Cell Calcium 49 (2011) 108–114

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Elevated intracellular calcium causes distinct mitochondrial remodelling and calcineurin-dependent fission in astrocytes

Andrew R. Tan, Andrew Yi Cai, Samineh Deheshi, Gordon L. Rintoul*

Department of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada V5A 1S6

ARTICLE INFO

Article history:
Received 24 July 2010
Received in revised form 1 December 2010
Accepted 2 December 2010
Available online 7 January 2011

Keywords:
Mitochondria
Astrocyte
Fission
Remodelling
Intracellular calcium

ABSTRACT

Disruptions of mitochondrial dynamics have been implicated in the pathogenesis of neurodegenerative diseases. The regulation mechanisms of mitochondrial dynamics have not been fully elucidated; however, calcium has been suggested to play a role. In the present study, we examined the role of intracellular calcium in regulating mitochondrial morphology and motility in cortical astrocytes employing different concentrations of a calcium ionophore. High levels of calcium caused a dramatic reduction in mitochondrial length, the result of two distinct phenomena: mitochondrial remodelling (or "rounding") and fission. Quantitative analysis revealed that mitochondrial remodelling/rounding was the predominant process. In addition, mitochondrial motility was reduced, as reported previously in neurons. By contrast, prolonged, more modest levels of intracellular calcium resulted in a reduction in mitochondrial length without significant effects upon mitochondrial motility. This calcium-induced reduction in mitochondrial length was not affected by the presence of calcineurin inhibitors; however, when mitochondrial fission events were specifically examined, calcineurin inhibitors had a significant inhibitory effect. This suggests that changes in mitochondrial length were primarily due to mitochondrial remodelling as opposed to fission. In the present study, we have therefore dissected the effects of calcium on mitochondrial motility, remodelling and fission. Our results suggest independent mechanisms for regulating these processes.

1. Introduction

It has been recognised for some time that mitochondria are dynamic organelles that undergo fission, fusion and translocation within cells [1]. Much attention has focused recently on the potential involvement of changes in mitochondrial dynamics in the pathogenesis of neurodegenerative disorders (reviewed in [2–6]). Intracellular Ca^{2+} levels have been implicated in regulating mitochondrial morphology and trafficking (reviewed in [7]); we have shown previously that influx of neurotoxic levels of Ca^{2+} results in reductions in mitochondrial movement and changes in mitochondrial morphology in glutamate-treated primary cortical neurons [8]. It has been shown recently that this cessation of mitochondrial motility is a function of the protein Miro [9,10]. A mechanism for Ca^{2+} mediated induction of mitochondrial fission has also been reported recently; Ca^{2+} binding to calcineurin results in the dephosphorylation of dynamin-related protein 1 (Drp1), which then migrates to mitochondria and induces fission [11,12]. However, in our study of glutamate-treated neurons, mitochondria appeared more "fragmented" after glutamate treatment, but the majority of mitochondrial morphology changes appeared to be due to remodelling or rounding of mitochondria as opposed to fission [8]. The quantification of fission/fusion/remodelling events in our previous study was hampered by the restricted localization and movement of mitochondria in the narrow processes of neurons. In many axons and dendrites, mitochondria frequently overlap, move over each other, stop, and reverse direction making mitochondrial morphology and discrete fission events difficult to quantify [13]. Other investigators examining Ca^{2+} mediated changes in mitochondrial morphology have reported fission [14] or remodelling [15,16], however the fact that both can occur simultaneously and contribute to mitochondrial morphology is under appreciated. In some reports both phenomena have been noted [8,17], although the relative contributions of fission and remodelling to mitochondrial morphology changes have not been examined. Moreover, the differences in the mechanisms by which Ca^{2+} can induce these two processes have not been investigated. In the present study we employed cortical astrocytes as a model system to examine mitochondrial dynamics, due to their flattened architecture in culture. In peripheral regions of these cells, mitochondria are more distinct, facilitating quantification of mitochondrial morphology changes.

Using this model system, changes in mitochondrial motility and morphology were induced with two different concentrations of the Ca^{2+} ionophore 4Br-A23187 and quantified.

* Corresponding author at: Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada V5A 1S6.
Tel.: +1 778 782 4801; fax: +1 778 782 3496.
E-mail address: gordon_rintoul@sfu.ca (G.L. Rintoul).

0143-4160/$ – see front matter.
doi:10.1016/j.ceca.2010.12.002

Higher concentrations of intracellular Ca^{2+} attenuated mitochondrial movement whereas more moderate increases did not. Increased intracellular Ca^{2+} also induced two distinct morphological phenomena in mitochondria: remodelling or "rounding" and fission, however remodelling was the predominant process at both tested calcium levels. More moderate and prolonged elevations of intracellular Ca^{2+} attenuated mitochondrial length primarily due to remodelling/rounding, an effect that was insensitive to calcineurin inhibitors, whereas Ca^{2+}-induced fission events were reduced by calcineurin inhibitors. In the present study, we have therefore dissected the effects of Ca^{2+} on mitochondrial motility, remodelling and fission. These results suggest independent mechanisms for regulating these processes. A previous abstract included portions of this work [18].

2. Materials and methods

2.1. Cortical astrocyte cultures

Post partum day 1 primary rat cortical astrocytes (provided by Dr. Michael Silverman, Simon Fraser University) were plated on 18 mm glass coverslips in 12 well plates (Corning) containing 1 mL per well of complete astrocyte media (basal medium eagle (Invitrogen) with 15% FBS (Hyclone), 200 μM Glutamax I (Invitrogen), 20 mM Glucose (Sigma), 100 units/mL Penicillin and 100 μg/mL Streptomycin (Invitrogen).

2.2. Live cell fluorescence imaging

The digital fluorescence imaging system (Nikon) consists of a Nikon TE-2000 microscope stand equipped with a Hamamatsu Orca-ER Camera (Hamamatsu), Lambda-LS xenon light source (Sutter), excitation and emission filter wheels with a Lamba-10-3 controller (Sutter). All treatments and imaging occurred in a HEPES-buffered salt solution (HBSS) containing (in mM): 137 NaCl, 5 KCl, 10 $NaHCO_3$, 20 HEPES, 5.5 glucose, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.4 $CaCl_2$, and 0.9 $MgSO_4$, pH adjusted to 7.4 (all reagents obtained from Sigma) at room temperature. Images and time lapse videos were captured employing a 40× extra-long-working-distance objective (Nikon). In many experiments, multiple fields of cells were examined using a motorized Prior OptiScan stage. Optical filters were purchased from Chroma Technology Corp. All data acquisition and analysis was carried out using SimplePCI software (Hamamatsu Corporation, Sewickley, PA, USA).

2.3. Measurement and modulation of intracellular Ca^{2+}

Intracellular Ca^{2+} levels were measured using the ratiometric indicator Fura-2. Astrocytes were incubated in 5 μM Fura-2 with 0.05% BSA in HBSS for 20 min in the dark at room temperature as described previously for other cell types [19]. The Fura-2 solution was then removed and the coverslip was washed twice with fresh HBSS. Using SimplePCI software, regions of interest (ROIs) were selected within individual astrocytes (excluding the nucleus) to measure emission at 510 nm upon excitation at 340 nm or 380 nm. Fura-2 fluorescence values, corresponding to changes in intracellular Ca^{2+} concentrations are expressed as a ratio of emissions at 340 nm/380 nm excitation, corrected for background fluorescence.

Astrocytes loaded with Fura-2 were treated with 10 μM 4Br-A23187, 1 μM 4Br-A23187, or vehicle control (anhydrous dimethyl sulfoxide, DMSO (Sigma)) in HBSS. The Ca^{2+} ionophore 4Br-A23187 (Sigma) was used to increase intracellular Ca^{2+} levels. Based on empirical experiments, it was determined that 1 μM of 4Br-A23187 would achieve moderate elevations of intracellular Ca^{2+} without toxicity. Pathophysiological levels of intracellular Ca^{2+} were achieved using 10 μM 4Br-A23187.

2.4. Transfection of cortical astrocyte cultures

In order to monitor mitochondrial dynamics, astrocytes were transfected with mitochondrially targeted enhanced yellow fluorescent protein mammalian expression DNA construct (mt-eYFP, generously provided by Dr. Roger Y. Tsien, University of California, San Diego), using a previously described Lipofectamine 2000 mediated protocol [20]. Briefly, for each glass coverslip of astrocytes, 0.75 μg of mt-eYFP and 1.12 μL of Lipofectamine 2000 (Invitrogen) were diluted in two separate tubes of 50 μL of basal medium eagle. After incubation at room temperature for 5 min, the mt-eYFP solution was mixed with the Lipofectamine 2000 solution and incubated at room temperature for 20 min. This transfection mixture was applied to one coverslip of astrocytes in 1 mL of complete astrocyte media in the well of a 12 well plate and incubated in a 37 °C incubator (Thermo Electron Corporation) with 5% CO_2. After overnight incubation, the media in the well was removed and replaced with 1 mL of complete astrocyte media. Fluorescence imaging of astrocytes was carried out 2 days after transfection.

2.5. Measurement of mitochondrial size, movement, and fission

For each treatment condition, the length of distinct mitochondria from a 255 × 255 pixel peripheral region of an astrocyte was measured using SimplePCI software. Due to the variability in mitochondrial morphology among astrocytes, values are reported as a percentage of the initial mean mitochondrial length.

Astrocytes display complex mitochondrial movement (see Section 3), as such we utilized a method to quantify bulk mitochondrial motility we have described previously [8] and which others have subsequently employed [21]. Briefly, 2 min time lapse videos, with a 10 s delay between frames were captured using SimplePCI. The fluorescence intensities of individual pixels in each successive image in the time lapse videos were monitored for increases or decreases, which provided a measure of mitochondrial motility (see for details [8]).

Fission was analysed by counting the number of mitochondria in a defined region pre- and post-treatment. The difference was subsequently normalized by dividing by the initial number of mitochondria in the region to determine the net percent change in mitochondria. An increase in the number of mitochondria in the analysed region indicates a net increase in mitochondrial fission. This analysis technique could be influenced by mitochondria moving into, or out of the analysed frame. However, in our treatment paradigms, mitochondrial translocation was halted (with high calcium loads), or did not significantly change (with more moderate calcium treatments) throughout the time course of the experiment. In both cases this would minimise the impact of mitochondrial motility on the fission analysis.

2.6. Exposure to calcineurin inhibitors

We then investigated if the calcineurin inhibitors Cyclosporin A (CsA) or FK506 (Alexis Biochemicals) would attenuate the effects of 4Br-A23187 on mitochondrial morphology and movement. Transfected astrocytes were treated with 1 μM 4Br-A23187 and 1 μM of CsA or 1 μM FK506 in HBSS. Vehicle controls were treated with 1 μM 4Br-A23187 and the appropriate volume of solvent (100% EtOH) used for CsA and FK506.

3. Results

3.1. The effect of increasing intracellular Ca^{2+} on mitochondrial dynamics

The Ca^{2+} ionophore 4Br-A23187 was employed to increase intracellular Ca^{2+} levels in primary cortical astrocytes. Two con-