (St. Louis, MO). The sources of the remaining chemicals have been reported previously (Rodeheaver et al., 1990). Stock solutions of all inhibitors were prepared daily in dimethylsulfoxide. All glassware was silanized and autoclaved before use. All media and buffers were sterilized by filtering before use.

Preparation and incubation of RPT. Rabbit RPT were isolated and purified as described by Rodeheaver et al. (1990) and suspended in an incubation buffer containing 1 mM alanine, 4 mM dextrose, 2 mM heptanoate, 4 mM lactate, 5 mM malate, 115 mM NaCl, 15 mM NaHCO3, 5 mM KCl, 2 mM NaH2PO4, 1 mM MgSO4, 1 mM CaCl2 and 10 mM HEPES (pH 7.4, 295 mOsm/kg). RPT suspensions (1 mg of cellular protein/ml) were incubated at 37°C in an orbital shaking water bath (180 rpm) under 95% air/5% CO2 (40 ml/min flow rate). All experiments contained a 15-min preincubation period with no experimental manipulations. EGTA (2 mM, pH 7.4), EGTA-AM (100 mM) or diluent (1% dimethylsulfoxide) was added immediately before antymycin A (1 pM), and the incubation continued for an additional 30 min. Aliquots of RPT suspensions were removed, and LDH release was determined. RPT suspensions were incubated for 30 min with the protease inhibitors before calpain activity was determined. Calpain inhibitor 1 or 2 (1 mM) or PD150606 (3–100 pM) was added 10 min before the addition of antymycin A or the calcium ionomycin (5 pM).

In situ calpain assay. Calpain activity was determined in RPT suspensions by measuring the release of the fluorescent product 7-amido-4-methyl coumarin (AMC) from the membrane permeant calpain substrate SLLVV-AMC (Sasaki et al., 1984; Wang et al., 1996b). Briefly, a 1-ml aliquot of RPT suspension was diluted with 3 ml of 37°C incubation buffer, and a 1.5-ml aliquot was placed in a thermostatically controlled 37°C stirred cuvette in a Hitachi F-2000 spectrofluorometer. Calpain substrate (50 pM) was added, and fluorescence (360 nm excitation, 430 nm emission) was monitored at one minute. The increase in fluorescence was linear between 7 and 30 min, with calpain activity determined between 7 and 11 min.

Calpain activity in cytosolic and membrane-associated fractions. This assay was based on the method of Edelstein et al. (1995) with the following modifications. Briefly, an aliquot of RPT was removed and centrifuged at 1000 × g for 1 min, and the supernatant was aspirated. The pellet was resuspended in imidazole buffer (63 mM imidazole, 10 mM 2-mercaptoethanol, 1 mM EDTA and 10 mM EGTA, pH 7.3) and incubated in the presence of digitonin (100 µM) for 10 min at 37°C. Digitonin permeabilizes the plasma membrane releasing the cytosolic contents. Under these conditions, LDH release is 94%. An aliquot is removed, the tube pelleted and supernatant is separated by centrifugation for 1 min at 1000 × g and the pellet is resuspended in imidazole buffer. Total calpain activity present in the supernatant and membrane-associated fraction was determined as follows: in Costar 24-well plates, 0.25 ml of supernatant or pellet was preincubated in imidazole buffer in the presence and absence of 3 mM CaCl2 for 5 min on an orbital shaker placed in a 37°C incubator. The samples incubated in the presence of CaCl2 were incubated in an imidazole-HCl buffer without EDTA and EGTA. Total volume in each well was 1 ml. After the preincubation, 50 µM SLLVV-AMC was added, and fluorescence was determined at 10, 20 and 30 min after substrate addition in a CytoFluor 2350 Fluorescence Measurement System (Perceptive Biosystems, Bedford, MA; 380 nm excitation; 460 nm emission). An AMC standard curve was included in each experiment, and calpain activity was determined as the time-dependent difference between the calcium-dependent fluorescence and the calcium-independent fluorescence. Activity was normalized to cytosolic or membrane-associated protein according to the method of Lowry et al. (1951).

Cl− and Ca++ influxes. Cl− and Ca++ influxes were determined by adding a tracer amount of 36Cl− (Na+) or 45Ca++ (2Cl−) to RPT suspensions 0 or 15 min after antymycin A addition (Miller and Schnellmann, 1993, 1995; Waters and Schnellmann, 1996). At 15 min later, aliquots were removed, and RPT was separated from the remaining buffer by rapid centrifugation through a layer of dibutylphosphate/dioctylphosphate (2:1). RPT 36Cl− and 45Ca++ contents were determined by resuspending the pellets in Triton X-100 solubilization buffer (100 mM Tris, 150 mM NaCl and 0.05% Triton X-100 at pH 7.5), and aliquots were taken for liquid scintillation spectrometry and protein determination. Extracellular 36Cl− and 45Ca++ were corrected for using the extracellular water marker [14C]dextran. RPT protein concentration was determined using the biuret method (Gornall et al., 1949).

Cell death. Cell death/lysis was assessed by measuring the release of LDH activity as described previously (Moran and Schnellmann, 1996).

Statistics. The data are presented as mean ± S.E.M. RPT suspensions isolated from one rabbit represented a single experiment (n = 1). Data were analyzed by analysis of variance, and multiple mean values were compared using Fisher’s protected LSD test with a level of significance of P < .05.

Results

Previous studies have demonstrated that exposure of rabbit RPT suspensions to anoxia or mitochondrial inhibitors increases Ca++ influx just before cell death/lysis (Jacobs et al., 1991; Weinberg et al., 1991). To document that Ca++ influx occurs during the late phase of cell injury in our model, a tracer amount of 45Ca++ was added simultaneously with antymycin A, and RPT 45Ca++ content determined. Ca++ influx did not increase above control values during the first 15 min of antymycin A exposure (fig. 2, top), a time frame during which ATP levels are depleted and Na+ influx and K+ efflux occur. In contrast, when a tracer concentration of 45Ca++ was added to RPT suspensions 15 min after antymycin A and RPT 45Ca++ content determined 15 min later, RPT 45Ca++ content increased 3.5-fold compared with controls (fig. 2, top). The calcium channel blocker nifedipine, but not verapamil, and chelation of intracellular Ca++ with EGTA-AM blocked the antymycin A-induced increase in RPT 45Ca++ content (fig. 2, bottom).

To confirm that the influx of extracellular Ca++ is necessary for cell death in our model, the extracellular Ca++ chelator EGTA, nifedipine, verapamil or EGTA-AM was added to RPT treated with antymycin A. Chelation of extracellular Ca++ or intracellular Ca++ with EGTA or EGTA-AM, respectively, decreased LDH release (table 1). Likewise, nifedipine inhibited antymycin A-induced LDH release in a concentration-dependent manner and inhibited LDH release when added 15 min after antymycin A (table 1). In contrast, verapamil was not cytoprotective and potentiated LDH release during which ATP levels are depleted and Na+ influx and K+ efflux occur. To determine whether calpains play a role in cell death, the effects of two calpain inhibitors on antymycin A-induced cell death were examined. A 30-min pretreatment with calpain inhibitor 1 or 2 was equally effective in blocking LDH release from RPT exposed to antymycin A (fig. 3, top). No differences were noted between calpain inhibitors 1 and 2. The calpain inhibitor PD150606 also inhibited antymycin A-induced LDH...
release in a concentration-dependent manner (fig. 3, bottom). In addition, PD150606 (100 \text{mM}) was cytoprotective against a variety of toxicant-induced injuries, including t-butyl hydroperoxide, bromohydroquinone and tetrafluoroethyl-L-cysteine (fig. 1, bottom). These results, as well as those reported previously (fig. 1, Schnellmann et al., 1994), demonstrate that calpain inhibitors are cytoprotective against diverse toxicant insults, strongly suggesting that calpains play a common and critical role in RPT cell death.

To document that calpain inhibitor 1 and PD150606 block calpain activity, calpain activity was measured in situ by adding the cell permeant calpain substrate SLLVY-AMC to RPT suspensions and monitoring the formation of the fluorescent product AMC over time. To test whether SLLVY-AMC was a substrate for other proteases under these conditions, a series of cysteine, serine and acid protease inhibitors were added to RPT, and calpain activity determined 30 min later. Protease inhibitors were added at their maximal non-toxic concentration (data not shown). E64d, leupeptin and pepstatin A had no effect on calpain activity, whereas 3,4-dichloroisocoumarin and N-p-tosyl-L-lysine chloromethyl ketone decreased calpain activity by \sim \text{10\%} (table 2). In contrast, calpain inhibitor 1 and PD150606 decreased calpain activity by \sim \text{62\%} and \sim \text{34\%}, respectively. These results suggest that SLLVY-AMC is hydrolyzed by calpains and other proteases in this assay, that calpain inhibitor 1 may inhibit calpains and other proteases and that PD150606 inhibits calpain activity. It is unlikely that lysosomal cysteine pro-
TABLE 2
Effects of serine, cysteine and aspartic acid protease inhibitors and calpain inhibitor 1 on calpain activity measured in situ in RPT suspensions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 0a</td>
</tr>
<tr>
<td>E64d (0.05 mM)</td>
<td>106 ± 6a</td>
</tr>
<tr>
<td>Leupeptin (0.5 mM)</td>
<td>98 ± 4a</td>
</tr>
<tr>
<td>Pepstatin A (0.2 mM)</td>
<td>97 ± 3b</td>
</tr>
<tr>
<td>3,4-Dichloroisocoumarin (0.2 mM)</td>
<td>89 ± 5b</td>
</tr>
<tr>
<td>N-p-tosyl-l-lysine chloromethyl ketone (0.1 mM)</td>
<td>82 ± 4b</td>
</tr>
<tr>
<td>Calpain inhibitor 1 (1 mM)</td>
<td>38 ± 4c</td>
</tr>
<tr>
<td>PD150606 (100 µM)</td>
<td>66 ± 6c</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. (n = 3).
Values with different superscripts are significantly different from one another (P < .05).

Cl⁻ influx also occurs in the late phase of cell injury and triggers the terminal cell swelling and lysis (Miller and Schnellmann, 1993, 1995; Waters and Schnellmann, 1996). To determine the temporal relationships among Ca²⁺ influx, calpain translocation and Cl⁻ influx, the effect of nifedipine, verapamil, calpain inhibitor 1 and PD150606 on antimycin A-induced Cl⁻ influx was examined. Antimycin A increased RPT 36Cl⁻ content by ~2.4-fold (fig. 7, top). Nifedipine, calpain inhibitor 1 and PD150606 blocked antimycin A-induced Cl⁻ influx. The use of calpain inhibitors prevented the translocation of calpains in the presence of antimycin A (fig. 6). These results suggest that extracellular Ca²⁺ influx mediates calpain translocation in the late phase of RPT cell injury.
Cl⁻ influx, whereas verapamil did not. Because nifedipine, calpain inhibitor 1 and PD150606 all inhibited the translocation of calpains to the membrane, these data suggest that Ca²⁺ influx results in calpain translocation to the membrane and in turn Cl⁻ influx.

To determine whether the calcium ionophore-induced extracellular Cl⁻ influx was calpain mediated, the effect of calpain inhibitor 1 and PD150606 on ionomycin-induced Cl⁻ influx was examined. Ionomycin increased RPT 36Cl⁻ content by ~4.1-fold (fig. 7, bottom). Calpain inhibition completely blocked ionomycin-induced Cl⁻ influx. We have previously shown that the Cl⁻ channel inhibitors NPPB, niflumic acid, IAA-94 and DPC inhibit antimycin A-induced cell death and Cl⁻ influx (Waters and Schnellmann, 1996). To determine whether Cl⁻ channel inhibitors also block calcium ionophore-induced Cl⁻ influx and cell death, the effects of NPPB, niflumic acid, IAA-94 and DPC on ionomycin-induced LDH release and Cl⁻ influx were examined. All four Cl⁻ channel inhibitors ameliorated ionomycin-induced LDH release (fig. 4) and Cl⁻ influx (fig. 7, bottom).

**Discussion**

The role of Ca²⁺ in cell death is controversial. Some studies have demonstrated increases in Ca²⁺ levels before cell death and/or that decreasing medium Ca²⁺ levels ameliorates anoxia- or toxicant-induced extracellular Ca²⁺ influx and cell death (Almeida et al., 1992; Choi, 1995; Harman and Maxwell, 1995; Rose et al., 1994; Takano et al., 1985; Trump and Berezovsky, 1995). Other studies have shown that Ca²⁺ does not increase early but late in the cell injury process just before the loss of calcium-sensitive dyes or cell death, suggesting that Ca²⁺ does not play a key role in cell death (Jacobs et al., 1991; Lemasters et al., 1987; Weinberg et al., 1991). With rabbit RPT subjected to mitochondrial inhibition, a model in which Ca²⁺⁻⁴ does not increase until just before cell death (Jacobs et al., 1991; Weinberg et al., 1991), we show that (1) extracellular Ca²⁺ influx occurs during the late phase of cell injury, (2) inhibition of extracellular Ca²⁺ influx blocks cell death, (3) chelation of intracellular Ca²⁺ blocks cell death and (4) inhibition of calpain activity blocks cell death produced by diverse toxicants. Thus, influx of extracellular Ca²⁺ in the late phase of RPT cell injury does indeed play a key role in cell death.

Because the influx of extracellular Ca²⁺ is an important event during RPT cell injury, additional studies were conducted to determine the pathway of Ca²⁺ entry. Nifedipine and verapamil are two dissimilar calcium channel blockers (Vanhoutte, 1987). Nifedipine inhibited antimycin A-induced Ca²⁺ influx and LDH release in a concentration-dependent manner, whereas verapamil was ineffective. Furthermore, nifedipine added 15 min after antimycin A, a time point after ATP depletion, Na⁺ influx and K⁺ efflux, was completely cytoprotective. These data are consistent with our observation that Ca²⁺ influx did not increase above control values during the first 15 min after antimycin A addition. Collectively, these results show that extracellular Ca²⁺ influx occurs in the late phase of cell injury through a nifedipine-sensitive pathway. Although a complete calcium channel has not been demonstrated in RPT (Yu, 1995), a nifedipine-sensitive Ca²⁺ entry pathway has been reported previously in rabbit RPT (McCart and O’Neil, 1991). Further studies are required to identify the Ca²⁺ entry pathway observed during cell injury.
Cytoprotection with Ca\textsuperscript{2+} channel blockers has been reported in other renal cell models subjected to anoxia or hypoxia. For example, Almeida et al. (1992) reported that verapamil transiently inhibited Ca\textsuperscript{2+} uptake and LDH release in rat RPT subjected to hypoxia and subsequently reported that verapamil may act intracellularly on the mitochondrion. In contrast, we did not observe cytoprotection with verapamil in rabbit RPT suspensions subjected to mitochondrial inhibition. The difference between our findings and those of Almeida et al. (1992) may reflect the different species used and/or the non-plasma membrane effects of verapamil in the rat. Rose et al. (1994) reported that methoxyverapamil decreased anoxia-induced Ca\textsuperscript{2+} influx and LDH release in rabbit RPT cells subjected to anoxia, whereas felodipine was protective by attenuating potassium loss during hypoxia. Possible explanations for the discrepancy between verapamil and methoxyverapamil include differences in potency, selectivity or actions at non-plasma membrane sites (Fleckenstein-Grun, 1992). It is unlikely that nifedipine attenuated potassium loss after mitochondrial inhibition because nifedipine was protective when added 15 min after antimycin A, a time point after potassium loss has occurred. These varying results with different Ca\textsuperscript{2+} channel blockers may also explain the conflicting actions of Ca\textsuperscript{2+} channel blockers seen in in vivo renal protection studies (Almeida et al., 1992; Rose et al., 1994).

Investigators have postulated that one potential mechanism of Ca\textsuperscript{2+}-induced cellular injury involves the activation of calpains (Bronk and Gores, 1993; Croall and Demartino, 1991; Edelstein et al., 1995; Nicotera et al., 1986; Saido et al., 1994; Wang and Yuen, 1994). However, the role of calpains in cell injury has been difficult to determine due to problems with calpain assays and the lack of specific calpain substrates and selective calpain inhibitors (Sasaki et al., 1984). We have shown that calpain inhibitor 2 and PD150606 are cytoprotective to RPT exposed to a group of diverse toxicants with different mechanisms of action (current results; Schnellmann, 1997; Schnellmann et al., 1994). Furthermore, the two inhibitors inhibited calpain activity as measured by an in situ calpain assay. Therefore, these results strongly suggest that calpains play a critical and common role in most types of necrotic renal cell death. The specific calpain isoform that is activated during the late phase of cell injury remains to be determined.

Because calpains are known to interact with a variety of intracellular substrates at both cytosolic and membrane sites (Saido et al., 1994), examination of the subcellular distribution of calpains during injury may indicate their site of action. In control samples, the subcellular distribution of calpain activity was ~33% and ~66% in the membrane-associated and cytosolic fractions, respectively. In RPT exposed to antimycin A, calpain activity translocated from cytosolic to membrane-associated fractions. Studies by Ostwald et al. (1993, 1994) reported similar distributions of calpain activity in normal rabbit hippocampal cells as well as calpain translocation after hypoxia. The observation that calpain translocation was inhibited by calpain inhibitor 1, PD150606 and nifedipine provides evidence that one calpain substrate involved in cell death is at or near the RPT plasma membrane.

Although the above data demonstrate that extracellular Ca\textsuperscript{2+} influx and calpains mediate necrotic cell death, the current study also provides evidence that calpains play a dual role in cell death. Calpain inhibition with both calpain inhibitor 1 and PD150606 not only blocked antimycin A-induced extracellular Ca\textsuperscript{2+} influx but also inhibited calcium-ionophore (ionomycin)-induced cell death. Furthermore, calpain inhibition with calpain inhibitor 1 or PD150606 and inhibition of extracellular Ca\textsuperscript{2+} influx with nifedipine blocked calpain translocation to the membrane. Collectively, these data suggest that calpains play a role both before and subsequent to extracellular Ca\textsuperscript{2+} influx. Thus, the mechanism of cytoprotection provided by calpain inhibitors probably involves both the inhibition of calpain-mediated extracellular Ca\textsuperscript{2+} influx and extracellular Ca\textsuperscript{2+} influx-mediated calpain translocation.

We have shown previously that the Cl\textsuperscript{−} influx that occurs during the late phase of RPT cell death/lysis is sensitive to Cl\textsuperscript{−} channel inhibitors (Waters and Schnellmann, 1996). The current study shows that nifedipine, calpain inhibitor 1 and PD150606 also inhibit this Cl\textsuperscript{−} influx. Furthermore, calpain inhibitor 1 and PD150606 blocked calcium ionophore (ionomycin)-induced Cl\textsuperscript{−} influx and cell death. The inhibition of Cl\textsuperscript{−} influx with the Cl\textsuperscript{−} channel inhibitors DPC, NPPB, IAA-94 and niflumic acid is consistent with previous results observed with antimycin A (Waters and Schnellmann, 1996).

In conjunction with the translocation observations, these studies suggest that during the late phase of cell injury, calpains are involved in substrate proteolysis at or near the plasma membrane that is associated with extracellular Cl\textsuperscript{−} influx.

Previous results combined with the current data have led us to propose the following sequence of events that lead to RPT cell death/lysis after mitochondrial inhibition (fig. 8). First, an increase in intracellular Ca\textsuperscript{2+}, levels triggers calpain-mediated extracellular Ca\textsuperscript{2+} influx through a nifedipine-sensitive Ca\textsuperscript{2+} influx (7, 8).

**Fig. 8.** Proposed sequence of events that occur after mitochondrial inhibition (antimycin A) in the late phase of cell injury that lead to rabbit RPT cell death.

- **Ca\textsuperscript{2+} influx (2, 3)**: The increase in cytosolic free Ca\textsuperscript{2+} activates calpains (1), calpain activation results in extracellular Ca\textsuperscript{2+} influx (2, 3) and the further increase in cytosolic free Ca\textsuperscript{2+} causes additional calpain activation (4) and translocation (5, 6) to the plasma membrane and Cl\textsuperscript{−} influx (7, 8). Note that the events illustrated are subsequent to ATP depletion, K\textsuperscript{+} efflux, Na\textsuperscript{+} influx and plasma membrane depolarization. The initial calpain activation may represent one calpain isozyme (1), and the subsequent calpain activation and translocation represent a second calpain isozyme (4–6). Alternatively, initial and subsequent calpain activations may represent the same isozyme activated to different degrees depending on the cytosolic free Ca\textsuperscript{2+} levels.
ine-sensitive pathway. This results in a large influx of extracellular Ca\(^{2+}\) that in turn mediates calpain activation and translocation that either directly or indirectly results in Cl\(^{-}\) channel opening. The resulting Cl\(^{-}\) influx triggers H\(_2\)O\(_2\) influx, causing cell swelling and death/lysis. The mechanisms and identification of the calpain isozyme or isozymes responsible for these effects remain to be elucidated.

In summary, we demonstrated that (1) chelation of extracellular or intracellular Ca\(^{2+}\) prevents cell death from mitochondrial injury, (2) the Ca\(^{2+}\) channel blocker nifedipine but not verapamil is cytoprotective and inhibits Ca\(^{2+}\) and Cl\(^{-}\) influxes, (3) antimycin A causes calpain translocation from cytosolic to membrane-associated cell fractions, (4) calpain inhibitor 1, PD150606 and nifedipine block antimycin A-induced calpain translocation, (5) calpain inhibitor 1 and PD150606 provide cytoprotection both before and subsequent to extracellular Ca\(^{2+}\) influx and (6) Cl\(^{-}\) influx during the late phase of cell injury is inhibited by nifedipine, calpain inhibitor 1 and PD150606.

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