Calpain 10: a mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction

David D. Arrington, Terry R. Van Vleet, and Rick G. Schnellmann

Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, South Carolina

Submitted 25 April 2006; accepted in final form 14 June 2006

Arrington, David D., Terry R. Van Vleet, and Rick G. Schnellmann. Calpain 10: a mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction. Am J Physiol Cell Physiol 291: C1159–C1171, 2006. First published June 21, 2006; doi:10.1152/ajpcell.00207.2006.—Calpains, Ca2+-activated cysteine proteases, are cytosolic enzymes implicated in numerous cellular functions and pathologies. We identified a mitochondrial Ca2+-inducible protease that hydrolyzed a calpain substrate (SLLVY-AMC) and was inhibited by active site-directed calpain inhibitors as calpain 10, an atypical calpain lacking domain IV. Immunoblot analysis and activity assays revealed calpain 10 in the mitochondrial outer membrane, intermembrane space, inner membrane, and matrix fractions. Mitochondrial staining was observed when COOH-terminal green fluorescent protein-tagged calpain 10 was overexpressed in NIH-3T3 cells and the mitochondrial targeting sequence was localized to the NH2-terminal 15 amino acids. Overexpression of mitochondrial calpain 10 resulted in mitochondrial swelling and autophagy that was blocked by the mitochondrial permeability transition (MPT) inhibitor cyclosporine A. With the use of isolated mitochondria, Ca2+-induced MPT was partially decreased by calpain inhibitors. More importantly, Ca2+-induced inhibition of Complex I of the electron transport chain was blocked by calpain inhibitors and two Complex I proteins were identified as targets of mitochondrial calpain 10, NDUFV2, and ND6. In conclusion, calpain 10 is the first reported mitochondrial calpain and is a mediator of mitochondrial dysfunction through the cleavage of Complex I subunits and activation of MPT.

MATERIALS AND METHODS

Mitochondrial isolation and fractionation. Kidney mitochondria were isolated from male Sprague-Dawley rats (250 g) and female New Zealand White rabbits (2 kg), as previously described (57, 69). Briefly, the kidney cortex was minced and homogenized in ice-cold buffer A (0.27 M sucrose, 5 mM Tris-HCl, and 1 mM EGTA, pH 7.4). Nuclei and cellular debris were pelleted by centrifugation at 600 g for 5 min. The supernatant was centrifuged at 7,700 g for 5 min, resulting in a crude mitochondrial pellet. The pellet was washed once in ice-cold 0.27 M sucrose and resuspended in buffer B (130 mM KCl, 9 mM Tris-Po43-, 4 mM Tris-HCl, and 1 mM EGTA, pH 7.4). Crude mitochondria were then layered onto a sucrose/Percoll gradient and centrifuged at 20,000 g for 20 min.

This research was supported by the Department of Defense (No. DAMD 17-01-1-0027) and the American Heart Association (0450013N). Address for reprint requests and other correspondence: R. G. Schnellmann, Dept. of Pharmaceutical Sciences, Medical Univ. of South Carolina, 280 Calhoun St., PO Box 250140, Charleston, SC 29425 (e-mail: schnell@musc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Purified mitochondria were subfractionated as described previously (70). Outer membrane rupture was achieved by hypotonic lysis in ice-cold buffer C (10 mM KH$_2$PO$_4$, pH 7.4) for 20 min at 0°C. Mitoplasts were separated from the supernatant by centrifugation at 7,700 g for 5 min. The outer membrane fraction was obtained by centrifugation of the supernatant at 54,000 g for 30 min. The resulting pellet was resuspended in ice-cold buffer D (300 mM sucrose, 1 mM EGTA, and 20 mM MOPS, pH 7.4) and sonicated five times in 30-s bursts. The inner membrane and matrix fractions were then separated by centrifugation at 54,000 g for 30 min. Outer and inner membrane fractions were resuspended in buffer D. Fraction purity was assayed via marker enzyme analysis (outer membrane, monoamine oxidase; intermembrane space, adenylate kinase; inner membrane, cytochrome oxidase; matrix, fumarase). The activities of monoamine oxidase (57a), adenylate kinase (12), cytochrome oxidase (49), and fumarase (72) were determined by standard methods. Fractions were frozen at −70°C for subsequent immunoblot analysis.

**Calpain activity.** Calpain activity was assayed spectrophotometrically using the calpain-specific substrate SLLVY-AMC (Bachem), as previously described (6). Whole, energized, mitochondria (200 μg) were diluted in buffer B and incubated with various concentrations of CaCl$_2$ in the presence of 50 μM SLLVY-AMC. Activity was measured under linear conditions as a function of AMC hydrolysis using excitation and emission wavelengths of 355 and 444 nm, respectively. Mitochondria incubated in the absence of substrate exhibited the same fluorescence as buffer B alone.

**Respiratory complex activity.** Complex I enzyme activity was measured as previously described (14). The assay medium was composed of antimycin A (2 μg/ml), ubiquinone (65 μM), NADH (130 μM), and KCN (2 mM) in a phosphate buffer (25 mM potassium phosphate, 5 mM MgCl$_2$, and 2.5 mg/ml BSA, pH 7.2). Mitochondria (20–50 μg) were then added and NADH oxidation was measured spectrophotometrically at 340 nm for 3–5 min before the addition of rotenone (2 μg/ml). Absorbance changes were measured for another 3 min and Complex I activity reported as the rotenone-sensitive NADH:ubiquinone oxidoreductase activity.

Complex II enzyme activity was measured as previously described (14). Briefly, mitochondria (20–50 μg) were preincubated in assay media (25 mM potassium phosphate and 5 mM MgCl$_2$, pH 7.2) and 20 mM succinate for 10 min at 30°C. Antimycin A (2 μg/ml), 2 mM KCN, 2 μg/ml rotenone, and 50 μM 2,6-dichlorophenolindophenol were added, and absorbance at 600 nm recorded for 3 min. The reaction was then initiated with ubiquinone (65 μM), and absorbance monitored for 3–5 min.

Complex III activity was measured as previously described (14). Briefly, 15 μM cytochrome c (III), 2 μg/ml rotenone, 0.6 mM dodecyl-b-D-maltoside, and 35 μM ubiquinol-2 were added to assay media (25 mM potassium phosphate, 5 mM MgCl$_2$, 2.5 mg/ml BSA (fraction V), 2 mM KCN, pH 7.2) and the nonenzymatic rate of reduction of cytochrome c measured for 1 min at 550 nm. To initiate the reaction, mitochondria (5–20 μg) were added, and the initial increase in absorbance measured for 2 min.

**Measurement of renal cortical mitochondria oxygen consumption.** Oxygen consumption was monitored as previously described (57) using a six-chambered oxyterometer and computer interface (model 928, Strathkelvin, Glasgow, UK). Renal cortical mitochondria (RCM) were suspended at ~1.3 mg mitochondrial protein/ml in mitochondrial incubation buffer with pyruvate/malate (5/5 mM) as the respiratory substrates. In some experiments, succinate (10 mM) or ascorbic acid/tetramethylphenylene diamine (TMPD; 50/50 mM) served as the respiratory substrates in the presence of 100 μM rotenone (a Complex I inhibitor) or 2.5 μM antimycin A (a Complex III inhibitor), respectively. Mitochondria were gassed (5% CO$_2$-95% O$_2$) for 5 min before treatments and measurement of respiration. The respiration chamber was maintained at 37°C and stirred magnetically. After the basal rate (state 4) of O$_2$ consumption was determined, ADP (final concentration = 1 mM) was injected to obtain state 3 respiration. Only mitochondria with respiratory control ratios (RCRs; state 3/state 4 ≥4) were used for experiments to ensure that test mitochondria were tightly coupled.

In some experiments, respiration measurements were performed in the presence or absence of 1 μM Ca$^{2+}$ over various time courses. For inhibition experiments, calpain inhibitors were added 30 min before the addition of 1 μM Ca$^{2+}$ and respiration measured after 5 min. Ca$^{2+}$ was added to buffer B in all experiments such that Ca$^{2+}$ concentrations were maintained at 1 μM.

**Mitochondrial swelling.** RCM swelling was assessed spectrophotometrically as previously described (1). Briefly, RCM were suspended at a final concentration of 1 mg/ml of mitochondrial protein in buffer B supplemented with pyruvate/malate (5/5 mM) and absorbance measured for 10 min at 540 nM. After basal measurements were taken, Ca$^{2+}$ was added (1 μM final sustained Ca$^{2+}$ concentration) and absorbance was monitored for an additional 5 min and swelling rates (ΔΔA/min) determined.

**Zymography.** Zymography was performed as previously described (5) with minor modifications. Zymogram gels were cast immediately before electrophoresis and consisted of a 10% non-denaturing acrylamide resolving gel and an 8% stacking gel. Resolving gels were copolymerized with the calpain substrates FITC-casein (10 mg/ml) or SLLVY-AMC (50 μM). Protein samples (200 μg) and purified porcine calpains 1 and 2 (2 μg) (Calbiochem) were loaded and subjected to electrophoresis in a non-denaturing running buffer (125 mM Tris base, 625 mM glycine, and 5 mM EGTA, pH 8.0) at 120 V for 2 h at 4°C. Gels were subsequently bathed in Ca$^{2+}$-incubation buffer (50 mM Tris·HCl, 5 mM CaCl$_2$, and 10 mM 2-mercaptoethanol, pH 7.0) overnight at 4°C and imaged on an Alpha Innotech imaging station fitted with a FITC filter.

**Immunoblot analysis.** Isolated mitochondrial fractions were subjected to SDS-PAGE (4–12% acrylamide) and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies to b-calm, μ-calpain, calpain 10, NDUFV2, DAP13, and ND6. The primary antibodies used were monoclonal rabbit anti-human m-calpain (1:1,000; Affinity Bioreagents), rabbit anti-human m-calpain (domain IV) (1:1,000; Calbiochem), rabbit anti-human μ-calpain (domain III-IV) (1:1,000; Abcam), 1:200 rabbit anti-rat calpain 10 (domain II; generously provided by Tom Shearer, Oregon Health and Science University, Portland, OR), rabbit anti-human calpain 10 (1:1,000; domain III, Abcam), rabbit anti-rat calpain 10 (1:1,000; domain II, Biogenesis), rabbit anti-NDUFV2 (1:200; generously provided by Dr. Yamaguchi, The Scripps Research Institute, La Jolla, CA), monoclonal mouse anti-human DAP13 (1 μg/ml; Genway Biotech), and monoclonal mouse anti-human ND6 (1 μg/ml; Molecular Probes). Antibody incubation was followed by a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1,000; Santa Cruz). Immunoreactive protein was visualized by enhanced chemiluminescence (Amersham) and imaged using an Alpha Innotech imaging station.

**Plasmid construction.** cDNA for human calpain 10a (BC004260) was obtained from ATCC in the pOTB7 shuttle vector. Full-length calpain 10 was amplified by PCR (sense: 5'-TGGAGGACCSCGC-GGACCGGAG-3'; antisense: 5'-TCATCAGCTGCAATGACGGAGAT-3') and cloned into pcDNA3.1-TOPO-TA-CT-GFP (pcDNA3.1-TS-GFP) to assess NH$_2$-terminal GFP (cytochrome oxidase IV signal sequence) were generous gifts from Dr. Douglas Sweet (Medical University of South Carolina, Charleston, SC).

Complementary DNA sequences coding for the N-terminal 15 amino acids of calpain 10 were generated, annealed, and ligated into pcDNA3.1-TOPO-TA-CT-GFP (pcDNA3.1-TS-GFP) to assess NH$_2$-terminal sufficiency for mitochondrial targeting. The negative control for this experiment was obtained via ligation of the above sequence
into pcDNA3.1-TOPO-TA-CT-GFP (pcDNA3.1-TSINV-GFP) in the reverse orientation.

Cell culture and transfection. NIH-3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum until confluent. Cells were split and plated onto 35-mm confocal dishes (MatTek) at a density of 250,000 cells/plate. At 70% confluence, cells were transiently transfected with 1 μg pcDNA3.1-CAPN10-GFP, pcDNA3.1-TS-GFP, or pcDNA3.1-TSINV-GFP plasmid constructs using Lipofectamine 2000 (Invitrogen). Selected plates were treated with 6 μM cyclosporine A, 5 mM 3-methyladenine, or vehicle (DMSO) 4 h after transfection. Cells were incubated for 24 h, and, when indicated, exposed to 50 nM MitoTracker Red (Molecular Probes) and/or 100 nM LysoTracker Red (Molecular Probes) for 20 min before confocal microscopy imaging. Cells were imaged using a Zeiss LSM 5 confocal microscope using multiple tracks to eliminate fluorescent cross-talk.

Statistical analysis. RCM isolated from one rabbit represent one experiment (n = 1). The appropriate ANOVA was performed for each data set by using SigmaStat statistical software. Individual means were compared with Fisher’s protected least-significant difference test, with P ≤ 0.05 being considered a statistically significant difference between mean values. Means with different lettered subscripts within groups are significantly different from each other, P ≤ 0.05. Linear regression was also performed by using SigmaStat statistical software.

RESULTS

Calpain activity in isolated mitochondria and submitochondrial fractions. To determine the presence of calpain activity, rabbit RCM were incubated with increasing concentrations of Ca^{2+} in the presence of the calpain substrate SLLVY-AMC. RCM cleavage of SLLVY-AMC occurred in the absence of added Ca^{2+}, was linear over time (data not shown), and increased in the presence of increasing Ca^{2+} concentrations (Fig. 1A) to a maximum of 120% of control at 10 μM Ca^{2+}.

![Fig. 1. RCM calpain activity.](http://ajpcell.physiology.org/)

A: coupled RCM (150 μg) were incubated with the calpain substrate SLLVY-AMC (50 μM) and increasing concentrations of CaCl_{2}, and fluorescence measured for 6 min at 25°C. B: RCM calpain activity was measured in 0.5% digitonin-permeabilized mitochondria in the presence of 1 μM CaCl_{2} and increasing concentrations EGTA. C: RCM calpain activity was measured in whole mitochondria in the presence of 1 μM CaCl_{2} and increasing concentrations of ruthenium red. D: RCM were pretreated for 20 min on ice with increasing concentrations of calpeptin (●), E-64 (○), and PD150606 (□), and subsequently assayed for 1 μM Ca^{2+}-induced hydrolysis of SLLVY-AMC. E: purified mitochondrial fractions (○), matrix (●), outer membrane (□), and inner membrane (△) were assayed for SLLVY-AMC hydrolysis in the presence of increasing Ca^{2+} concentrations (0–10 mM). Data are expressed as means ± SE (N = 3). Means with different lettered subscripts within each group are significantly different from each other, P < 0.05.
EGTA to RCM permeabilized with 0.5% digitonin, in the presence of 1 μM Ca\(^{2+}\), produced a maximal 50% decrease in SLLVY-AMC hydrolysis (Fig. 1B). Blockade of the Ca\(^{2+}\) uniporter with ruthenium red also inhibited Ca\(^{2+}\)-induced hydrolysis of SLLVY-AMC in RCM (Fig. 1C). Ca\(^{2+}\)-activated SLLCY-AMC hydrolysis was inhibited in a concentration-dependent manner by the calpain inhibitors calpeptin and E-64, but not by the calpain inhibitor PD150606 (Fig. 1D). These data reveal that RCM exhibit basal calpain activity, that exogenous Ca\(^{2+}\) increases calpain activity in a ruthenium red-sensitive manner, that calpain activity exists in the absence of Ca\(^{2+}\), and that the calpain inhibitors calpeptin and E-64, but not PD150606, blocked the calpain activity. Because ruthenium red blocks Ca\(^{2+}\) uptake across the mitochondrial inner membrane and the increase in calpain activity produced by exogenous Cu\(^{2+}\) was ruthenium red sensitive, we suggest that the calpain activity is located in the mitochondrial matrix. Because the calpain activity was insensitive to PD150606, which binds to domain IV of typical calpains (67), we suggest that the RCM calpain lacks the penta-EF-hand domain and is a member of the atypical calpain family.

To determine which sub mitochondrial fraction(s) contain calpain activity, RCM were fractionated into outer membrane, inter membrane space, inner membrane, and matrix fractions. Marker enzymes (monoamine oxidase, outer membrane; adenylate cyclase, inter membrane space; cytochrome oxidase, inner membrane; fumarase, matrix) were used to assess mitochondrial fraction purity (Table 1). Ca\(^{2+}\)-activated hydrolysis of SLLVY-AMC was observed in each fraction with the outer membrane having the highest activity and the matrix the least on a per mg protein basis. Maximal activity was observed at 2 mM Ca\(^{2+}\) in the outer membrane and at 10 mM for the remaining three fractions (Fig. 1E). These results reveal that calpain activity is present in multiple mitochondrial fractions.

FITC-casein zymography is a technique commonly used to evaluate calpain activity in cell lysates. We employed this technique to evaluate calpain activity in rabbit RCM fractions and in the cytosol from renal cortical cells. Equal amounts of total protein (200 μg) from cytosolic, mitochondrial, and submitochondrial fractions were loaded into individual zymogram gels. Two bands of activity were observed in the cytosol corresponding to calpains 1 and 2 (Fig. 2A), both known to exist in the cytosol of renal proximal tubule cells (44). However, no bands were detected in the sample lanes for whole mitochondria or mitochondrial subfractions, suggesting that the mitochondria harbored no endogenous calpain 1 or 2, that the mitochondrial preparation was free of cytosolic contamination, and that mitochondrial calpain exhibits a substrate specificity different from that of the typical calpains. We modified this assay to evaluate mitochondrial calpain activity by replacing FITC-casein with SLLVY-AMC (a known mitochondrial calpain substrate in vitro) as the calpain substrate. AMC is liberated by proteolytic activity to generate fluorescent bands in the gel. Purified calpains 1 and 2 were used as controls and fluorescent bands were identified in the presence of Ca\(^{2+}\) (Fig. 2B). No fluorescent bands were observed in the outer membrane and intermembrane space fractions in the presence of Ca\(^{2+}\). In contrast, one strongly fluorescent band and two weaker bands were observed in the matrix fraction. These results reveal three distinct calpain activities in the matrix fraction that are Ca\(^{2+}\)-inducible and are different than calpains 1 or 2. No activity was observed in the outer membrane, inner membrane, or intermembrane space fractions.

Identification of calpain 10 in mitochondrial subfractions. The protein localization algorithm MITOP2 (http://ihg.gsf.de/mitop2/start.jsp) was used to determine which of the 15 calpain isoforms would be predicted to localize to mitochondria. While a negative score using this algorithm does not exclude the possibility of mitochondrial localization, this algorithm only returned a positive score for calpain 10. Cytosolic and RCM fractions from rabbit and rat kidney cortex were probed for calpain 10 with three separate antibodies directed against different calpain 10 domains using immunoblot analysis. The presence of calpains 1 and 2 also were examined. A 75 kDa band was identified in all rabbit and rat RCM fractions using all three calpain 10 antibodies, albeit at different levels (Fig. 3A). An immunoreactive band also was identified in the cytosol using an antibody against calpain 10. Immunoblots probed with antibodies against calpains 1 and 2 revealed immunoblotting results for all calpain isoforms.

**Table 1. Relative specific activity of marker enzymes in mitochondrial subfractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Monoamine Oxidase</th>
<th>Adenylate Kinase</th>
<th>Cytochrome Oxidase</th>
<th>Fumarase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer membrane</td>
<td>100</td>
<td>9±2</td>
<td>3±1</td>
<td>9±2</td>
</tr>
<tr>
<td>Inter membrane</td>
<td>20±1</td>
<td>100</td>
<td>5±2</td>
<td>18±4</td>
</tr>
<tr>
<td>Inner membrane</td>
<td>11±2</td>
<td>21±5</td>
<td>100</td>
<td>17±3</td>
</tr>
<tr>
<td>Matrix</td>
<td>1±1</td>
<td>19±3</td>
<td>2±1</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5. Enzyme activities were determined in the mitochondrial subfractions as described in MATERIALS AND METHODS. The highest specific activity was normalized to 100% for each marker enzyme tested.
active proteins against their respective purified proteins and cytosol but not in any of the RCM fractions (Fig. 3A). These results reveal that calpain 10 also resides in the mitochondria in at least two species (rabbit and rat), and is present in all RCM fractions. Furthermore, the bands of immunoreactivity are consistent with the largest calpain 10a isoform (75 kDa) (37), indicating the absence of import signal cleavage.

The strong fluorescent band identified in the matrix sample following zymography was eluted and subjected to SDS/PAGE and immunoblot analysis using an anti-calpain 10 antibody (Fig. 3B). These results reveal that the mitochondrial matrix calpain activity observed during zymography is calpain 10.

Calpain 10 is targeted to mitochondria via an NH₂ terminal targeting peptide. Human calpain 10a was subcloned into a TOPO-TA-CT-GFP vector (Invitrogen) to produce a calpain 10-GFP fusion protein containing an intact NH₂-terminus and a COOH-terminal GFP moiety. This construct was chosen to avoid modification of the NH₂ terminus, which could result in the loss of putative mitochondrial localization motifs.

NIH-3T3 cells were transfected with the pcDNA3.1-CAPN10-GFP construct, exposed to MitoTracker and/or LysoTracker Red, and imaged via confocal microscopy. Cells also were transfected using GFP constructs specific for cytosol (data not shown) or mitochondria (Fig. 4A) to serve as positive GFP controls for these compartments.

During initial experiments, we observed two distinct populations of cells after transfection with the CAPN10-GFP construct. All transfected cells demonstrated GFP expression that was localized to intracellular compartments with some being punctuate in nature and others exhibiting larger globular patterns of GFP expression. The latter of these two groups of cells exhibited altered cellular morphology, decreased plate adhesion, and the formation of autophagocytic vesicles as shown by costaining with LysoTracker Red (Fig. 4B). The former of these two groups of cells exhibited normal cellular morphology, increased plate adhesion, and contained swollen mitochondria (Fig. 4C) compared with mitochondrially targeted GFP controls (Fig. 4A). These data conclusively show the targeting of calpain 10 to the mitochondria and suggest that calpain 10 may play a role in mitochondrial dysfunction and/or swelling as evidenced by the changes seen in mitochondrial morphology.

To test the hypothesis that the NH₂ terminus of calpain 10 is responsible for mitochondrial localization, oligonucleotides coding for the first 15 NH₂-terminal amino acid residues (Fig. 5B) were annealed and ligated into the TOPO-TA-CT-GFP
vector. Negative controls included the same oligonucleotides inserted in the reverse orientation. NIH-3T3 cells transfected with these constructs displayed cytosolic targeting and mitochondrial targeting for the reverse and forward orientations, respectively (Fig. 5A). These results reveal that the NH$_2$-terminal 15 amino acids of calpain 10 are sufficient for mitochondrial targeting. We also analyzed the NH$_2$ terminus of calpain 10 using LaTeX software package TeXtopo designed by 10.220.33.3 on June 2, 2017 http://ajpcell.physiology.org/ Downloaded from AJP-Cell Physiol • VOL 291 • DECEMBER 2006 • www.ajpcell.org
to display peptide sequences in an alpha helix representation (Fig. 5B). The results revealed that the NH$_2$-terminus of calpain 10 can form the classic mitochondrial targeting motif, the amphipathic helix.

**Mitochondrial swelling induced by overexpression of calpain 10 is sensitive to cyclosporine A and 3-methyladenine.** As discussed above, overexpression of calpain 10 resulted in the conversion of the normal branched reticular mitochondrial morphology of control cells (Fig. 4A) to that of smaller, round and swollen organelles (Fig. 4C). Thus we hypothesized that the calpain 10-induced mitochondrial swelling may be sensitive to cyclosporine A [an inhibitor of MPT (62)] or 3-methyladenine [an inhibitor of MPT and autophagocytosis (62)]. To test this hypothesis, cells were transfected with the calpain 10-GFP construct, treated with cyclosporine A or 3-methyladenine, and the mitochondria was examined by confocal microscopy. Cyclosporine A and 3-methyladenine preserved the normal branched mitochondrial morphology and integrity (Fig. 4, D and E, respectively) compared with cells expressing calpain 10-GFP in the absence of MPT inhibitors, suggesting that calpain 10 may mediate mitochondrial swelling, in part, through the formation of the MPT pore. However, MPT formation was not explicitly examined in these experiments and it is possible that the mitochondrial fragmentation observed in our studies could also result from mitochondrial depolarization, electron transport chain dysfunction, or changes in mitochondrial fission or fusion proteins.

To further examine the possible role of calpain 10 in MPT, RCM were treated with 1 μM Ca$^{2+}$ in the presence and absence of cyclosporine A or calpeptin, and RCM swelling determined. The addition of Ca$^{2+}$-induced RCM swelling, which was blocked by cyclosporine A (Fig. 6A). Mitochondrial pretreatment with calpeptin blocked ~30% of Ca$^{2+}$-induced RCM swelling, indicating a role for calpain 10 in the formation of the MPT pore (Fig. 6B).

**Calpain 10 mediates Ca$^{2+}$-induced mitochondrial dysfunction.** As shown in Fig. 4B, calpain 10 overexpression resulted in a fragmented mitochondrial morphology. This type of organelar derangement is seen not only during MPT, but is also characteristic of mitochondria with damaged or impaired components of the electron transport chain (ETC) (41). Thus calpain 10 may mediate ETC dysfunction. To test this hypothesis, RCM were isolated and suspended in incubation buffer containing the model Complex I substrates pyruvate/malate (5/5 mM). Mitochondrial oxygen consumption was then measured over time after treatment with 1 μM Ca$^{2+}$ in the presence of 1 mM ADP. State 3 respiration was unchanged under normoxic and hypoxic conditions in the absence of Ca$^{2+}$ (Fig. 7A), and all future respiratory measurements were performed under normoxic conditions. The addition of 1 μM Ca$^{2+}$ produced a time-dependent decrease in mitochondrial state 3 respiration (Fig. 7B), and all subsequent experiments were conducted following the incubation of RCM with 1 μM Ca$^{2+}$ for 5 min. Experiments including inhibitors were performed after a 30 min preincubation period with specific calpain inhibitors. Pretreatment of RCM with increasing concentrations of the active-site calpain inhibitors calpeptin and E-64, but not the domain IV calpain inhibitor PD150606 preserved mitochondrial state 3 respiration (Fig. 7C). Higher concentrations of E-64 did not produce enhanced state 3 respiration, most likely due to its poor membrane permeability. Simultaneous measurement of state 3 dysfunction and mitochondrial calpain activity demonstrated a strong correlation between these two end points (Fig. 7D).

To determine the site of ETC dysfunction, isolated RCM were suspended in incubation buffer containing pyruvate/malate (Complex I substrates), succinate/rotenone (Complex II substrate/Complex I inhibitor), or ascorbate-TMPD/antimycin A (Complex IV electron donor/Complex III inhibitor). Mitochondrial state 3 respiration was then measured after treatment with 1 μM Ca$^{2+}$ for 5 min. Respiratory deficits were observed only in the treatment group containing the Complex I substrates (Fig. 8A). To verify these findings, we performed specific Complex I, II, and III activity assays and determined that only Complex I activity was significantly decreased after Ca$^{2+}$ exposure (Fig. 8B) and that this activity could be preserved by pretreatment with calpeptin. These findings show that Ca$^{2+}$-induced respiratory dysfunction is limited to Complex I under these conditions and that electron transport in Complexes II-IV remains intact.

**Calpain 10 cleaves mitochondrial complex I subunits.** Complex I of the ETC is composed of 46 individual subunits. We sought to determine the complex I protein sub-strate(s) of calpain 10. To do this, we analyzed each of the 46 Complex I subunits for possible calpain cleavage motifs using the PEST-FIND algorithm (http://srs.nchc.org.tw/emboss-bin/emboss.pl?_action=input&app=pestfind). The PEST-FIND algorithm is useful for the identification of possible calpain and proteasome substrates, but does not predict actual cleavage sites within protein targets. Subsequent to PEST analysis, four protein species were identified as potential calpain substrates; NDUFV2, DAP13, NDUF57, and ND6. Antibodies were available and obtained for NDUFV2, DAP13, and ND6. RCM were isolated and pretreated with various inhibitors for 30 min before the addition of Ca$^{2+}$. RCM were treated with 1 μM Ca$^{2+}$ for 5 min, pelleted by centrifugation, and resuspended in buffer deficient in Ca$^{2+}$ and containing a protease inhibitor cocktail (Sigma) to stop all proteolytic reactions. Aliquots were then subjected to immunoblot analysis using antibodies against NDUFV2, DAP13 and the ND6 subunits of Complex I. The calpain inhibitors ALLN and calpeptin completely inhibited NDUFV2 and ND6 hydrolysis while pretreatment with the
serine protease inhibitor bestatin did not (Fig. 8C). DAP13 did not undergo hydrolysis (Fig. 8C). Pretreatment with ruthenium red also blocked NDUFV2 and ND6 degradation (Fig. 8C), suggesting that calpain 10-mediated proteolysis of Complex I subunits occurs in the matrix. Our results demonstrate Ca\(^{2+}\)-induced calpain-dependent proteolysis of the Complex I subunits NDUFV2 and ND6, but not of the DAP13 subunit.

**DISCUSSION**

Calpains are ubiquitously expressed throughout eukaryotic organisms and are involved in numerous cellular functions (30). Within mammals, calpains 1, 2, 4, 5, 7, 10, 12, and 13 are ubiquitously expressed, whereas calpains 3, 6, 8, 9, and 11 have more tissue-specific distributions (37), implicating a diverse set of roles for calpain family members. However, the physiological and pathological roles of most calpain isoforms have not been examined. While calpains are thought to be cytosolic proteins, investigators have reported calpain-like activities in isolated mitochondria (1, 4, 9, 19, 31, 52, 60). Using multiple approaches, we identified a resident mitochondrial calpain as calpain 10 and determined that it plays a role in mitochondrial dysfunction.

Ma et al. (45) first cloned calpain 10 in 2001 and proposed that this calpain isoform was localized to the cytosol and translocated to the nucleus after increases in cellular Ca\(^{2+}\). Our data reveal that calpain 10 is present in the cytosol and mitochondria, while significant nuclear staining is absent under nonstimulated conditions (Fig. 4C). Ma et al. also proposed that this calpain isoform was localized to the cytosol and translocated to the nucleus after increases in cellular Ca\(^{2+}\). Our data reveal that calpain 10 is present in the cytosol and mitochondria, while significant nuclear staining is absent under nonstimulated conditions (Fig. 4C). However, in the present study, we have not evaluated the effects of Ca\(^{2+}\) overload on calpain 10 subcellular localization. We believe that our cloning strategy avoids the complications and cross-reactivity of antibodies used for immunohistochemistry and provides a clearer picture of the subcellular localization of calpain 10.

Many mitochondrial matrix-targeted proteins contain either a NH\(_2\)- or COOH-terminal signaling motif (25, 58). This motif most commonly takes the form of an NH\(_2\)-terminal amphipathic helix containing positively charged residues on one half of the helix and hydrophobic residues on the other (58). We
determined the presence of such a motif in calpain 10 using LaTeX software package TeXtopo (Fig. 5B), designed to display peptide sequences in a helical representation (10). We tested the hypothesis that the NH2-terminal 15 amino acids of calpain 10 are responsible for mitochondrial localization, and found that cells expressing GFP conjugated to the NH2-terminal 15 amino acids localized to the mitochondria whereas cells expressing GFP conjugated to the same nucleotides inserted in the reverse orientation remained in the cytosol.

Investigators have previously reported a Ca2+-inducible calpain-like activity in mitochondria (1, 9, 52, 60). Our data reveal that calpain activity is present in intact mitochondria and is increased following Ca2+ addition. Because Ca2+ is concentrated in the mitochondrial matrix, and ruthenium red blocked the Ca2+-induced increase in calpain activity, we propose that the majority of Ca2+-inducible calpain 10 activity in mitochondria is localized to the matrix. This idea is supported by the experiment in which calpain 10 was identified in the matrix fraction following zymography. Using permeabilized mitochondria and Ca2+ chelation, we observed that ~50% of the mitochondrial calpain 10 activity was Ca2+-dependent. It is unclear whether the remaining cysteine protease activity in the matrix was due to Ca2+-independent calpain 10 activity or due to the activity of another cysteine protease. One tempting explanation is that some of the Ca2+-independent activity seen in isolated RCM subfractions may come from one of the remaining 7 calpain 10 splice variants (all of which carry the mitochondrial targeting signal). It is also possible that calpain 10 is less Ca2+-dependent than the typical calpains due to its lack of a Domain IV or that it is dually regulated by both calcium and some sort of secondary protein modification such as phosphorylation (an event shown to activate calpain 2) (29).

Fig. 6. Mitochondrial calpain mediates Ca2+-induced MPT. A: cyclosporine A pretreatment inhibits mitochondrial swelling induced by 1 μM Ca2+. Basal mitochondrial swelling was monitored for 10 min, after which Ca2+-induced swelling for an additional 5 min. B: RCM were pretreated with calpeptin for 30 min and mitochondrial swelling induced by 1 μM Ca2+. Swelling was measured as indicated above. Data are expressed as means ± SE (N = 3). P < 0.05, means with different lettered subscripts within each group are significantly different from each other.

Fig. 7. Mitochondrial calpain mediates Ca2+-induced respiratory dysfunction. State 3 mitochondrial respiration was measured using isolated RCM (1.5 mg/ml) in the presence and absence of 1 μM Ca2+ and various calpain inhibitors. A: state 3 mitochondrial respiration was measured in the absence of Ca2+ under conditions of normoxia and hypoxia in the presence and absence of the Complex I substrates pyruvate/malate (5/5 mM). B: state 3 mitochondrial dysfunction was measured as a function of time in the presence of the Complex I substrates pyruvate/malate (5/5 mM) and 1 μM Ca2+. C: RCM were pretreated for 30 min with a variety of dissimilar calpain inhibitors. RCM were then challenged with 1 μM Ca2+ for 5 min and state 3 mitochondrial respiration measured. The calpain inhibitors calpeptin and E-64 protected against state 3 dysfunction, whereas PD150606 did not. The solid and dashed lines represent mitochondrial state 3 respiration in the absence and presence of 1 μM Ca2+, respectively. D: mitochondrial state 3 dysfunction is positively correlated with increases in mitochondrial calpain activity (r² = 0.93). Data are expressed as means ± SE (N = 3). Means with different-lettered subscripts within each group are significantly different from each other, P < 0.05.
More research is necessary to evaluate the relationship between Ca\textsuperscript{2+} and calpain 10 using purified protein.

Interestingly, a recent report from Garcia et al. (28), has proposed that calpain 1 is localized to both the cytosol and mitochondrial fractions of rat brain cortex and of SH-SY5Y human neuroblastoma cells. Our data are not consistent with the mitochondrial localization of calpain 1 in kidney mitochondria. For example, we assayed for calpain 1 in purified mitochondria and cytosol by both immunoblot and FITC-casein zymography. While calpain 1 was identified in the cytosol it was not present in mitochondria. Furthermore, calpain 1 does not have an identifiable mitochondrial targeting signal as determined by the MITOP2 algorithm, although this does not completely exclude the possibility of mitochondrial localization. Finally, the “typical” calpain inhibitor PD150606 (a membrane permeable inhibitor) blocks calpain 1 but did not block calpain activity in mitochondria. Excluding model differences, one possible explanation may be that calpain 1 can associate with the mitochondrial outer membrane as described for the Golgi and endoplasmic reticulum (36), but is incapable of penetrating into the mitochondrial interior.

Using isolated RCM fractions, we observed calpain activity and calpain 10 protein in the outer membrane, intermembrane space, inner membrane, and matrix fractions. These results support the work of others who have reported calpain activity in more than one mitochondrial fraction (9). However, using zymography, Ca\textsuperscript{2+}-inducible calpain 10 activity was primarily observed in the matrix fraction and increases in calpain activity.

Fig. 8. Complex I subunits NDUFV2 and ND6 are proteolytic targets of the mitochondrial calpain. A: isolated RCM were treated with 1 \textmu M Ca\textsuperscript{2+} for 5 min in the presence of Complex I substrates pyruvate/malate (5/5 mM), Complex II substrate and inhibitor succinate/rotenone (10 mM/100 \textmu M), or Complex IV electron donor and Complex III inhibitor ascorbate/rotenone (5/0.5 mM/2.5 \textmu M). B: Complex I, II, and III enzyme activities were measured in isolated RCM as outlined in the text. Mitochondria were pretreated with calpeptin (30 \textmu M) or diluent for 30 min and subsequently exposed to 1 \textmu M Ca\textsuperscript{2+} for 5 min. Data are expressed as means ± SE (N = 3). Means with different subscripts within each group are significantly different from each other, P < 0.05. C: isolated RCM were pretreated with various inhibitors for 30 min and then exposed to 1 \textmu M Ca\textsuperscript{2+} for 5 min. Samples were then prepared for Western blot analysis and were probed with antibodies against the Complex I subunits NDUFV2, ND6, and DAP13. Results are representative of three independent experiments.
following Ca\(^{2+}\) addition were ruthenium red-sensitive, suggesting matrix calpain 10 activity. While the reasons for these differences are not known, we propose that Ca\(^{2+}\)-inducible calpain 10 activity in intact mitochondria is primarily the result of matrix calpain 10 and that calpain 10 may be inactive in the remaining fractions or regulated in a Ca\(^{2+}\)-independent manner.

Ca\(^{2+}\) activation of calpains is important for physiological functions, but excessive Ca\(^{2+}\) can produce abnormal proteolytic activity and cell injury and death (44) (See Fig. 9). MPT is a form of mitochondrial dysfunction produced by Ca\(^{2+}\) overload, decreased adenine nucleotide concentrations, decreased mitochondrial membrane potential, and increased oxidative stress, and is characterized by the opening of a pore and mitochondrial swelling (2, 33, 43). Calpain 10 overexpression induced mitochondrial fragmentation and swelling, consistent with MPT (50) and this altered mitochondrial morphology was blocked by two MPT inhibitors. In addition, high levels of calpain 10 expression induced mitochondrial autophagy, a process blocked by 3-methyladenine and thought to be stimulated by MPT induction (42, 43, 55). We demonstrated 30% inhibition of MPT by the calpain inhibitor calpeptin, which agrees with Gores et al. (1, 31), who reported a liver mito-

Deficiencies or mutations in various protein subunits of Complexes I-IV have been identified and linked to clinical syndromes (27). Complex I subunit mutations are perhaps the most common and account for 33% of all respiratory chain disorders (64). In addition, Ricci et al. (53) have shown that the proteolysis of Complex I subunits can also contribute to ETC dysfunction by demonstrating the caspase-mediated cleavage of the 75 kDa NDUFV1 subunit. In the current study, we have identified two Complex I subunits, NDUFV2 and ND6, which undergo calpain-mediated hydrolysis. The NDUFV2 subunit is a nuclear-encoded 24-kDa protein found in the matrix arm of Complex I and is required for Complex I activity (3, 40). Defects in this protein have been identified previously and result in encephalopathies and bipolar disorder (11, 68). The ND6 subunit, a 20-kDa protein encoded by the mitochondrial genome, is a transmembrane protein known to assist in Complex I assembly, is required for Complex I activity, and whose mutations are associated with Leber’s hereditary optic neuropathy (7, 8, 17, 22). Thus, similar to the mitochondrial calpain-induced hydrolysis of ND6 and NDUFV2 and the associated Complex I dysfunction, genetic alterations in these proteins result in inhibition of Complex I. Interestingly, Koopman et al. (41), have shown that single subunit mutations in Complex I not only reduce enzyme activity of the complex but are often associated with alterations in mitochondrial morphology; a phenomenon we observed with calpain 10 overexpression.

In summary, we have identified the endogenous mitochondrial calpain as calpain 10 and that it plays a role in mitochondrial dysfunction and that calpain inhibition with calpeptin protects against Complex I dysfunction. We sought to determine potential Complex I protein substrate(s) of calpain 10 using the PEST-FIND algorithm (http://srs.nchc.org). Four Complex I proteins were identified and three proteins were tested (NDUFV2, DAP13, and ND6). Ca\(^{2+}\)-induced inhibition of ETC was associated with hydrolysis of NDUFV2 and ND6 but not DAP13. Further, pretreatment with ruthenium red also blocked NDUFV2 and ND6 degradation, suggesting that Ca\(^{2+}\)-induced calpain 10-mediated proteolysis of NDUFV2 and ND6 subunits occurs in the mitochondrial matrix.

The mitochondrial ETC provides a mechanism for the generation of ATP through the oxidative phosphorylation of ADP. As previously reported (65), mitochondrial Ca\(^{2+}\) overload results in decreased ETC activity. Our results reveal that Complex I is the most sensitive complex to Ca\(^{2+}\)-induced mitochondrial dysfunction and that calpain inhibition with calpeptin protects against Complex I dysfunction.
drial viability. While the physiological and pathological functions of calpain 10 have not been studied extensively, it has been linked to ryanodine-induced apoptosis, GLUT4 vesicle translocation, pancreatic β-cell exocytosis, cataractogenesis, hypertriglyceridemia, and is genetically linked to Type II diabetes, a disease associated with mitochondrial dysfunction (18, 34, 39, 45, 46, 51). Mitochondria are increasingly being thought of as cell death checkpoints at which signals for necrosis and apoptosis are sent for downstream processing. It will be exciting to elucidate the role of calpain 10 in orchestrating these events and how we may be able to manipulate this system for therapeutic intervention in disease states dominated by the dysregulation of cellular Ca2+ homeostasis.

ACKNOWLEDGMENTS

We thank Dr. Tom Shearer for the generous gift of the domain IIa calpain 10 antibody, Dr. Eric Beitz for technical assistance with the LaTeX TeXtopo software, and Drs. Douglas Sweet and Geri Youngblood for help in troubleshooting the subcloning of calpain 10.

Present address for T. R. Van Vleet: Bristol-Myers Squibb, 2400 W. Lloyd Expressway, P3, Evansville, IN 47721.

REFERENCES

C1171


