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An improved method for isolation of mitochondria from cell lines that enables reconstitution of calcium-dependent processes

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Abstract

Optimum cytosolic calcium concentrations support balanced mitochondrial respiration. However, the concentration of cytosolic Ca^{2+} varies among cell types and excess cytosolic Ca^{2+} can cause mitochondrial dysfunction, particularly when the mitochondria are isolated from cell lines. We optimized a mitochondrial isolation protocol using a human retinal cell line to delude any excess Ca^{2+} and thereby minimize structural damage. We carefully measured Ca^{2+} uptake in part via monitoring the regulated mitochondrial soluble adenylate cyclase-protein kinase A (sAC-PKA) pathway. Here, we measured mitochondrial Ca^{2+} -dependent PKA activity using cAMP ELISAs, and O_2 consumption levels during mitochondria states II – IV with XF96^e high-resolution respirometry. We found that 3 nM Ca^{2+} increased cAMP levels and produced optimal state III respiration. These results demonstrate that optimized isolation of mitochondria from cell lines using calcium denudation provides the best platform for the study of Ca^{2+} -dependent regulation of mitochondrial signaling.

INTRODUCTION

Mitochondria produce much of the cell's ATP and thus are often referred to as “the powerhouse of the cell”. Mitochondrial respiration is affected by extra- and intracellular physiological stimuli. These stimuli often elevate cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$). Calcium levels in tissues are tightly regulated. The extracellular Ca^{2+} concentration is typically in the range of 1–4 mM, whereas the homeostatic range of the $[\text{Ca}^{2+}]_i$ is 4–100

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Author Contributions

Masaaki Ishii: conceptualization, methodology, experimental data acquisition and analysis, original draft preparation. Gyda Beeson: Data curation, writing- Original draft preparation. Craig Beeson: writing- reviewing and editing, supervision. Bärbel Rohrer: conceptualization, writing- Original draft preparation, supervision.

nM. However, the optimal $[Ca^{2+}]_i$ varies depending on the cell type. Cytosolic calcium effects the efficiency of ATP production via the tricarboxylic acid cycle (TCA) cycle coupled to the electron transport chain (ETC) [1–3], with transient increases of $[Ca^{2+}]_i$ stimulating mitochondrial respiration. In contrast, persistent high levels of $[Ca^{2+}]_i$ cause cellular damage leading to cell death [1]. Specifically, cellular stress such as ischemia, reperfusion, and oxidative stress result in mitochondrial calcium overload, promoting the release of apoptosis-inducing factor, cytochrome-c, [1]. However, the basis for the optimal $[Ca^{2+}]_i$, and the mechanisms for its regulation within a given cell type are not always clear.

Cytosolic Ca^{2+} uptake and release occurs via plasma membrane Ca^{2+} exchangers. Mitochondrial Ca^{2+} uptake occurs via the mitochondrial calcium uniporter (MCU) from the cytosol and endoplasmic reticulum (ER). The Ca^{2+} uptake into the mitochondrial matrix is regulated via the calcium sensor proteins “MICU1” and “MICU2” that control MCU gating [4]. Recently, it has been reported that soluble adenylyl cyclase protein kinase A (sAC-PKA) signaling inside mitochondria regulates ATP production and it also regulates mitochondrial Ca^{2+} uptake [5, 6]. More recently, several G-protein coupled receptors that can use calcium as second messengers, such as cannabinoid receptors CB-1 [7], melatonin MT1 receptors [8], GABA_B receptors [9], and 5-HT receptors 5-HT₄ [10] have been identified in the mitochondrial membranes. Therefore, in order to understand GPCR function in mitochondria and Ca^{2+} -mediated cross-talk between GPCR activity and sAC-PKA signaling, it is essential to investigate optimal mitochondrial Ca^{2+} concentrations.

Retinal Pigment Epithelial (RPE) cells are the pigmented cells situated between the photoreceptors and the choroidal blood supply. The RPE has several functions; it composes the blood-retinal barrier, performs specialized ion transport, phagocytoses photoreceptor outer segments, generates the visual chromophore 11-*cis* retinal, and participates in the immune response to conserve a healthy neural retina. Cellular damage such as oxidative stress or inflammation induces $[Ca^{2+}]_i$ elevation and RPE cell dysfunction. Thus, to understand the physiological mitochondrial Ca^{2+} range and its effects on RPE function, it is important to understand how $[Ca^{2+}]_i$ effects respiration and mitochondrial signaling.

In this study, we report an optimized method for mitochondrial isolation derived from ARPE-19 cell cultures to test the direct effect of Ca^{2+} on respiration of isolated mitochondria. During the initial disruption of cells, membrane permeable BAPTA-AM and EDTA in the buffer was used to deplete much of the Ca^{2+} and thereby minimize subsequent Ca^{2+} -mediated organelle damage. We then titrated the $[Ca^{2+}]_m$ to determine optimal mitochondrial respiratory states as measured with XF^e96 high-resolution respirometry. Our findings demonstrate that the optimized calcium level of 3 nM in the extra mitochondrial buffer led to the highest efficiency of mitochondrial respiration and that 3 nM Ca^{2+} activated sAC-PKA signaling via direct measurement of cAMP.

MATERIALS AND METHODS

Mitochondrial isolation

We modified a mitochondrial isolation procedure reported previously [11] for use in ARPE-19 cells cultured on 100 mm culture dishes (100% confluency, $\sim 1.7 \times 10^6$ cells).

Cells were washed with 0.1 M PBS three times, and incubated in DMEM containing 1 mM EDTA and 10 μ M BAPTA-AM for 30 minutes to chelate Ca^{2+} in the medium and the intracellular compartment, respectively. Cell swelling was induced by exposure to hypo-osmotic lysis buffer (6 mL, 10 mM NaCl, 1.5 mM MgCl_2 , 10 mM Tris-HCl, pH 7.5) for 6–10 minutes. The cells were then scraped and collected into a 15 mL centrifuge tube to which 4 mL of the $\times 2.5$ MS homogenization buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, 2.5 mM EDTA, pH 7.5) was added. The cell membranes were disrupted with a Dounce homogenizer with a “B” pestle, gently stroked 5–7 times. The homogenate was centrifuged ($1300 \times g$, 5 min, 4 $^{\circ}\text{C}$), and the supernatant collected into a new tube, a process that was repeated 4 times. The final supernatant was centrifuged ($15000\text{--}17000 \times g$ 15 minutes, 4 $^{\circ}\text{C}$), and the mitochondria were pelleted. $1\times$ MS homogenization buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added to the pellet, collected into new tube, and the mitochondria were pelleted again. The final mitochondrial pellet was collected and suspended in the appropriate buffers: x1-Mitochondrial Assay Solution (MAS $\times 1$) buffer for XF^e96 respirometric assays or RIPA buffer for protein extraction.

Confocal imaging of the isolated mitochondria

To confirm the purity of the mitochondrial preparation, the isolated mitochondria were labeled with fluorescent dyes (all from Invitrogen, Carlsland, CA USA) specific for mitochondria (MitoTracker Green, 1 μ M and MitoTracker Deep Red, 1 μ M), ER (ER-Tracker White-Blue, 1 μ M), nuclei (Hoechst 33342, 0.1 μ M), and lysosomes (LysoTracker Deep Red, 2 μ M) for 30 minutes at 37 $^{\circ}\text{C}$. A phase contrast image of the isolated mitochondria spread onto the XF^e96 assay plate was captured with a built-in microscope imaging system (Incucyte Zoom; Essen Bioscience, Ann Arbor, MI) after a completed XF^e96 Seahorse assay experiment. Confocal images were captured with a spinning disk confocal microscope, the UltraView VoX 3D Live Imaging System, running Volocity software (Perkin Elmer, Wokingham, UK).

XF^e96 respirometric experiment

Mitochondria isolated from two 100 mm dishes (100% confluent; $\sim 1.7 \times 10^6$ cells) were used per 96-well XF^e96 plate. Plates were coated with Cell-TakTM (Corning, Tewksbury, MA USA) as per the manufacturer's protocol. MASx1 buffer (70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 2 mM HEPES, 1 mM EGTA, and 0.2 % (w/v) fatty acid-free BSA, pH 7.0) and mitochondrial substrates were added (5 mM succinic acid, 5 mM maleic acid, 5 mM glutamic acid and 10 mM pyruvic acid). The 96-well XF^e96 plate (Agilent Technologies, Santa Clara, CA, USA) was centrifuged ($4700 \times g$, 20 min, 4 $^{\circ}\text{C}$) to adhere mitochondria onto the bottom of the plate. The injection ports were loaded with 25 μ L solutions composed of MAS3x buffer (210 mM sucrose, 660 mM mannitol, 30 mM KH_2PO_4 , 15 mM MgCh, 6 mM HEPES, pH 7.0) and compounds to give final concentrations (after injection) of: 4 mM adenosine 5'-diphosphate (ADP, Sigma, St. Louis, MO, USA), 2.5 μ M oligomycin, 6 μ M carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Tocris, Pittsburgh, PA, USA), or 0 (Ca^{2+} free), 1, 3, and 10 nM CaCl_2 (Sigma-Aldrich, St. Louis, MO, USA) as the final concentration. The oxygen consumption ratio (OCR) measurements were performed using a XF^e96 high-resolution

respirometry assay system (Agilent Technologies, Santa Clara, CA, USA). The assays were separated into two procedures as mitochondria have a limited time to support optimal respiration upon isolation: (1) injection of ADP to produce state III respiration, and (2) injection of FCCP to measure the maximum uncoupled respiration rate. The OCR data in each well was normalized with the value of the mitochondrial coverage measured from phase contrast image (Fig. 1A) measured with a microscope imaging system (Incucyte Zoom; Essen Bioscience, Ann Arbor, MI) and data were incorporated using the XF^e96 Wave software (Agilent Technologies, Santa Clara, CA, USA).

Mitochondrial Protein extraction

Isolated mitochondria were mixed with 200 μ L RIPA buffer containing protease inhibitor cocktail (1:100, Sigma-Aldrich, P2714), 10 mM phenylmethane sulfonyl fluoride (PMSF) and Phosphatase inhibitor (1 tablet in 10 mL, Thermo Scientific, IL USA), placed into a 1.5 mL tube and homogenized with a pestle. Protein concentration was measured by NanoDrop (Thermo Scientific, Walham, MA, USA).

cAMP ELISA assay

The cAMP levels as well as indirect PKA activity of isolated mitochondria was assayed using the Monoclonal Anti-cAMP Antibody Based Direct cAMP ELISA Kit (NewEast Bioscience, Malvern PA, USA), according to the manufacturer's instructions. Isolated mitochondria were incubated in 1.0 mL MASx1 buffer containing mitochondrial substrates (5 mM succinic acid, 5 mM maleic acid, 5 mM glutamic acid, 10 mM pyruvic acid, and 4 mM ADP), to which different Ca²⁺ concentrations (0, 1, 3, 10 nM) were added. In some instances, 5 μ M KH7 (a selective inhibitor of soluble adenylyl cyclase; Tocris, Pittsburgh, PA, USA) was added to the mitochondria before Ca²⁺ stimulation. A 500 μ g mitochondrial protein sample was loaded in each well of the provided 96-well microplate.

Data analysis

Results from the cAMP ELISA assays, XF^e96 Seahorse assays, and the imaging data were saved as a text file and read into the GraphPad Prism Statistical analysis software (La Jolla, LA, USA). Data are reported as the mean $\pm$ S.E.M. Statistical significance was determined using a One-tailed Student's t-test, and ANOVA. Significance was set $P < 0.05$; n represents the number of independent experiments.

RESULT

Mitochondrial isolation

We modified a published mitochondrial isolation protocol [11] for ARPE-19 monolayer cultures. A protein concentration was selected to provide mitochondrial coverage on XF^e96 Seahorse plates measured by phase contrast imaging of 87 ± 6 % (mean $\pm$ SD, $n=6$, Fig. 1A). Cross-contamination of the preparation with other organelles, specifically with fragmented nuclei, endoplasmic reticulum and lysosomes was examined with organelle specific dyes. The amount of fluorescence as examined by confocal microscopy indicated an almost pure mitochondrial population (MitoTracker Green, MTG or MitoTracker Deep Red, MTRD), with minimal cross-contamination with lysosomal fragments (LysoTracker Deep Red;

LTDR) but no nuclear profiles (Hoechst 33342) or ER fragments (ERTracker, ERTR) (Fig. 1B–E).

cAMP level measurements depended on mitochondrial Ca^{2+} concentrations

A sAC-cAMP-PKA signaling cascade present within mitochondria modulates OXPHOS in a calcium-dependent manner. Thus, we measured baseline cAMP levels in ARPE-19 cell mitochondria and examined cAMP levels affected by different Ca^{2+} concentrations to estimate the PKA activation levels. The sAC-cAMP-PKA signaling cascade was initiated by adding 4 mM ADP to the assay buffer. Specificity for sAC activity was confirmed by measuring cAMP levels in the presence of the sAC inhibitor, KH7. After preliminary experiments to identify an appropriate dose range, four different concentrations of Ca^{2+} , 0, 1, 3 and 10 nM were tested. cAMP measurements based on a sensitive ELISA identified the baseline levels in the absence of calcium at ~24 pmol/mg protein. The 1 nM Ca^{2+} treatments showed an elevation of the cAMP levels, with peak levels at 3 nM, followed by a decrease at 10 nM Ca^{2+} . In comparison with the baseline levels at 0 nM Ca^{2+} , the cAMP levels at 3 nM indicated a ~10% increase (Figure 2, $p < 0.05$). The addition of 5 μM KH7 (a selective inhibitor of soluble adenylyl cyclase) completely eliminated the dose response to Ca^{2+} .

Seahorse XF^e96 high-resolution respirometry assay for isolated mitochondria

Seahorse respirometry assays were subsequently used to identify the Ca^{2+} effect on the respiratory states of isolated mitochondria. We designed two separate experimental assays to examine mitochondrial function. One was measuring the baseline of mitochondrial oxygen consumption ratio (OCR), adding Ca^{2+} at 0, 1, 3, and 10 nM followed by challenge with 4 mM ADP to measure *state III* respiration, which was followed with 6 μM oligomycin to simulate *state IV* respiration (Table S1). Another assay was used to measure the OCR by adding Ca^{2+} at 0, 1, 3, and 10 nM followed by 6 μM FCCP to simulate the *state IIIu* respiration (Table S2).

After Ca^{2+} injection, OCR increased in mitochondria exposed to 1, 3 and 10 nM, but not 0 nM Ca^{2+} (Fig. 3A). The slope of the 3 nM Ca^{2+} injection from the baseline was higher than that of the other Ca^{2+} injections (Fig. 3B). The OCR of *state III* respiration showed a gradual increase from 0 to 1 nM, peaking at 3 nM with a ~50% increase over baseline, followed by a decreased response at 10 nM. To estimate the efficiency of the electron transport to the ATP-synthase, we calculated the ratio of OCR (baseline – oligomycin) as the proton leak (Fig. 3C). Interestingly, at 3 nM Ca^{2+} , proton leak was minimal, and appropriately 30% smaller than that of 0 nM. Again, the inhibition of sAC with 5 μM KH7 eliminated this effect induced by different Ca^{2+} concentration.

Addition of FCCP tests for the maximal respiratory capacity of the mitochondria. Next, we investigated the FCCP uncoupling effect at different Ca^{2+} concentrations independently from that tested for the *state III* respiration (Fig. 4). As with the *state II-III* response, OCR was significantly higher at 3 nM Ca^{2+} in response to 6 μM FCCP, when compared to that in the presence of 0, 1 and 10 nM Ca^{2+} , with the maximal respiratory response at 3 nM showing an ~50% increase over baseline. This gradient was eliminated by 5 μM KH7.

DISCUSSION

The intracellular Ca^{2+} concentration affects cellular homeostasis and mitochondrial metabolism. In this study, we investigated Ca^{2+} effects on mitochondrial metabolism in isolated mitochondria derived from ARPE-19 cells. However, to do so, it was necessary to optimize the method for mitochondrial isolation to prevent calcium damage of the mitochondria during the isolation procedure. Specifically, we showed that the use of membrane permeable BAPTA-AM and EDTA in the isolation buffer was suitable to deplete Ca^{2+} and prevent mitochondrial Ca^{2+} overload and subsequent damage. This depletion of intra-mitochondrial calcium subsequently allows for the titration of $[\text{Ca}^{2+}]_m$ to determine optimal levels for calcium-dependent processes.

OXPHOS in response to substrate is regulated in part by the sAC-cAMP-PKA signaling cascade present within mitochondria, which is modulated by calcium. We found an optimum Ca^{2+} concentration for both cAMP production and mitochondrial respiration with a bell-shaped curve around 3 nM Ca^{2+} . Specifically, we showed that, *state III respiration*, the FCCP uncoupled response, and proton leak, all showed biphasic changes as expected given that Ca^{2+} is required for multiple enzymatic activities.

In normal cellular homeostasis, 1–5 mM extra cellular Ca^{2+} is required, intracellular Ca^{2+} is 1–500 nM, depending on the cell type [2]. For example, Fink and colleague showed that 450 nM Ca^{2+} present the optimum concentration for ATP production in isolated mouse forelimb muscle mitochondria [12]. In contrast, in our study, we tested ARPE-19 cell mitochondria from 1 to 10 nM, and confirmed 3 nM as the optimum concentration for mitochondrial respiration based on FCCP response, *state IIIu* respiration and minimal proton leak. As summarized by Tarasov and colleagues [13], there are a number of Ca^{2+} sensitive enzymes, including FAD-glycerol phosphate dehydrogenase, pyruvate dehydrogenase phosphatase, NAD⁺-isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase and the F1-FO ATP synthase as well as others. Also, the Ca^{2+} is required by the pyruvic acid dehydrogenase (PAD) in TCA cycle [14]. It would be of great interest to further characterize how mitochondrial respiration in different cell types is regulated by, or adjusts to different cytosolic Ca^{2+} concentrations.

Here we also characterized the Ca^{2+} dependence of cAMP production in mitochondria. cAMP is generated by sAC in the matrix and activates PKA by binding to its regulatory domain. Mitochondrial PKA has multiple predicted targets in mitochondria. The complex I protein, NDUFS4, is phosphorylated by PKA [15], as is cytochrome-c dehydrogenase in complex III. Complex I and III are both electron carriers, pumping protons across the inner mitochondrial membrane and building the electrochemical potential required to make ATP. PKA also phosphorylates the ATP-synthase, the final enzyme in the ETC, which utilizes the proton gradient to generate ATP. .

Finally, our results suggest that Ca^{2+} concentrations need to be exquisitely controlled in order to study their role in signaling. In RPE cells, at cytoplasmic/mitochondrial Ca^{2+} concentrations higher than 3 nM, the complex I activity and maximal mitochondrial respiratory capacity are reduced and the proton leak is increased. Thus, long-term exposure

to higher Ca^{2+} concentrations are expected to result in a loss of the proton gradient balance between inner- and inter membrane of the mitochondria, eventually leaving to calcium overload, cytochrome-c release and cell death. Also, Saunders et al. reported that 200 μM Ca^{2+} exposing on the isolated mitochondria induced mitochondrial swelling measured with the UV-VIS spectrometry immediately [16]. Therefore, higher Ca^{2+} concentration than the optimum level could be a mitochondrial stress on mitochondrial respiratory function. Thus, we would argue that isolation conditions that significantly deplete Ca^{2+} will leave the mitochondria in a state better suited for the study of Ca^{2+} -mediated signaling events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of isolated mitochondrial preparation.

(A) Phase contrast image of mitochondria plated on XFe96 Seahorse assay plate. (B, C) Fluorescence confocal microscopy images of isolated mitochondria on 96-well glass bottom microplates stained with markers for specific organelles, confirm purity of the preparation. (B) Triple-labeling for mitochondria (MTG; green), nuclear (Hoechst; blue) and lysosomal fragments (LTDR; red). (C) Double-labeling for mitochondria (MTDR; red) and endoplasmic reticulum fragments (ERTR; blue). (D) 3 independent preparations were analyzed and the number of fluorescent dots per area calculated. (E) The same data as in D was normalized and expressed as percent. The Scale bar in A is 200 μm , in B and C is 20 μm . Data is expressed as mean $\pm$ SEM, n=3

Figure 2. Calcium dependence of mitochondrial cAMP concentration.

cAMP concentration in 1 mg mitochondrial protein was assessed using a highly sensitive cAMP ELISA assay. cAMP concentrations peaked at 3 nM Ca^{2+} , an effect that was soluble adenylate cyclase (sAC) dependent (sAC inhibitor KH7 eliminated the regulation by Ca^{2+}). Data is expressed as mean $\pm$ SEM, n=3

Figure 3. Mitochondrial stage III respiration and proton leak are regulated by calcium. XF⁹⁶ high resolution respiratory assays were utilized for isolated mitochondria. (A) Averaged traces of oxygen consumption rate (OCR) changes in response to Ca²⁺, substrate (ADP), and stage III respiration inhibition (oligomycin). (B, C) Average responses for ADP drive and proton leak determine Ca²⁺ sensitivity and dependence on soluble adenylate cyclase (sAC) activity. KH7 is a sAC inhibitor. (B) Average OCR changes in response to ADP injection, calculated between baseline and the ADP peak. (C) Estimated proton leak, calculated between baseline and the oligomycin response. Data is expressed as mean $\pm$ SEM, n=3

Figure 4. Mitochondrial maximal respiratory capacity is regulated by calcium.

XF⁹⁶ high resolution respiratory assays were used to characterize the response to the mitochondrial uncoupler carbonilcyanide p-trifluoromethoxyphenylhydrazine (FCCP), also known as the maximal respiratory capacity. Ca^{2+} and substrate (ADP) were provided with the recording buffer. (A) Averaged traces of OCR changes in response to FCCP. (B) Average FCCP-elicited responses reveal Ca^{2+} sensitivity and dependence on soluble adenylate cyclase (sAC) activity. KH7 is a sAC inhibitor. Data is expressed as mean $\pm$ SEM, $n=3$