Biochemical and Biophysical Research Communications 404 (2011) 424–427

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Visualization and quantification of endoplasmic reticulum Ca^{2+} in renal cells using confocal microscopy and Fluo5F

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ARTICLE INFO

Article history:
Received 23 November 2010
Available online 3 December 2010

Keywords:
Calcium
Endoplasmic reticulum
Confocal microscopy
Kidney
Fluo5f

ABSTRACT

Sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+} is the most abundant store of intracellular Ca^{2+}, and its release is an important trigger of physiological and cell death pathways. Previous work in our laboratory revealed the importance of ER Ca^{2+} in toxicant-induced renal proximal tubular cell (RPTC) death. The purpose of this study was to evaluate the use of confocal microscopy and Fluo5F, a low affinity Ca^{2+} indicator, to directly monitor changes in RPTC ER Ca^{2+}. Fluo5F staining reflected ER Ca^{2+}, resolved ER structure, and showed no colocalization with tetramethyl rhodamine methyl ester (TMRM), a marker of mitochondrial membrane potential. Thapsigargin, an ER Ca^{2+} pump inhibitor, decreased ER fluorescence by 30% and 55% at 5 and 15 min, respectively, whereas A23187, a Ca^{2+} ionophore caused more rapid ER Ca^{2+} release (55% and 75% decrease in fluorescence at 5 and 15 min).

Carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler, added at the end of the experiment, further decreased ER fluorescence after thapsigargin treatment, revealing that thapsigargin did not release all ER Ca^{2+}. In contrast, FCCP did not decrease ER fluorescence after A23187 treatment, suggesting complete ER Ca^{2+} release. ER Ca^{2+} release in response to A23187 or thapsigargin resulted in a modest but significant decrease in mitochondrial membrane potential. These data provide evidence that confocal microscopy and Fluo5F are useful and effective tools for directly monitoring ER Ca^{2+} in live cells.

1. Introduction

Ca^{2+} acts as a universal second messenger and regulates numerous cellular functions including metabolism, motility, and transport [1]. Loss of Ca^{2+} homeostasis is critical to many disease processes and is a major component of cell death pathways including necrosis, apoptosis, and autophagy [2–8]. Sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+} is the most abundant store of intracellular $Ca^{2+}$and its disruption often initiates the deleterious cascade of events leading to cell death and dysfunction [5–11].

Traditional methods of measuring of ER Ca^{2+} are indirect or require difficult probe loading techniques (i.e. membrane permeabilization, microinjection, or fused cell hybrids). For example, human embryonic kidney cells require 1 h of dye loading (2 μM Fluo-3 at room temperature) and extracellular Ca^{2+} chelation using BAPTA or EGTA, just to monitor increases in cytosolic Ca^{2+} as an indirect measure of ER Ca^{2+} stores [12]. To monitor ER Ca^{2+} directly, Montero and Robert et al. engineered an ER-targeted aequorin chimera, a Ca^{2+}-sensitive photoprotein with a lower affinity for Ca^{2+}. However, in HeLa and skeletal muscle cells, the aequorin chimera was rapidly saturated by the high Ca^{2+} concentrations within the ER and required a non-physiological reduction of ER Ca^{2+} to detect changes in Ca^{2+} stores [13,14].

Primary cultures of renal proximal tubular cells (RPTC) are highly aerobic, gluconeogenic, and exhibit robust transport capacity, making them ideal for the study of kidney tubular function and injury [15,16]. Previous work in our laboratory revealed the importance of ER Ca^{2+} in toxicant-induced kidney injury; although, the mechanism by which Ca^{2+} plays such a pivotal role is not completely understood [5,7,9,10,17,18].

Confocal microscopy is a useful method for the visualization and quantification of fluorophores at the subcellular level in living cells, and is compatible with most Ca^{2+} indicators. Chemical fluorescent (UV and visible-wavelength excitation fluorescent indicators) and

Abbreviations: ER, endoplasmic reticulum; EGTA, ethylene glycol tetraacetic acid; RPTC, renal proximal tubular cell; TMRM, tetramethyl rhodamine methyl ester; Fluo5F, Fluo-5F-AM; LSCM, laser scanning confocal microscopy; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; SERCA, sarcoplamic/endoplasmic reticulum ATPase; DAG, diacylglycerol; IP3, inositol triphosphate; DMSO, dimethyl sulfoxide.

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.11.137

bioluminescent calcium indicators (Ca^{2+} binding photoproteins and GFP-based Ca^{2+} indicators) differ in their characteristics (loading requirements, excitation/emission spectrum, permeability, compartmentalization, relative brightness, and Ca^{2+} affinity) and have inherent drawbacks (i.e., dye leakage, cytotoxicity, bleaching, autofluorescence, intracellular buffering, and lack of ion specificity)[19]. Fluo5F is a chemical fluorescent Ca^{2+} indicator with a lower affinity for Ca^{2+} ($K_d \sim 2.3$ μM), limited cytotoxicity and bleaching, and high Ca^{2+} specificity, making it ideal for studying ER Ca^{2+}. The purpose of this study was to evaluate the use of confocal microscopy and Fluo5F for directly monitoring changes in ER Ca^{2+} in RPTC.

2. Materials and methods

2.1. Materials

Female New Zealand White rabbits (1.5–2.0 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). Tetramethyl rhodamine methyl ester (TMRM) and Fluo-5F-AM were purchased from Molecular Probes, Invitrogen (Carlsbad, CA). All other chemical and materials were obtained from Sigma Chemical (St. Louis, MO).

2.2. Isolation of rabbit RPTC and culture conditions

Rabbit RPTC were isolated using the iron oxide perfusion method and grown to confluence in 35 mm tissue culture dishes under improved conditions as previously described [15,16]. The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 medium (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM L-glutamine, 1 μM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μM) were added daily to fresh culture medium.

2.3. RPTC loading

RPTC were loaded with 2 μM Fluo-5F-AM (Fluo5F) and 100 nM TMRM for 20 min at 37° C, washed twice with 37° C phosphate buffered saline, and media was replaced. Then, 1 μM Fluo5F and 40 nM TMRM were added to the media to maintain dye equilibrium and incubated for 30 min at 37° C prior to imaging. The TMRM loading protocol was modified from Lemasters and Ramshesh [20].

2.4. Microscopic imaging and analysis

RPTC were imaged on a Leica Microsystems, TCS SP2 AOBS laser scanning confocal microscope (LSCM) using standardized pinhole, gain, and black level settings. A 63 × 0.9 NA water-immersion objective was used in an upright microscope configuration. All images were acquired at 8-bit resolution and at 1024 × 1024 with a line averaging of two.

RPTC were monitored for 10 min prior to treatment to establish baseline. Then, RPTC were treated and monitored an additional 15 min prior to Carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 1 μM) addition to depolarize mitochondria. Images were acquired in 1 min intervals during the course of experiments. To quantify data, images were analyzed using Adobe Photoshop. Mean intensities of images in the green and red channel after background subtraction was interpreted as a quantitative measure of ER Ca^{2+} and mitochondrial membrane potential, respectively. Values are graphed as the percent mean intensity of each subsequent image vs. mean intensity of the image taken at time zero. Images comprise of 7–10 cells.

2.5. Statistical analysis

RPTC isolated from one animal represents one experiment ($N = 1$). Data are presented as means ± SE, and multiple means were compared using Student–Newman–Keuls test at each time point. Means with values $p < 0.05$ were considered statistically significant.

3. Results and discussion

To investigate the use of confocal microscopy to monitor ER Ca^{2+} dynamics in RPTC, Fluo5F fluorescence was monitored as described above (Fig. 1A). Fluo5F fluorescence revealed a reticular pattern around nuclei void of staining. The peripheral granularity of staining and scarcity of staining at the cell margins provided evidence that the probe is not fluorescing in the cytosol. To confirm that Fluo5F was not in the mitochondria, RPTC were loaded with tetramethyl rhodamine methyl ester (TMRM), a marker of polarized mitochondria. TMRM staining (Fig. 1B) had a punctuate/tubular pattern unlike Fluo5F staining with no co-localization (Fig. 1C), confirming that Fluo5F does not reflect mitochondrial Ca^{2+}, the second most significant storage/buffering compartment for intracellular Ca^{2+} [4].

We investigated the use of Fluo5F to monitor real-time changes in RPTC ER Ca^{2+}. TMRM was used to measure mitochondrial membrane potential. In control cells, both Fluo5F and TMRM fluorescence intensity, morphology, and resolution were maintained over time (Fig. 2). Carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler, was added at the end of each experiment to cause mitochondrial depolarization and indirectly ER Ca^{2+} release, due to ATP depletion and cessation of sarcoplasmic/ER Ca^{2+} ATPase (SERCA) activity.

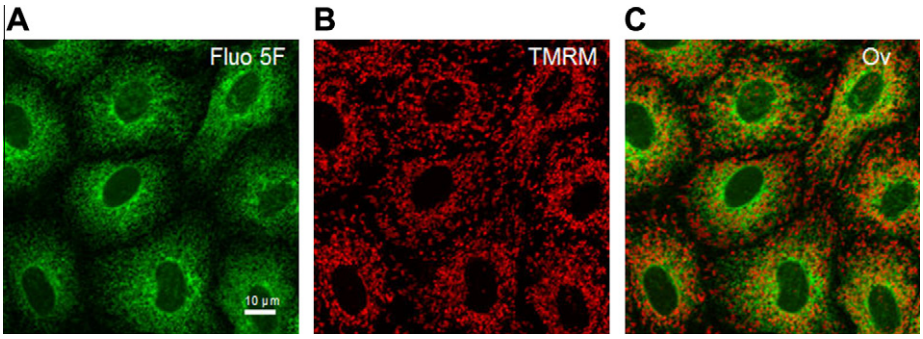

Fig. 1. Morphology of Renal Proximal Tubule Cells (RPTC) co-loaded with Fluo5F and TMRM using confocal microscopy. RPTC were co-loaded with Fluo5F (2 μM) and TMRM (100 nM) as described in Section 2. Green fluorescence of Fluo5F and red fluorescence of TMRM were imaged by laser scanning confocal microscopy. The white bar represents 10 μm. Fluo5F fluorescence (A) represents endoplasmic reticulum (ER) Ca^{2+}, TMRM fluorescence (B) represents polarized mitochondria, and there was no co-localization (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)