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Direct monitoring of mitochondrial calcium levels in cultured cardiac myocytes using a novel fluorescent indicator protein, GCaMP2-mt

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ABSTRACT

Background: An opening of the mitochondrial permeability transition pore (MPTP), which leads to loss of mitochondrial membrane potential ($\Delta\Psi_m$), is the earliest event that commits a cell to death. Mitochondrial matrix calcium ($[Ca^{2+}]_m$) is considered to be a critical regulator of MPTP, but direct monitoring of $[Ca^{2+}]_m$ is difficult with previously-reported sensors. We developed a novel fluorescent indicator for $[Ca^{2+}]_m$, GCaMP2-mt, by adding a mitochondrial targeting sequence to a high signal-to-noise Ca^{2+} sensor protein GCaMP2, and monitored dynamic changes in oxidant-induced cardiac myocyte death.

Methods and results: GCaMP2-mt was transduced into neonatal rat cardiac myocytes using a recombinant adenovirus. We confirmed that GCaMP2-mt colocalized with tetramethylrhodamine ethyl-ester, a fluorescent indicator of $\Delta\Psi_m$. We monitored oxidant-induced responses of $[Ca^{2+}]_m$ and $\Delta\Psi_m$ using time-lapse confocal microscopy. The response of $[Ca^{2+}]_m$ was synchronous with that of cytosolic calcium and was divided into three kinetically-distinct phases; the first phase, during which $[Ca^{2+}]_m$ maintained its baseline level; the second phase, during which $[Ca^{2+}]_m$ showed a rapid and sudden increase; and the third phase, during which $[Ca^{2+}]_m$ continued to increase at a slower rate until the collapse of $\Delta\Psi_m$. The third phase was likely to be mediated through a mitochondrial Ca^{2+} uniporter, because it was modulated by uniporter-acting drugs. Importantly, there was a remarkable cellular heterogeneity in the third phase, and $\Delta\Psi_m$ loss occurred in an all-or-none manner depending on the cellular $[Ca^{2+}]_m$ level with a clear cut-off value.

Conclusions: Direct monitoring of $[Ca^{2+}]_m$ using GCaMP2-mt provides deeper insight into the mechanism of cardiac myocyte death.

1. Introduction

Mitochondria are crucial regulators of life and death in a variety of cells [1,2] and play pivotal roles in cardiac myocyte death in response to a variety of stress, such as myocardial ischemia/reperfusion injury [3,4]. An opening of the mitochondrial permeability transition pore (MPTP), which leads to the loss of mitochondrial inner membrane potential ($\Delta\Psi_m$), mitochondrial swelling, and subsequent irreversible mitochondrial dysfunction, is the earliest event that commits the cell to death [5]. Mitochondrial matrix Ca^{2+} ($[Ca^{2+}]_m$) is considered to play an important role in this process through regulation of the opening of MPTP [6,7].

The influx of Ca^{2+} into the mitochondrial matrix is mainly supported by a Ca^{2+} electrophoretic mechanism, termed the mitochondrial Ca^{2+} uniporter (MCU), whereas Na^+/Ca^{2+} and Na^+/H^+ exchangers are responsible for Ca^{2+} efflux [8]. Under pathological conditions, such as ischemia/reperfusion, when cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) becomes excessively high, the increase in $[Ca^{2+}]_c$ leads to the mitochondrial Ca^{2+} overload, thereby inducing the opening of MPTP and subsequent cell death [9].

Although $[Ca^{2+}]_m$ is considered to be a critical regulator of MPTP, direct monitoring of $[Ca^{2+}]_m$ is difficult with previously reported sensors due to limited signal intensity or low targeting efficiency. Among the fluorescent Ca^{2+}-sensitive dyes, rhodamine derivative rhod-2 has been used to discriminate between cytosolic and mitochondrial Ca^{2+} [10]. The cytosolic fraction of rhod-2 usually remains significant, even with the use of specific loading protocols [11]. Mitochondrially-targeted aequorin, a luminescent Ca^{2+} sensitive protein, resolved this problem in terms of targeting efficiency, but its signal is too weak for single-cell Ca^{2+} imaging [12]. Genetically-encoded Ca^{2+} probes have been developed in recent years and hold

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great promise in this regard [13–15]; however, partial mis-targeting, low response amplitude, or inadequate temperature stability still remain to be addressed [16].

We have developed a novel fluorescent indicator for $[Ca^{2+}]_m$, GCaMP2-mt, by adding the mitochondrial targeting sequence of cytochrome oxidase to a high signal-to-noise Ca^{2+} sensor protein GCaMP2 [17]. GCaMP2 is an improved version of the prototype GCaMP [18]. We successfully visualized $[Ca^{2+}]_m$ in cultured cardiac myocytes with excellent spatial and temporal resolution using GCaMP2-mt and monitored dynamic changes in oxidant-induced cardiac myocyte death.

2. Materials and methods

All procedures were performed in accordance with the regulations of the Kyoto University Animal Experimentation Committee, which conform to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health.

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma, unless otherwise stated.

2.2. Design of GCaMP2-mt and generation of recombinant adenovirus

We created an adenovirus carrying GCaMP2-mt. GCaMP2-mt was constructed by adding the mitochondrial targeting sequence of cytochrome oxidase (subunit VIII) to GCaMP2. The recombinant adenovirus was generated using the AdEasy system (Qbiogene: Irvine, CA, USA). In brief, the GCaMP2-mt cDNA insert was subcloned into a pShuttle-CMV vector, which contained a cytomegalovirus (CMV) promoter to drive expression of the gene of interest. The recombinant adenoviral plasmids were generated by homologous recombination of pShuttle-CMV with pAdEasy in BJ5183 cells. Then, the PacI linearized adenoviral construct was transfected into QBI293A cells. Transfected cells were harvested when the cells had almost peeled off. After three freeze–thaw cycles, the lysate was used for large scale production of adenovirus in QBI293A cells. Virus was purified by double CsCl centrifugation and subsequently dialyzed. The final concentration of virus product was assessed by plaque forming assays on QBI293A cells.

2.3. Primary culture of neonatal rat cardiac myocytes

A primary culture of cardiac myocytes was prepared from one- to two-day-old Wistar rats (Shimizu Laboratory Supplies: Kyoto, Japan) and cultured as described previously [19]. In brief, the hearts were removed, and the ventricles were minced into small fragments, which were digested by trypsin dissociation. The dissociated cells were preplated for 1 h to enrich the culture with myocytes. The non-adherent myocytes (approximately, 30 to 50 million cells per isolation) were then plated in plating medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 5% fetal calf serum, penicillin (100 U/mL), streptomycin (100 mg/mL), and 2 μg/mL vitamin B12. The final myocyte cultures contained >90% cardiac myocytes at partial confluence. The cells were maintained at 37 °C in the presence of 5% CO_2 in a humidified incubator. Bromodeoxyuridine (0.1 mmol/L) was included in the medium for the first three days after plating to inhibit fibroblast growth. Cultures were then placed in serum-free DMEM containing vitamin B12 24 h before drug treatment.

2.4. Expression of GCaMP2-mt

Neonatal rat cardiac myocytes were transduced with the adenovirus on Day 4–6 of culture. The multiplicity of infection was ~10. Transductions were carried out in culture medium for 1 h at 37 °C. For both HeLa cells and cardiac myocytes, experiments were carried out at least 24 h after transduction.

2.5. Calibration of GCaMP2-mt

GCaMP2-mt was calibrated in permeabilized HeLa cells and cardiac myocytes. Cells expressing GCaMP2-mt were permeabilized with saponin (100 μg/mL) in the intracellular solution (mmol/L): KCl 100, $MgCl_2$ 1, HEPES 50, EGTA 0.2, and mounted on a microscope stage before perfused with the intracellular solution containing Ca^{2+}. Fluorescence measurements (LSM510, Zeiss) were taken upon excitation at 488 nm and the data were fitted using the following equation for $[Ca^{2+}] = K_d \times (F - F_{min})/(F_{max} - F)$ where F_{max} and F_{min} were the maximum and minimum fluorescence values observed in high and low Ca^{2+} respectively.

2.6. Loading of cells with fluorescent indicators

To monitor $\Delta\Psi_m$ and $[Ca^{2+}]_m$ simultaneously, cells expressing GCaMP2-mt were loaded with tetramethylrhodamine ethyl-ester (TMRE; 100 nmol/L) (Molecular Probes, Eugene, OR, USA) at 37 °C for 20 min. To monitor $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ simultaneously, the cells were loaded with 2 μmol/L Rhod-4, AM (AAT Bioquest, CA, USA) at 37 °C for 20 min.

2.7. Confocal time-lapse analysis

Cells plated on 35-mm glass-bottom dishes (1.0 to 1.5 million cells per dish) were maintained at 37 °C in the presence of 5% CO_2 with a heater platform installed on a microscope stage and were placed in serum-free DMEM. After the desired temperature was reached, time-lapse confocal microscopy was started at 1-min intervals using a 40× objective lens. Images were obtained using laser scanning confocal microscope (LSM510, Zeiss). GCaMP2-mt was excited using the 488 nm line of an argon laser and detected at 500–530 nm. TMRE and Rhod-4 were excited using the 543 nm line of a helium/neon laser and detected at 565–615 nm. Ten to fifteen cells were randomly selected in each scan by drawing regions around individual cells, and the green and red fluorescence intensity was monitored sequentially. The results are representative of at least three independent experiments, and we have confirmed the reproducibility of these findings.

2.8. Image analysis

Post-acquisition image analysis was performed using ImageJ software (http://www.rsb.info.nih.gov/ij/). From the image sequences, regions of interest were drawn over part of an individual cell, and fluorescence signals within these regions were collected over time. $\Delta\Psi_m$, $[Ca^{2+}]_m$, and $[Ca^{2+}]_c$ were monitored using mean TMRE, GCaMP2-mt and Rhod-4 brightness within the regions, respectively.

2.9. Statistical analysis

Quantitative data are presented as the mean ± SEM. Comparisons were carried out using either unpaired Student's *t* test or one-way ANOVA with Bonferroni's least significant difference as the post-hoc test. A level of $P<0.05$ was accepted as statistically significant.

3. Results

3.1. Subcellular localization and calibration of GCaMP2-mt

GCaMP2-mt was targeted to mitochondria by adding the mitochondrial targeting sequence of cytochrome oxidase subunit VIII to GCaMP2 (Fig. 1A). This probe was transduced into HeLa cells or primary cultures of rat cardiac myocytes using a recombinant adenovirus. GCaMP2-mt fluorescence showed excellent colocalization with TMRE in both HeLa cells (Fig. 1B (i)) and cardiac myocytes (Fig. 1B (ii)), as observed by confocal microscopy. These results confirmed the high targeting efficiency of this probe to mitochondria.

For Ca^{2+} calibration of GCaMP2-mt, permeabilized HeLa cells expressing GCaMP2-mt were perfused with intracellular solution containing 1 μmol/L ionomycin and 4 μmol/L carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). GCaMP2-mt fluorescence was measured under a range of Ca^{2+} at pH 7.0, 7.5 and 8.0. Fig. 1C shows the Ca^{2+} titration of GCaMP2-mt, which was fitted with a monophasic curve. Apparent K_d for Ca^{2+} and Hill coefficient were 185 nM and 2.0 (pH7.0), 136 nM and 1.6 (pH 7.5), and 124 nM and 1.6 (pH 8.0), respectively; changing pH of the intracellular solution did not significantly affect the affinity of GCaMP2-mt for Ca^{2+}. The affinity of GCaMP2-mt for Ca^{2+} was comparable to that of GCaMP2 (K_d 146 nM) [17] and was higher than that of previously reported mitochondria-targeted genetically-encoded Ca^{2+} indicators (220 nM–2 μM) [14–16]. Fig. 1D shows the pH sensitivity of GCaMP2-mt. Apparent pKa values with and without Ca^{2+} were 7.4 and 7.7 respectively. We also performed $Ca^{2\pm}$ calibration of GCaMP2-mt in cardiac myocytes (K_d 189 nM, Hill coefficient 2.7; at pH 7.5) (Fig. 1E).

3.2. Histamine-induced changes of GCaMP2-mt fluorescence

Then, we tested the capability of GCaMP2-mt as the sensor for changes in $[Ca^{2+}]_m$. Fig. 2A illustrates the responses of GCaMP2-mt induced by histamine (100 μmol/L) in HeLa cells. As soon as histamine was applied, GCaMP2-mt showed a rapid increase in fluorescence intensity (F/F_0 1.60 ± 0.12) followed by a gradual return to the baseline