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Review

Mitochondria: The calcium connection

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ABSTRACT

Calcium handling by mitochondria is a key feature in cell life. It is involved in energy production for cell activity, in buffering and shaping cytosolic calcium rises and also in determining cell fate by triggering or preventing apoptosis. Both mitochondria and the mechanisms involved in the control of calcium homeostasis have been extensively studied, but they still provide researchers with long-standing or even new challenges. Technical improvements in the tools employed for the investigation of calcium dynamics have been–and are still–opening new perspectives in this field, and more prominently for mitochondria. In this review we present a state-of-the-art toolkit for calcium measurements, with major emphasis on the advantages of genetically encoded indicators. These indicators can be efficiently and selectively targeted to specific cellular sub-compartments, allowing previously unavailable high-definition calcium dynamic studies. We also summarize the main features of cellular and, in more detail, mitochondrial calcium handling, especially focusing on the latest breakthroughs in the field, such as the recent direct characterization of the calcium microdomains that occur on the mitochondrial surface upon cellular stimulation. Additionally, we provide a major example of the key role played by calcium in patho-physiology by briefly describing the extensively reported–albeit highly controversial–alterations of calcium homeostasis in Alzheimer's disease, casting lights on the possible alterations in mitochondrial calcium handling in this pathology.

1. Introduction

Calcium-based signalling is a universal mechanism through which extracellular messengers modify the activity of target cells. Cells can decode Ca^{2+} signals based on the characteristics of the intracellular changes in Ca^{2+} concentration (amplitude, duration, frequency and localization) and generate outputs as diverse as proliferation or death. To this end, eukaryotic cells have evolved a complex Ca^{2+} toolkit that encompasses proteins capable of detecting changes in Ca^{2+} levels (and thus trigger a signalling cascade through effectors), as well as complex homeostatic mechanisms that include Ca^{2+} channels on the plasma membrane and organelles, Ca^{2+}-buffering proteins, and systems for Ca^{2+} extrusion and sequestration [1–5].

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in basal, resting conditions is a steady state that depends exclusively on the equilibrium between the influx and efflux rates at the plasma membrane (PM) level. Any change in the activity or concentration of other members of the Ca^{2+} toolkit (e.g., buffers, organelle pumps, etc.) affect cytosolic Ca^{2+} levels only transiently and have no effect on the long-term concentration of this ion. Surprisingly, this basic and essential concept is not obvious to all investigators in the field (see the recent contribution by Rios where these concepts are described in an elegant, accurate and formally impeccable way [6]). Cytoplasmic Ca^{2+} signals (i.e., increases in $[Ca^{2+}]_c$) may be due to (i) Ca^{2+} entry from the extracellular matrix (through the PM), or (ii) Ca^{2+} release from intracellular stores, or a combination of both. At the end of the signal, basal $[Ca^{2+}]_c$ levels are regained through the action of pumps or antiporters at the expense of energy consumption. Organelles must be refilled as well through their specific Ca^{2+} uptake systems that take advantage of selective pumps (SERCA, SPCAs) or exchangers.

Although the endoplasmic reticulum (ER) and its specialized form in muscle (the sarcoplasmic reticulum, SR) is generally considered the

Abbreviations: Aβ, amyloid beta; $\Delta\Psi_m$, inner mitochondrial membrane potential; APP, amyloid precursor protein; BRET, bioluminescence resonance energy transfer; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{ER}$, endoplasmic reticulum Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial Ca^{2+} concentration; CaM, calmodulin; CCE, capacitative calcium entry; CFP, cyan fluorescent protein; Cyp D, cyclophilin D; mCU, mitochondrial calcium uniporter; ER, endoplasmic reticulum; FAD, familiar Alzheimer disease; FRET, fluorescence resonance energy transfer; GECI, genetically encoded Ca^{2+} indicator; GFP, green fluorescent protein; IMM, inner mitochondrial membrane; IMS, intermembrane space; KO, knock out; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; OMM, outer mitochondrial membrane; PM, plasma membrane; PMCA, plasma membrane Ca^{2+} ATPase; PS1, Presenilin-1, PS2, presenilin-2; PTP, permeability transition pore; RyR, ryanodine receptor; RR, Ruthenium Red; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; SPCA, secretory pathway Ca^{2+} ATPase; SR, sarcoplasmic reticulum; VOCC, voltage-operated Ca^{2+} channel; YFP, yellow fluorescent protein

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main intracellular Ca^{2+} store, almost all other organelles play a role in Ca^{2+} signalling [3,5]: mitochondria (see below), the Golgi apparatus [7], secretory vesicles [8], lysosomes [9], endosomes [10] and peroxisomes [11,12].

Much attention has been dedicated over the last decades to the involvement of mitochondria in Ca^{2+} homeostasis: mitochondria are known since the late 1960s to be endowed with the capacity of accumulating Ca^{2+} in an energy-dependent way (through the so-called Ca^{2+} uniporter, mCU) and in the 1970s the existence of antiporters capable of releasing Ca^{2+} from these organelles was firmly established (Na^+ dependent and independent Ca^{2+} exchangers). The kinetic characteristics of mitochondrial Ca^{2+} transport are well known, but the proteins involved have yet to be identified at the molecular level [13–15], although some candidates have been presented recently for the mCU [16,17] and the Na^+/Ca^{2+} antiporter [18].

For a long time, however, due to the low affinity of the mitochondrial Ca^{2+} uptake system under physiological conditions (K_d around 10–20 μM) this process was considered to take place only at pathophysiological [Ca^{2+}], i.e., in conditions of cellular Ca^{2+} overload [3,19–21]. A number of observations, in contrast with this over-simplified view, were largely overlooked by the scientific community for quite some time. In particular, it was known that mitochondrial key functions (matrix dehydrogenases in particular) are strongly affected by [Ca^{2+}] within the matrix [22]. In addition, it was well known that cytosolic Ca^{2+} can influence the activity of mitochondrial enzymes located on the outer surface of the inner mitochondrial membrane (IMM), such as glycerophosphate dehydrogenase, or the malate-aspartate shuttle and glutamate/malate dependent respiration through activation of the aspartate-glutamate carriers [23,24]. It was, however, the demonstration that physiological cytosolic Ca^{2+} increases are accompanied by similar and larger mitochondrial ones that made the process of mitochondrial Ca^{2+} regulation one of wide interest. Finally, the discovery that excess Ca^{2+} uptake by mitochondria triggers a bioenergetic failure of the organelle through the opening of the permeability transition pore (PTP, [25]), release of cytochrome *c* and of other proapototic factors and cellular death by apoptosis or necrosis, has definitively put the study of mitochondrial Ca^{2+} homeostasis at the centre of interest of a vast group of scientists [26].

Pivotal in this renewed interest in mitochondrial Ca^{2+} homeostasis was the development of Ca^{2+} indicators that enable the accurate quantification of the concentration of this cation in the matrix of organelles in living cells. Such probes, either synthetic or natural, exhibit quantifiable changes in some physicochemical property, most commonly an alteration in the spectrophysical properties (fluorescence intensity and/or wavelength shift), that are proportional to [Ca^{2+}]. Numerous probes are currently in use, with each possessing advantages and disadvantages. At present, the wide choice of such Ca^{2+} indicators offers a solution for almost any biological problem. The genetically encoded Ca^{2+} indicators (GECIs) are, in our opinion, the best choice because of their highly selective targeting to subcellular compartments, long-term expression when integrated into the genome, and marginal or no toxicity.

In this contribution, we will first briefly review the state-of-the-art concerning the different types of Ca^{2+} probes and their use in living cells, with emphasis on the newly generated GECIs and their use in the determination of Ca^{2+} handling by organelles. We will then address mitochondrial Ca^{2+} handling and its relevance in physiology and pathology, also focusing on recent findings by our group in cell models of Alzheimer disease.

2. Ca^{2+} measurements in living cells

The 1960s and 1970s saw the identification of some organic compounds (murexides and azo dyes in particular) that were able to change spectral properties upon Ca^{2+} binding. However, these probes had no fixed stoichiomestry, exhibited low signal-to-noise ratio, and had to be laboriously delivered into cells by microinjection [27]. Given that these dyes could, in the best case, be trapped only within the cytosol, they could only provide indirect information of organelle Ca^{2+} handling. The invention in the early 1980s by Tsien and coworkers of a new family of synthetic fluorescent Ca^{2+} indicators that could be easily delivered to cells by incubation with their acetoxymethyl ester derivatives represented a breakthrough in the field of Ca^{2+} imaging ([28–30], Table 1). Tens of Ca^{2+} indicators have been generated based on the same principles (most of them by Tsien's group) and the available probes presently cover a wide range of Ca^{2+} affinities and spectral properties. For example, some indicators only increase the emitted fluorescence upon Ca^{2+} binding (e.g., Calcium-Green, Fluo-3), whereas in other cases Ca^{2+} causes a shift in either the excitation (Fura-2) and/or emission spectra (Indo-1) [27]. Since the esterases that hydrolyze the AM forms are primarily (though not only) located in the cytoplasm, these indicators cannot be specifically targeted to organelles or subcellular compartments, with Rhod-2 being the only possible exception. Trapping of the fluorescent dyes in organelles does occur under some conditions, but this is never selective (again with the exception of Rhod-2). Recently it has been shown that selective labelling of organelles with these dyes can be obtained by over-expressing esterases in specific locations within the cell [31]. This interesting approach, however, has not gained much popularity yet. The only Ca^{2+} probe of this family that shows some specificity in subcellular trapping (to the mitochondrial matrix) is Rhod-2. Rhod-2 in fact possesses, in both the AM and hydrolyzed form, a delocalized positive charge that favours its accumulation inside the mitochondrial matrix due to the negative membrane potential across the inner membrane of this organelle. In most cases, however, partial mislocalization of Rhod-2 in the cytoplasm and other compartments is observed and specific protocols had to be devised to take this problem into account. Also, given that Rhod-2 responds to Ca^{2+} rises with an increase in fluorescence intensity, ratiometric imaging is not feasible and artefacts due to different dye loading or organelle movements cannot be easily compensated for [32]. Other concerns raised by these indicators are the toxicity of the de-esterification reaction products (acetate and formaldehyde) and, in some cases (e.g., fura-2 and indo-1), the photodamage that can result upon prolonged excitation with high energy photons [27,33,34].

The targeting problem was elegantly solved with GECIs, because the transfected proteins can be selectively targeted to the subcellular compartment of interest by including in their sequence specific signal peptides [35–39]. GECIs can be expressed under the control of inducible or tissue specific promoters, and therefore the amount of indicator and type of cell can be modified or selected. All available GECIs are based on two proteins present in the jellyfish *Aequorea victoria*: aequorin and green fluorescent protein (GFP). GECIs can be subdivided in bioluminescent (based on aequorin) and fluorescent probes (based on GFP and its mutants).

Bioluminescent GECIs take advantage of the emission of a photon in the reaction of peroxidation of the cofactor coelenterazine, which is catalyzed by aequorin in the presence of Ca^{2+}. The rate of photon emission is proportional to the [Ca^{2+}], thus enabling the determination at any instant of the local [Ca^{2+}] in the environment where the protein is localized. Furthermore, as no excitation is needed (photon emission is the result of a chemical reaction), in experiments employing aequorin the background noise is essentially due to instrumental noise and thus very low. Initially, aequorin had to be laboriously extracted from jellyfish [40], but cloning of its cDNA not only permitted heterologous cell expression, but also allowed genetic engineering of the protein to modify the Ca^{2+} affinity and to deliver it to specific organelles [27,35,36,41,42]. For example, targeting of aequorin to subcellular compartments allowed to demonstrate that mitochondria in living cells respond to physiological changes of [Ca^{2+}] in the cytoplasm [43], to unravel the ER–mitochondria relationship [44,45] and to prove that the Golgi apparatus acts as a releasable Ca^{2+} reservoir [7]. The main disadvantages of aequorin-based