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Imaging of mitochondrial Ca^{2+} dynamics in astrocytes using cell-specific mitochondria-targeted GCaMP5G/6s: Mitochondrial Ca^{2+} uptake and cytosolic Ca^{2+} availability via the endoplasmic reticulum store

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Abstract

Mitochondrial Ca^{2+} plays a critical physiological role in cellular energy metabolism and signaling, and its overload contributes to various pathological conditions including neuronal apoptotic death in neurological diseases. Live cell mitochondrial Ca^{2+} imaging is an important approach to understand mitochondrial Ca^{2+} dynamics. Recently developed GCaMP genetically-encoded Ca^{2+} indicators provide unique opportunity for high sensitivity/resolution and cell type-specific mitochondrial Ca^{2+} imaging. In the current study, we implemented cell-specific mitochondrial targeting of GCaMP5G/6s (mito-GCaMP5G/6s) and used two-photon microscopy to image astrocytic and neuronal mitochondrial Ca^{2+} dynamics in culture, revealing Ca^{2+} uptake mechanism by these organelles in response to cell stimulation. Using these mitochondrial Ca^{2+} indicators, our results show that mitochondrial Ca^{2+} uptake in individual mitochondria in cultured astrocytes and neurons can be seen after stimulations by ATP and glutamate, respectively. We further studied the dependence of mitochondrial Ca^{2+} dynamics on cytosolic Ca^{2+} changes following ATP stimulation in cultured astrocytes by simultaneously imaging mitochondrial and cytosolic Ca^{2+} increase using mito-GCaMP5G and a synthetic organic Ca^{2+} indicator, x-Rhod-1, respectively. Combined with molecular intervention in Ca^{2+} signaling pathway, our results demonstrated that the mitochondrial Ca^{2+} uptake is tightly coupled with inositol 1,4,5-trisphosphate receptor-mediated Ca^{2+} release from the endoplasmic reticulum and the activation of

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Author contributions

HL conducted molecular biology, transfection and 2-P imaging of astrocytes. XW cultured primary neurons isolated from mouse embryo and neuronal mitochondrial imaging. NZ was involved in 2-P imaging and analysis. MKG was responsible for culturing astrocytes. VP provided scientific input and edited the manuscript. SD conceived the study, supervised and participated in designing the experiments, and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interests.

G protein-coupled receptors. The current study provides a novel approach to image mitochondrial Ca^{2+} dynamics as well as Ca^{2+} interplay between the endoplasmic reticulum and mitochondria, which is relevant for neuronal and astrocytic functions in health and disease.

Keywords

Mitochondrial Ca^{2+} uptake; Mitochondrial matrix; IP_3 5-phosphatase; ER-mitochondrial communication; ATP

1. Introduction

Mitochondria are known as the powerhouses of the cell for energy production. They also regulate metabolic processes and are used as Ca^{2+} sinks that can take up large amounts of Ca^{2+} to buffer local or bulk cytosolic Ca^{2+} rises; mitochondrial Ca^{2+} uptake affects oxidative phosphorylation and stimulates ATP production under physiological conditions [1–4]. Mitochondrial Ca^{2+} uptake is also a determinant to induce necrotic and apoptotic cell death in several brain disorders [5–7]. Mitochondrial Ca^{2+} overloading opens mitochondrial permeability transition pore (mPTP); a sustained mPTP open state initiates apoptotic cell death, while transient openings of the pore may serve as a physiological mechanism to remove Ca^{2+} rapidly from mitochondria [8]. Therefore, studying mitochondrial Ca^{2+} dynamics in living cells is important for understanding the (patho)physiological function of mitochondria, yet there are limited probes available to readily image these dynamics.

Although many synthetic organic Ca^{2+} probes have been used for cellular Ca^{2+} imaging (for review see [9,10]), the major disadvantage of these probes is the partitioning between mitochondria, other organelles and the cytosol, making the measurements difficult. For example, even the most widely used mitochondrial dye Rhod-2, which preferentially locates to the mitochondrial matrix (MM) (for review see [10,11]), also shows partial localization in the cytosol with associated errors in measurements that are difficult to correct. Other problems generally associated with synthetic organic Ca^{2+} probes are the cytotoxicity resulting from the de-esterification reaction products and photodamage due to a necessity for prolonged exposure to high energy excitation light [12]. Genetically encoded Ca^{2+} indicators (GECIs) provide substantial advantages over synthetic organic Ca^{2+} probes due to their high sensitivity, brightness and dynamic range of fluorescence. For these exact reasons, fluorescence intensity-based GCaMP Ca^{2+} indicators have recently emerged as major GECIs [13–16]. For instance, GCaMP5 and GCaMP6 can readily report on cytosolic Ca^{2+} increases in a neuron after triggering a single action potential [15,16]. In this study, we implemented cell- and organelle-specific targeting of GCaMP5G and GCaMP6s to assess mitochondrial Ca^{2+} dynamics in primary cultured astrocytes and neurons. Our results demonstrate that GCaMP5/6 are suitable probes for detecting Ca^{2+} uptake in individual mitochondria in both astrocytes and neurons.

There are multiple signaling pathways that increase cytosolic Ca^{2+} and regulate Ca^{2+} homeostasis in astrocytes (for review see [17,18]). In addition to the widely acknowledged G-protein coupled receptors (GPCRs), ionotropic P2X [26,27] and NMDA receptors [27], channels containing transient receptor potential (TRP) A1, C1 and C3 [19–22], and

plasmalemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger [23–25] also contribute to Ca^{2+} dynamics in astrocytes. It has been shown in many cell types that Ca^{2+} released from the ER, mediated by inositol 1,4,5-trisphosphate receptor (IP_3R) upon GPCR activation, is effectively taken up by mitochondria [28–30]. As IP_3R -mediated Ca^{2+} release after the activation of GPCRs is the primary form of Ca^{2+} signaling pathway in astrocytes [31–35], we further studied its influence on mitochondrial Ca^{2+} uptake in astrocytes by simultaneously imaging mitochondrial and cytosolic Ca^{2+} signals following ATP stimulation using mito-GCaMP5G and x-Rhod-1, respectively. Combined imaging and pharmaco-molecular interventions demonstrate that mitochondrial Ca^{2+} signal is tightly coupled to IP_3R -mediated Ca^{2+} release from the ER in astrocytes.

2. Materials and methods

2.1. Ethical approval

All procedures were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Missouri Animal Care Quality Assurance Committee and by the University of Alabama, Birmingham Institutional Animal Care and Use Committee. The procedures also conform to the principles of UK regulations, as described elsewhere [36].

2.2. Primary cultured astrocytes and neurons

Astrocytic cell cultures were prepared using a previously described procedure [37]. Briefly, visual cortices of 0–2-day old C57BL/6J mice pups were isolated, treated with papain (20 IU/ml; Sigma) in the presence of l-cysteine (0.2 mg/ml) for 1 h at 37 °C, neutralized with trypsin inhibitor (type II-O, 10 mg/ml; Sigma) for 5 min at room temperature (22–25 °C), and triturated in cell culture media. The resulting cell suspension was plated into 25 cm² tissue culture flasks. One hour post plating, the cell suspension was replaced with fresh culture media containing α -minimum essential medium (without phenol red; Life Technologies) supplemented with fetal bovine serum (FBS; 10%, v/v; Hyclone), sodium bicarbonate (14 mM), sodium pyruvate (1 mM), d-glucose (20 mM), L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) (pH 7.35). Cells were maintained at 37 °C in a 95% air/5% CO_2 atmosphere incubator for 1–2 weeks to obtain cell growth and proliferation to ~60% confluency after which the cell cultures were purified for astrocytes using a previously described procedure [38]; flasks were shaken twice on an orbital shaker, first for 1.5 h followed by two exchanges with culture media and then again for 18 h, at 37 °C and 260 rpm. These astrocytes (>95% pure as quantified by the anti-glial fibrillary acidic protein, GFAP, immunoreactivity) were subsequently subcultured at 37 °C in a 95% air/5% CO_2 atmosphere incubator and fed every 48 h with fresh Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS as described previously [39]. Astrocytes used for imaging were cultured on plain glass coverslips (12 mm in diameter; Fisher Scientific) [40].

Cortical neurons were prepared from embryonic day 15/16 (E15/16) C57BL/6J mice as in our previous study [41]. Briefly, cortical tissues from embryos were dissociated by mild mechanical trituration after digestion with trypsin. The dissociated cells were plated onto poly-L-lysine-coated (100 $\mu\text{g}/\text{ml}$; Sigma) glass cover-slips in 24-well culture plates with

DMEM/F12 (Life Technologies) supplemented with 10% heat-inactivated FBS (Atlanta Biological) for 4 h; the medium was then exchanged with Neurobasal Media (Life Technologies) containing 2% B27 serum free supplements (Life Technologies). The cultures were maintained at 37 °C in a 95% air/5% CO₂ atmosphere incubator. Experiments were conducted within 9–11 days *in vitro* (DIV). Our procedure yielded high purity of cultured neurons [41].

2.3. DNA constructs for mitochondria-targeted expression of GCaMP5/6 and other transgenes, and DNA transfection in cultured astrocytes and neurons

Mitochondrial matrix (MM)-targeting sequence (mito-) ATGT CCGTCCTGAC GCCGCTGCTG CTGCGGGGCT TGACAGGCTC GGCCCGGCGG CTCCCAGTGC CGCGCGCCAA GATCCATTCG TTG [42] was inserted in the backbone of AAV plasmid pZac2.1 containing either astrocytic gfaABC₁D [40] or neuronal CaMKII [43] promoter. The cistrons of interest, GCaMP5G/6s or monomeric red fluorescence protein (mRFP), were subcloned into the cloning sites to form new plasmids that can target transgene expression in mitochondria of either astrocytes or neurons (Suppl Fig. 1A). Similarly, mRFP alone (control) or bicistronic IP₃ 5-phosphatases (5ppases; either the wild-type or R343A and R343A/R350A mutated forms [44]) – the internal ribosomal entry site (IRES) – DsRed were subcloned in pZac2.1 plasmid containing the gfaABC₁D promoter for expression in the cytosol of astrocytes (Suppl. Fig. 1D). The above DNA plasmids were transfected in astrocytes or neurons using lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's instructions and our previous studies [40,41]. Astrocytes and neurons expressing the proteins of interest were used 1–2 days after transfection.

Supplementary Fig. 1 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ceca.2014.09.008>.

2.4. 2-Photon (2-P) imaging of mitochondrial and cytosolic Ca²⁺ signals in cultured astrocytes and neurons

For cytosolic Ca²⁺ imaging in astrocytes, cells cultured on glass coverslips were incubated for 45 min with 0.5 ml fluo-4 AM or x-Rhod-1 AM (each at 1 µg/ml, diluted from a stock solution of 1 µg/µl in dimethyl sulfoxide (DMSO)) in artificial cerebrospinal fluid (ACSF) containing (in mM): 120 NaCl, 10 Hepes, 3.1 KCl, 2 CaCl₂, 1.3 MgCl₂, and 10 glucose (pH 7.4). After incubation, excess fluo-4 AM or x-Rhod-1 AM was washed with ACSF and the cells were incubated in ACSF for additional 30 min to allow for the deesterification of the dye. Time-lapse Ca²⁺ imaging was performed using an upright 2-P microscope (Prairie Technologies, WI) with a 40×/NA0.8 Olympus water immersion objective. One advantage in using 2-P imaging is that a single wavelength (here 850 nm) can simultaneously excite two distinct fluorophores (here x-Rhod-1 and mito-GCaMP5G) because of the broad excitation spectra of 2-P excitation for many fluorescent dyes [45]. Excitation was generated by a pulse laser beam from a Chameleon Ti:Sapphire Ultra I laser (Coherent, CA). Emission was detected using two photo-multiplier tubes (PMTs). ATP or glutamate was applied to the cells through a perfusion system equipped with a pinch valve that can control the duration of application. Images were acquired every 2 s for 180 s. All the raw fluorescence images had pixel intensities without saturation and within the PMT's dynamic range (0–4095).

2.5. Data analysis of the Ca²⁺ signal

Analysis of the cytosolic and mitochondrial Ca²⁺ signals in astrocytes and neurons was done as described in our previous studies [32,33,40]. Briefly, the fluorescence signals were quantified by measuring the mean pixel intensities of the circular regions of interest (ROI) (either big regions with a diameter of 6.8 μ m or small regions with a diameter of 1.8 μ m, the latter containing a single mitochondrion) using MetaMorph software (Universal Imaging, CA). To test the spatial difference in mitochondrial Ca²⁺ uptake, we measured four small regions around the nuclei of astrocytes. Ca²⁺ changes were calculated by Origin software (OriginLab Corporation, MA) and expressed as F/F_0 values vs. time, where F_0 is the baseline fluorescence and F is the baseline subtracted fluorescence; all data was prior background subtracted using fluorescence values from a region of coverslip containing no cells.

Summary data of mitochondrial and cytosolic Ca²⁺ signals are expressed as mean $\pm$ standard error of mean. Statistical comparisons were made by Student's *t*-test for two groups or one-way ANOVA followed by Bonferroni's test for multiple groups and *p*-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Imaging mitochondrial Ca²⁺ in astrocytes and neurons using mito-GCaMP5G after receptor activation

Previously, we developed an AAV approach using an astrocytespecific promoter gfaABC₁D to express transgenes in astrocytes in mouse brain [40]. Here, we subcloned gfaABC₁D and CaMKII promoters [40,43] combined with MM-targeting sequence (mito-) [42] and GCaMP5G Ca²⁺ indicator in AAV expressing plasmid pZac 2.1, *i.e.*, pZac2.1-gfaABC₁D-mito-GCaMP5G and pZac2.1-CaMKII-mito-GCaMP5G for imaging mitochondrial Ca²⁺ in astrocytes and neurons, respectively (Suppl. Fig. 1A) [40]. To validate mito-GCaMP5G expression, we co-transfected pZac2.1-gfaABC₁D-mito-mRFP with pZac2.1-gfaABC₁D-mito-GCaMP5G, or pZac2.1-CaMKII-mito-mRFP with pZac2.1-CaMKII-mito-GCaMP5G in primary cultured astrocytes or neurons, respectively. Two days after transfection, bright GCaMP5G fluorescence with mitochondrial pattern, colocalized with mito-mRFP, was observed in cultured astrocytes and neurons (Suppl. Fig. 1B and C). Since this mitochondria-targeting sequence was used to target green fluorescent protein to visualize mitochondria in cultured mammalian cells [19,31], the colocalization of mito-mRFP and mito-GCaMP5G in cultured astrocytes and neurons indicates that mito-GCaMP5G is expressed in MM and can be used for mitochondrial Ca²⁺ imaging.

Next, we assessed the capability of mito-GCaMP5G to detect mitochondrial Ca²⁺ increase elicited by receptor activation in astrocytes and neurons after ATP (100 μ M) and glutamate (50 or 100 μ M) stimulations, respectively. Such stimulations lead to increases in cytosolic Ca²⁺ levels in astrocytes/neurons, and due to mitochondrial buffering one would expect Ca²⁺ uptake in these organelles during times of high cytosolic Ca²⁺ levels. Indeed, ATP and glutamate caused a large increase of mito-GCaMP5G fluorescence in astrocytes and neurons (Fig. 1A, B, E and F, respectively). Analysis of the Ca²⁺ signal in a region with a diameter

of 6.8 μm near the nuclei showed that the average peak values of F/F_0 were 2.31 ± 0.49 ($n = 6$) for cultured astrocytes (100 μM ATP) and 2.90 ± 0.40 ($n = 5$) for cultured neurons (50 μM glutamate) transfected with mito-GCaMP5G. Recent evidence suggests that there may be fundamental differences in Ca^{2+} buffering and metabolism between astrocytic and neuronal mitochondria [46,47]. Although enormously informative, such bulk experiments have not garnered experimental evidence at single mitochondrial level to the above notion. As GCaMP5G has a brighter fluorescence and a higher sensitivity [15] compared to other GECIs, thus making it possible to image Ca^{2+} uptake in individual mitochondria in astrocytes and neurons, we set up to assess whether mitochondrial Ca^{2+} signal differs between astrocytes and neurons. We analyzed fluorescence changes in 4 individual mitochondria in an astrocyte following ATP stimulation and a neuron following glutamate stimulation. Clear increase in GCaMP5G fluorescence in individual mitochondria was observed in astrocytes (Fig. 1C and D) and neurons (Fig. 1G and H). Of note, neuronal mitochondrial Ca^{2+} increases were sustained post-stimulation, *i.e.* displayed plateau for a prolonged time, while astrocytes show transient mitochondrial Ca^{2+} increases, indicating differential capabilities of these neural cells to handle mitochondrial Ca^{2+} ; similar mitochondrial Ca^{2+} increases were obtained not only with ATP, but also when astrocytes were exposed to glutamate, presumably due to the stimulation of group I metabotropic glutamate receptors (mGluRs) [48,49] (Suppl. Fig. 2). Furthermore, ATP caused similar mitochondrial Ca^{2+} dynamics when astrocytes were transfected to express mito-GCaMP6s (Suppl. Fig. 3). Of note, GCaMP6s is a newer version of GCaMP indicator, previously shown to be more sensitive than GCaMP5G and other GCaMP6 variants for cytosolic Ca^{2+} imaging [16]. Thus, our results show that mito-GCaMP5G can be used to monitor mitochondrial Ca^{2+} dynamics with single mitochondrion resolution in astrocytes and neurons.

Supplementary Figs. 2 and 3 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ceca.2014.09.008>.

3.2. Ru360 inhibits ATP-induced mito-GCaMP5G fluorescence increase in astrocytes

The mitochondrial buffering of Ca^{2+} is an interplay between the uptake from and release of Ca^{2+} into the cytosol, which is a consequence of the concentration gradients between these two cellular compartments. Indeed, this is a fairly complex process and we decided to focus on the uptake of mitochondrial Ca^{2+} and its governance by the cytosolic Ca^{2+} in astrocytes. It is widely accepted that Ca^{2+} uptake by mitochondria occurs through mitochondrial Ca^{2+} uniporter (mCU) [50], which can be blocked using ruthenium 360 (Ru360). To assess the mitochondrial uptake of Ca^{2+} via mCU, we loaded mito-GCaMP5G transfected astrocytes with x-Rhod-1 to simultaneously image mitochondrial and cytosolic Ca^{2+} , respectively. Application of ATP (100 μM) robustly stimulated fluorescence increases of both x-Rhod-1 and mito-GCaMP5G in astrocytes (Fig. 2A–E). In addition, normalized fluorescence increases of x-Rhod-1 and mito-GCaMP5G showed matching kinetics of cytosolic and mitochondrial Ca^{2+} signals (Fig. 2F) suggesting a close interplay between the ER and mitochondria in astrocytes. Application of Ru360 (1 μM) abolished ATP-induced mito-GCaMP5G fluorescence increase in astrocytes (Fig. 3A–B and D–F), while the x-Rhod-1 fluorescence was slightly increased (Fig. 3A, C and E–G). These results directly

demonstrate that Ca^{2+} accumulation in astrocyte MM following high cytosol loads occurs through mCU, a finding consistent with previous experiments using bulk cytosolic recordings [51]. As ATP can robustly stimulate Ca^{2+} increase through the activation of P2Y receptors *in vitro* and *in vivo* [32,33,40], which leads to the release of Ca^{2+} from the ER to the cytosol via IP_3 Rs, these results also indicate that ATP-induced mitochondrial Ca^{2+} uptake is highly correlated with IP_3 R-mediated Ca^{2+} release from the ER.

3.3. Interplay between cytosolic and mitochondrial Ca^{2+} signals in astrocytes

In mammalian cells, cytosolic and mitochondrial Ca^{2+} increases after G_q -coupled GPCR stimulations are highly correlated due to close contacts between the ER and mitochondria [29,30]. We further investigated the relationship between IP_3 R-mediated Ca^{2+} increase in the cytosol, originating from the ER, and mitochondrial Ca^{2+} uptake in cultured astrocytes. We transfected astrocytes to express IP_3 5-phosphatase (5ppase), that, due to IP_3 hydrolysis, can diminish IP_3 R-mediated Ca^{2+} release from the ER [44], along with the bicistronic expression of a red fluorescent marker DsRed. Using Fluo-4, a spectrally compatible Ca^{2+} indicator, we monitored cytosolic Ca^{2+} levels in transfected astrocytes. We observed that 5ppase inhibited ATP-stimulated cytosolic Ca^{2+} increase in astrocytes (Fig. 4B and E), while the single mutant R343A 5ppase and the double mutant R343A/R350A 5ppase that have reduced enzymatic activity to hydrolyze IP_3 did not inhibit ATP-induced cytosolic Ca^{2+} increase (Fig. 4C–E), similar to the control experiments using mRFP-expressing astrocytes (Suppl. Fig. 1D and Fig. 4A and E).

Next we tested whether this modulation of the cytosolic Ca^{2+} levels by 5ppase affected mitochondrial Ca^{2+} uptake. Astrocytes expressing 5ppase did not exhibit Ca^{2+} uptake in mitochondria after ATP stimulation (Fig. 5A and D). However, the expression of the 5ppase mutants had no effect on Ca^{2+} uptake in mitochondria (Fig. 5B–D), a finding consistent with the preservation of cytosolic Ca^{2+} signal in these conditions (Fig. 4C–E). These results directly demonstrate the dependence of mitochondrial Ca^{2+} dynamics on the interplay of these organelles with the ER via cytosolic Ca^{2+} availability and that mitochondrial Ca^{2+} signals are highly coupled with IP_3 R-mediated Ca^{2+} released from the ER.

4. Discussion

In the present study, we constructed plasmids, in a viral vector backbone, for mammalian expression of mitochondria-targeted GCaMP5G/6s GECIs under cell type-specific promoters to image mitochondrial Ca^{2+} uptake in primary cultured astrocytes and neurons. Combined with synthetic organic Ca^{2+} indicators for cytosolic Ca^{2+} imaging, pharmacological approach to inhibit mCU and molecular intervention to disrupt IP_3 3R-mediated Ca^{2+} signaling pathway using 5ppase, we further used this approach to study the interplay of mitochondrial Ca^{2+} dynamics with Ca^{2+} variations in the cytosol in astrocytes, particularly those associated with the release of this cation from the ER store. Our results demonstrated that GCaMP5G/6s can be selectively targeted to astrocytic and neuronal mitochondria when mitochondrial targeting sequence was inserted, and significant fluorescence increase in individual mitochondria can be revealed after ATP and glutamate stimulations in astrocytes and neurons, respectively. Our results also demonstrated that

mitochondrial Ca^{2+} uptake was tightly coupled to IP_3R -mediated Ca^{2+} release from the ER to the cytosol in astrocytes.

Other GECIs have been used for cytosolic and mitochondrial Ca^{2+} imaging, latter by targeting them to MM. Aequorin, a bioluminescent probe, was one of the first GECIs and is still used for mitochondrial Ca^{2+} imaging in live cells [42,52]. However, studies using aequorin were primarily carried out at the cell population level because of the low amount of light emitted and the ‘consumption’ of the probe itself, *i.e.*, coelenterazine, a noncovalently bound chromophore. Other GECIs based on fluorescence emission in the cytosol such as Ratiometric Pericam (RP), Camgroos (intensity-based), Yellow Cameleons (YCs) (FRET-based), RCaMP1e (intensity-based) have improved sensitivity, however, their dynamic ranges are relatively small [53–55]. GCaMPs were used for cytosolic Ca^{2+} imaging due to their high dynamic range, sensitivity and response speed [13,15,16]. Despite the wide use of GCaMPs in imaging cytosolic Ca^{2+} signals in brain cells, to our knowledge, no study has been reported using GCaMP5G/6s to study mitochondrial Ca^{2+} dynamics in astrocytes and neurons. The improved performance of GCaMP5G/6s as compared to GCaMP2/3 makes them uniquely suitable for imaging mitochondrial Ca^{2+} [15,16]. Our study showed that mito-GCaMP5G/6s can image mitochondrial Ca^{2+} uptake after the activation of GPCRs in a single cell and mitochondrion level in astrocytes and neurons. GECIs have unique advantages over synthetic organic Ca^{2+} indicators. They can target specifically to subcellular compartments including mitochondria by adding a targeting sequence to the indicators. They can also be selectively expressed in specific cell types such as astrocytes and neurons for *in vivo* imaging. Moreover, they can be expressed for a long time with marginal or no toxicity when integrated into the genome; therefore, using viral transduction and transgenic mice, repeated and long-term imaging can be performed in the same cells previously imaged [40,56–59]. Thus, mitochondrial Ca^{2+} signaling with physiological changes and pathological progression can be monitored. Nevertheless, one drawback using intensity-based Ca^{2+} indicators, including mito-GCaMP5G/6s in this study, is that they can only measure the relative change of Ca^{2+} from basal levels, and the absolute Ca^{2+} concentration cannot usually be determined accurately.

ER-mitochondrial Ca^{2+} interplay has been studied in other cell types by direct stimulation with IP_3 and ATP in permeabilized and intact cells [28–30,60,61], and in intact astrocytes [51], but not directly by measuring mitochondrial Ca^{2+} in astrocytes which we have done here. Astrocytes are not electrically excitable cells in the central nervous system and generally mediate Ca^{2+} signaling through the release of Ca^{2+} from the ER via opening of IP_3R following the stimulation of GPCRs and subsequent liberation of IP_3 [31,62,63]. It has been shown that Ca^{2+} released from the ER mediated by IP_3R activation will be taken up by MM and thus stimulates mitochondrial energy metabolism [1,3,4,29,30]. Studies from other cell lines such as Hela and RBL-2H3 suggest that the ER and mitochondria have intimate contacts [29,30]. The spatial distribution of intracellular organelles might differ in different cell types. Therefore, in the present study, we initially investigated the interplay between the ER and mitochondria using ATP stimulation and by simultaneously imaging cytosolic (as a measure of Ca^{2+} released from the ER via IP_3R) and mitochondrial Ca^{2+} signals using x-Rhod-1 and mito-GCaMP5G in intact astrocytes. Our results show that the rise times of

mitochondrial Ca^{2+} and cytosolic Ca^{2+} after ATP stimulation are essentially the same with the temporal resolution used. Disruption of IP_3R -mediated Ca^{2+} signaling pathway by molecular intervention affected Ca^{2+} increase in mitochondria after the activation of GPCRs in astrocytes. When mito-GCaMP5G was cotransfected with WT 5ppase, mitochondrial Ca^{2+} increase was absent after ATP stimulation, but when mito-GCaMP5G was cotransfected with two mutants of 5ppase, *i.e.*, R343A and R343A/R350A 5ppase mutants, which lack enzymatic activity, the mitochondrial Ca^{2+} increase was not affected compared to the control condition. These results indicate that mitochondria can uptake the Ca^{2+} released (IP_3R -mediated) from the ER into the cytosol, and cytosolic and mitochondrial Ca^{2+} levels are highly coupled in astrocytes pointing to close structural arrangements between the ER and mitochondria, with the cytosol serving as an intermediary conduit for Ca^{2+} delivery. Compared with studies using permeabilized cells and direct IP_3 stimulation, our approach using intact astrocytes and molecular expression of 5ppase to disrupt IP_3R -mediated Ca^{2+} signaling pathway should reveal the more realistic spatio-temporal coupling (within technical limits of resolution) of GPCR-stimulated Ca^{2+} communication between the ER and mitochondria in intact cells. Although the current study focuses on the effect of mitochondrial Ca^{2+} dynamics and the relationship between Ca^{2+} released from the ER and its uptake by mitochondria following GPCR stimulation using ATP as an agonist, the Ca^{2+} entry from the extracellular space via plasmalemmal routes (for review see [17,18]; also see introduction for more references) will likely affect mitochondrial Ca^{2+} dynamics in astrocytes as well.

It is worth mentioning that in a subset of astrocytes, we observed Ca^{2+} oscillations after ATP stimulation. It appears that Ca^{2+} oscillations in astrocytes are dependent on agonist concentration and the state of cells, for example, morphology and connectivity as observed in other studies [64,65]. Since we only analyzed Ca^{2+} increase of the first peak, the oscillations should not affect our interpretation of the data.

In summary, mito-GCaMP5G/6s can be used to image mitochondrial Ca^{2+} uptake and handling in neurons and astrocytes with sufficient sensitivity to resolve Ca^{2+} change at single mitochondrial level. Using simultaneous imaging of cytosolic and mitochondrial Ca^{2+} , and molecular intervention, our study reveals that IP_3R -mediated Ca^{2+} increase in the cytosol is highly coupled with mitochondrial Ca^{2+} uptake in astrocytes. As astrocytes exhibit enhanced cytosolic Ca^{2+} signaling and intercellular waves *in vivo* in many neurological diseases in mouse models including status epilepticus, ischemia, traumatic brain injury and Alzheimer diseases [32,33,35,66,67], AAV constructs in the current study provide a valuable tool for mitochondrial Ca^{2+} imaging and to investigate ER-mitochondrial interplay in the brains of live mice under physiological and pathological conditions using viral transduction; these studies, of course, would be of future interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Stimulated mito-GCaMP5G fluorescence increase at single mitochondrion level in cultured astrocytes and neurons. (A and B) 2-P images of mito-GCaMP5G expression in an astrocyte (A) and its response to 100 μ M ATP stimulation at indicated times (B). (C) Images of mito-GCaMP5G in the four individual mitochondria in the astrocyte shown in (A) at the different time points before and after ATP stimulation. (D) The time courses of mito-GCaMP5G fluorescence changes in the four individual mitochondria shown in (C), plotted as $\Delta F/F_0$. (E and F) 2-P images of mito-GCaMP5G expression in a neuron (E) and its response to 100 μ M glutamate stimulation at indicated times (F). (G) Images of mito-GCaMP5G in the four individual mitochondria in the neuron shown in (E) at the different time points before and after glutamate stimulation. (H) The time courses of mito-GCaMP5G fluorescence changes in the four individual mitochondria shown in (G), plotted as $\Delta F/F_0$. Astrocytes or neurons were transfected with pZac2.1-gfaABC₁D-mito-GCaMP5G or pZac2.1-CaMKII-mito-GCaMP5G, respectively. The red arrows in (D) and (H) indicate the time points

corresponding to the images in (B and C) and (F and G), respectively. The pseudocolor scales are a linear representation of the fluorescence intensities in this and other figures.

Fig. 2.

Simultaneous cytosolic and mitochondrial Ca^{2+} imaging in cultured astrocytes stimulated with ATP. (A) 2-P images of astrocytes expressing mito-GCaMP5G (left) and loaded with x-Rhod-1 (middle) for simultaneous mitochondrial and cytosolic Ca^{2+} imaging, respectively; the merged images are shown in the right panel. (B and C) Simultaneous 2-P imaging of mitochondrial Ca^{2+} with mito-GCaMP5G (B) and cytosolic Ca^{2+} with x-Rhod-1 (C) in astrocytes shown in (A) at the different time points shown in response to 100 μM ATP. (D and E) The time courses of Ca^{2+} changes in mitochondria (D) and cytosol (E) in the same four regions marked by circles in A (left and middle). (F) A subset of time courses (between the two vertical dashed lines; normalized to the peak F/F_0) of mitochondrial and cytosolic Ca^{2+} increases in (D and E) showing the matching kinetics of mitochondrial and cytosolic Ca^{2+} increase after ATP stimulation. The red arrows in (D) and (E) indicate the time points corresponding to the images in (B) and (C), respectively.

Fig. 3.

The effect of Ru360 on mito-GCaMP5G fluorescence signals in cultured astrocytes. (A) 2-P images of astrocytes expressing mito-GCaMP5G (left) and loaded with x-Rhod-1 (middle) for simultaneous mitochondrial and cytosolic Ca^{2+} imaging, respectively, in the presence of 1 μM RU360; the merged images are shown in the right panel. (B and C) Simultaneous 2-P images of mitochondrial Ca^{2+} with mito-GCaMP5G (B) and cytosolic Ca^{2+} with x-Rhod-1 (C) of the astrocyte shown in (A) at the different time points indicated in response to 100 μM ATP in the presence of Ru360. (D and E) The time courses of Ca^{2+} changes in

mitochondria (D) and cytosol (E) in the same four regions marked by circles in A (left and middle). Notice that ATP-stimulated mitochondrial Ca^{2+} increase is completely blocked by Ru360 (D), while cytosolic Ca^{2+} appears markedly increased (E), as compared to Fig. 2D and E, respectively. The red arrows indicate the time points corresponding to the images in (B) and (C). (F and G) Summary of the effect of Ru360 on mitochondrial (F) and cytosolic (G) Ca^{2+} increases. Bars represent average peak values, along with their standard error of means, of F/F_0 analyzed from untreated astrocytes ($n = 5$ cells) and those treated ($n = 6$ cells) with Ru360. $*p < 0.05$, Student's t-test.

Fig. 4.

Inhibition of ATP-induced cytosolic Ca^{2+} increase in cultured astrocytes by 5ppase. (A–D) (Upper panels) Cultured astrocytes transfected to express mRFP, a cytosolic expression marker (A, middle; control), or WT 5ppase (B, middle), R343A (C, middle) or R343A/A350A (D, middle) mutants of 5PPase along with bicistronic DsRed, and subsequently loaded with the Ca^{2+} indicator fluo-4 AM (left). The right panels show the merged images. (Lower panels) 2-P images of cytosolic Ca^{2+} responses to 100 μM ATP in transfected astrocytes (left) at the indicated time points shown in the four subcellular regions (circles) and their time courses of cytosolic Ca^{2+} signal in response to ATP stimulation (right). Notice that astrocytes transfected with WT 5PPase do not exhibit Ca^{2+} increase after ATP stimulation (B). The red arrows indicate the time points corresponding to the images in the lower panels. Scale bar in (A–D) is 0.5 for F/F_0 and 25 s for time. (E) Summary of the effect of various forms of 5ppase on cytosolic Ca^{2+} increase after ATP stimulation. Other

annotations are the same as in Fig. 3. Data were collected from astrocytes transfected with mRFP ($n = 4$ cells), or with WT 5ppase ($n = 4$ cells), R343A 5ppase ($n = 5$ cells) or R343A/R350A 5ppase ($n = 8$ cells), respectively, along with bicistronic DsRed. $*p < 0.05$ one-way ANOVA test followed Bonferroni's test compared with other conditions.

Fig. 5.

Control of mitochondrial Ca^{2+} uptake by IP_3R -mediated Ca^{2+} release from the ER in cultured astrocytes. (A–C) (Upper panels) 2-P images of cultured astrocytes co-expressing mito-GCaMP5G (left) and WT 5ppase (A, middle), R343A (B, middle) or R343A/A350A (C, middle) mutants of 5ppase with bicistronic DsRed. The right panels show the merged images. (Lower panels) 2-P images of mitochondrial Ca^{2+} responses in transfected astrocytes (left) at the indicated time points in the four regions (circles) and their time courses of cytosolic Ca^{2+} signal in response to ATP stimulation (right). Notice that astrocytes expressing WT 5ppase do not exhibit mitochondrial Ca^{2+} increase after ATP stimulation (A). The red arrows indicate the time points corresponding to the images in the lower panels. Scale bar in (A–C) is 1 for F/F_0 and 25 s for time. (D) Summary of the effect of various forms of 5ppase on mitochondrial Ca^{2+} increase after ATP stimulation. Other annotations are the same as in Fig. 3. Data were collected from astrocytes transfected with mito-GCaMP5G alone (control, $n = 6$ cells), and astrocytes cotransfected with mito-GCaMP5G and WT 5ppase ($n = 5$ cells), R343A 5ppase ($n = 7$ cells) or R343A/R350A 5ppase ($n = 8$ cells), respectively, along with bicistronic DsRed. $*p < 0.05$ one-way ANOVA test followed by Bonferroni's test compared with other conditions.