Calcium-dependent Spontaneously Reversible Remodeling of Brain Mitochondria

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Natalia Shalbuyeva, Tatiana Brustovetsky, Alexey Bolshakov, and Nickolay Brustovetsky

From the Department of Pharmacology and Toxicology, Stark Neuroscience Research Institute, Indiana University School of Medicine, Indianapolis, Indiana 46202

An exposure of cultured hippocampal neurons expressing mitochondrially targeted enhanced yellow fluorescent protein to excitotoxic glutamate resulted in reversible mitochondrial remodeling that in many instances could be interpreted as swelling. Remodeling was not evident if glutamate receptors were blocked with MK801, if Ca\(^{2+}\) was omitted or substituted for Sr\(^{2+}\) in the bath solution, if neurons were treated with carbonylcyanide p-trifluoromethoxyphenylhydrazone to depolarize mitochondria, or if neurons were pretreated with cyclosporin A or N-methyl-4-isoleucine-cyclosporin (NIM811) to inhibit the mitochondrial permeability transition. In the experiments with isolated brain synaptic or nonsynaptic mitochondria, Ca\(^{2+}\) triggered transient, spontaneously reversible cyclosporin A-sensitive swelling closely resembling remodeling of organelles in cultured neurons. The swelling was accompanied by the release of cytochrome c, Smac/DIABLO, Omi/HtrA2, and AIF but not endonuclease G. Depolarization with carbonylcyanide p-trifluoromethoxyphenylhydrazone or inhibition of the Ca\(^{2+}\) uniporter with Ru360 prevented rapid onset of the swelling. Sr\(^{2+}\) depolarized mitochondria but failed to induce swelling. Neither inhibitors of the large conductance Ca\(^{2+}\)-activated K\(^{+}\) channel (charybdotoxin, iberiotoxin, quinine, and Ba\(^{2+}\)) nor inhibitors of the mitochondrial ATP-sensitive K\(^{+}\) channel (5-hydroxydecanoate and glibenclamide) suppressed swelling. Quinine, dicyclohexylcarbodiimide, and Mg\(^{2+}\), inhibitors of the mitochondrial K\(^{+}/H^+\) exchanger, as well as external alkalization inhibited a recovery phase of the reversible swelling. In contrast to brain mitochondria, liver and heart mitochondria challenged with Ca\(^{2+}\) experienced sustained swelling without spontaneous recovery. The proposed model suggests an involvement of the Ca\(^{2+}\)-dependent transient K\(^{+}\) influx into the matrix causing mitochondrial swelling followed by activation of the K\(^{+}/H^+\) exchanger leading to spontaneous mitochondrial contraction both in situ and in vitro.

In the last 2 decades, the mitochondrial permeability transition (mPT)\(^3\) made a long journey from an experimental artifact to a potentially crucial target in the treatment of various diseases (1). The mPT is defined as a sudden increase in permeability of the inner mitochondrial membrane (IMM) in response to mitochondrial Ca\(^{2+}\) overload and/or oxidative stress (2–4). There are numerous indications that the mPT has complex regulation and may exist in low and high conductance modes (5–9). A growing body of evidence implicates the mPT in various pathologies associated with the elevation of cytosolic Ca\(^{2+}\) and/or oxidative stress (1, 10). Stroke accompanied by brain ischemia with neurons suffering from glucose/oxygen deprivation and exposure to high excitotoxic concentrations of glutamate is one such pathological condition. Overstimulation of glutamate receptors in stroke causes massive Ca\(^{2+}\) influx into neurons where mitochondria play a crucial role in clearance of elevated Ca\(^{2+}\) (11–14). However, the active role of mitochondria in sequestration of cytosolic Ca\(^{2+}\) comes with a high price. Excessive Ca\(^{2+}\) accumulation causes the mPT accompanied by its main manifestations, depolarization and mitochondrial swelling (3).

The pivotal role of the mPT in neuronal damage emerged in the 1990s when several groups reported a protective role of cyclosporin A (CsA), an inhibitor of the mPT, in studies of the mechanisms of excitotoxicity (15–22). The protection with CsA is widely considered a hallmark of the mPT involvement in cellular or mitochondrial injury (1, 23). Lately, the role of the mPT in the mechanisms of excitotoxicity was questioned largely because of the lack or inconsistency of CsA-imposed protection (24–28). However, it is well known that the protective effects of CsA could be easily overridden by increasing the concentrations of the mPT inducers/activators (29–31). Thus, the lack of CsA protection might not necessarily indicate the lack of the mPT.

Many studies of mitochondrial involvement in excitotoxicity in situ are focused on mitochondrial depolarization that precludes oxidative phosphorylation restricting the energetic capacity of the cell (32). Deregulation of calcium homeostasis

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1 Present address: Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, Baltiyskaya Str. 8, 125315 Moscow, Russia.

2 To whom correspondence should be addressed: Dept. of Pharmacology and Toxicology, Indiana University School of Medicine, 635 Barnhill Dr., Medical Science Bldg., Rm. S49, Indianapolis, IN 46202. Tel.: 317-278-9229; Fax: 317-274-7714; E-mail: nbrous@iupui.edu.

3 The abbreviations used are: mPT, mitochondrial permeability transition; mito-eYFP, mitochondrially targeted enhanced yellow fluorescent protein; KHX, K\(^{+}/H^+\) exchanger; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; \(\Delta\psi\), mitochondrial membrane potential; TPP\(^{+}\), tetraphenylphosphonium cation; CsA, cyclosporin A; CSH, cyclosporin H; NMDDA, N-methyl-D-aspartic acid; MK801, (±)-5-methyl-10,11-dihydro-SN-dibenzo[a,d]cyclohepten-5,10-imine; DCCD, N,N’-dicyclohexylcarbodiimide; TEM, transmission electron microscopy; BSA, bovine serum albumin; EndoG, endonuclease G; NIM811, N-methyl-4-isoleucine-cyclosporin; AIF, apoptosis inducing factor.
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that disturbs intracellular signaling and activates processes of cell degradation is another favorite area of these studies (33), although only a few of the early studies paid attention to mitochondrial morphological changes associated with glutamate insults (16, 24, 34, 35). Excessive mitochondrial swelling appears not to be a less dangerous process than depolarization, which potentially leads to rupture of the OMM and release of mitochondrial apoptogenes that amplify apoptotic signal transduction (36–38). Massive Ca\textsuperscript{2+} influx into the cytosol and subsequent Ca\textsuperscript{2+} sequestration by mitochondria during glutamate challenge result in distinct changes in mitochondrial morphology that, in most cases, could be characterized as swelling of the organelles (16, 24, 38). However, the definitive role of the mPT in mitochondrial morphological changes was not firmly established in the early studies. Later, glutamate-induced alterations in mitochondrial morphology were found to be CsA-insensitive, and that argued against involvement of the classical mPT (39). In another study, an application of NMDA to cultured hippocampal neurons caused mitochondrial swelling, but whether this swelling was associated with the mPT remained unclear (38).

In our previous paper (37), we showed that brief exposure of cultured neurons to an excitotoxic concentration of glutamate causes partial release of cytochrome c into the cytosol in a CsA-sensitive manner. The sensitivity to CsA linked this release to the mPT. It was proposed that mitochondrial swelling in situ followed by the rupture of the OMM underlies the release of cytochrome c. In this study, we demonstrated a CsA-sensitive, Ca\textsuperscript{2+}-dependent mitochondrial remodeling in cultured neurons treated with excitotoxic glutamate. Furthermore, we compared this remodeling in situ with morphological changes in isolated brain mitochondria in vitro. Remarkably, in both cases the remodeling was spontaneously reversible and had numerous common features suggesting common mechanisms in situ as well as in vitro. This finding provided an opportunity to use isolated brain mitochondria as a well-defined, cell-free model system for a detailed analysis of the mechanisms of mitochondrial remodeling in cultured neurons under excitotoxic conditions.

EXPERIMENTAL PROCEDURES

Cell Culturing—Primary cultures of hippocampal neurons were prepared from postnatal day 1 rat pups according to approved protocols from the Institutional Animal Care and Use Committees and procedures published previously (40). Briefly, neurons were plated on glass-bottomed Petri dishes without preplated glia as described previously. For all platings, 35 μg/ml uridine plus 15 μg/ml 2-deoxy-5-fluorouridine was added 24 h after plating to inhibit proliferation of non-neuronal cells. Cultures were maintained in a 5% CO\textsubscript{2} atmosphere at 37 °C in Eagle’s medium supplemented with 10% NuSerum (BD Biosciences) and 27 mM glucose.

Transfection of Primary Hippocampal Neurons—To visualize mitochondria within live cells, hippocampal neurons were transfected in suspension during plating using electroporator BTX 630 ECM (Harvard Apparatus, Holliston, MA) with a plasmid encoding mitochondrially targeted enhanced yellow fluorescent protein (eYFP, generously provided by Dr. Roger Tsien, University of California, San Diego). This procedure usually provided 15–20% transfection efficacy with <1% efficacy with commercial cationic lipid liposomes. Neurons were imaged 10–12 days after transfection. Supplemental Fig. 1 demonstrates typical bright field and fluorescence images of cultured hippocampal neurons expressing mito-eYFP at 11 days in vitro.

Fluorescence Microscopy—A Nikon Eclipse TE2000-U inverted microscope equipped with objective Nikon CFI SuperFluor 100× 1.3 NA and Metamorph 6.3 software (Molecular Devices, Downingtown, PA) were used to visualize neuronal mitochondria targeted with eYFP. In all experiments neurons were perfused using ValvBank 8 perfusion system (AutoMate Scientific, San Francisco). The standard perfusion solution contained 139 mM NaCl, 3 mM KCl, 0.8 mM MgCl\textsubscript{2}, 1.8 mM CaCl\textsubscript{2}, 10 mM HEPES, pH 7.2, 5 mM glucose, 20 mM sucrose. Neurons were illuminated at 480 ± 20 nm. Fluorescence was collected through a 505 nm dichroic mirror and a 535 ± 25 nm emission filter by a CCD camera CoolSNAP_HQ (Photometrics, Tuscon, AZ). Images were taken at room temperature every 15 s during the time course of the experiment.

Isolation of Brain, Liver, and Heart Mitochondria—To isolate mitochondria, male Sprague-Dawley rats, 225–250 g (Harlan, Indianapolis, IN), were sacrificed by decapitation according to an approved protocol from the Institutional Animal Care and Use Committee. Nonsynaptic mitochondria derived from the single rat brain were isolated in mannitol/sucrose medium and purified on a discontinuous Percoll gradient (37, 41). Synaptic mitochondria were isolated from synaptosomes by the nitrogen cavitation method using a nitrogen cell disruption bomb, model 4639 (Parr Instrument Co., Moline, IL), cooled on ice (42) with some modifications. Briefly, the synaptosomes obtained during preparation of nonsynaptic mitochondria were transferred in a cooled plastic beaker and placed into the nitrogen bomb on ice under 1,000 psi for 13 min. The synaptosomes were layered on top of the discontinuous Percoll gradient (26/40%) and centrifuged at 31,000 × g for 28 min in a Beckman SW–41 rotor. The mitochondrial fraction in the interface between Percoll layers was transferred into a fresh tube, diluted 1:5 with medium containing 394 mM sucrose, 0.1 mM EGTA, 10 mM HEPES, pH 7.4, and centrifuged at 31,000 × g for 20 min. The pellet was resuspended in 0.5 ml of the latter medium and kept on ice. Liver and heart mitochondria were isolated and purified in the same way as nonsynaptic brain mitochondria except the lower level in the discontinuous density gradient contained 60% Percoll (43). Mitochondrial protein concentration was measured by the Bradford method (44) with BSA as a standard.

Measurements of Light Scattering, Membrane Potential (Δψ), and Mitochondrial Respiration—Mitochondrial swelling was evaluated by following changes in light scattering of mitochondrial suspension at 525 nm with an incident beam under 180° in a 0.3-ml chamber at 37 °C and continuous stirring. Δψ (mitochondrial membrane potential) was monitored by following the distribution of tetraphenylphosphonium cation (TPP\textsuperscript{+}) between the external medium (initially 1.8 μM TPP\textsuperscript{+}-Cl) and the mitochondrial matrix with a TPP\textsuperscript{+}-sensitive electrode (45) in the standard incubation medium containing 125 mM KCl, 10
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mm HEPES, pH 7.4, 0.5 mm MgCl₂, 3 mm KH₂PO₄, 0.1% BSA (free from fatty acids) supplemented with 3 mm pyruvate and 1 mm malate unless stated otherwise. Where it is stated, experiments have been performed in hypotonic KCl-based medium (75 mm KCl) or K⁺-free medium containing either 125 mm N-methyl-D-glucamine or 125 mm choline chloride or 215 mmmannitol plus 70 mm sucrose. A decline in the external TPP⁺ concentration corresponded to mitochondrial polarization, whereas a rise in the TPP⁺ concentration in the medium corresponded to depolarization. Mitochondrial respiration was recorded in the standard 125 mm KCl-based incubation medium at 37 °C under continuous stirring. Rat liver mitochondria were incubated with 3 mM glutamate plus 1 mM malate, all other mitochondrial preparations with 3 mM pyruvate plus 1 mM malate. The 0.3-ml incubation chamber was equipped with a Clark-type oxygen electrode and a tightly closed lid. The slope of the O₂ electrode trace corresponded to the respiration rate. The representative respiratory traces and averaged respiratory control ratios (the ratio of respiratory rates in the presence of 300 μM ADP and in the presence of 1 μM oligomycin, RCR_Oligo) are shown in supplemental Fig. 2. In all experiments with isolated mitochondria, the concentration of mitochondrial protein in the chamber was 0.1 mg/ml. All data traces shown are representative of at least three separate experiments.

Monitoring of Matrix pH—To monitor the matrix pH, mitochondria were loaded with 10 μM 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein-AM in the dark at room temperature for 20 min. Mitochondria were then washed twice in the medium for resuspension. The standard incubation medium was supplemented with 50 mm HEPES to minimize fluctuations of the external pH (46). The measurements were performed under continuous stirring in a PerkinElmer Life Sciences LS 55 luminescence spectrometer equipped with a bio-kinetics accessory attachment using excitation/emission wavelengths of 500 and 535 nm, respectively.

Transmission Electron Microscopy—Samples for transmission electron microscopy (TEM) were taken prior to Ca²⁺ addition, at the top of mitochondrial swelling, and at the end of recovery as indicated in Fig. 3a. Purified brain mitochondria were incubated in the standard 125 mm KCl-based medium supplemented with 3 mM pyruvate plus 1 mM malate at 37 °C prior to fixation in 2% paraformaldehyde plus 2% glutaraldehyde in 0.05 M phosphate buffer in the same incubation medium at room temperature for 15 min. Mitochondria were then centrifuged at 15,800 × g for 5 min, and the supernatant was discarded. The pellet was layered with a fresh solution of 2% paraformaldehyde plus 2% glutaraldehyde in 0.1 M phosphate buffer and left overnight at 4 °C. The samples were postfixed in 1% osmium tetroxide for 1 h and dehydrated through a series of graded ethyl alcohols before embedding in the resin Embed®812 (Electron Microscopy Sciences, Fort Washington, PA). Thick sections were cut on an Ultratoc UCT microtome (Leica, Bannockburn, IL), and then thin sections were cut using a diamond knife (Diatom, Electron Microscopy Sciences) at 70–90 nm and stained with uranyl acetate and lead citrate. Digital electron micrographs were obtained using a Tecnai G12BioTwin electron microscope (FEI, Hillsboro, OR) equipped with an AMT 2.6 × 2.6k digital CCD camera. To quantitatively assess the morphological changes, five different TEM micrographs representing randomly chosen areas in the grid were selected and analyzed in a blinded fashion using the approach described previously (47). Total mitochondrial population was categorized into three groups according to their morphology as follows: (i) condensed, (ii) swollen, and (iii) orthodox. Mitochondria with characteristics bridging morphologic groups were assigned to the lower category. Mitochondria were counted, and morphological distribution was statistically analyzed using one-way analysis of variance followed by Bonferroni’s post-test (GraphPad Prism, 4.0).

Immunoblotting—Release of mitochondrial apoptogenic proteins, including cytochrome c, Smac/DIABLO, Omi/HtrA2, endonuclease G (EndoG), and AIF, was assessed using Western blotting in supernatants obtained through incubation of mitochondria in 75 mm KCl-based medium for 15 min at 37 °C with or without Ca²⁺. Western blotting was performed as described previously (48). During the course of this project, we found that BSA used in the incubation medium interferes with detection of the released AIF in the supernatants. Therefore, the release of AIF was studied in the BSA-free incubation medium. The release of proteins from mitochondria treated with alamethicin (30 μg/ml) was used as a positive control. However, we could not detect release of EndoG even after treatment of mitochondria with alamethicin. In this case, we used solubilized mitochondria as a positive control.

Statistics—Statistical analyses of experimental data consisted of one-way analysis of variance followed by Bonferroni’s post-test (GraphPad Prism, 4.0, GraphPad Software, San Diego). Data represent mean ± S.E. of at least three separate, independent experiments.

RESULTS

Reversible mPT-linked Mitochondrial Remodeling in Cultured Neurons—A brief exposure of cultured neurons expressing mito-eYFP to excitotoxic concentration of glutamate (500 μM, 3 min) resulted in rapid mitochondrial remodeling that in many instances could be interpreted as swelling of organelles. We found similar remodeling in response to high glutamate in all 63 examined individual neurons (57 culture dishes, 9 separate platings). Before glutamate treatment, most of the mitochondria in the neuronal soma appeared as elongated, thread-like structures (Fig. 1a). After incubation with glutamate, many mitochondria looked rounded and presumably swollen (Fig. 1b). MK801 (20 μM), an inhibitor of glutamate/NMDA receptor, prevented glutamate-induced mitochondrial morphological alterations (not shown). After glutamate removal, mitochondria recovered their shape within 30 min in all examined cells (Fig. 1c). Prolonged exposure to high glutamate (500 μM, >5 min) caused irreversible morphological changes. An application of lower glutamate concentration (100 μM, 20 min) evoked variable neuronal responses causing delayed alterations in mitochondrial morphology in some neurons (in 17 of 39 examined cells, 34 dishes, 5 separate platings). In the experiments with neurons treated with 500 μM glutamate for 3 min, preliminary mitochondrial depolarization with carbonylcyanide-p-trifluoromethoxyphenylhydrazone (supplemental Fig. 3), replacing Ca²⁺ for Sr²⁺ (supplemental Fig. 4), or omission of
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**FIGURE 1. Glutamate-induced Ca^{2+}-dependent morphological changes in neuronal mitochondria are preventable by CsA.** a–f, fluorescent images of cultured hippocampal neurons expressing mitochondrially targeted eYFP. a–c, neurons were treated with vehicle. d–f, neurons were preincubated with 3 μM CsA for 20 min before glutamate treatment. Cells were then treated with 500 μM glutamate and 10 μM glycine for 3 min in the presence of 3 μM CsA (b and e), followed by washing in the glutamate-free solution containing 3 μM CsA and 20 μM MK801 for 30 min (c and f). MK801, an inhibitor of the NMDA-type glutamate receptor, was used during washing to prevent residual activation of the glutamate receptors (38). Here and in all other experiments, only freshly prepared solutions of CsA were used.

Ca^{2+} in the perfusion buffer (not shown) precluded glutamate-induced morphological changes. Glutamate also failed to change mitochondrial morphology in the presence of 3 μM CsA, an inhibitor of the mPT (Fig. 1, d and e). The lower CsA concentrations (0.5–1 μM) did not provide a distinct and consistent protection. The relatively high CsA concentration was used to overcome CsA binding to cytosolic cyclophilins and ensure a sufficient delivery of CsA to mitochondrial cyclophilin D (49). In the presence of 3 μM CsA we did not detect overt changes in mitochondrial morphology in response to glutamate (500 μM glutamate, 3 min) in 35 of 37 examined neurons (36 dishes, 5 separate platings). It has to be mentioned that in our experiments CsA itself influenced mitochondrial appearance making mitochondria look somewhat grainy (Fig. 1 d). To distinguish between the action of CsA on the mPT and calcineurin, we used N-methyl-4-isoleucine-cyclosporin (NIM811), a nonimmunosuppressive analog of CsA (generously provided by Novartis Pharma AG, Basel, Switzerland), which did not inhibit calcineurin (50). NIM811 (1 μM) prevented glutamate-induced morphological changes in neuronal mitochondria (Fig. 2). In addition, we used cyclosporin H (CsH, 3 μM; Axxora, San Diego) instead of CsA to confirm involvement of the mPT in mitochondrial swelling in cultured neurons. It is known that CsH does not bind well to cyclophilin D, and hence it does not block pore opening (51). In our experiments, CsH appeared to be ineffective in preventing mitochondrial morphological changes in glutamate-treated neurons (supplemental Fig. 5). These data confirmed the role of the mPT in glutamate-induced, Ca^{2+}-dependent remodeling of neuronal mitochondria. Altogether, the observations made with cultured cells suggested that the exposure of neurons to excitotoxic concentration of glutamate resulted in reversible mitochondrial remodeling in situ apparently associated with an induction of the mPT.

**Transient, Spontaneously Reversible Mitochondrial Swelling in Vitro**—The whole-cell experiments with cultured neurons implicated the mPT in mitochondrial morphological alterations following a glutamate insult. However, the complexity of cell organization and regulation represented a significant obstacle in the study of the mechanisms of mitochondrial remodeling. Therefore, we questioned whether isolated brain mitochondria are capable of the Ca^{2+}-dependent, spontaneously reversible remodeling and, consequently, could be used in elucidation of this phenomenon.

In our experiments we used Percoll gradient-purified brain mitochondria as a well defined cell-free experimental model that allows a deeper insight into the mechanisms of Ca^{2+}-induced mitochondrial alterations in cultured cells (27). We examined responses to Ca^{2+} in both nonsynaptic and synaptic brain mitochondria. Nonsynaptic mitochondria represented organelles from neuronal somas as well as from glial cells. Synaptic mitochondria were recovered from synaptosomes and, therefore, predominantly originated from neuronal synaptic terminals. Both nonsynaptic and synaptic mitochondria incubated in the standard 125 mM KCl-based medium responded to Ca^{2+} by a transient decrease in light scattering that could be interpreted as an indication of reversible mitochondrial swelling (Fig. 3). The transient decreases in light scattering were observed regardless of the substrates used. The reversible mitochondrial swelling was also observed in hypo-osmotic KCl-based media (75, 50, or 25 mM KCl) (not shown). The recovery phase diminished or entirely disappeared with increasing Ca^{2+} dosage (Fig. 3). We also examined whether the K^{+}-selective ionophore valinomycin could produce reversible swelling in brain mitochondria, as it was shown to produce in heart mito-
Indeed, valinomycin produced a reversible decrease of light scattering in suspension of nonsynaptic brain mitochondria indicative of reversible mitochondrial swelling (Fig. 3d). A similar result was obtained with synaptic mitochondria (not shown). In line with previous observations, rat heart mitochondria treated with low concentrations of valinomycin experienced transient, spontaneously reversible swelling, whereas rat liver mitochondria treated with valinomycin underwent sustained swelling without spontaneous recovery (supplemental Fig. 6). Sr$^{2+}$, another divalent cation that did not

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Neurons were preincubated with 1 mM NIM811 for 20 min before glutamate treatment (a). Cells were then treated with 500 μM glutamate and 10 μM glycine for 3 min in the presence of 1 μM NIM811 (b), followed by washing in the glutamate-free solution containing 1 μM NIM811 and 20 μM MK801 for 30 min (c). MK801, an inhibitor of the NMDA-type glutamate receptor, was used during washing to prevent residual activation of the glutamate receptors (38). In all experiments, only freshly prepared solutions of NIM811 were used.
induce mitochondrial remodeling in situ, failed to produce a decrease of light scattering in the experiments with isolated mitochondria but at a high dose caused sustained depolarization (Fig. 4). The Sr\(^{2+}\)-induced depolarization was insensitive to CsA or to ADP and ATP and thus could not be firmly linked to the mPT (not shown). In contrast to brain mitochondria, rat liver and heart mitochondria responded to Ca\(^{2+}\) by CsA-sensitive sustained decrease of light scattering accompanied by depolarization (Fig. 5). In all three tested K\(^+\)-free media, mannitol/sucrose, N-methyl-D-glucamine, and choline chloride, Ca\(^{2+}\) produced swelling of liver and heart mitochondria without spontaneous recovery (supplemental Fig. 7). The additional experiments were performed to test whether shrinkage of liver and heart mitochondria can be observed if CsA is added after swelling takes place. With both liver and heart mitochondria, CsA failed to reverse swelling even in the presence of 20 \(\mu\)M cytochrome c (supplemental Fig. 8). In both cases, the recovery of \(\Delta\psi\) was below detection levels. Cytochrome c was added to compensate its possible loss because of swelling of mitochondria in saline medium (54).

TEM and morphometric analysis substantiated results obtained in light scattering assay with isolated brain mitochondria. We distinguished three types of mitochondria based on their morphology as follows: (i) condensed, with electron-dense, dark cristae and strong vacuolization of contracted matrices; (ii) swollen, with expanded, more transparent matrices; and (iii) orthodox, with smaller size and evenly spread gray matrices (Fig. 6, a–c). This categorization was used for morphometric analysis (Fig. 6d). TEM revealed that most untreated mitochondria appeared to be in the condensed state (Fig. 6a). Ca\(^{2+}\)-induced swelling in a rather uniform fashion throughout the mitochondrial population followed by spontaneous shrinkage (Fig. 6, b and c). This might suggest that the mPT in brain mitochondria occurs as an “all-or-nothing” phenomenon as proposed for liver mitochondria (29). Interestingly, after recovery most mitochondria appeared to be in the new, orthodox state (Fig. 6c). Thus, results obtained with TEM provided evidence for the mitochondrial swelling-contraction cycle and validated results obtained in light scattering assay.

An overstimulation of glutamate/NMDA receptors causes massive influx of not only Ca\(^{2+}\) but also Na\(^{+}\), leading to ele-
vated Na⁺ concentration in the cytosol (55–57). In the NaCl-based medium, Ca²⁺ also produced reversible mitochondrial swelling similar to swelling in the KCl medium but with smaller amplitude and slower kinetics (Fig. 7a). Additionally, Ca²⁺ caused rapid swelling in choline chloride- or N-methyl-D-glucamine-based media (Fig. 7b). However, in these cases the recovery phase was not observed.

As with the reversible swelling, isolated brain mitochondria treated with Ca²⁺ experienced sustained depolarization (Fig. 8a). CsA added prior to mitochondria prevented both swelling and sustained depolarization, thus unequivocally linking these phenomena to the mPT. The transient depolarization in the presence of CsA was because of Ca²⁺ influx into mitochondria and could be inhibited by Ru360, an inhibitor of Ca²⁺ uniporter (not shown). An increase in light scattering in the presence of CsA might be due to formation of hydroxyapatite complex in mitochondrial matrices (58, 59). CsA, added at the point in time when maximal decrease of light scattering was reached, significantly accelerated the recovery phase but failed to recover Δψ (Fig. 8b). This observation suggested that CsA more efficiently inhibited processes affecting mitochondrial morphology than depolarization. In addition, these experiments indicated that the CsA-induced closure of the mPT pore facilitated recovery of mitochondrial volume.

Depolarization of isolated mitochondria with mitochondrial toxins inhibited swelling (Fig. 8c). The existing paradigm suggests that mitochondrial swelling during the mPT occurs...
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FIGURE 8. Cyclosporin A, mitochondrial depolarization, or blockade of the mitochondrial Ca$^{2+}$ uniporter with Ru360 prevented the rapid onset of the Ca$^{2+}$-dependent spontaneously reversible swelling. Cyclosporin A accelerated the recovery phase of the reversible swelling but failed to restore $\Delta\psi$. 1.2 $\mu$mol of Ca$^{2+}$/mg of protein was added to mitochondria as indicated.

|               | Mitochondria in neuron | Isolated mitochondria |
|---------------|------------------------|------------------------|
| Activation by Ca$^{2+}$ | +          | +          |
| Reversibility   | +          | +          |
| Inhibition by depolarization | +    | +          |
| Inhibition by Ru360 | ND*    | +          |
| Lack of activation by Sr$^{2+}$ | +    | +          |
| Release of cytochrome c | +    | +          |
| Inhibition by CsA | +    | +          |

* ND indicates not determined.

because of passive movement of solutes into mitochondria down their concentration gradient (60, 61). In this case, depolarization should not inhibit swelling. Alternatively, if swelling depends on electrochemical K$^+$ influx, then depolarization could suppress swelling. However, the swelling was also inhibited in mitochondria treated with Ru360, an inhibitor of the Ca$^{2+}$ uniporter (Fig. 8d). There was no recovery phase in either case. Apparently, mitochondrial depolarization inhibited reversible swelling both in vitro and in situ because of inhibition of Ca$^{2+}$ uptake by mitochondria.

Taken together, all these findings suggested that the Ca$^{2+}$-dependent, spontaneously reversible swelling in isolated brain mitochondria had numerous common features with mitochondrial remodeling in cultured neurons (Table 1), and thus might have common mechanisms underlying reversible morphological changes in both cases.

The inhibition of the Ca$^{2+}$-dependent reversible swelling with CsA linked this phenomenon to the mPT. However, activation of the mitochondrial large conductance Ca$^{2+}$-activated K$^+$ channel (62, 63), recently found in brain mitochondria (64), could also contribute to mitochondrial swelling induced by Ca$^{2+}$. Because glutamate/NMDA-induced Ca$^{2+}$ influx into neurons and Ca$^{2+}$-evoked mitochondrial depolarization decrease ATP content (27, 36, 65), the mitochondrial ATP-sensitive K$^+$ channel might also be activated and involved in the Ca$^{2+}$-induced swelling of brain mitochondria. However, neither inhibitors of the mitochondrial ATP-sensitive K$^+$ channel (60–500 $\mu$M 5-hydroxydecenoate or 20–100 $\mu$M glibenclamide) nor inhibitors of the mitochondrial large conductance Ca$^{2+}$-activated K$^+$ channel (charybdotoxin or iberiotoxin, both 10–200 nM, or 50–200 $\mu$M Ba$^{2+}$ or 50–800 $\mu$M quinine (63)) inhibited the Ca$^{2+}$-induced, spontaneously reversible swelling (not shown). Thus, the mitochondrial K$^+$-selective channels apparently were not involved in the Ca$^{2+}$-dependent reversible swelling.

Mitochondrial swelling might activate the KHX that extrudes K$^+$ from the matrix (54, 66, 67) and, therefore, might contribute to the recovery phase of the transient swelling. To test this hypothesis, we used various concentrations of Mg$^{2+}$, quinine, and DCCD, known to inhibit the KHX activity (67). We also used incubation media with a different pH, assuming that a lower external pH should favor K$^+$/$H^+$ exchange by providing better conditions for $H^+$ influx into the matrix associated with K$^+$ extrusion from mitochondria. Indeed, an increase in the medium pH suppressed mitochondrial contraction, whereas acidification of the medium accelerated it (Fig. 9a). The kinetics of the swelling phase remained unchanged at various pH values. We could not detect inhibition of the swelling at a lower pH even with the lower Ca$^{2+}$ pulses (supplemental Fig. 9). Although this observation contradicts the existing paradigm, a recent report indicates that in contrast to de-energized brain mitochondria, where acidosis inhibits the mPT in energized mitochondria, acidosis can promote the mPT because of an increased rate of $P_i$ uptake, which is important for the mPT induction (68). Furthermore, the authors proposed that the increased rate of $P_i$ uptake may overcome the inhibitory effect of acidic pH. Thus, the lack of inhibition of the mPT at a lower pH in energized mitochondria is not unusual. All three tested inhibitors of the KHX (Mg$^{2+}$, quinine, and DCCD) suppressed recovery of mitochondria after swelling (Fig. 9). None of these agents affected the swelling phase. Although these inhibitors are not sufficiently specific to draw a final conclusion, their effects imply that the activation of the KHX underlies mitochondrial shrinkage following Ca$^{2+}$-induced swelling.

To determine a possible role of $\Delta$pH as a driving force for the K$^+$/H$^+$ exchange, we performed experiments with brain moto-
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FIGURE 10. Ca\(^{2+}\)-induced release of apoptogenic proteins from isolated brain nonsynaptic mitochondria. The release of mitochondrial apoptogenes into the incubation medium was evaluated by Western blotting. Mitochondria (Mito) incubated with 3 mM pyruvate and 1 mM malate were treated with various amounts of CaCl\(_2\) (50 mM protein) as indicated by numbers near the immunoblots. As shown earlier, a decrease in the medium tonicity (75 instead of 125 mM KCl) favored the Ca\(^{2+}\)-induced release of cytochrome c, presumably because of better osmotic balance between incubation medium and mitochondrial matrix (37). A decrease of medium osmolarity did not influence response to Ca\(^{2+}\) as judged by the light scattering assay. Therefore, in the protein release experiments we used the 75 mM KCl-based medium. a, cytochrome c (Cyt c) release was induced by various amounts of Ca\(^{2+}\). For comparison, maximal cytochrome c release induced by alamethicin (Alam) is shown. b, the Ca\(^{2+}\)-induced release of Smac/DIABLO, apoptosis inducing factor (AIF), and Omi/HtrA2 are shown. Endonuclease G (EndoG) was not released from brain mitochondria in response to Ca\(^{2+}\) or in response to alamethicin. In this case, solubilized mitochondria were used as a positive control for EndoG detection.

to detect rapid matrix alkalization (supplemental Fig. 10). However, given these conditions Ca\(^{2+}\) failed to cause mitochondrial swelling in our experiments (not shown). With intermediate Pi concentration (1.5 mM), Ca\(^{2+}\) produced spontaneously reversible swelling and transient alkalization of mitochondrial matrix (supplemental Fig. 10). The transient nature of the alkalization might be because of dissipation of ΔpH in the K\(^+\)/H\(^+\) exchange or because of increased H\(^+\) permeability of the IMM. In the standard incubation medium with 3 mM Pi, Ca\(^{2+}\) produced spontaneously reversible swelling, but we did not detect alkalization in response to Ca\(^{2+}\) pulse. This might be explained by rapid ΔpH compensation because of Pi/Hi(OH\(^-\)) exchanger activity. The compensatory fluxes of Pi and H\(^+\) make the effect of pH rather complex. It is conceivable that K\(^+\) leaves mitochondria in exchange for a proton, but Pi leaves with a proton making the overall process pH-neutral. Thus, it remains unclear to what extent ΔpH contributes to the driving force for the K\(^+\)/H\(^+\) exchange following the Ca\(^{2+}\)-induced swelling.

What are the potential consequences of the reversible swelling observed in this study? Does it lead to the release of mitochondrial apoptogenes and to what extent? To answer these questions we tested the release of apoptogenes into the supernatants obtained before and after completion of the mitochondrial swelling-contraction cycle. The Ca\(^{2+}\)-induced perturbations in mitochondrial morphology were accompanied by a partial release of cytochrome c and Smac/DIABLO and, to a lesser extent, Omi/HtrA2 and AIF (Fig. 10). On the other hand,
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EndoG, another mitochondrial apoptogenic protein, was not released under these circumstances. Moreover, we did not detect EndoG release even after treatment of mitochondria with alamethicin, an antibiotic that completely eliminated barrier properties of the OMM (37). Presumably, the release of EndoG required additional factors such as active caspases. Thus, despite the transient nature of the Ca\(^{2+}\)-induced swelling, the release of mitochondrial apoptogenes was readily detectable. Keeping in mind a proportion of mitochondria that experienced sustained swelling without recovery (about 2%, see Fig. 6d) and the extent of the release of cytochrome c and Smac/DIABLO (Fig. 10), it seemed very likely that at least a fraction of those mitochondria that experienced transient, reversible swelling contributed to the release. On the other hand, it remains unclear whether this conclusion could be extended to a much less prominent release of AIF and Omi/HtrA2 that might occur from mitochondria with irreversible damage.

DISCUSSION

In this paper, we demonstrated a reversible, mPT-related remodeling of mitochondria in cultured neurons exposed to excitotoxic glutamate. Furthermore, we showed that isolated Percoll gradient-purified brain synaptic and nonsynaptic mitochondria also can undergo spontaneously reversible remodeling (swelling) in response to Ca\(^{2+}\) pulse. In contrast to brain mitochondria, liver and heart mitochondria challenged with Ca\(^{2+}\) pulses experienced sustained swelling without spontaneous recovery. Thus, the Ca\(^{2+}\)-induced spontaneously reversible remodeling might be a unique feature of brain mitochondria. We further propose that the same mechanisms underlie the Ca\(^{2+}\)-dependent reversible remodeling in isolated mitochondria and in cultured neurons treated with excitotoxic glutamate.

Indeed, a conspicuous similarity between reversible remodeling of mitochondria in cultured neurons and in suspension of isolated brain mitochondria suggests common mechanisms causing morphological changes both in situ and in vitro. The common characteristics of mitochondrial remodeling in situ and in vitro are summarized in Table 1. In both cases, mitochondrial remodeling was triggered by Ca\(^{2+}\), was prevented by depolarization and/or inhibition of Ca\(^{2+}\) influx into mitochondria, and was not evident when Ca\(^{2+}\) was substituted for Sr\(^{2+}\). Of note, similar to Ca\(^{2+}\), Sr\(^{2+}\) readily enters neurons through the nonspecific channel of the activated glutamate/NMDA receptor and is taken up by mitochondria (12, 69–71). In both cases, in situ (37) and in vitro, mitochondrial remodeling (swelling) was accomplished by the release of cytochrome c. Most importantly, CsA prevented glutamate-induced changes in mitochondrial morphology both in situ and in vitro linking this phenomenon to the mPT. Interestingly, CsA appeared to be more effective in suppression of mitochondrial swelling than in prevention of depolarization.\(^4\) Thus, we believe that it is highly likely that we deal with the same mPT-related phenomenon both in situ and in vitro.

Ca\(^{2+}\)-induced swelling of liver or brain mitochondria could be reversed following Ca\(^{2+}\) chelation by EGTA (72, 73). This observation echoes the study by Dubinsky and Levi (16) in which the authors showed that mitochondrial swelling in cultured neurons induced by the calcium ionophore 4Br-A23187 could be reversed by incubation of cells in calcium-free medium containing EGTA. In primary forebrain neurons, glutamate induced a reversible decrease in length and a simultaneous increase in roundness of mitochondria (39). In cultured hippocampal neurons, NMDA stimulation resulted in a reversible fragmentation and swelling of mitochondria (38). In the same study, electron microscopy of cryosections of rapidly frozen neurons confirmed mitochondrial swelling induced by NMDA stimulation (38). Thus, glutamate/NMDA-triggered Ca\(^{2+}\) influx into neurons may cause reversible mitochondrial remodeling that could be interpreted as swelling. In this study, we linked this reversible remodeling to the mPT by demonstrating protection with CsA and Nim811.

Valinomycin can induce spontaneously reversible swelling in heart mitochondria incubated in KCl-based medium (52, 53). The swelling induced by valinomycin occurs because of electrogenic K\(^+\) uptake followed by a water influx (61). The reversibility of this swelling relied on volume-dependent activation of the KHX (54, 66, 67). We found similar valinomycin-induced reversible swelling in isolated brain mitochondria. The reversibility of the Ca\(^{2+}\)-induced swelling of brain mitochondria observed in our experiments resembles the swelling caused by valinomycin. It is tempting to speculate that the electrogenic K\(^+\) influx, a mechanism of valinomycin-induced swelling (61), might also take place when brain mitochondria are treated with Ca\(^{2+}\). A similar model was proposed earlier for the Ca\(^{2+}\)-induced swelling in liver mitochondria (74). However, currently we do not have sufficient experimental data to support this hypothesis. It is believed that during the mPT mitochondria swell because of an osmotic imbalance leading to passive diffusion of ions or small molecules into the matrix down their concentration gradient (60, 61). The opening of the mPT pore allows equilibration of all solutes of less than 1.5 kDa leaving a colloidal osmotic pressure exerted by the matrix proteins, and it is believed that this leads to mitochondrial swelling. The electrogenic K\(^+\) influx during the mPT is considered unlikely because of rapid depolarization that accompanies the onset of the mPT and precludes \(\Delta\varphi\)-driven cation uptake (61). Yet it seems conceivable that at least in the first moments of the mPT onset, electrogenic K\(^+\) influx might contribute to swelling as it was shown to do in liver mitochondria (74).

Regardless of the mechanisms of swelling, it seems reasonable to propose that the recovery phase after Ca\(^{2+}\)-induced swelling occurs because of activation of the KHX. Indeed, the mitochondrial swelling might activate the KHX (54, 66, 67) leading to contraction of organelles. The experiments with quinine, DCCD, and Mg\(^{2+}\), inhibitors of the KHX (67), provided evidence in favor of this hypothesis indicating that the contraction of swollen mitochondria observed in our experiments might occur because of swelling-induced activation of the mitochondrial KHX. The KHX does not discriminate monovalent cations and therefore might catalyze Na\(^+\)/H\(^+\) exchange in NaCl-based media together with a more selective Na\(^{+}\)/H\(^+\) exchanger (3). Ca\(^{2+}\) also induced mitochondrial swelling when K\(^+\) was substituted for larger organic cations such as choline or

\(^4\) N. Shalbuyeva and N. Brustovetsky, unpublished observations.
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N-methyl-D-glucamine. However, in these cases, the recovery phase was abolished so that swelling had a sustained, irreversible character. This finding suggests that not only swelling but also an influx of K\(^+\) into the mitochondrial matrix is necessary for the active operation of the KHX and consequent contraction of swollen mitochondria. If our reasoning concerning the role of the KHX in the recovery of mitochondrial volume is correct, then we have to conclude that the KHX in brain mitochondria does not transport large organic cations. In this, brain mitochondria differ from liver mitochondria where the KHX apparently transports large organic cations like tetrapropylammonium (75).

The recovery phase was also suppressed by external alkalization and activated by external acidification consistent with the inward direction of H\(^+\) flux in the K\(^+\)/H\(^+\) exchange. However, this conclusion contradicts earlier reports that external alkalization favors activation of the KHX in liver mitochondria (66, 76). Thus, either the KHX in brain mitochondria have different pH dependence or changes of the pH\(_{\text{ex}}\) influence other processes, such as stabilization of the mPT pore in the open state, which is facilitated by alkalization (29, 77). Indeed, it is conceivable that the kinetics of the recovery phase could reflect a balance between the activity of the KHX and a spontaneous closure of the mPT pore. Presumably, the larger Ca\(^{2+}\) pulses or alkaline pH favored the open state of the pore and therefore suppressed the recovery phase. Accordingly, CsA added at the maximum amplitude of swelling triggered closure of the mPT pore and thus substantially accelerated the recovery phase. On the other hand, it must be kept in mind that Mg\(^{2+}\) and quinine are inhibitors of the mPT (77, 78). Therefore, if their action would be primarily directed at the mPT, one might expect acceleration of the recovery phase similar to the CsA effect. However, this was not the case. Thus, their suppressing effects on the recovery phase are more consistent with the inhibition of the KHX.

The rupture of the OMM and release of mitochondrial apoptogenes appears to be the main detrimental consequence of the excessive mitochondrial swelling (37, 79–83). Whether the release of apoptogenes occurs from the entire mitochondrial population or from just a fraction of mitochondria with damaged OMM is not clear. The response of brain mitochondria to Ca\(^{2+}\) was reported to be heterogenic (84). However, in our experiments Ca\(^{2+}\) induced mitochondrial morphological changes in rather uniform fashion consistent with the observation published previously (73). Following a moderate Ca\(^{2+}\) pulse, mitochondria underwent a swelling-contraction cycle leading to the transition of a vast majority of mitochondria from condensed to swollen and then to orthodox configuration. However, it is difficult to establish from our data whether efflux of apoptogenes represents a total escape of these proteins from some mitochondria or a partial loss from all mitochondria in the population. Nevertheless, we presume that during a swelling-contraction cycle, the release of apoptogenes occurred from both mitochondrial populations experiencing transient and sustained swelling. At the same time, the relative contribution of these subpopulations to the release is hard to determine.

Taken together, the findings presented in this study suggest that in brain Ca\(^{2+}\) can cause mPT-related transient remodeling because of sequential activation of K\(^+\)(Na\(^+\)) influx and K\(^+\)(Na\(^+\)) extrusion, leading to mitochondrial swelling and contraction, respectively. In this model, the K\(^+\) influx could be driven by either osmotic imbalance or Δψ in the first moments of the mPT onset contributing to the rapid remodeling (swelling) and depolarization. Mitochondrial depolarization and the increase of matrix K\(^+\) concentration presumably preclude further K\(^+\) influx. The increase of matrix K\(^+\) enforces water influx causing mitochondrial swelling. Consequently, mitochondrial swelling activates the KHX that extrudes excessive K\(^+\) accompanied by osmotically obliged water causing mitochondrial contraction. Parallels between mitochondrial behavior in situ and in vitro suggest that in both cases similar mechanisms can be involved in mitochondrial response to Ca\(^{2+}\).

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