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Calcium, mitochondria and apoptosis studied by fluorescence measurements

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ABSTRACT

Among the many unsolved problems of calcium signalling, the role of calcium elevations in apoptotic and necrotic cell death has been a focus of research in recent years. Evidence has been presented that calcium oscillations can effectively trigger apoptosis under certain conditions and that dysregulation of calcium signalling is a common cause of cell death. These effects are regularly mediated through calcium signal propagation to the mitochondria and the ensuing mitochondrial membrane permeabilization and release of pro-apoptotic factors from mitochondria to the cytoplasm. The progress in this area depended on the development of (1) fluorescent/luminescent probes, including fluorescent proteins that can be genetically targeted to different intracellular locations and (2) the digital imaging technology, fluorescence-activated cell sorting and fluorescent high throughput approaches, which allowed dynamic measurements of both $[Ca^{2+}]$ in the intracellular compartments of interest and the downstream processes. Fluorescence single cell imaging has been the only possible approach to resolve the cell-to-cell heterogeneity and the complex subcellular spatiotemporal organization of the cytoplasmic and mitochondrial calcium signals and downstream events. We outline here fluorometric and fluorescence imaging protocols that we set up for the study of calcium in the context of apoptosis.

1. Introduction

The extracellular Ca^{2+} concentration is >1 mM, whereas the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) is ≈100 nM at the resting state. $[Ca^{2+}]$ in the mitochondrial matrix ($[Ca^{2+}]_m$) and nuclear matrix is similar to $[Ca^{2+}]_c$. By contrast, the endoplasmic reticulum (ER) (sarcoplasmic reticulum (SR) in muscle cells) shows high Ca^{2+} concentration (100–500 μM) as compared to $[Ca^{2+}]_c$. The activity of the plasma membrane Ca^{2+} pumps (PMCA) and the sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCAs) is primarily responsible for the maintenance of the large $[Ca^{2+}]$ gradients. Opening of the ER/SR Ca^{2+} release channels, IP_3 receptors (IP3Rs) and ryanodine receptors (RyRs) results in Ca^{2+} mobilization from the ER/SR that manifests as a decrease of $[Ca^{2+}]_{ER}$ and an increase of $[Ca^{2+}]_c$ [1]. Activation of plasma membrane Ca^{2+} channels allows Ca^{2+} to enter along the concentration and electrical gradients and also yields a $[Ca^{2+}]_c$ increase. The mitochondrial matrix is separated from the cytoplasm by two membranes and the inner mitochondrial membrane (IMM) has a very limited permeability to ions. However, conditions of elevated $[Ca^{2+}]_c$ evoke activation of the Ca^{2+} uniporter that mediates $\Delta\Psi_m$-driven Ca^{2+} uptake to the mitochondria [2,3]. Despite the presence of robust Ca^{2+} buffering capacity in the mitochondrial matrix, $[Ca^{2+}]_c$ spikes yield rapid elevations in $[Ca^{2+}]_m$ from 100 nM to at least micromolar and in some cases over hundred micromolar [4]. Due to the relatively low affinity of the uniporter, the global $[Ca^{2+}]_c$ rise (≈1 μM) established by ER Ca^{2+} release and Ca^{2+} entry during physiological $[Ca^{2+}]_c$ signals results in only a slow increase in $[Ca^{2+}]_m$. However, long-lasting global $[Ca^{2+}]_c$ signals may result in accumulation of vast amounts of Ca^{2+} in the mitochondria. Alternatively, mitochondria close to the ER or plasma membrane may sense the large and local $[Ca^{2+}]_c$ increases in proximity to the activated Ca^{2+} channels and exhibit a $[Ca^{2+}]_m$ rise closely coupled to the rise of the $[Ca^{2+}]_c$ signal [5].

In physiological conditions, calcium signal propagation to the mitochondria results in stimulation of ATP production through activation of the Ca^{2+}-sensitive dehydrogenases and yields feed back effects on cytoplasmic calcium signalling (Fig. 1A). However, Ca^{2+} overloading of the mitochondria triggers permeability transition pore (PTP) opening that appears as uncoupling, IMM reorganization, mitochondrial swelling and finally, outer mitochondrial membrane (OMM) permeabilization [6–9]. Disruption of the OMM barrier induces apoptosis by stimulating the release of apoptosis promoting factors from the mitochondrial intermembrane space (IMS) to the cytoplasm (cyto *c*, AIF, Smac/DIABLO, OMI/HtrA2, pro-caspases, etc.) and by impairing mitochondrial function [10,11]. The PTP is thought to be a multi-protein complex that is assembled at the contact sites between the IMM and OMM but the exact molecular composition of the PTP has not been elucidated [12]. In the presence of various stress factors (e.g. ceramide, arachidonic acid, reactive oxygen species (ROS) etc.) the PTP becomes sensitized to physiological $[Ca^{2+}]_m$ spikes that in turn, trigger mitochondrial membrane permeabilization and apoptosis instead of a metabolic response (Fig. 1B) [13–17].

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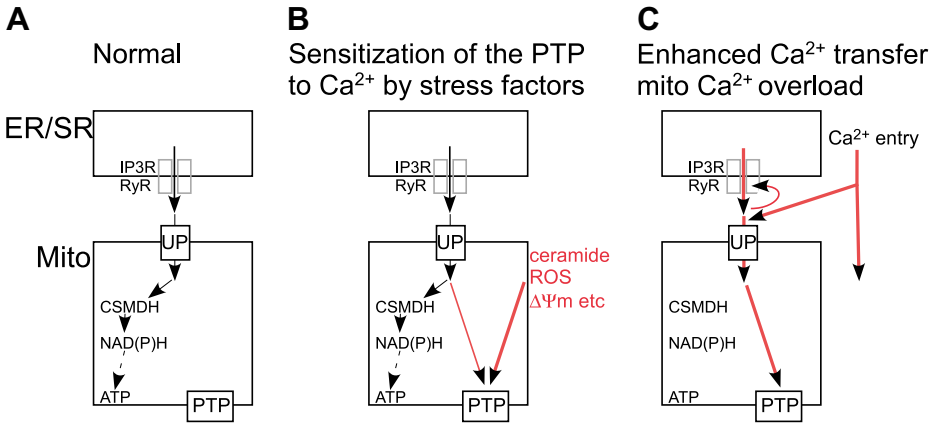

Fig. 1. Schematic representation of the ER/SR-mitochondrial Ca^{2+} transfer and the intramitochondrial control of energy metabolism and PTP opening by Ca^{2+} under different conditions: (A) Normal, calcium oscillations activate the Ca^{2+}-sensitive mitochondrial dehydrogenases (CSMDH) to enhance ATP production; (B) Mitochondria are exposed to factors that increase the sensitivity of the PTP towards Ca^{2+}; (C) Increased mitochondrial Ca^{2+} transfer leads to PTP opening during physiological calcium oscillations. UP—mitochondrial Ca^{2+} uniporter.

Alternatively, conditions that enhance the Ca^{2+} mobilization from the ER/SR or recruit massive Ca^{2+} entry may augment the mitochondrial Ca^{2+} uptake to cause alone activation of the PTP and apoptosis (Fig. 1C) [18]. These conditions may include mutations in the IP3R/RyR or in their associated proteins (e.g. huntingtin) [19], posttranslational modifications of IP3R/RyR and pathologic activation of the NMDA receptors by glutamate, glutamate excitotoxicity [20]. Furthermore, narrowing the ER-mitochondrial gap by synthetic interorganellar linkers also causes increased Ca^{2+} transfer to the mitochondria and promotes mitochondrial overload and apoptosis [21]. Another remarkable feature of the activation of PTP by Ca^{2+} is that the opening of the PTP permits release of the accumulated Ca^{2+} that is subsequently taken up by adjacent mitochondria (Fig. 1C). This mechanism may result in a regenerative response that spreads throughout the entire mitochondrial population of the cell as a propagated wave [22,23]. Notably, mitochondrial Ca^{2+} may also recruit ROS formation and ROS formation and release from the mitochondria can also form a regenerative mechanism for driving mitochondrial waves [24,25]. PTP-dependent intermitochondrial calcium signaling has been reported to recruit mitochondria to the apoptotic process [26]. As a high density of mitochondria facilitates communication between neighboring organelles, the local signalling machinery can be utilized effectively in the large myotubes of heart and skeletal muscle that are among the cells most abundant in mitochondria. Thus, the calcium signal→ $[Ca^{2+}]_m$ rise→ PTP opening→OMM permeabilization axis efficiently triggers the execution of apoptosis and is likely to be relevant in tissue damage under a variety of stress conditions and genetic alterations.

This review describes experimental approaches that can be used for analysis of the role of calcium signalling in apoptosis. These methods target primarily the mitochondrial phase of apoptosis. Direct measurements of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ in both intact and permeabilized cells became feasible using Ca^{2+} sensitive fluorophores and fluorescent proteins. Monitoring of the $\Delta\Psi_m$ allows detection of the PTP opening and can be conducted using potentiometric dyes. Cytochrome *c* release from the mitochondria can be assessed by monitoring the distribution of cytochrome *c*-GFP (cyto *c*-GFP) between mitochondria and cytosol. Caspase activation, phosphatidylserine (PS) exposure and nuclear apoptosis can also be monitored using fluorescent probes. Application of these probes in fluorometry or in fluorescence imaging allows dynamic measurements of the respective parameters in real-time. Furthermore, fluorescence imaging also offers resolution of the individual cell responses and the intracellular spatiotemporal pattern of the measured parameters. Combination of these capabilities is extremely valuable for the study of calcium signalling in apoptosis and is a distinctive feature of fluorescence imaging. However, other approaches like luminescence measurements of genetically targeted reporters (e.g. Ca^{2+} and ATP) [27] and fluorescence activated cell sorting [28] have their own edge and provide complementary information.

2. Methods

2.1. Cells and reagents

Cell Culture:

1. Human hepatoma cell line HepG2 (ATCC) and rat cardiac muscle cell line H9c2 (ATCC).
2. Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco), 1 mM Sodium Pyruvate (Gibco), 2 mM L-glutamine (Biowhittaker), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Biowhittaker).
3. Solution of Trypsin (0.25%) and EDTA (1 mM) (Gibco).
4. Poly-D-lysine-coated 25 mm round glass coverslips.
5. Cyto *c*-GFP expressing cells were generated by transfection using plasmid DNA [29] and Lipofecta-AMINE 2000 (Invitrogen) [26].
6. For the studies with myotubes H9c2 cells were grown to reach confluency (1 week on average) and subsequently for an additional 3–7 days to allow differentiation [30].

Assay Buffers:

1. Extracellular medium with 2% bovine serum albumin (BSA) (2% BSA/ECM) consisting of 121 mM NaCl, 5 mM $NaHCO_3$, 10 mM Na–Hepes, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM glucose, pH 7.4.
2. Extracellular medium with 0.25% BSA (0.25% BSA/ECM) consisting of 121 mM NaCl, 5 mM $NaHCO_3$, 10 mM Na–Hepes, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM glucose (BSA), pH 7.4.
3. Ca^{2+} free extracellular buffer (Na–Hepes–EGTA), containing 120 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 0.2 mM $MgCl_2$, 0.1 mM EGTA, 20 mM Hepes–NaOH pH 7.4.
4. Intracellular medium (ICM) composed of 120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 5% Dextran, 20 mM Hepes–Tris pH 7.2 supplemented with 1 μ/ml of each of antipain, leupeptin and pepstatin (Sigma). To lower the ambient $[Ca^{2+}]$, the ICM was