Invited review

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Mitochondria as biosensors of calcium microdomains

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Summary The notion that the agonist-dependent increases in intracellular Ca^{2+} concentration, on ubiquitous signalling mechanism, occur with a tightly regulated spatio-temporal pattern has become an established concept in modern cell biology. As a consequence, the concept is emerging that the recruitment of specific intracellular targets and effector system mechanisms depends on exposure to local [Ca^{2+}] that differs substantially from the mean [Ca^{2+}]. A striking example is provided by mitochondria, intracellular organelles that have been overlooked for a long time in the field of calcium signalling due to the low affinity of their Ca^{2+}- uptake pathways. We will summarize here some of the evidence indicating that these organelles actively participate in Ca^{2+} homeostasis in physiological conditions (with consequences not only for the control of their function, but also for the modulation of the complexity of calcium signals) because they have the capability to respond to microdomains of high [Ca^{2+}] transiently generated in their proximity by the opening of Ca^{2+} channels.© Harcourt Publishers Ltd

INTRODUCTION

Over a century ago it was discovered that calcium ions play a key role in cell signalling when their role in controlling cardiac-muscle contraction was directly demonstrated. However, it has only been in the last 2 decades that scientists have found that the vast majority of cell types translate the information conveyed by a variety of stimuli through an increase in intracellular Ca^{2+} concentration. This is achieved via the rapid flow of Ca^{2+} into the cytosol from high-capacity sinks, such as the extracellular space or intracellular organelles (e.g. the endoplasmic and sarcoplasmic reticula), which are endowed with high Ca^{2+} content, via the opening of highly selective channels. The large expansion in our knowledge on calcium signalling was made possible by Tsien et al.'s development of fluorescent indicators, highly specific for this cation and easily trappable in virtually every cell type [1]. Thanks to their efficacy and ease of use, these tools in most cases replaced other techniques used for the study of calcium signalling (photoproteins, metallochromic indicators, etc.) and can now be regarded as the method of choice for measuring cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$). Moreover, the rapid development of imaging technology has made single-cell analysis of Ca^{2+} signalling a relatively easy task. This methodological approach was used to demonstrate that in most cell types Ca^{2+} signals occur with a complex spatio-temporal pattern, such as localized increases, waves orderly diffusing throughout the cell, as well as repetitive spiking of $[Ca^{2+}]_c$ increases, a phenomenon known as '$[Ca^{2+}]_c$ oscillations'. The modes, and regulatory mechanisms, of this complex signalling route have been thoroughly investigated and will not be reviewed here; however, they were used as the starting point for the work we will present, since they obviously imply the need for 'previleged' local signalling routes, occurring via the generation of local $[Ca^{2+}]_c$ rises. Thus, although other examples are available, we will focus on mitochondrial calcium signalling, essentially summarizing, for reasons of brevity, the work of our group. Indeed, local Ca^{2+} signalling is essential for recruiting this organelle into the realm of Ca^{2+} homeostasis, with a direct consequence not only for the control of mitochondrial function, but also for the diffusion of the Ca^{2+} signal throughout the cell and for the orderly activation of Ca^{2+}-regulated cell functions.

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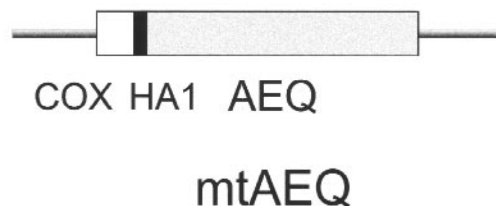
TARGETED AEQUORIN: A SPECIFIC PROBE FOR MITOCHONDRIAL Ca^{2+}

In order to measure Ca^{2+} within the mitochondria in a highly specific manner, we devised a novel approach, based on the specific targeting of the Ca^{2+}-sensitive photoprotein aequorin [2]. Aequorin is a well-known Ca^{2+} probe (it allowed, for example, the first demonstration of $[Ca^{2+}]_c$ oscillations in living cells) that in recent years, however, has been largely superseded by the fluorescent dyes due to the difficulty of loading into the cells. The isolation of the aequorin cDNA [3] not only made recombinant expression possible, thus circumventing the need for traumatic measures of introducing the photoprotein into the cells, but also allowed for its molecular engineering. Thus, it was possible to construct an aequorin molecule specifically targeted to the mitochondrial matrix, by fusing the photoprotein to the information allowing mitochondrial proteins to reach their correct destination. This approach was then utilized for the construction of chimeric aequorins targeted to various other compartments; they will not be dealt in this chapter and the reader is referred for review to [4].

By fusing in frame the aequorin cDNA with that encoding a mitochondrial presequence (i.e. the targeting information of a resident protein encoded by nuclear genes, that is removed after the protein is imported into the mitochondria), we obtained a fusion protein, that retained the functional properties of aequorin (and hence its Ca^{2+}-dependent luminescence) but, when expressed in mammalian cells, was virtually all localized in the mitochondrial matrix [2]. Figure 1 shows a schematic map of the chimeric cDNA and the immunolocalization of the recombinantly expressed photoprotein, as revealed by staining with an antibody that recognizes the HA1 tag appended to the photoprotein.



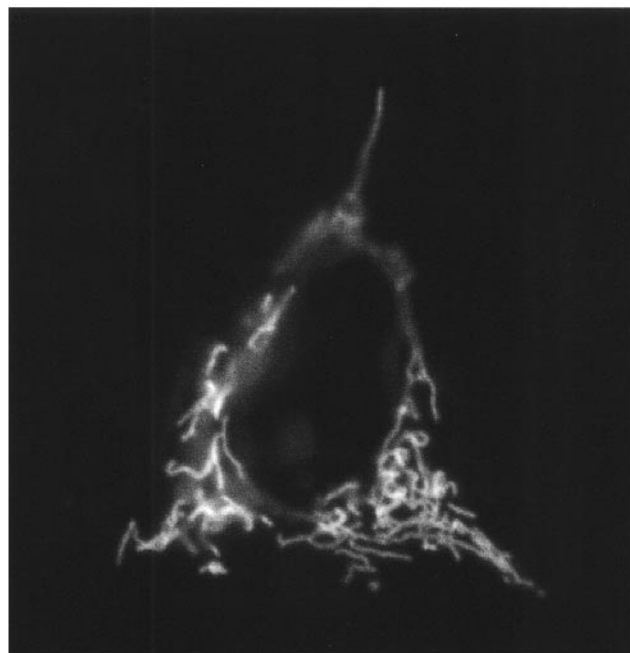


Fig. 1 cDNA map and immunolocalization of mitochondrially targeted aequorin (mtAEQ). On top, the schematic map is shown of the chimeric cDNA. Coding and non-coding regions are represented as boxes and lines respectively. The portions encoding the mitochondrial presequence, the epitope tag and aequorin are in white, black and grey respectively. On the bottom, an immunofluorescence image of a transfected HeLa cell is shown. Staining with a monoclonal antibody recognizing the HA1 tag was revealed with a TRIC-conjugated secondary antibody. The image, acquired on an inverted epifluorescence microscope, was captured with a back-illuminated CCD camera (Pricenton Instruments) using the Metamorph software (Universal Imaging).

MITOCHONDRIAL Ca^{2+} TRANSPORT: GENERAL CONCEPTS

With this tool, we then carried out direct measurements of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) in living cells. Before reviewing the experimental data, what was known about mitochondrial Ca^{2+} transport at the time these experiments were started will be briefly summarized (for a review, see [5–7]) and thus the expected results. While the mitochondrial outer membrane is freely permeable to ions and molecules up to 1000 Daltons MW, the inner membrane is tightly sealed to all ions, except for specific transporters. Ca^{2+} uptake occurs down its electrochemical gradient contributed to by the membrane potential (negative inside) generated by the respiratory chain. Under physiological conditions, Ca^{2+} uptake does not depend on ATP hydrolysis, but rather on the presence of an electrogenic transporter, the so-called 'Ca^{2+} uniporter' (presumably a gated channel). While its molecular nature is unknown, indeed, none of the mitochondrial Ca^{2+} transport pathways has yet been cloned; its kinetic properties, and the sensitivity to inhibitors, such as Ruthenium Red and lanthanides, have been well established through extensive biochemical work on isolated mitochondria. As to the release, two biochemical pathways have been characterized that catalyse the exchange of Ca^{2+} with either Na^+ or H^+ [8,9] and keep matrix $[Ca^{2+}]$ away from electrochemical equilibrium (a membrane potential of ~180 mv would predict a $[Ca^{2+}]_m$ value at equilibrium higher than 0.1 M). The very low affinity of the uniporter (under physiological Mg^{2+} concentrations the Kd is >10 μM) while preventing a futile

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cycling of Ca^{2+} across the mitochondrial membrane, led to the prediction that mitochondrial Ca^{2+} uptake would be negligible not only at rest, but also during the transient increases to ~1–2 μM that occur in the cytoplasm of a stimulated cell. Thus, it was generally assumed that mitochondria, although capable of accumulating Ca^{2+}, would not significantly participate in physiological conditions, but would rather act as low affinity buffers in cases of calcium overload (i.e. in a variety of pathophysiological conditions).

AGONIST STIMULATION EVOKES LARGE INCREASES IN MITOCHONDRIAL Ca^{2+} CONCENTRATION

It thus came as a surprise when the direct measurement of $[Ca^{2+}]_m$ in HeLa cells (a representative trace is shown in Fig. 2) revealed that, during a physiological stimulation, the Ca^{2+} signal is rapidly extended to the mitochondria [2,10]. Indeed, when the cells were triggered with histamine, an agonist coupled via a Gq protein to the generation of inositol 1,4,5-trisphosphate, and, thus, to the release of Ca^{2+} from the intracellular store, a transient rise in $[Ca^{2+}]_m$ occurred, that rapidly surged to a peak well above the values occurring in the cytoplasm (~10 μM vs 2 μM in the cytoplasm). Later studies demonstrated that this response was not an isolated finding in this cell type, but rather represented the typical response of mitochondria in a wide variety of cells, e.g. endothelial and epithelial cells [10], fibroblasts [11], skeletal myotubes [12], insulin-secreting cells [13] and neurons, to name a few. While unexpected, this response was indeed due to uptake into the mitochondrial matrix; if the electrochemical proton gradient was collapsed with a protonophore such as carbonylcyanide p-(trifluoro-methoxy) phenylhydrazone (FCCP), a procedure that has little effect on the cytoplasmic Ca^{2+} signal (not shown), the $[Ca^{2+}]_m$ rise was almost entirely abolished.

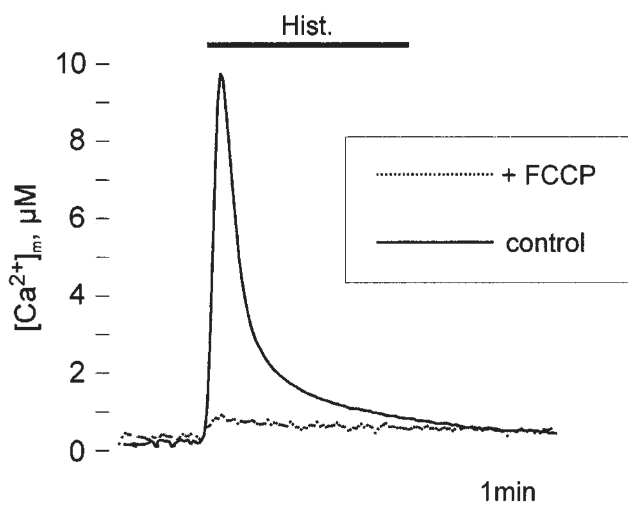

Fig. 2 $[Ca^{2+}]_m$ monitoring in mtAEQ-transfected HeLa cells. Transfection with mtAEQ, aequorin reconstitution, luminescence detection and calibration into $[Ca^{2+}]$ values was carried out as previously described [31]. Where indicated, the cells were challenged with the IP3-generating agonist histamine. In the dotted trace, the cells were treated with the uncoupler FCCP (added 1 min before histamine, and maintained in the perfusion medium throughout the experiment).

So, why would mitochondria accumulate Ca^{2+} during an agonist stimulation that raised $[Ca^{2+}]_c$ to <2 μM for less than 1 min? Various reasons would, at least in principle, explain this result. The first is that the cytoplasmic Ca^{2+} rise was drastically underestimated. This was unlikely, given that our $[Ca^{2+}]_c$ estimate agreed with a wide number of similar estimates obtained in HeLa cells and other cell types by a number of laboratories. Moreover, when two radically different probes for cytoplasmic $[Ca^{2+}]$were employed (the fluorescent indicator Fura-2 and transfected untargeted aequorin), very similar estimates were obtained [14]. A second explanation was that the affinities calculated in isolated organelles were much lower than those occurring in vivo (e.g. for the loss of a soluble accessory protein of factor). The following experiment ruled out this possibility. We permeabilized the cells with digitonin, then perfused a saline solution mimicking the cytoplasmic milieu (and with known buffered $[Ca^{2+}]$): at $[Ca^{2+}]$ similar not only to those of a resting, but also to those of a stimulated cell, mitochondrial Ca^{2+} uptake was very slow. However, if IP3 was directly added to the perfused cell, a rapid mitochondrial Ca^{2+} uptake was evoked, similar to that observed in intact cells [10]. Thus, in these conditions the affinity of the uniporter proved low, but not for the loss of a constitutive 'activating' factor. Indeed, the experiment also hinted to the correct explanation. This, and other experiments, that for brevity we cannot show, suggested that mitochondria were highly responsive to the release of Ca^{2+} from the intracellular Ca^{2+} store (the endoplasmic reticulum [ER], as if they were closely positioned to this organelle. Thus, upon opening of IP3-gated channels, they would be able to sense not the average $[Ca^{2+}]_c$ rise, but rather the much higher concentration occurring in the proximity of the open channels. In other words, mitochondria appear to participate in calcium signalling because they sense, and decode, a microdomain, the amplitude and occurrence of which is overlooked by the classical measurements of bulk cytoplasmic Ca^{2+} concentration.

MITOCHONDRIA ARE IN CLOSE OPPOSITION TO THE ER, THE MAIN INTRACELLULAR Ca^{2+} STORE

To validate this notion, we pursued two types of evidence. The first was to directly demonstrate the close apposition of the two compartments by high-resolution

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microscopy techniques. For this purpose, we needed two fluorescent probes capable of selectively labelling the two compartments in vivo in a clearly distinct way and an imaging system endowed with high spatial resolution. As to the probes, we took advantage of a fluorescent protein that has rapidly gained widespread application in cell biology studies: green fluorescent protein (GFP). By fusing GFP to either a mitochondrial presequence (i.e. that employed successfully for targeting aequorin to the mitochondria) or to an ER retention signal (a portion of the heavy chain of immunoglobulin), we obtained targeted fluorescent probes, capable of specifically labelling the two organelles [15]. In order to be able to distinguish the fluorescence of the two organelles in the same cell, we used GFP moieties with different spectral properties: GFP(S65T) that shares with wild-type GFP the emission of green light but lacks the UV excitation peak and is ~6-fold more fluorescent when excited with blue light for the ER, and GFP(Y66H, Y145F), a mutant that emits blue fluorescence when excited with UV light for the mitochondria. As to the imaging system, preliminary experiments carried out with conventional wide-field and confocal microscopy showed that, given the rapid motion of organelles inside the cell and the need for three-dimensional (3D) rendering for solving their intricate structure, a serial stack of images needed to be acquired in a very short time in order to obtain high-resolution pictures. This was achieved with the special apparatus developed by the Biomedical Imaging Group of the University of Massachussetts, that is based on a low-noise, high-sensitivity back-illuminated cooled CCD camera (which allows acquisition times of 5–20 ms) and a fast motorization of the z plane [16]. With this apparatus, stacks of 40 images

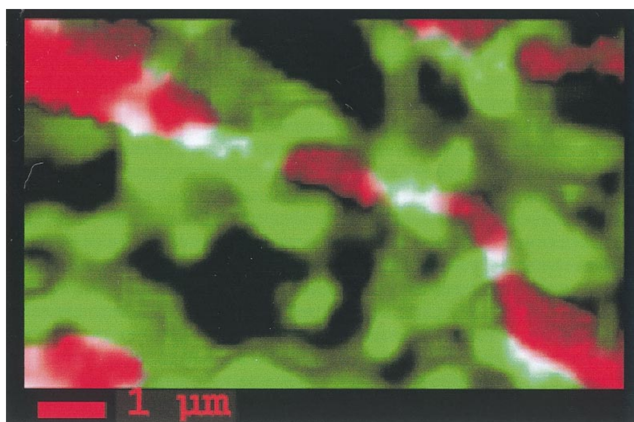

Fig. 3 High-resolution 3D imaging of ER and mitochondria (from [17], with permission). The 3D images were reconstructed from computationally deblurred data acquired with a high-speed wide-field fluorescence microscope, as described in detail in [16]. The mitochondrial and ER image are in red and green pseudocolors respectively, while the white pixels represent those in which the two images overlap.

0.25 μm spacing the whole thickness of a cell) can be obtained in <1 s. By rapidly alternating the exciting wavelengths, the complete stacks of the blue (mitochondria) and green (ER) fluorophores were obtained in 2–3 s [17]. The following application of deconvolution algorithms allowing the removal of out-of-focus haze yielded a 3D fluorescence image of unprecedented resolution (pixel size ~80 nm) as shown in Figure 3. In the image, mitochondria and ER are shown in red and green pseudocolors respectively, while the white pixels are those in which the two images could not be resolved. The image obtained through the analytical system is, thus, fully compatible with the hypothesis that the formation of microdomains with high [Ca^{2+}] sensed by the mitochondria depends on the proximity to ER.

AN AEQUORIN CHIMERA LOCALIZED IN THE MITOCHONDRIAL INTERMEMBRANE SPACE REPORTS LARGER [Ca^{2+}] RISES THAN THOSE OF THE BULK CYTOPLASM

The picture emerging from the morphological data is that of a mitochondrial reticulum finely intertwined with the organelle (the ER) acting as the calcium store, and coming in a number of close contacts where local, 'low-affinity' signalling could occur. If this is the case, one would expect that a calcium probe located at the mitochondrial surface could reveal higher [Ca^{2+}] changes than those of the bulk cytoplasm. For this purpose, a probe needed to be located between the outer mitochondrial membrane, which is freely permeable to ions and small molecules, and the ion-impermeable inner membrane, where the low-affinity mitochondrial Ca^{2+} uniporter is located. We thus constructed a new aequorin chimera, denominated 'mitochondrial intermembrane space' aequorin (mimsAEQ). To construct this chimera, the cDNA encoding HA1-tagged aequorin was fused in frame with that encoding glycerol phosphate dehydrogenase (GPD) [18], an integral protein of the inner mitochondrial membrane, with a large C-terminal tail (~70% of the molecule) protruding into the intermembrane space. A schematic map of the final construct is shown in Figure 4A, and the expected topology is graphically depicted in Figure 4B. The encoded polypeptide includes, from the N- to the C-terminus, aa 1–626 of GPD (thus eliminating the EF-type Ca^{2+}-binding site), the HA1 tag and aequorin. The final construct was transiently expressed in HeLa cells, and evidence for correct localization was sought by immunocytochemistry (not shown).

Figure 4C shows the [Ca^{2+}] measurements performed with mims AEQ, and their comparison with the cytoplasmic data [17]. Where indicated, the cells were treated with histamine, evoking, in both cell domains, a [Ca^{2+}] rise. It is apparent, however, that the $[Ca^{2+}]_{mims}$ rise was

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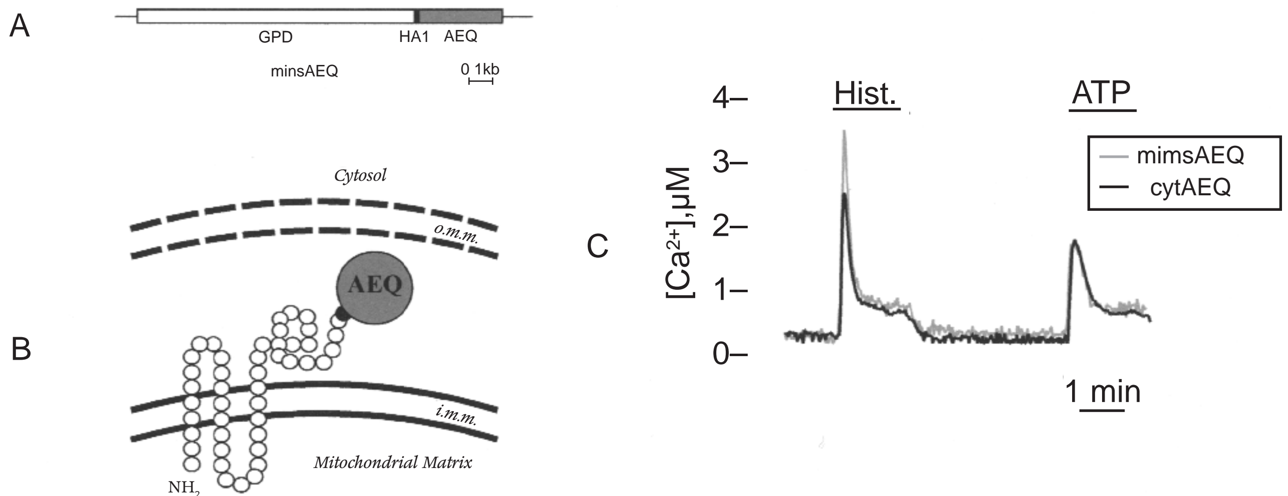

Fig. 4 Monitoring [Ca^{2+}] in the mitochondrial intermembrane space (MIMS). (A) Schematic map of the mimsAEQ cDNA. Coding and non-coding regions are represented as boxes and lines respectively. In the coding region, the portions encoding glycerol-phosphate dehydrogenase (GPD), the HA1 epitope (HA1) and aequorin (AEQ) are white, black and grey respectively. (B) Putative subcellular distribution of the mimsAEQ chimera, based on the topology of GPD: the aequorin moiety is expected to be exposed in the mitochondrial intermembrane space, i.e. between the ion-permeable outer membrane (o.m.m.) and the ion-impermeable inner membrane (i.m.m.). (C) The effect of histamine on the [Ca^{2+}] of the cytosol ([Ca^{2+}]$_c$) and mitochondrial intermembrane space ([Ca^{2+}]$_{mims}$). The traces show the monitoring of the [Ca^{2+}] of the two compartments in parallel batches of HeLa cells, transiently transfected with the appropriate aequorin chimera (cytAEQ or mimsAEQ). Where indicated, the cells were treated first with 100 μM histamine (Hist.), then with 100 μM ATP.

significantly larger (peak value 3.5 vs 2.5 μM). After histamine washout, the cells were stimulated with a second IP3-generating agonist, ATP. Also in this case [Ca^{2+}]$_c$ and [Ca^{2+}]$_{mims}$ rises were evoked that were, however, indistinguishable in amplitude (peak value ~1.5 μM). Thus, this experiment provides two pieces of information. The first is that, indeed, larger [Ca^{2+}] rises are evoked at the surface of mitochondria. The second is that the difference between the data obtained from the two aequorin chimeras that cannot be observed during the second stimulation, is not due to a generalized difference but rather to the contribution of a small fraction of mimsAEQ that is exposed to exceedingly large [Ca^{2+}] (and thus is totally discharged in the first stimulation). In other words, the scenario revealed by the mimsAEQ data, well in agreement with the morphological data, suggests that upon agonist stimulation large increases in [Ca^{2+}] are sensed by mitochondria at defined 'close apposition sites' with the ER.

A FINAL MODEL

A final model, that takes into account the above-mentioned and previously published data, is shown in Figure 5. Mitochondria, organelles with low affinity for Ca^{2+} uptake, are closely apposed to the source of the calcium signal. It should be stressed here that what we have investigated in detail and presented here is mitochondrial calcium signalling in a non-excitable cell, the HeLa cell line, demonstrating that mitochondria sense the release of Ca^{2+} from the ER via the IP3 receptor due to a 'close functional coupling' with the ER. However, it should be remembered that rapid and large mitochondrial Ca^{2+} responses are a general theme, common to cells utilizing radically different Ca^{2+} signalling routes. Indeed, in neurons, large [Ca^{2+}]$_m$ rises are observed upon opening of the plasma membrane NMDA or voltage-gated channels Rosario Rizzuto and Tullio Pozzan, unpublished data), and in myotubes upon release via the Ca^{2+} channels of the sarcoplasmic reticulum (the RyR) [12]. Thus, it is highly likely that the scheme described below should be by no means limited to IP3-mediated signalling, but rather extended, with different molecular actors and/or Ca^{2+} sources, to most cell types, thus providing a general notion of mitochondria as low-affinity players of the Ca^{2+} game recruited by microdomains.

In HeLa cells, when the IP3 generated upon agonist stimulation allows the opening of the ER Ca^{2+} channels, a microdomain is generated in proximity of mitochondria, that allows rapid and efficient accumulation of Ca^{2+} into mitochondria at the apposition sites. This rapid Ca^{2+} uptake, and the following diffusion to the rest of the matrix, plays a key role in priming the organelle to the increased metabolic needs of a stimulated cell. Indeed, classical biochemical studies dating over two decades ago [19], then confirmed by the direct measurement of NADH [20] and ATP [21] levels in vivo, showed that a [Ca^{2+}] rise in the mitochondrial matrix exerts a direct

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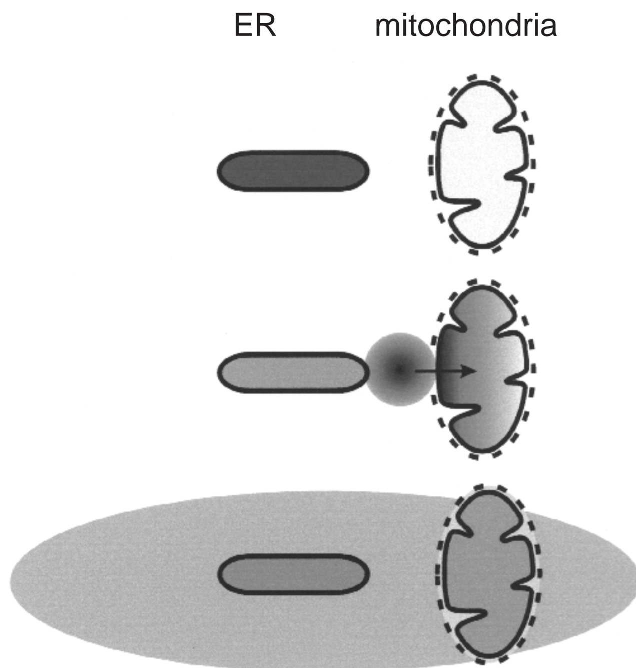

Fig. 5 Schematic model of the ER/mitochondria calcium cross-talk. At rest, the ER is endowed with a $[Ca^{2+}]$ of ~0.5–1 mM, whereas the $[Ca^{2+}]$ of the mitochondrial matrix is similar to that of the bulk cytosol (top panel). When inositol 1,4,5-trisphosphate (IP_3) is produced following the binding of an agonist to a Gq-coupled receptor, Ca^{2+} is rapidly released from the ER via the InsP3 receptor, and a microdomain of high $[Ca^{2+}]$ is generated at the ER/mitochondria contacts. The high local $[Ca^{2+}]$ allows rapid Ca^{2+} uptake via the low-affinity mitochondrial uniporter, thereby causing a major rise in the $[Ca^{2+}]$ of the matrix (middle panel). The dissipation of the microdomain at the mouth of the IP3-gated channel drastically reduces mitochondrial Ca^{2+} uptake (thereby preventing mitochondrial Ca^{2+} overload) and extends the Ca^{2+} signal to the bulk cytosol, thus activating the Ca^{2+}-sensitive effectors. The early rise in matrix Ca^{2+}, and presumably the diffusion through the mitochondrial network, allows the timely activation of mitochondrial metabolism (lower panel).

effect on mitochondrial function, by stimulating three Ca^{2+}-sensitive enzymes of the Krebs cycle (Pyruvate, isocitrate and oxoglutarate dehydrogenases) (for a review, see [22,23]). The ER/mitochondrial cross-talk in the early stages of cell signalling, however, is most likely not restricted to the purpose of controlling mitochondrial activity. Indeed, numerous experimental evidence supports the view that mitochondrial Ca^{2+} uptake also influences the dynamics of cytoplasmic signalling. In *Xenopus* oocytes, it was shown that mitochondrial Ca^{2+} uptake can modulate the shape and velocity of IP3-induced Ca^{2+} waves [24]. More recently, also in mammalian cells mitochondrial Ca^{2+} uptake in the microenvironment of Ca^{2+} channels has been shown to tune the kinetics and amplitudes of the $[Ca^{2+}]_c$ changes caused by the opening of ER or plasma-membrane channels [25–27]. At the end of this 'privileged' local signalling, the Ca^{2+} hotspot then diffuses toward the cytoplasm, with two consequences: the first is that the 'activation' signal reaches the cytoplasmic effectors, thus evoking the functional, energy-consuming response (e.g. contraction, secretion, movement, etc.). The second is that mitochondrial Ca^{2+} uptake drastically slows down, thus preventing mitochondrial Ca^{2+} overload and/or futile cycling across the mitochondrial membrane. The importance of such an arrangement has been directly demonstrated by the elegant work of Hajnoczky et al. [20], who, using fluorescent probes for $[Ca^{2+}]_c$, $[Ca^{2+}]_m$ and NADH, were able to compare the effect on mitochondrial Ca^{2+} signalling and activity in single cells in two conditions: the rapid, pulsatile signal of $[Ca^{2+}]_c$ oscillations and large sustained $[Ca^{2+}]_c$ increases evoked by supramaximal stimulation or SERCA inhibitors treatment. In the former case, each $[Ca^{2+}]_c$ rise was paralleled by a $[Ca^{2+}]_m$ spike, and an increase in NADH levels. Indeed, due to a kinetic delay in the deactivation step, a rapid pace of $[Ca^{2+}]_c$ oscillations was decoded by mitochondria into a sustained activation of organelle activity. Conversely, a large sustained rise in $[Ca^{2+}]_c$ caused a major rise in $[Ca^{2+}]_m$ that, however, rapidly declined as soon as the microdomain faded away (and the energy drain on mitochondria due to Ca^{2+} cycling collapsed the driving force for accumulation). As a result, the increase in NADH levels was only transient.

LOCAL Ca^{2+} SIGNALLING DEFECTS IN MITOCHONDRIAL GENETIC DISORDERS

Finally, the complexity of mitochondrial Ca^{2+} uptake, and its strong dependence on a transient, local signalling, may, at least in principle, place it at risk in a number of pathophysiological conditions, in which either organelle distribution or the driving force for Ca^{2+} accumulation is impaired. Recently, one such example has been directly demonstrated. Mitochondrial diseases include a number of genetic disorders, sharing an alteration in mitochondrial structure and/or function, but differing for molecular mechanism and cellular pathogenesis. The ease of isolation and analysis of the mitochondrial genome (mtDNA) has allowed the identification of various deletions and mutations affecting tRNAs and/or structural genes encoded by mtDNA (for a review, see [28]). However, the knowledge of the molecular defects has not been paralleled by a comparable understanding of the cellular events deriving from the molecular lesion (and, thus, of the specificity of the different syndromes). Using transmitochondrial cell lines (i.e. cells deprived of their mtDNA and repopulated with mutated mtDNAs of interest) [29], we have recently shown a calcium signalling defect in cells harboring the mutation of the disease myoclinic epilepsy with ragged red fibres (MERRF) (clinically characterized by myoclonic epilepsy with the morphological hallmark of ragged red fibras [30]. These cells carry a

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mutation in the mtDNA tRNAlys, that causes a global impairment of mitochondrial protein synthesis (thus including the respiratory chain complexes, with ensuing defect in the generation of the mitochondrial membrane potential). As a consequence, mitochondrial Ca^{2+} signals are drastically reduced, but can be restored by treating the cells with blockers of the mitochondrial efflux pathways. The correction of the calcium signalling defect also enhances the capability of cells to increase ATP production upon agonist stimulation.

CONCLUSION

In conclusion, the role of mitochondria in calcium signalling is now being re-evaluated, thanks to the demonstration that the spatio-temporal complexity of $[Ca^{2+}]$ increases generates conditions in which these organelles, endowed with low-affinity transport systems, can also rapidly accumulate large amounts of Ca^{2+}. While this mitochondrial renaissance is leading to novel, exciting information and new insights into the dynamics of Ca homeostasis, we wish to conclude by reminding readers that they also provide a more general example of how local signals may recruit unexpected partners in the complex scenario of Ca signalling.

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REFERENCES

1. Tsien RY, Pozzan T, Rink TJ. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *Nature* 1982; **295:** 68–71.
2. Rizzuto R, Simpson AWM, Brini M, Pozzan T. Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. *Nature* 1992; **358:** 325–328.
3. Inouye S, Noguchi M, Sakaki Y et al. Cloning and sequence analysis of cDNA for the luminescent protein aequorin. *Proc Natl Acad Sci USA* 1985; **82:** 3154–3158.
4. De Giorgi F, Brini M, Bastianutto C et al. Targeting aequorin and green fluorescent protein to intracellular organelles. *Gene* 1996; **173:** 113–117.
5. Lehninger AL, Carafoli E, Rossi CS. Energy-linked ion movements in mitochondrial systems. *Adv Enzymol Relat Areas Mol Biol* 1967; **29:** 259–320.
6. Carafoli E. Intracellular calcium homeostasis. *Annu Rev Biochem* 1987; **56:** 395–433.
7. Pozzan T, Rizzuto R, Volpe P, Meldolesi J. Molecular and cellular physiology of intracellular Ca^{2+} stores. *Physiol Rev* 1994; **74/3:** 595–636.
8. Rizzuto R, Bernardi P, Favaron M, Azzone GF. Pathways for Ca^{2+} efflux in heart and liver mitochondria. *Biochem J* 1987 **246:** 271–277.
9. Crompton M, Kunzi M, Carafoli E. The calcium-induced and sodium-induced effluxes of calcium from heart mitochondria. Evidence for a sodium-calcium carrier. *Eur J Biochem* 1977; **79:** 549–558.
10. Rizzuto R, Brini M, Murgia M, Pozzan T Microdomains with high Ca^{2+} concentration that are sensed by neighboring mitochondria. *Science* 1993; **262:** 744–747.
11. Rizzuto R, Bastianutto C, Brini M et al. Mitochondrial Ca^{2+} homeostasis in intact cells. *J Cell Biol* 1994; **126:** 1183–1194.
12. Brini M, De Giorgi F, Murgia M et al. Subcellular analysis of Ca^{2+} homeostasis in primary cultures of skeletal myotubes. *Mol Cell Biol* 1997; **8:** 129–143.
13. Rutter GA, Theler JM Murgia M et al. Stimulated Ca^{2+} influx raises mitochondrial free Ca^{2+} to supramicromolar levels in a pancreatic β-cell line. *J Biol Chem* 1993; **268:** 22385–22390.
14. Brini M, Marsault R, Bastianutto C et al. Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$): a critical evaluation. *J Biol Chem* 1995; **270:** 9896–9903.
15. Rizzuto R, Brini M, De Giorgi F et al. Double labelling of subcellular structures with organelle-targeted GFP mutants in vivo. *Curr Biol* 1996; **6:** 183–188.
16. Rizzuto R, Carrington W, Tuft RA. Digital imaging microsocopy of living cells. *Trends Cell Biol* 1998; **8:** 288–292.
17. Rizzuto R, Pinton P, Carrington W et al. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* 1998; **280:** 1763–1766.
18. Brown LJ, MacDonald MJ, Lehn DA, Moran SM. Sequence of rat mitochondrial glycerol-3-phosphate dehydrogenase cDNA. Evidence for EF-hand calcium-binding domains. *J Biol Chem* 1994; **269:** 14363–14366.
19. Denton RM, Randle PJ, Martin BR. Stimulation by calcium ions of pyruvate dehydrogenase phosphate phosphatase. *Biochem J* 1972; **128:** 161–163.
20. Hajnoczky G, Robb-Gaspers LD, Seitz MB, Thomas AP. Decoding of cytosolic calcium oscillations in the mitochondria. *Cell* 1995; **82:** 415–424.
21. Jouaville LS, Pinton P, Bastianutto C et al. Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming. *Proc Natl Acad Sci* USA, in press. 1999
22. McCormack JG, Halestrap AP, Denton RM. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* 1990; **70:** 391–425.
23 Hansford RG. Physiological role of mitochondrial Ca^{2+} transport. *J Bioenerg Biomembr* 1994; **26:** 495–508.
24. Jouaville LS, Ichas F, Holmuhamedov EL. et al. Synchronization of calcium waves by mitochondrial substrates in *Xenopus laevis* oocytes. *Nature* 1995; **377:** 438–441.
25. Csordas G, Thomas AP, Hajnoczky G. Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. *Embo J* 1999; **18:** 96–108.
26. Landolfi B, Curci S, Debellis L et al. Ca^{2+} homeostasis in the agonist-sensitive internal Ca^{2+} store: Functional interactions between mitochondria and the ER measured in situ in intact cells. *J Cell Biol* 1998; **142:** 1235–1243.
27. Hoth M, Fanger CM, Lewis RS. Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. *J Cell Biol* 1997; **137:** 633–648.
28. Wallace DC. Mitochondrial DNA mutations in diseases of energy metabolism. *J Bioenerg Biomembr* 1994; **26:** 241–50.
29. King MP, Attardi G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* 1989; **246:** 500–503.
30. Brini M, Pinton P, King MP et al. A calcium signaling defect in the pathogenesis of a mitochondrial DNA inherited oxidative phosphorylation deficiency. *Nature Med* 1999; **5:** 951–954.
31. Rizzuto R, Brini M, Bastianutto C et al. Photoprotein mediated

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measurement of [Ca^{2+}] in mitochondria of living cells. *Meth Enzymol* 1995; **260:** 417–428.

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