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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

Table 1
Commonly used Ca^{2+} indicators (chemically synthesized) and GECIs.

	Response to [Ca^{2+}]	λ excitation[a] (nm)	λ emission[b] (nm)	K_d for Ca^{2+} μM (*in situ*)[c]	Observations	REF
Quin-2	Fluorescence intensity	340	495	0.115	NST[d]	[30]
Indo-1	Ratiometric	345	400 / 480	0.25	NST[d] Ratiometric emission 400(Ca^{2+} bound)/480 (Ca^{2+} free)	[180]
Fura2	Ratiometric	340 /380	510	0.224	NST[d] Ratiometric excitation 340 (Ca^{2+} bound)/380 (Ca^{2+} free)	[180]
Calcium Green-1	Fluorescence intensity	504	532	0.19 (0.93)	NST[d]	[34]
Oregon Green 488 BAPTA-1	Fluorescence intensity	488	520	0.17 (0.43)	NST[d]	[34]
Fluo-3	Fluorescence intensity	488	520	0.39 (0.81)	NST[d]	[32,34]
Fluo-4	Fluorescence intensity	488	520	0.345 (1)	NST[d]	[32,34]
Rhod-2	Fluorescence intensity	540	580	1–0.570	Tends to accumulate in the mitochondrial matrix	[32,181]
Aequorins	Increase rate of photon emission (luminescence)		~470	7	Aequorins with different affinities for Ca^{2+} are available	[41,182]
GFP-Aequorin	Increased BRET		510	Similar to Aeq		[47,50]
Camgaroo-1	Fluorescence intensity	490	514	7		[52]
Camgaroo-2	Fluorescence intensity	490	514	5.8 (8)		[57,183]
R-Pericam				1.7		[51]
R-Pericam mt	Ratiometric	400 (Ca^{2+} free) 490 (Ca^{2+} bound)	514	(11)	Targeted to the mitochondrial matrix	[38,45]
2mt8RP				(2)	Targeted to the mitochondrial matrix	[38]
R-Pericam nu				(2.5)	Targeted to nucleus	[45]
Inverse pericam	Fluorescence intensitiy	490	514	0.2 (0.9)		[51,183]
GCaMP1.6	Fluorescence intensity	490	514	1.23 (0.64)		[184,185]
GCaMP2	Fluorescence intensity	490	514	n.d. (0.75)		[185]
Cameleons	Ratiometric (FRET changes)	430	460–500 (CFP) 525–580 (YFP)	Citrine / cpv[e] D1: 0.8, 60 / – D2: 0.8; 26/ 0.03, 3 D3: 1.2 / 0.6 (0.49) D4: 195/ 64		[60,185]
Tn-L15	Ratiometric (FRET changes)	430	460–500 (CFP) 525–2580 (YFP)	0.72(0.36) M		[61,185]
Tn-XL	Ratiometric (FRET changes)	430	460–500 (CFP) 525–2580 (YFP)	2.5 (0,77) M		[62,185]
Tn-XXL	Ratiometric (FRET changes)	430	460–500 (CFP) 525–580 (YFP)	0.8 (reduced *in situ*)		[61–63]

[a] The wavelength most often employed for excitation.
[b] The wavelength most often employed for emission.
[c] Reported in vitro affinity for Ca^{2+} and, when available, the corresponding apparent K_d determined *in situ* are given between brackets. Please note that the K_d may change from *in vitro* to *in situ* conditions and also between different compartments.
[d] NST: not selectively targetable. Targeting of esterases has been recently proposed to obtain selective accumulation of these dyes into specific organelles.
[e] For these probes, the different *in vitro* affinities of the variants with citrine or circular permuted venus (cpv) are given for the 4 cytosolic designs.

probes are: (i) the fact that upon photon emission aequorin is "consumed" and accordingly the amount of responsive probe decreases with time, the faster the higher the [Ca^{2+}] [37,46]; (ii) the low amount of photons emitted (less than 1 photon/molecule); and (iii) the necessity of a cofactor, coelenterazine, to reconstitute the active Ca^{2+} indicator.

In the jellyfish, GFP emits fluorescence after energy transfer from Ca^{2+} bound aequorin, in a process known as bioluminescence resonance energy transfer (BRET). BRET probes can be generated by fusing GFP and aequorin, and changes in [Ca^{2+}] result in modifications of GFP fluorescence intensity [47]. An advantage of this GECI over aequorin is that because GFP fluorescence is red shifted compared to the photons emitted by aequorin, absorption of light from tissues is less important and the quantum efficiency is slightly higher [48]. Indeed, GFP-aequorin has been used not only in cell cultures, but also *in vivo*, both in *Drosophila melanogaster* [49], and in mammals, where it has been successfully employed to monitor [Ca^{2+}] changes within cytoplasm and mitochondria in the free moving mice [50].

The GECIs based on GFP can be distinguished in probes containing one or two GFP variants fused to a Ca^{2+} sensor, usually calmodulin (CaM). A first group of such probes containing one GFP molecule (the prototype being "camgaroos") exhibits only a change in fluorescence intensity upon Ca^{2+} binding to the sensor. Another group, the prototype being "ratiometric pericams," consists of a circularly permuted YFP sandwiched between CaM and its interacting peptide from myosin light chain kinase, M13. Ca^{2+} binding to CaM results in change in the excitation spectrum of circularly permuted YFP and accordingly the [Ca^{2+}] can be directly obtained from the ratio between two excitation wavelengths [51]. In all these probes, GFP fluorescence is quite sensitive to pH changes [52,53]. A major problem in all the above-mentioned probes is that the Ca^{2+} sensors is represented by CaM, which is widely expressed in all cells and participates in a variety of signalling events. Therefore, concerns were raised on the possible interference of the probe with endogenous targets. Recently Zou and co-workers [54] generated a GECI similar to pericam in which only the CaM loop III and its flanking helices were inserted in the EGFP sequence, in the attempt to override the potential problem of interference with endogenous CaM-dependent reactions. Other solutions to this problem have been found, see below.

A third group of GFP-based GECIs exhibits a change in fluorescence resonance energy transfer (FRET) between two mutants of GFP upon Ca^{2+} binding to the sensor. In 1997, two such FRET-based probes were

independently generated. In one case, Romoser and co-workers [55] inserted the CaM interacting peptide M13 between a blue (FRET donor) and green (FRET acceptor) variants of GFP. In this probe FRET is maximal at low [Ca^{2+}] and energy transfer between the two GFP mutants is abolished when the Ca^{2+}–CaM complex binds to the M13 and increases the distance between the donor and acceptor molecules. The other sensor was generated in the group of Tsien and was named "cameleon." In this case, CaM and M13 were fused in tandem between the blue and green GFP mutants: binding of Ca^{2+} to CaM resulted in wrapping around of CaM to M13 and this led to an increase in FRET between the two GFPs [56]. Many improvements to the original cameleons were introduced in the following years to enhance the dynamic range of the FRET changes and to decrease the interference with medium composition. First, the blue and green GFP mutants were substituted by the more suitable FRET pair cyan and yellow fluorescent proteins (CFP and YFP). Given that YFP is quite sensible to pH, a new family of cameleons was generated in which YFP was substituted by the mutant "citrine" that is much less pH sensitive [57]. Finally, Miyawaki's group produced a novel YFP called Venus, that bears mutation causing a decrease in pH and [Cl^-] sensitivity and a faster maturation of the chromophore at 37 °C [58]. The use of these new YFPs and of their circularly permuted counterparts in cameleons substantially improved the dynamic range of the FRET changes upon Ca^{2+} binding [59]. Cameleons in which the Ca^{2+} binding sites of CaM were purposely mutated were also produced, resulting in a family of FRET based probes with vastly different Ca^{2+} affinities.

Finally, in an elegant work by Palmer and co-workers [60], interference from endogenous CaM was drastically decreased by engineering both CaM and M13. In particular, by means of computational analysis of the binding interface between CaM and M13, new peptide pairs aimed at abolishing the interaction between the M13 with endogenous CaM were generated by mutagenesis. Probes with different Ca^{2+} affinities were also generated by this approach. Thus, new cameleons bearing both citrine (D1, D2, D3, D4) or circularly permuted Venus (D-cpv) with sensitivities covering a 100 fold range of [Ca^{2+}] (K_ds between 0.6 and 60 μM) were produced, all highly insensitive to endogenous CaM. For example, D1 is suitable to measure [Ca^{2+}] ranging from 1 μM up to 300 μM, whereas D2cpv would be more indicated to detect modest elevations above basal Ca^{2+} levels and D4cpv is ideal for subcellular compartments with very high [Ca^{2+}] [60].

A different approach to avoid interference by endogenous CaM was also devised by Heim and co-workers, who chose as Ca^{2+} sensor murine troponin-C from skeletal muscle, the rationale being that troponin-C is specifically expressed in skeletal muscle and should not have endogenous binding proteins in other cell types. The first generation of these probes (Tn-L5) was later improved by molecular rearrangement of the Ca^{2+} sensor portion, thus reducing the interference of Mg^{2+} while maintaining a good FRET performance with a K_d for Ca^{2+} of 800 nM. The improved probe, termed Tn-XXL was successfully expressed in hippocampal organotypic slices and also *in vivo*. The authors performed Tn-XXL electroporation *in utero* or virus injection in adult animals and were able to perform single cell measurements both in cell cultures and *in vivo*. Noteworthy, the probe expressed *in vivo* via viral infection was stable over time and repeated experiments could be performed through a cranial window several weeks after the initial injection of the viral vector [61–63].

As pointed out previously, GECIs (and therefore cameleons) can be efficiently localized to subcellular compartments by fusion with specific targeting sequences [35,60,64]. However, as far as targeting of cameleons to the mitochondrial matrix is concerned, the initial results were disappointing. Introduction at the N-terminal end of cytochrome *c* oxidase subunit VIII (COX-VIII) targeting sequence, that is very efficient in localizing camgaroos and pericams to the mitochondrial matrix, resulted in very poor localization of the cameleons in the mitochondria, while the majority of the probe was mislocalized to the cytosol. The problem was largely overcome by inserting multiple targeting sequences in tandem at the N-terminus of the cameleon; in cells transfected with cameleon containing 8 repeats of COX-VIII almost no mislocalization of the probe was observed, but at the expense of affecting mitochondrial shape and viability; 2 and 4 repeats appear to be the best compromise between localization and toxicity [38,60].

FRET-based sensors are ideally suited for single and multi-photon confocal microscopy, given that most commercial apparatuses are routinely equipped with the possibility of monitoring contemporarily two or more emitted wavelengths while alternating two exciting laser beams is technically and economically highly demanding [65,66].

Several strategies are available to deliver the probes to cells, tissues, and organisms, from transfection techniques to viral constructs. Cameleons have been efficiently delivered to cultured cells and *in vivo* expression has been obtained by either the use of viruses or *in utero* electroporation [67]. Recently, transgenic animals with cameleon expressed under the control of a tissue/cell specific promoter [68] or under TET control [69] have also been generated.

3. Microdomains: the mitochondria–ER connection

The central role of mitochondria in cellular Ca^{2+} homeostasis is now an established concept in cell biology. Ca^{2+} entry into the mitochondrial matrix is mediated by an inwardly rectifying ion channel, the mCU [70]. Recently, it has been suggested that more than one mCU may be expressed by mitochondria [71,72] and an alternative Ca^{2+} influx pathway (a Ca^{2+}/H^+ antiport) has been proposed by Jiang and coworkers [16]. Ca^{2+} entry through the mCU is driven by the membrane potential (negative inside; $\Delta\Psi_m$) that exists across the IMM. Since $[Ca^{2+}]_c$ in resting condition is around 100 nM and the $\Delta\Psi_m$ due to respiratory chain activity is ~ −180 mV, the prediction is that at electrochemical equilibrium, $[Ca^{2+}]_m$ could reach values as high as 0.1 M [73]. However, this value is never reached due to the existence of efflux mechanisms that couple Ca^{2+} extrusion to H^+ or Na^+ entry down their electrochemical gradient (see below). Thus, the steady state Ca^{2+} level in the mitochondrial matrix is far from electrochemical equilibrium due to a futile cycle of Ca^{2+} entry and exit across the IMM. The energy cost of this cycle is relatively modest, since the mCU has a low Ca^{2+} affinity (K_d around 10–20 μM) [74] and thus the Ca^{2+} uptake rate, particularly under resting conditions, is extremely slow. Based on this consideration, it was long assumed that mitochondrial Ca^{2+} uptake was of little importance in cell physiology since $[Ca^{2+}]_c$, even upon stimulation, reaches values far below the [Ca^{2+}] required for strong mCU activation. Therefore, mitochondrial Ca^{2+} uptake was regarded as a safeguard mechanism put in action when pathological elevations of Ca^{2+} occur. This idea was initially challenged by the demonstration that mitochondrial oxidative metabolism was dependent on the matrix [Ca^{2+}], due to the Ca^{2+} dependency of three matrix dehydrogenases (for a recent review see [22]). The breakthrough in our understanding of the importance of mitochondrial Ca^{2+} uptake in physiology, however, was the demonstration (using genetically encoded Ca^{2+} probes targeted to the mitochondrial matrix) that $[Ca^{2+}]_m$ could reach values up to tens or hundreds micromolar upon $[Ca^{2+}]_c$ rises of a few micromolar [43,75]. In order to reconcile *in vitro* experiments that unambiguously proved the low Ca^{2+} affinity of mCU with the data obtained by measurement of $[Ca^{2+}]_m$ in intact cells, the so-called "microdomain hypothesis" was proposed [43,44]. According to this hypothesis, the fast mitochondrial Ca^{2+} uptake in intact cells depends on the close apposition between mitochondria and the sites of Ca^{2+} release/influx. In other words, the mCU in intact living cells can locally experience a [Ca^{2+}] that is much higher than that measured in the bulk cytosol, i.e., high enough to ensure a strong activation of the uptake mechanism. The hypothesis was supported by a large body of indirect evidence, though the existence and amplitude of high Ca^{2+} microdomains on the surface of mitochondria was demonstrated directly only very recently (see below Section 3.1).

3.1. The molecular machinery of Ca^{2+} uptake and release of mitochondria

Despite intense investigations on the mCU for almost 50 years, the molecular identity of this gated channel is still a matter of debate. Recently, Graier and co-workers [17] proposed that uncoupling proteins UCP2 and 3 are fundamental components of the mCU. In particular, they showed that over-expression of these proteins causes an increase, while their silencing or mutagenesis cause a decrease, in mitochondrial Ca^{2+} accumulation. Moreover, mitochondria isolated from liver of UCP2$^{-/-}$ mice did not show a Ruthenium Red (RR)-sensitive Ca^{2+} uptake. However, mitochondria from UCP2 or 3 expressing cells of *Saccharomyces cerevisiae* failed to show a RR-sensitive Ca^{2+} uptake, thus highlighting that these proteins alone cannot constitute the mCU. On the other hand, these findings have been challenged by measuring Ca^{2+} uptake in heart and liver mitochondria [76]. This latter group showed that, notwithstanding the higher expression of UCPs in heart, there is no significant difference in mitochondrial Ca^{2+} uptake rate between heart and liver and, most importantly, no effect of UCPs inhibitors on Ca^{2+} uptake by mitochondria was observed. Moreover, in conflict with Graier's group data, no Ca^{2+} uptake variation was measured in mitochondria from neither UCP2$^{-/-}$ nor UCP3$^{-/-}$ mice. The effect on mitochondrial Ca^{2+} entry upon UCPs silencing or over-expression in HeLa cells was not reproduced by several groups (see for example [16]).

Other candidates to play the role of mCU have been proposed in the last years, in particular a mitochondrial Ryanodine receptor (RyR), that was identified and characterized only in heart mitochondria [77,78]. How a protein endowed with a specific targeting sequence for the ER/SR membrane could end up into the inner mitochondrial membrane remains mysterious. The possibility that this RyR in the heart mitochondrial fraction represents a contamination from the bona fide RyR of the SR has not been completely excluded.

In the last months major interest has received the report that the IMM protein Letm1 could be a Ca^{2+}/H^+ antiporter, catalyzing the uptake of Ca^{2+} into mitochondria. This protein was identified as a possible player in mitochondrial [Ca^{2+}] homeostasis by a genome-wide RNAi high-throughput screening in Drosophila S2 cells stably expressing the mitochondrial targeted Ca^{2+} and H^+ probe Pericam [16]. Letm1 was firstly characterized as a gene involved in Wolf–Hirschhorn syndrome, a disease due to hemizygous deletion of a set of genes on chromosome 4 [79]. In particular, lack of Letm1 is thought to be the cause of the seizures that characterize this syndrome [80]. Up until the report of Jiang and co-workers, Letm1 (and its yeast homolog, Mdm38) was believed to be the mitochondrial H^+/K^+ antiport. This conclusion is supported by a number of strong data in yeast, mammals and Drosophila [81–83]. In their recent paper, Jiang and co-workers, on the contrary, demonstrate in intact and permeabilized cells that Letm1 down-regulation reduces mitochondrial Ca^{2+} increases (at low [Ca^{2+}]) and that its over-expression enhances the organelle Ca^{2+} uptake. Moreover, they showed that partially purified Letm1 can recreate a RR- sensitive Ca^{2+} uptake when reconstituted in liposomes. Surprisingly Jiang and co-workers concluded that Letm1 is not the mCU or one of its key components, but rather an additional Ca^{2+} uptake mechanism, a $1Ca^{2+}/1H^+$ antiport. This latter conclusion is particularly surprising, given that it has been firmly established that Ca^{2+} uptake into the mitochondria results in the net transfer of 2 positive charges across the membrane. Our biased opinion is that, as hypothesized by McQuibban and colleagues [83], the effect on mitochondrial Ca^{2+} transport of Letm1 down regulation/over-expression is a secondary effect of a decreased K^+/H^+ exchange activity.

Several reports have been published in the last few years describing the modulation of mCU activity by protein kinases: PKD [84] and PKC isoform β/δ have been reported to inhibit it, while PKC isoform ζ activates it [85]. Studies with the p38 MAPK inhibitor SB202190 suggested that also this kinase can inhibit mCU (but see [86] for an alternative explanation). Additional evidence in support of a role of p38 MAPK has been reported by the group of Spat [87]. A major problem in interpreting these results is that the mCU is believed to be a protein of the IMM, thus insulated from the catalytic activity of cytosolic kinases such as p38, PKC and PKD by the protein-impermeable outer mitochondrial membrane.

There is also some evidence that the mCU can be slowly modulated by Ca^{2+} itself. For example a few minutes pre-incubation in Ca^{2+} of de-energized mitochondria results in a substantial activation of Ca^{2+} uptake upon providing an energy source [88]. Moreau and colleagues [89] suggested that this process of activation needs a Ca^{2+}–CaM intermediate, since use of CaM inhibitors impair mCU activation. Given the well known side effects of most CaM inhibitors, this latter conclusion must be taken with caution.

Finally a word of warning as to the use, in living cells, of RR and its purified form Ru360 as an inhibitor of mCU. RR can bind many proteins with different affinities (e.g., it is a good inhibitor of the RyR) and given that these compound possesses six positive charges it should not be easily permeable across the PM. Although several reports have been published reporting indirect evidence suggesting that extracellularly added RR or Ru360 can inhibit mitochondrial Ca^{2+} uptake of intact cells, in our hands addition of RR or Ru360 to intact cells has never resulted in a specific inhibition of mitochondrial Ca^{2+} uptake.

As to the efflux mechanisms, Ca^{2+} can be extruded from the matrix by Na^+-dependent or independent mechanisms. The Na^+-dependent mechanism exchanges $1Ca^{2+}$ with $3Na^+$ and thus is electrogenic, similarly to the PM isoform [90]. This antiport is mainly found in excitable tissues, e.g., brain and heart, and it is inhibited by CGP37157 [91]. Recently, strong evidence has been provided that the Na^+/Ca^{2+} exchanger isoform NCLX is the long-sought protein responsible for the mitochondrial Na^+-dependent Ca^{2+} efflux [18]. The Na^+-independent mechanism couples the extrusion of 1 Ca^{2+} to the entry of 2(3) H^+ and it is predominant in non-excitable cells [92]. The low conductance mode of the mitochondrial megachannel or permeability transition pore (PTP) can be also considered as a non-saturating mechanism for Ca^{2+} efflux from mitochondria. When open, PTP allows the passage of ions and molecules up to 1.5 kDa molecular weight, including Ca^{2+}. PTP opening is caused by conditions of mitochondrial Ca^{2+} overload, especially when these occur in concurrence with oxidative stress, high levels of phosphate and adenine nucleotide depletion (for a recent review see [25,93]. Upon PTP opening and thus mitochondrial $\Delta\Psi$ loss, Ca^{2+} can exit also through the mCU.

As described above, the "Ca^{2+} microdomain" hypothesis explains the paradox of a low Ca^{2+} affinity mCU and a highly efficient mitochondrial Ca^{2+} uptake under physiological conditions, by assuming that a mitochondrial subpopulation is positioned in very close proximity to Ca^{2+} release sites from ER or Ca^{2+} entry sites from PM where [Ca^{2+}] can reach values higher than those in the bulk cytosol. Since the formulation of the hypothesis, many data supporting it have been reported. For example the existence of mitochondria–ER close appositions was demonstrated by high resolution microscopic analysis of living cells expressing mitochondrial and ER-targeted GFP mutants; electron microscopy analysis of quickly frozen samples has also confirmed that mitochondria are often closely apposed to either the ER or the PM [94]; through an aequorin targeted to the intermembrane space of mitochondria, a [Ca^{2+}] slightly higher than the cytosolic one was measured upon cellular stimulation [44]. Based on these and other findings, it has been suggested that mitochondria can be divided into two populations, one located in close contact to Ca^{2+} release sites and thus rapidly accumulating Ca^{2+} upon cellular stimulation, one that experiences smaller Ca^{2+} levels, close to that of the bulk cytosol [45], and takes up Ca^{2+} much more slowly. The proximity of mitochondria to Ca^{2+} release sites is also supported by the demonstration that a physical linker between the two organelles exists [95] and also by the identification of proteins that can act as tethers, like grp75, a chaperone linking VDAC1 and IP_3R [96], and mitofusin-2, that is expressed in ER and mitochondria surfaces and forms homodimers or heterodimers with mitofusin-1 [97,98]. Moreover, an ER–mitochondria tethering

complex made up of 4 different proteins was also recently identified in *Saccharomyces cerevisiae* by an elegant screen based on a synthetic protein complementing for mitochondria–ER tethering defects [99]. However, despite the fact that the "Ca^{2+} microdomain" hypothesis was taken for granted by most investigators thanks to the indirect experimental evidence mentioned above, a direct proof of Ca^{2+} microdomains existence on mitochondrial surface was reached only very recently by our group [100]. In order to monitor in the very same cell OMM and bulk cytosolic Ca^{2+} variations upon cellular stimulation a nuclear and a OMM new generation FRET-based Ca^{2+} sensors ([60], see section 2) were generated. In cells co-transfected with OMM and nuclear targeted Ca^{2+} probes (challenged with an IP_3 generating stimulus) we found that the mean Ca^{2+} rise in the two compartments appears almost indistinguishable (Fig. 1A). However, a more sophisticated statistical analysis of the images reveals the existence of small OMM, but not nuclear, regions whose [Ca^{2+}] reaches values as high as 15–20 μM (Fig. 1B). An estimation made on the first 4 s after the stimulation shows that at least 10% of OMM surface is covered by these hot spots during the transient increase in cytosolic Ca^{2+} elicited by IP_3 production. Classical epifluorescence and TIRF experiments were then combined in order to monitor the generation of high Ca^{2+} microdomains in mitochondria located near plasma membrane. By this approach we could show that Ca^{2+} hot spots on the surface of mitochondria occur upon opening of voltage operated calcium channels (VOCCs), but not upon capacitative Ca^{2+} entry (CCE). From these data and also from those of Korzeniowski and colleagues [101], it can be concluded that upon CCE activation the close apposition between Orai1 channels (located in the PM; [102]) and STIM1 (located in ER "punctae" near PM; [103]) does not allow mitochondria to be in sufficient proximity to the mouth of the PM channels to sense the local [Ca^{2+}] hot spots. The main mechanisms participating in mitochondrial Ca^{2+} homeostasis and Ca^{2+} microdomains generation are summarized in Fig. 2.

4. Mitochondrial Ca^{2+} handling and cell death

In the last decade, it has become clear that mitochondria, in addition to their role as the cell powerhouse, have a key role in several other processes of major importance in cell physiology and pathology [25,26,104,105]. Notably, great attention has been paid to the role of mitochondria in cell death. Indeed, although the extrinsic pathway for apoptosis may or may not involve mitochondria, in the intrinsic pathway these organelles perform a pivotal role: they release a number of proapototic factors from the IMS that initiate the executor caspase cascade (for review see [26,106]).

A main mechanism leading to the release of proapototic factors in the cytosol is the formation of the "permeability transition pore," also known as mitochondrial megachannel (see also section 3.1). PTP has been characterized functionally in a number of cell types. Remarkably, its opening is favoured by excessive Ca^{2+} accumulation by mitochondria (Ca^{2+} overload). The protein composition of PTP is still under debate, although there is evidence that several OMM, IMS and IMM proteins are involved in its regulation, namely: adenine nucleotide translocase, hexokinase, peripheral-type benzodiazepine receptor, voltage dependent anion channel, mitochondrial creatine kinase and cyclophilin D (Cyp D) [26]. Surprisingly, in cells from knock out (KO) mice of many of these proteins, PTP opening appears normal, with the notable exception of cells from Cyp D KO mice. Genetic ablation of Cyp D, in fact, leads to a delayed Cyclosporin A-insensitive PTP activation in response to Ca^{2+} overload. PTP opening triggered by stimuli other than Ca^{2+} overload, however, is similar in wild type and Cyp D KO

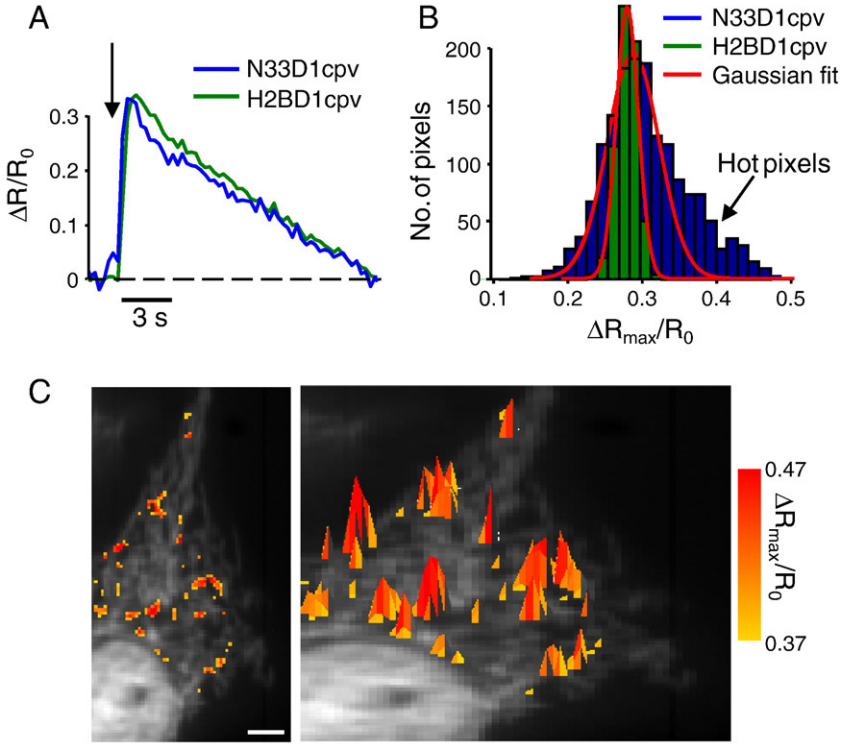

Fig. 1. A pixel by pixel correlation analysis uncovers Ca^{2+} microdomains on OMM upon cellular stimulation. HeLa cells co-transfected with nuclear-(H2BD1cpv) and OMM-(N33D1cpv) targeted cameleons were treated with the IP_3-coupled stimulus histamine (arrow in A). (A) The average [Ca^{2+}] rise (here expressed as $\Delta R/R_0$) of nucleus (green trace) and OMM (blue trace) are very similar. R is the ratio between YFP and CFP emission, R_0 is the ratio at $t=0$ and ΔR the difference between R at any given time and R_0. (B) The maximum $\Delta R/R_0$ value ($\Delta R_{max}/R_0$) reached by each single pixel of the nucleus (green) or OMM (blue) during the first 4 s after histamine stimulation is expressed as a histogram of numerousness. The Gaussian fit (red trace) of the distribution is superimposed to highlight the right tail due to hot-spot formation on OMM upon cellular stimulation (see [100] for details). (C) Yellow-to-red color representation of $\Delta R_{max}/R_0$ spatial distribution of pixels in 2D (left) and 3D (right), superimposed to YFP fluorescence image. Only pixels that during the 4 s after the histamine challenge have a $\Delta R_{max}/R_0$ exceeding 125% the $\Delta R/R_0$ of the whole compartment were color-coded. Scale bar, 5 μm.

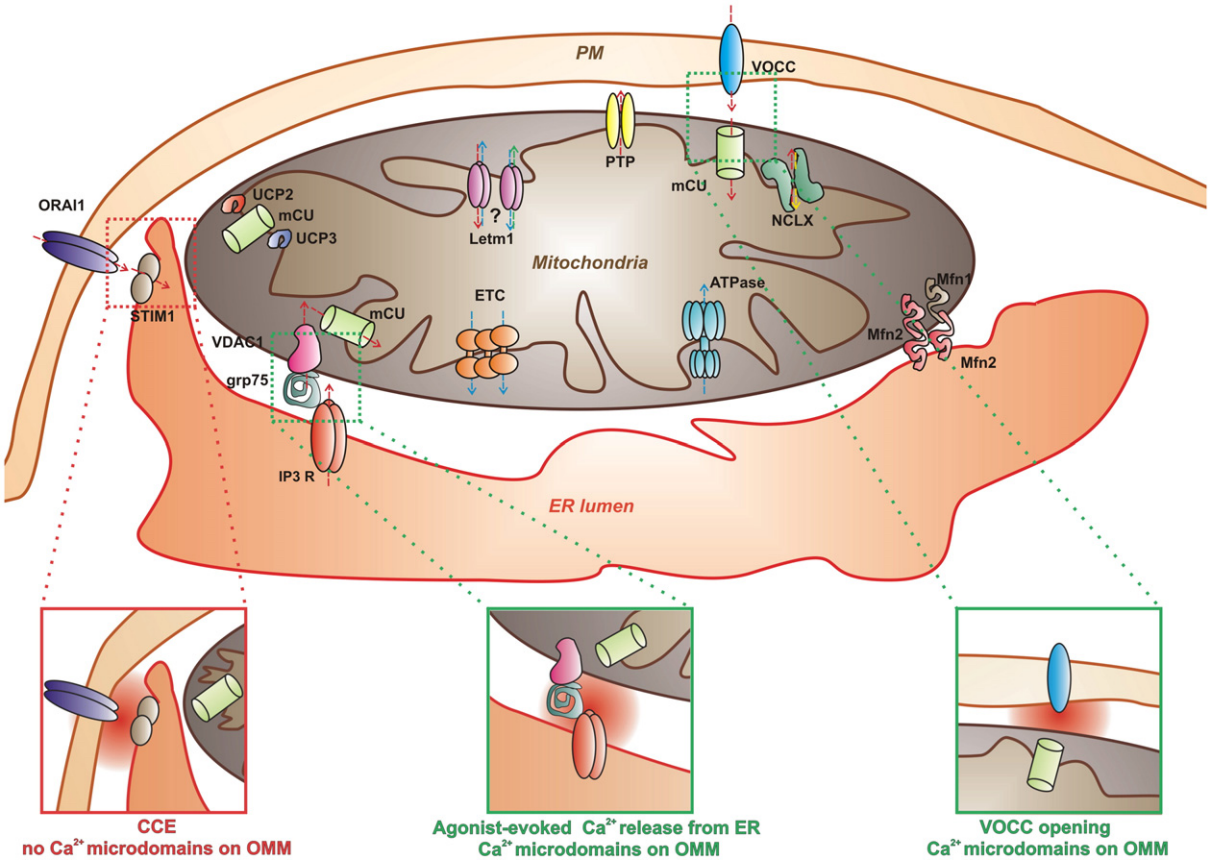

Fig. 2. Mitochondrial Ca^{2+} toolbox. Arrows indicate ion fluxes. Red arrows, Ca^{2+}; blue arrows, H^+; yellow arrow, Na^+; green arrow, K^+. ETC, electron transport chain. In the case of Letm1, both hypotheses on its function are shown. The insets represent the main mechanisms causing a $[Ca^{2+}]_m$ rise: the color of the square shaping each inset indicates if that $[Ca^{2+}]_m$ rise occurs without (green) or with (red) generation of Ca^{2+} microdomains on the OMM. See text for details.

cells, suggesting that Cyp D plays a regulatory and not a structural role in this megachannel. [107–110].

Apoptosis is modulated by a number of proteins of the Bcl-2 family, among which the classical proapototic proteins Bax and Bak and the antiapoptotic Bcl-2 and Bcl-X_L. These proteins reside in the ER, cytosol and mitochondria as homo o heterodimers. As mentioned above, during apoptosis permeabilization of the OMM occurs with release from IMS of cytochrome c and other proapoptotic factors. Of interest, proapoptotic proteins such as Bcl-2 affect ER–mitochondrial Ca^{2+} crosstalk, as the over-expression of Bcl-2 reduces the Ca^{2+} content of the ER [111] making the cells resistant to apoptosis. Similarly, genetic ablation of the proapoptotic proteins Bax and Bak (that drastically increases the resistance to death signals) also results in a dramatic reduction in ER Ca^{2+} content (and consequently in a reduction of the Ca^{2+} that can be transferred to mitochondria) [112]. Moreover, several different approaches resulting in decreases of ER Ca^{2+} content protect cells from apoptosis while, vice versa, an increase in Ca^{2+} within the ER favours apoptosis triggered by a number of stimuli [113].

As to the mechanisms leading to OMM permeabilization a pivotal role is played by Bid and by the PTP [25,114]. While Bid- (and other BH3-only proapoptotic proteins) dependent OMM permeabilization is insensitive to mitochondrial Ca^{2+}, in many other situations the apoptotic cascade (and OMM permeability) somehow relies on mitochondrial Ca^{2+} overload. The concept of mitochondrial Ca^{2+} overload, however, does not necessarily refer solely to a very large increase in $[Ca^{2+}]_m$. In fact very large, but short lasting, increases in matrix Ca^{2+} may occur under several physiological conditions without detrimental consequences for cell survival; rather, much smaller, but prolonged, increases of Ca^{2+} may activate the apoptotic machinery. Finally, most relevant for physiopathology, mitochondria can function as coincidence detectors for the apoptotic process, i.e. if a rise of Ca^{2+} not toxic in itself (and actually beneficial because it increases the efficiency of ATP synthesis) occurs synchronously with another toxic event, it may synergize with this insult and turn a beneficial process into a death stimulus [115,116].

Mitochondria are thus emerging as crucial players in the pathology of many different diseases, both as primary or secondary executioners. Indeed, apart from the primary cause, that is frequently far away from mitochondria and even in the extracellular matrix (see below), many diseases show as final step a general Ca^{2+} deregulation that in turn causes mitochondrial Ca^{2+} overload and cell death through PTP opening and mitochondria swelling. For instance, Gandhi and co-workers found impaired Ca^{2+} efflux from mitochondria through the Na^+/Ca^{2+} exchanger in neurons lacking PINK1, a serine threonine kinase implicated in autosomal recessive early-onset parkinsonism. This led to increased Ca^{2+} uptake capacity, decreased membrane potential, and increased ROS production, all leading to early triggering of the PTP opening and concomitant neuronal death [117]. Blockade of this final process, no matter the original cause, is thus emerging as an effective therapeutic strategy also *in vivo*. Indeed, genetic ablation or pharmacological inhibition of Cyp D, a main regulator of the PTP, has been shown to decrease the mitochondrial alterations and ameliorate the pathology both in the case of Collagen VI deficiency and in the Scgd$^{-/-}$ mouse, a model for severe dystrophia [118,119]. In the following section, we will review in more detail the relationship between altered Ca^{2+} homeostasis and mitochondria in Alzheimer's disease.

4.1. Alzheimer's disease

AD is the most common human neurodegenerative disease, currently affecting more than 20 million people worldwide. Although AD cases are mostly sporadic, a small percentage (presumably less than

1%) is dominantly inherited. Three genes responsible for the Familial Alzheimer's Disease (FAD) were identified in the early 1990s: current statistics show that approximately 80% of FAD cases are due to mutations in the presenilin-1 (PSEN-1) gene (with the most aggressive phenotype and the earliest onset), 14% to mutations in the Amyloid-Precursor Protein (APP) gene, and only 6% to mutations in the presenilin-2 (PSEN-2) gene. Albeit accounting only for a minor percentage of the total AD cases, FAD-mutations in these three genes provided useful tools and models to investigate the mechanisms behind the pathology. Indeed, sporadic and familial AD have indistinguishable histopathological phenotypes, including the two AD hallmarks, i.e., extracellular amyloid plaques, composed by aggregates of amyloid β (Aβ) peptides derived from APP cleavage (see below), and intracellular neurofibrillary tangles, formed by filaments of hyperphosphorylated tau protein [120–122].

APP is initially cleaved by two mutually exclusive enzymes, α- or β-secretase, and subsequently by γ-secretase. Presenilin-1 and presenilin-2 (PS1 and PS2) are the catalytic core of the γ-secretase complex and the pathway that implies β- and γ-cleavage is called "amyloidogenic". Indeed the cleavage of APP by β and γ-secretase leads to the generation of multiple Aβ peptides, mostly Aβ40 and Aβ42, the latter being the most noxious and aggregation-prone [123,124]. Although initially PS-FAD mutations were reported to increase Aβ peptides generation, especially of the toxic Aβ42 species, in the last few years several data drastically changed this view suggesting a total decreased γ-secretase activity in the presence of PS-FAD mutants. Mutations would impair the cleavage efficiency of PSs both reducing the total Aβ production and promoting less precise cuts of APP stub with a major generation of Aβ42 and thus a shift in Aβ42/Aβ40 ratio towards the more toxic and amyloidogenic species (as reviewed in [125] and in [126]). It should be also mentioned here that, although FAD is a central nervous system pathology, APP and PSs are ubiquitously expressed. Indeed, PSs play a key role in processes other than APP cleavage, e.g., in the Notch signalling pathway, cell adhesion, intracellular trafficking regulation, GSK3β modulation, and Ca^{2+} signalling (see [127,128]).

It is noteworthy that alterations in Ca^{2+} homeostasis in sporadic AD patients started being reported in the middle of the 1980s, albeit in contrasting ways [129–131]. Moreover, the link between AD and Ca^{2+} dysregulation became more evident with studies on FAD mutations, especially in PSs, that have revealed strong–and controversial–effects on Ca^{2+} signalling (for recent reviews see [132–134].

The first hypothesis on FAD-linked Ca^{2+} dysregulation is the so-called Ca^{2+} overload hypothesis, stating that FAD-PSs should lead to an increased Ca^{2+} content in cellular stores and consequently to an exaggerated Ca^{2+} release upon stimulation [135–138]. A major support to the Ca^{2+} overload hypothesis came from the demonstration that PSs can form low conductance Ca^{2+}-leak channels in the ER membrane essential in the maintenance of a physiological $[Ca^{2+}]$ inside ER; the permeability of the channels to ions was reported to be impaired by FAD mutations, thus leading to an increase in $[Ca^{2+}]_{ER}$ and to a bigger cytosolic Ca^{2+} increase upon stimulation [139,140].

An alternative model is that proposed recently by Cheung and co-workers [141]. These authors have shown that PSs (and especially FAD-linked PSs mutants) positively modulate IP_3Rs, sensitizing the receptors to low concentration of IP_3. According to this model, the final effect of mutated PSs is to augment Ca^{2+} release from the ER at low, physiological, agonist concentrations [141]. Notably, Cheung et al. reported that FAD-PSs cause a slight, but measurable decrease in $[Ca^{2+}]_{ER}$ [141]. Increase opening probability of IP_3R (at low IP_3 concentrations) has been very recently demonstrated also in B lymphoblast from FAD patients by nuclear patch clamp electrophysiology [142]. Hypersensitivity of the IP_3 signalling pathway in cells from FAD models was also suggested by previous works in intact neurons [143], and evidence for abnormal hyperactivity and/or increased expression of the other ER Ca^{2+}-releasing channel, the RyR, has been also reported [144–147]. In addition, Carafoli's group reported an interaction between PS2 and the Ca^{2+}-binding protein DREAM, leading to reduced ER Ca^{2+} levels by acting on IP_3R expression [148].

A third group of laboratories, including our own, have found that PSs (and more effectively FAD-PS2 expression) substantially reduce $[Ca^{2+}]_{ER}$. For example, we showed that in human fibroblasts from FAD patients, as well as in primary mouse cortical neurons over-expressing FAD-PS1/2, there is a clear reduction of Ca^{2+} release from intracellular stores when compared to controls. Moreover, by employing ER- and Golgi-targeted aequorins to directly evaluate the $[Ca^{2+}]$ within these compartments, it was clearly shown that in PS2- and, to a lower extent, in PS1-transfected cells there is a reduction in $[Ca^{2+}]$ within the stores [149–151]. Consistent with these findings, recent data demonstrated a Ca^{2+} deficit in neurons from *D. melanogaster* expressing FAD-PS1/2 compared to control neurons, an effect that seems to require CaM [152]. Last, but not least, while Tu and colleagues [139] reported that MEF cells knock down for PS1 and 2 have an increased $[Ca^{2+}]_{ER}$, Kasri et al. [153] reported, using the same cells, a reduced Ca^{2+} content within the stores, and data from our lab showed no significant difference between wild type and PS1/PS2 KO cells in terms of $[Ca^{2+}]_{ER}$ [154]. The reduced level of $[Ca^{2+}]$ in the stores of FAD-PSs over-expressing cells has been attributed to an increased Ca^{2+} leak through IP_3Rs and/or RyRs (thus in agreement with the results of [141]) and to a reduction in the SERCA activity (see below) [154].

Indeed, the SERCA has been proposed to be a major target of PSs: La Ferla and colleagues [155], based on a series of indirect data, suggested that the SERCA is regulated by PSs (in particular PS2) and hyper-activated by FAD-PS1. Opposite results were obtained however considering PS2 FAD mutants and directly measuring ER Ca^{2+} uptake with ER-targeted aequorin [154]: (FAD)-PS2 over-expression results in a strong inhibition of SERCA activity and appears to be a major component of the PS2 dependent reduction of $[Ca^{2+}]_{ER}$.

Given the contradictory experimental findings reported above, it is difficult to draw a firm and unbiased conclusion about the role of PSs on ER Ca^{2+} homeostasis. On the one hand, the groups of Bezprozvanny and La Ferla have provided strong evidence, though often indirect, supporting the hypothesis that FAD linked mutations lead to ER Ca^{2+} overload, due to a reduced leak and/or stimulation of the SERCA. On the other hand, our group and that of Foskett, by direct investigation of the activity of the channels, of the Ca^{2+} pumps, and of the $[Ca^{2+}]$ within the lumen of organelles, have generated data showing that mutated PSs tend to reduce the Ca^{2+} level in the ER, either by increasing the sensitivity to IP_3 or by inhibiting the activity of the SERCA, or a mixture of both. The reason for these experimental discrepancies remains at present unknown and is particularly intriguing since, in many cases, the exact same cell types were used by the different groups. Most relevant (and again unlike the results obtained by the groups of Bezprozvanny and La Ferla), our data indicate that, in terms of Ca^{2+} handling by the ER, there is a major difference between PS2 and PS1 (wild type and FAD linked), with PS1 having a minor, almost negligible effect on ER Ca^{2+} levels. These striking and unexpected differences between PS1 and PS2 on ER Ca^{2+} handling have recently been independently confirmed by Carafoli's group (E. Carafoli's personal communication).

The scenario becomes even more complex when considering the effects of Aβ peptides. These have been proposed to affect cellular Ca^{2+} homeostasis in many different (and also controversial) ways. A detailed discussion of this topic is beyond the scope of this review but the reader is referred to recent literature on the matter [132,133,156]). We should mention, however, some recent reports that address specific aspects of this issue: altered Ca^{2+} regulation was found in spines and dendrites in neurons proximal to Aβ deposits [157]; Aβ oligomers were reported to cause Ca^{2+} entry in neurons (and consequent mitochondrial Ca^{2+} overload) [158], as well as toxic activation of ionotropic glutamate

receptor [159]; inhibitory effects of Aβ peptides on PMCA have also been described [160]. Moreover, massive Ca^{2+} transfer between ER and mitochondria has been reported upon exposure of cultured cortical neurons to Aβ (and prion protein) [161].

An open problem in the field of AD and Ca^{2+} dys-homeostasis that has been only superficially investigated thus far is mitochondrial Ca^{2+} handling. As discussed above, any defect in cytosolic Ca^{2+} release/clearance inevitably impinges on mitochondria and affects their activity [5]. The above-mentioned AD-linked alteration in Ca^{2+} signalling should be considered also from the mitochondrial perspective, especially because mitochondrial deficits have already been reported in most neurodegenerative diseases [162] and more specifically in AD [163,164]. Alteration in mitochondrial morphology and/or distribution has been found in neurons from brains of AD patients [165,166] and as a consequence of Tau (both in its wild type and mutated forms, [167,168]; APP [169] and FAD-Presenilin-1 [170] expression in different experimental models, and also in an Aβ42-over-expressing *D. melanogaster* [171]. In addition, FAD PS1 has been reported to exacerbate the mitochondrial toxicity of several stimuli [172,173] and mitochondrial deficits appear to be early events in the development of AD [174]. Of particular interest on this topic is the recent report that PSs are enriched in ER Mitochondria-Attached Membranes (MAMs) [175]; i.e. domains of the ER that closely and preferentially interact with mitochondria and are endowed with key players for Ca^{2+} handling [176–178].

We have recently focussed our attention on this aspect and we have found that PS2 over-expression increases the interaction between ER and mitochondria, an effect that is greater for FAD variants; as a consequence, a favoured Ca^{2+} transfer between ER and mitochondria can be measured upon over-expression of (FAD)-PS2 in cell lines. The pathophysiological consequences of this new and unexpected action of PS2 are still under investigation, but it is possible to speculate that this favoured interaction could potentially result in a toxic mitochondrial Ca^{2+} overload (see [95]) or, alternatively, in a compensatory phenomenon ensuring proper Ca^{2+} signalling to mitochondria in the presence of a reduced $[Ca^{2+}]_{ER}$ (see [154]); on the other hand, an enhanced recruitment of mitochondria close to ER could be also a consequence of a dysfunction in Ca^{2+} handling by the store, since mitochondria dynamics are known to be tightly regulated by Ca^{2+} (as reviewed in [179]).

5. Conclusions

Fifty years of research on mitochondria and their role in Ca^{2+} homeostasis have clarified the main players of this process and the principal functions for which they are responsible, ranging, as discussed in this review, from the regulation of cell's energy supply to the control of the intrinsic pathway of apoptosis. However, mitochondria and their Ca^{2+} handling machinery are still extremely intriguing and continue to tease investigators towards a deeper comprehension of their multifaceted nature. The tremendous improvements achieved in the measurement of intracellular Ca^{2+}, mainly due to the optimization of GECIs, have significantly contributed to the advancements in these studies. The use of these GECIs *in vivo*, although still complex and technically demanding, seems to be particularly promising for the future of this field, opening completely new insights in the investigation of Ca^{2+} dynamics in physiology and also in the numerous diseases where cellular (and in particular mitochondrial) Ca^{2+} homeostasis is deregulated, both as primary cause or as a secondary effect of the pathology.

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