Swelling of mitochondria in cultured rat hippocampal astrocytes is induced by high cytosolic Ca^{2+} load, but not by mitochondrial depolarization

Stefan Kahlert, Georg Reiser*

Medizinische Fakultät der Otto-von-Guericke-Universität Magdeburg, Institut für Neurobiochemie, Leipziger Str. 44, 39120 Magdeburg, Germany

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Abstract The influence of cytosolic Ca^{2+} load and of mitochondrial membrane potential change on mitochondrial morphology was investigated in cultured rat hippocampal astrocytes. The uncoupler FCCP, applied together with oligomycin, depolarized mitochondria rapidly but did not change their morphology. Depolarization was associated with a moderate cytosolic $[Ca^{2+}]_i$ rise of up to 0.3 μM. Only high cytosolic Ca^{2+} load (above a threshold of 50 μM), which was evoked by application of the ionophore 4-Br-A23187 in Ca^{2+}-containing medium, caused drastic change of mitochondrial morphology. The shape change from the typical rod-like to a spherical shape, indicating mitochondrial swelling, was associated with depolarization. Cyclosporin A sensitivity suggests involvement of permeability transition. Thus, a dramatic cytosolic $[Ca^{2+}]_i$ rise is required to induce mitochondrial swelling and depolarization. A large but still moderate $[Ca^{2+}]_i$ rise evoked by physiological stimulation, however, has no comparable effect.

Key words: Mitochondrial potential; Mitochondrial morphology; Permeability transition pore; Calcium homeostasis; Apoptosis

1. Introduction

The cytosolic $[Ca^{2+}]_i$ concentration influences the capacity of mitochondria to generate ATP [1]. Mitochondria, on the other hand, also modulate cellular Ca^{2+} homeostasis [2]. Within a physiological range, the Ca^{2+} concentration acts as a regulator of mitochondrial oxidative phosphorylation [3]. Moreover, mitochondria play an important role in the cellular fate. If Ca^{2+} concentrations exceed the maximally tolerated level irreversible changes are triggered which lead to cell death [4]. One important consequence of a disturbance of Ca^{2+} homeostasis is the opening of a pore in mitochondria with high conductance that allows the passage of molecules of up to 1.5 kDa through the mitochondrial inner membrane. Mitochondrial permeability transition pore (PTP) opening leads to mitochondrial swelling. Such swelling has been shown with isolated mitochondria as a response to exposure to high Ca^{2+} concentrations [5,6]. Swelling causes destruction of the outer mitochondrial membrane and release of proteins from intermembrane space such as cytochrome *c* [7]. Over the last few years the release of cytochrome *c* from mitochondria was established to be a key process necessary for mitochondria-dependent apoptosis [8,9]. Nevertheless, there are strong indications that mitochondrial swelling is not the only mechanism that is responsible for cytochrome *c* release. The Ca^{2+}-independent mode of cytochrome *c* release involves members of the Bcl-2 protein family, such as BAX, and is not linked to a change in mitochondrial shape [10,11].

Experiments using isolated mitochondria have been instrumental for our understanding of the basic molecular mechanisms of mitochondrial functions or dysfunctions. Nevertheless, more complex systems are needed to fully understand the regulatory role of mitochondria in intact cells. For various modes of insults, indications for swelling of mitochondria have already been reported, for instance after oxidative stress in cultured mouse astrocytes [12] or upon mitochondrial depolarization in osteosarcoma cells [13]. However, only live imaging of mitochondria in cells, which has not yet been done under such conditions, can clarify the physiological relevance of these findings. The change in mitochondrial shape, which has indeed characteristics typical for mitochondrial swelling, is linked to the opening of the mitochondrial PTP [14]. In the present study we used high resolution fluorescence imaging of live hippocampal astrocytes for determining the role of Ca^{2+} and mitochondrial depolarization in mitochondrial shape change.

2. Materials and methods

2.1. Materials

The culture medium TNB-100 and stabilized glutamine (*N*-acetyl-L-alanyl-L-glutamine) were from Biochrom (Berlin, Germany), Ultroser G serum substitute was from Gibco (Eggenstein, Germany), tetramethylrhodamine ethyl ester perchlorate (TMRE) and MitoTracker Green FM from Molecular Probes (Eugene, OR, USA), antibodies against glial fibrillary acidic protein (GFAP), NeuN from Chemicon (Mannheim, Germany) and Ru360 from Calbiochem (Bad Soden, Germany). All other chemicals were from Sigma.

2.2. Cell cultures

Cells were prepared as described previously [15]. Briefly, hippocampi of 1 day old rat pups were isolated and cut into slices in ice-cold preparation medium. Preparation medium contained 1 mM pyruvate, 10 mM glucose, 6 μg/ml DNase I type IV, 1 mg/ml bovine serum albumin, 2 mM stabilized glutamine, and 1% (v/v) antibiotics mixture

*Corresponding author. Fax: (49)-391-6713097.
E-mail address: georg.reiser@medizin.uni-magdeburg.de (G. Reiser).

Abbreviations: PTP, permeability transition pore; FCCP, carbonyl cyanide *p*-(trifluoro)methoxyphenylhydrazone; TMRE, tetramethylrhodamine ethyl ester perchlorate; GFAP, glial fibrillary acidic protein; HBSS, Hanks' buffered salt solution; CsA, cyclosporin A

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(penicillin/streptomycin) in HEPES-buffered PBS buffer (10 mM HEPES, 120 mM sodium gluconate, 20 mM NaCl). Slices were digested in preparation medium supplemented with trypsin type XI for 7 min at 37°C. Digestion was stopped by adding trypsin inhibitor and slices were triturated twice. The resulting cell suspension was plated on glass coverslips coated with poly-L-lysine yielding a density of 150 cells/mm^2 and cultured in TNB-100 medium, 5% horse serum and 2% Ultroser G. Cells were fed at a 3 day interval and finally 24 h before the experiment. Cultures were used within 12–15 days after plating at a density of 50–100 cells/mm^2 for experiment. They contained $>95\%$ GFAP-positive mature astrocytes and a minor amount of neurons which stained positively with NeuN.

2.3. Measurement of mitochondrial shape and potential

Mitochondrial shape in rat hippocampal astrocytes was visualized by loading the cells with the MitoTracker Green FM, excited at 485 nm and detected at 520 nm. Immediately after loading a typical, rod-like morphology of mitochondria was visible, which was stable at least for the usual period of observation (approximately 15 min). Because of the three-dimensional structure of mitochondria, any out-of-focus signal gives a fuzzy background staining in the cell body. This problem could be circumvented by confocal microscopy. Nevertheless, only conventional video fluorescence microscopy was suitable for solving this dilemma, because its recording speed is high enough and this system has a low enough excitation light intensity. The exposure time of the video camera, set at 20–50 ms, was fast enough to cope with spontaneous jittery movements of mitochondria in the cytosol. Furthermore, we found that both long exposure to excitation light and high excitation light intensity can alter the structure of mitochondria (data not shown). These technical limitations could be overcome by the recording situation employed here.

Fluorescence was detected with an imaging system (TILL Photonics, Planegg, Germany) attached to a Zeiss Axioscope. Mitochondrial potential was monitored with the cationic dye TMRE (100 nM). In those experiments all perfusion solutions were supplemented with the dye. TMRE was excited at 555 nm and fluorescence intensity (F) was detected at >590 nm. Due to loss of autoquenching in less polarized mitochondria, depolarization is seen as an increase in fluorescence intensity. Depolarization was quantified by dividing the actual fluorescence intensity by the initial value of fluorescence intensity (F_0).

Cytosolic Ca^{2+} was measured as described previously [15] using fura-2 fluorescence ratio at 340 nm and 380 nm (R). Ca^{2+} values are given as R/R_0 by normalizing R by the value obtained at 1.5 min after the beginning of the experiment.

For the experiments the cells were bathed in Hanks' balanced salt solution (HBSS; 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 132 mM NaCl, supplemented with 1.26 mM $CaCl_2$ and 10 mM HEPES, pH 7.3) which, by continuous superfusion, was completely exchanged within 0.5 min. All experiments were performed at 36°C. Inhibitors and agonists were applied by addition to the superfusate. Ca^{2+}-free HBSS was prepared by omitting $CaCl_2$ and adding 0.6 mM EGTA. For experiments in Ca^{2+}-free conditions cells were allowed to equilibrate for 5 min in EGTA-containing Ca^{2+}-free medium. Mitochondrial respiration was inhibited by uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM). Metabotropic receptors were stimulated by ATP (10 μM). Ionophore 4-Br-A23187 was applied at 10 μM, if not otherwise indicated.

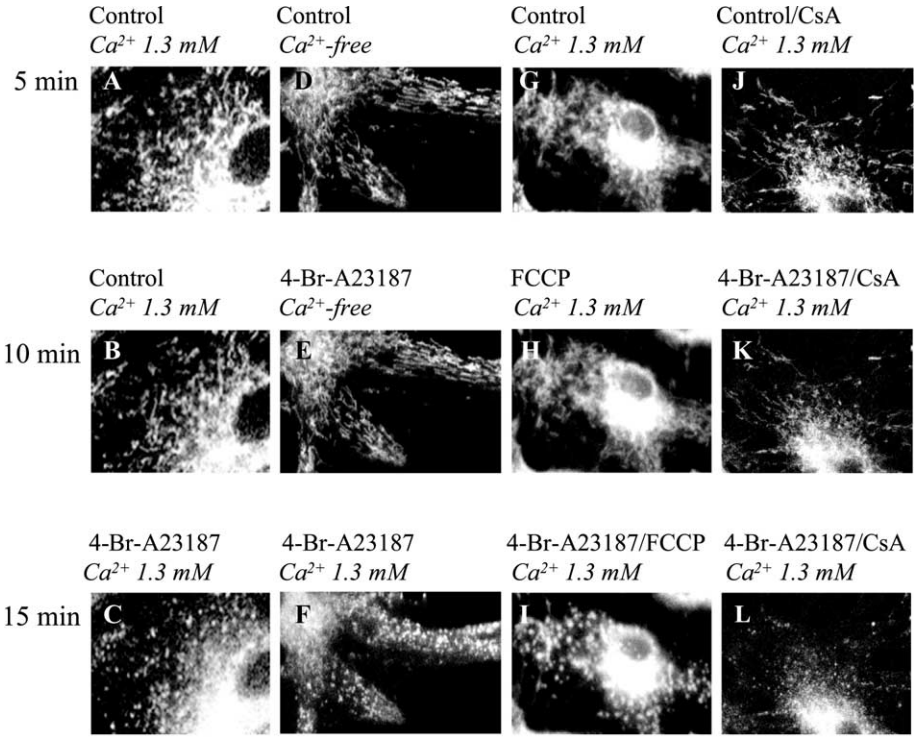

Fig. 1. Change in mitochondrial shape in rat astrocytes in response to cytosolic Ca^{2+} rise and mitochondrial depolarization. In four different astrocytes (A–C, D–F, G–I, J–L, respectively) mitochondria were labeled with MitoTracker Green FM (200 nM for 10 min). Cells were then incubated during three consecutive steps of 5 min each. The total time of incubation is indicated besides the respective row of pictures. Initial shape (A) did not change after 10 min superfusion with buffer (B), but displayed mitochondrial swelling after exposure to ionophore 4-Br-A23187 (10 μM) in 1.26 mM Ca^{2+}-containing buffer (C). Ionophore in the absence of extracellular Ca^{2+} was without influence on mitochondrial shape (E, in comparison to initial shape in the control in D), but subsequent addition of Ca^{2+} led to a characteristic punctuated pattern of swelling (F). Depolarization of mitochondria with FCCP (10 μM) had almost no effect (H, in comparison to initial pattern in G), and subsequent addition of ionophore in the presence of extracellular Ca^{2+} caused swelling of mitochondria (I). CsA (25 μM) added 5 min before ionophore in Ca^{2+}-containing medium prevented shape change (K, in comparison to control in J), but after further incubation with 4-Br-A23187 in Ca^{2+}-containing buffer the protective effect was abolished, seen by mitochondrial swelling (L). 10 μM CsA was similarly protective (not shown). Examples shown are representative for 20–30 cells observed in at least three independent experiments.

3. Results and discussion

Mitochondrial shape was monitored in hippocampal astrocytes loaded with MitoTracker Green FM. In control cells, mitochondria which were frequently concentrated around the nucleus displayed typical rod-like structures. Examples for control staining are given in Fig. 1A,D,G,J. Technical details of image recording conditions are described in Section 2. The mitochondrial morphology remained stable under control superfusion conditions for at least 10 min (Fig. 1B). High cytosolic Ca^{2+} loads were evoked by application of the ionophore 4-Br-A23187. The resulting intracellular $[Ca^{2+}]_i$ concentrations exceed 50 μM [16]. Challenge with Ca^{2+} ionophore caused a change of the mitochondrial morphology to a punctuated pattern, as shown in Fig. 1C. Such change of mitochondrial shape has been identified previously as mitochondrial swelling [14]. In astrocytes, swelling of mitochondria mediated by Ca^{2+} ionophore could only be induced in the presence of Ca^{2+} in the extracellular medium. After application of 10 μM 4-Br-A23187 in Ca^{2+}-free, EGTA-containing buffer the structure of mitochondria remained unchanged (Fig. 1E vs. D).

Similarly, there was no change of mitochondrial shape upon application of the uncoupler FCCP in combination with oligomycin (Fig. 1H in comparison with G). We have shown previously that FCCP can increase $[Ca^{2+}]_i$ in the range of up to 300 nM in rat hippocampal astrocytes, which was most likely due to depletion of the mitochondrial Ca^{2+} pool

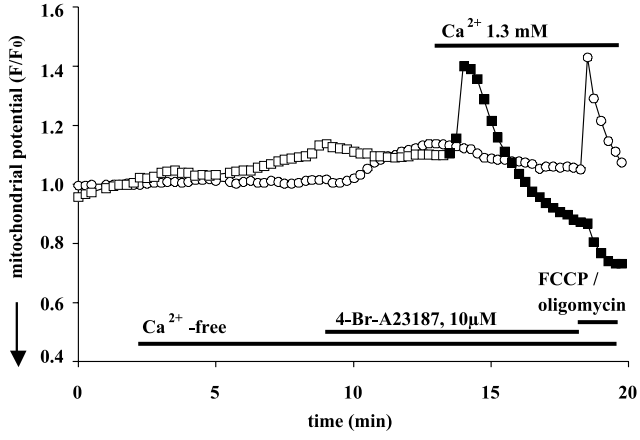

Fig. 2. Mitochondrial depolarization mediated by cytosolic Ca^{2+} load in rat hippocampal astrocytes treated with Ca^{2+} ionophore. Mitochondrial potential was measured by fluorescence signal (F) of TMRE. 4-Br-A23187 (10 μM) was added to two samples of cells. In experiment 1 (open circles) in the absence of extracellular Ca^{2+} mitochondria remained polarized with addition of ionophore. This is shown by the following depolarization (increase in fluorescence) caused by FCCP (10 μM)/oligomycin (10 μM). In experiment 2 (open squares), in the presence of the ionophore, at the filled squares perfusion was switched to normal Ca^{2+} (1.26 mM)-containing buffer. Depolarization indicated by a dequenching signal was seen. Subsequent application of FCCP/oligomycin had no further dequenching effect. The enhanced fluorescence decrease in sample 2 seen after addition of FCCP/oligomycin is probably due to a minor residual mitochondrial polarization. TMRE (100 nM) was present in all solutions. Fluorescence signal of TMRE (F) was normalized to fluorescence observed at 1.5 min after the start of the experiment (F_0). Representative traces of three independent experiments (20–40 cells per experiment) are shown.

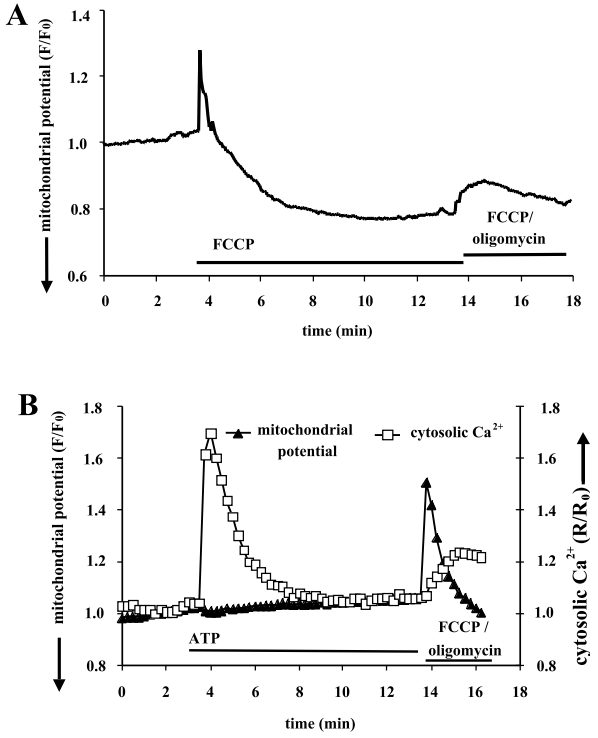

Fig. 3. Sensitivity of mitochondrial potential to FCCP and to ATP-mediated Ca^{2+} release from endoplasmic reticulum. Mitochondrial potential was measured by fluorescence signal (F) of TMRE, and cytosolic Ca^{2+} was measured by fura-2 fluorescence ratio. A: FCCP (10 μM) caused depolarization (dequenching signal of TMRE). Due to partial depolarization only, TMRE was accumulated again into mitochondria causing a drop in fluorescence by quenching. Subsequent additional application of oligomycin (10 μM) evoked a small dequenching peak. B: Cytosolic Ca^{2+} (open squares) was transiently increased by ATP up to 800 nM [16] by release from the endoplasmic reticulum by P2Y receptor stimulation, but there was no influence on the mitochondrial potential (filled triangles). Dequenching peak (mitochondrial depolarization) was observed with FCCP/oligomycin. Cytosolic Ca^{2+} is indicated by normalized fluorescence ratio (R/R_0). Representative traces of three independent experiments (20–40 cells per experiment) are shown.

[15]. Thus, the FCCP-mediated depolarization was obviously not sufficient to mediate swelling of mitochondria in intact cells. A similar lack of influence of FCCP has been observed in hepatoma MH1C1 cells [17].

Furthermore, we could demonstrate that opening of the mitochondrial PTP was involved in mitochondrial swelling, because application of cyclosporin A (CsA; 25 μM), given 5 min before the ionophore in Ca^{2+}-containing medium, suppressed any shape change during 5 min of exposure (10 min of total observation time; Fig. 1K). However, even in the presence of this high amount of CsA swelling was only delayed (Fig. 1L), but was not prevented during prolonged incubation with ionophore. This observation is in accordance with results found with isolated brain mitochondria, showing that high Ca^{2+} concentrations above a distinct Ca^{2+} threshold were able to overcome the protective effect of CsA [18]. Possibly, an additional CsA-insensitive mechanism comes into action to induce swelling. Moreover, it is noteworthy that CsA might also affect other targets besides PTP, such as the Na^+/Ca^{2+} exchanger [19]. At the end of each experimental recording we

could consistently evoke mitochondrial swelling by ionophore-evoked high Ca^{2+} load, as demonstrated in Fig. 1C,F,I,L.

It was recently reported that MitoTracker dyes can also modulate the mitochondrial potential [20]. However, as shown in Fig. 1G,H, the mitochondrial structure in astrocytes was not affected by massive changes of mitochondrial potential over the time period of recording. Therefore, the change in mitochondrial shape, which is obviously associated with the opening of the PTP, was mediated exclusively by very high cytosolic Ca^{2+} load, but certainly not by a minor depolarization induced by MitoTracker Green itself. This high Ca^{2+} load could be caused by Ca^{2+} ionophore in normal (1.26 mM) Ca^{2+}-containing buffer.

In further experiments, mitochondrial polarization was monitored by measuring the accumulation of TMRE. The cationic dye TMRE was present in the incubation medium at a concentration of 100 nM throughout the experiment. Application of 4-Br-A23187 (10 μM) in the absence of extracellular Ca^{2+} had absolutely no effect on accumulation of the cationic dye and, thus, did not affect polarization of mitochondria (Fig. 2, open squares). After addition of Ca^{2+} (1.26 mM), however, a typical depolarization peak was observed (Fig. 2, filled squares). Addition of Ca^{2+} led to a massive breakdown of the mitochondrial potential which was nearly complete. Completeness was indicated by the lack of depolarization after a subsequent FCCP/oligomycin application (Fig. 2, filled squares). A depolarization was found with FCCP/oligomycin in Ca^{2+}-free EGTA buffer (Fig. 2, open circles). The fluorescence change showed a transient kinetics. This is due to the self-quenching effect of TMRE at high concentrations. Therefore, the decline after the peak is unlikely to represent repolarization, but is due to washout of TMRE which is no longer trapped in mitochondria. When FCCP was applied without oligomycin, incomplete depolarization of the mitochondria was obtained, indicated by the fact that a small peak still appeared upon addition of FCCP in combination with oligomycin (Fig. 3A). The remaining polarization is most likely attributed to activity of the reverse mode of the ATP synthase.

FCCP-induced increase in cytosolic $[Ca^{2+}]_i$ of up to 300 nM [16] caused by depletion of mitochondria was not accompanied by a change of mitochondrial shape. Therefore, increases of $[Ca^{2+}]_i$ in this range that are mediated by receptor stimulation with agonists like ATP or by inhibition of sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA pumps) with cyclopiazonic acid [10] were tested in further experiments. Obviously the Ca^{2+} load mediated by ATP (up to 800 nM; quantified in [16]) was not sufficient by itself to depolarize the mitochondria (Fig. 3B) and to induce a change of the mitochondrial shape (results not shown).

Based on results from experiments using isolated brain mitochondria we previously derived a model [7] which suggested two distinct Ca^{2+} thresholds for impairment of brain mitochondria. A massive cytosolic Ca^{2+} rise was required for complete depolarization of the mitochondria and rupture of the outer mitochondrial membrane. That rupture is the requirement for necrotic cell death. On the other hand, cytochrome *c*, a mediator of apoptosis, was released from isolated brain mitochondria at a lower threshold, micromolar cytosolic Ca^{2+} levels. In the latter case the mitochondria remained intact. Here we corroborate this model by in vitro determinations using intact cells. We found that a change of mitochondrial morphology, known as swelling in isolated mitochondria, can be induced only by drastic Ca^{2+} load, i.e. above 50 μM cytosolic $[Ca^{2+}]_i$. Below this threshold, mitochondria are still functionally intact, act as energy supply for cellular processes and buffer Ca^{2+}, as described recently for hippocampal neurons [21]. Dramatic changes in cytosolic Ca^{2+} concentration were found in pathological situations of glutamate excitotoxicity. Increasing extracellular glutamate concentrations were accompanied by increasing cytosolic Ca^{2+} loads in cortical neurons [22]. Interestingly, CsA was protective against glutamate-induced excitotoxicity only within a distinct range of glutamate in intact neuronal cells [18]. It was further reported that CsA was able to repolarize isolated brain mitochondria, if Ca^{2+}-mediated depolarization was induced by Ca^{2+} loads below 50 μM. However, above this threshold, CsA was unable to restore mitochondrial polarization [18]. Opening of the mitochondrial PTP in intact hippocampal astrocytes leading to mitochondrial swelling requires $[Ca^{2+}]_i$ levels which are much higher than those concentrations reached under physiological conditions or with moderate pathological stimuli.

In conclusion, we have shown that in rat hippocampal astrocytes mitochondrial swelling associated with the opening of the mitochondrial PTP can only be achieved by a dramatic imbalance of Ca^{2+} homeostasis. This cannot be induced by application of physiological stimuli, such as ATP. Even the mitochondrial uncoupler FCCP evoking partial or, when given in combination with oligomycin, complete depolarization did not cause mitochondrial swelling.

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