Endoplasmic reticulum/mitochondria calcium cross-talk

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Abstract. The interaction of mitochondria with the endoplasmic reticulum (ER) Ca^{2+} store plays a key role in allowing these organelles to rapidly and effectively respond to cellular Ca^{2+} signals. In this contribution, we will briefly discuss: (i) old and new concepts of mitochondrial Ca^{2+} homeostasis; (ii) the relationship between mitochondrial 3D structure and Ca^{2+} homeostasis; (iii) the modulation by cytoplasmic signalling pathways; and (iv) new data suggesting that mitochondria and ER Ca^{2+} channels are assembled in a macromolecular complex in which the inositol-1,4,5-trisphosphate receptor directly stimulates the mitochondrial Ca^{2+} uptake machinery.

2007 New perspectives on mitochondrial biology. Wiley, Chichester (Novartis Foundation Symposium 287) p ••–••

Mitochondrial Ca^{2+} uptake: an old history of a new concept

A long-standing biological observation is the capacity of energized mitochondria to transport Ca^{2+} across the ion-impermeable inner membrane. Indeed, the translocation of protons by the respiratory chain complexes establishes a large electrochemical gradient, mostly composed of an electrical gradient ($\Delta\Psi$), that represents a large thermodynamic force for Ca^{2+} accumulation into the mitochondrial matrix. Work carried out in isolated mitochondria in the 1960s and 70s characterized the fundamental properties of Ca^{2+} transport in mitochondria, which, apart from the recent electrophysiological demonstration that the mitochondrial Ca^{2+} uniporter (MCU) is a bona fide channel (Kirichok et al 2004), still represent the current knowledge of the process. Ca^{2+} is accumulated into the matrix through a Ruthenium red-sensitive electrogenic route (the MCU) and re-extruded, in exchange with monovalent cations (H^+ or Na^+), by two antiporters that prevent the attainment of electrical equilibrium (that would imply, for a mitochondrial membrane potential, $\Delta\Psi_m$, of 180 mV and a cytosolic Ca^{2+} concentration of 0.1 μM, accumulation of Ca^{2+} into the matrix up to 0.1 M). It was thus logical to assume that mito-

chondria were loaded with Ca^{2+}, possibly releasing it in a number of physiological and/or pathological conditions.

However, when in the 1980s Ca^{2+} homeostasis emerged as a ubiquitous signalling route, characterized by a remarkable spatiotemporal and molecular complexity, the role of mitochondria took the opposite route, and was greatly downplayed to a that of a low-affinity, high capacity sink coming into action only in the case of major cellular overload with Ca^{2+} (i.e. in pathophysiological conditions). Indeed, it became apparent that the endoplasmic reticulum (ER), and not the mitochondria, are the source of rapidly mobilizable Ca^{2+}: it possesses a Ca^{2+} pump for accumulating Ca^{2+}, and the inositol-1,4,5-trisphosphate receptor (IP_3R) for rapidly releasing it upon cell stimulation. At the same time, mitochondria did not appear an important target of the released Ca^{2+}, as the $[Ca^{2+}]$ reached in the cytoplasm (2–3 μM at the peak) appeared well below those required for rapid accumulation by the low-affinity MCU.

This situation was completely reversed when novel gene-encoded targeted probes allowed to unambiguously measure the $[Ca^{2+}]$ of the mitochondrial matrix ($[Ca^{2+}]_m$). This was first achieved by targeting to mitochondria a Ca^{2+}-sensitive photoprotein, aequorin, that allowed to demonstrate that a rapid $[Ca^{2+}]_m$ peak, reaching values well above those of the bulk cytosol, parallels the $[Ca^{2+}]$ rise evoked in the cytoplasm by cell stimulation (Rizzuto et al 1992). Similar conclusions could be reached also with fluorescent indicators, such as the positively charged Ca^{2+} indicator rhod-2 (that accumulates within the organelle) (Jou et al 1996) and the more recently developed GFP-based fluorescent indicators (Nagai et al 2001). With the latter probes, endowed with a much stronger signal than the photoprotein, single cell imaging of organelle Ca^{2+} can be carried out. Thus, it is possible to match the accurate estimates of $[Ca^{2+}]_m$ values, obtained with the photoprotein, with detailed spatiotemporal analyses of $[Ca^{2+}]_m$ transients. With these tools in hands, not only the notion was confirmed that mitochondria promptly respond to cytosolic $[Ca^{2+}]$ rises, but also that the $[Ca^{2+}]_c$ oscillations, the typical response to agonists of many cell types, are paralleled by rapid spiking of $[Ca^{2+}]_m$, thus specifically decoding a frequency-mediated signal within the mitochondria, as clearly shown in hepatocytes (Thomas et al 1995), cardiomyocytes (Trollinger et al 1997) and HeLa cells (Rizzuto et al 1994).

The rediscovery of the process of mitochondrial Ca^{2+} uptake has been paralleled by the appreciation of its role in regulating widely diverse cellular functions. Within the matrix two radically different effects can be triggered by a $[Ca^{2+}]$ rise. The first, as originally proposed by Denton, McCormack and Hansford in the 1960s, is the activation of three key metabolic enzymes (the pyruvate, α-ketoglutarate and isocitrate dehydrogenases), thus stimulating aerobic metabolism when a cell is stimulated to perform energy-consuming processes in the cytosol (e.g. contraction, secretion, etc.). Indeed, the direct measurement of mitochondrial Q1

ATP levels with a targeted chimera of the ATP-sensitive photoprotein luciferase demonstrated that the Ca^{2+} signal within the mitochondria is responsible for the enhanced ATP production, an effect that lasts longer than the Ca^{2+} signal itself, highlighting a novel form of cellular 'metabolic memory' (Jouaville et al 1999). In some conditions, however, a Ca^{2+} signal within the mitochondria may trigger cell death. The alteration of the Ca^{2+} signal reaching the mitochondria and/or the combined action of apoptotic agents or pathophysiological conditions (e.g. oxidative stress) can induce a profound alteration of organelle structure and function (Szalai et al 1999, Pinton et al 2001). As a consequence, proteins normally retained in the organelle, such as an important component of the respiratory chain, cytochrome c, (Kluck et al 1997, Yang et al 1997), as well as more recently discovered proteins, such as AIF (Susin et al 1999) and Smac/Diablo (Du et al 2000), are released into the cytoplasm, where they activate effector caspases and drive cells to apoptotic cell death. In relation to this effect, the anti-oncogene Bcl-2 was shown to reduce the steady state Ca^{2+} levels in the ER (and thus dampen the pro-apoptotic Ca^{2+} signal) (Pinton et al 2000, Foyouzi-Youssefi et al 2000).

On the cytosolic side, mitochondrial Ca^{2+} uptake exerts two different effects. In the first, the spatial clustering of mitochondria in a defined portion of the cell represents a physiological 'fixed spatial buffer' that prevents (or delays) the spread of cytoplasmic Ca^{2+} waves, as elegantly demonstrated in pancreatic acinar cells. In these cells, the Ca^{2+} response to a low-dose agonist stimulation is restricted to the apical pole (where it causes granule secretion) by the action of a mitochondrial 'firewall' located between the apical and basolateral portions of the cell (Tinel et al 1999). Mitochondria however can affect cytosolic Ca^{2+} signalling through a different mechanism. Indeed, since they are located in close proximity to ER (or plasma membrane) channels and avidly take up part of the Ca^{2+} released upon stimulation, they reduce the $[Ca^{2+}]$ in the critical microenvironment of the open Ca^{2+} channel. In this way, they modulate the feedback effect of Ca^{2+} (negative or positive, depending on the Ca^{2+} concentrations and channel isoform) on the channel itself, and thus the duration and amplitude of ER Ca^{2+} release. This mechanism was first demonstrated in Xenopus oocytes, in which the energization state (and thus the capacity to accumulate Ca^{2+}) was shown to influence the spatiotemporal pattern of the typical propagating Ca^{2+} waves induced by IP_3 (Jouaville et al 1995). Then, a series of studies in mammalian cells confirmed the notion and demonstrated its importance in shaping the Ca^{2+} responses to agonists of cells as diverse as hepatocytes (Thomas et al 1995) astrocytes (Boitier et al 1999) or BHK cells (Landolfi et al 1998). In addition, the concept has been extended to the control of plasma membrane channels, as in the case of the relief of the Ca^{2+}-dependent inhibition of CRAC channels, i.e. the influx pathway triggered by the drop of $[Ca^{2+}]$ in intracellular stores (Hoth et al 1997, Gilabert & Parekh 2000).

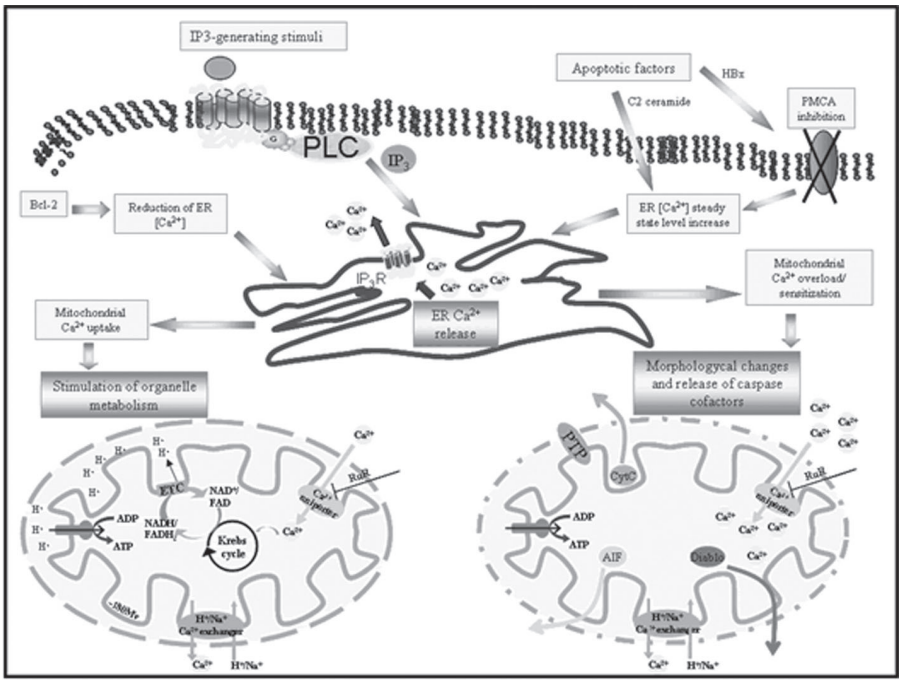

FIG. 1. Mitochondrial [Ca^{2+}] rise can trigger two radically different effects: stimulation of aerobic metabolism or cell death. IP_3-generating stimuli at cell membrane provide IP_3R opening, causing Ca^{2+} release from ER. Mitochondria closely located near ER channels take up part of Ca^{2+} released through the mitochondrial Ca^{2+} uniporter (MCU). Ca^{2+} in the matrix activates key metabolic enzymes of Krebs cycle thus stimulating organelle metabolism. Apoptotic factors exert their effects increasing the amount of Ca^{2+} released from ER, directly (such as the lipid mediator of apoptosis C2-ceramide) or indirectly, i.e. the Herpetic B virus X protein HBx which inhibits PMCA activity. Mitochondrial Ca^{2+} overload determines PTP opening and release of mitochondrial proteins such as Cytochrome C, AIF or Smac/Diablo in the cytosol, triggering activation of apoptotic pathways. Conversely, Bcl-2 exerts its anti-apoptotic effect reducing ER steady state level, thus preventing mitochondrial Ca^{2+} overload.

The regulation of mitochondrial Ca^{2+} homeostasis

Given the growing interest in the process of mitochondrial Ca^{2+} homeostasis, the focus of this contribution is the clarification of the mechanism that finely tune the Ca^{2+} transfer from the ER to the mitochondria. Indeed, a dynamic membrane-bound organelle with a compound protein machinery for Ca^{2+} uptake and release implies that various mechanisms could cooperate in regulating the Ca^{2+} responsiveness of mitochondria. We will discuss our own data, pointing to three different mechanisms:

— the three dimensional structure of the organelle
— signalling pathways activated by extracellular stimuli
— protein–protein interactions within macromolecular complexes assembled at ER–mitochondria contacts.

The 3D structure of mitochondria

The observation that mitochondria in different cell types, or within the same cell in different physiological or pathological conditions, can form a largely connected network extended throughout the cell or represent an isolated oval-shaped organelle is as old as the microscopic identification of this organelle. However, only in the recent years has the control of mitochondrial shape emerged as a tightly regulated cellular process, with a dedicated molecular machinery. Indeed, not only the proteins promoting mitochondrial fusion or fission have been identified and shown to be membrane-associated GTP-binding mechanochemical enzymes, but also these proteins and their activities have been shown to play an important role in determining the sensitivity of cells to apoptotic challenges. While this confers further interest in the activity of these proteins, the mechanism through which alterations of mitochondrial shape can affect mitochondrial participation in apoptosis is still debated. The modulation of Ca^{2+} signals represented an obvious possibility, so we investigated Ca^{2+} and apoptotic changes in HeLa cells in which the 3D structure of mitochondria was modified through the recombinant expression of the mitochondrial fission factor dynamin-related protein 1 (Drp1) (Frank et al 2001). Ca^{2+} homeostasis in the cytosol and in mitochondria was investigated by two approaches, i.e. the co-transfection of aequorin and the measurement of $[Ca^{2+}]$ in the whole cell population (thus obtaining an accurate estimate of the global phenomenon) and the co-transfection of GFP-based recombinant Ca^{2+} probes that allow us to carry out single cell imaging experiments with high spatial and temporal resolution. The imaging results showed that waves of $[Ca^{2+}]_m$ originate from distinct sites (most likely corresponding to the contacts with the ER) and travel across the network. In fragmented mitochondria, wave diffusion is blocked, mitochondrial Ca^{2+} increases are smaller, and thus the sensitivity to Ca^{2+}-dependent apoptotic challenges (such as the lipid mediator of apoptosis C2-ceramide) is reduced (Szabadkai et al 2004).

Modulation by cytosolic signalling pathways

On this topic, we will describe only one interesting example, but we remind the reader that several other pathways besides that described here are emerging that affect mitochondrial Ca^{2+} responsiveness (see for example the different effects of the various PKC isoforms on cytosolic and mitochondrial Ca^{2+} homeostasis;

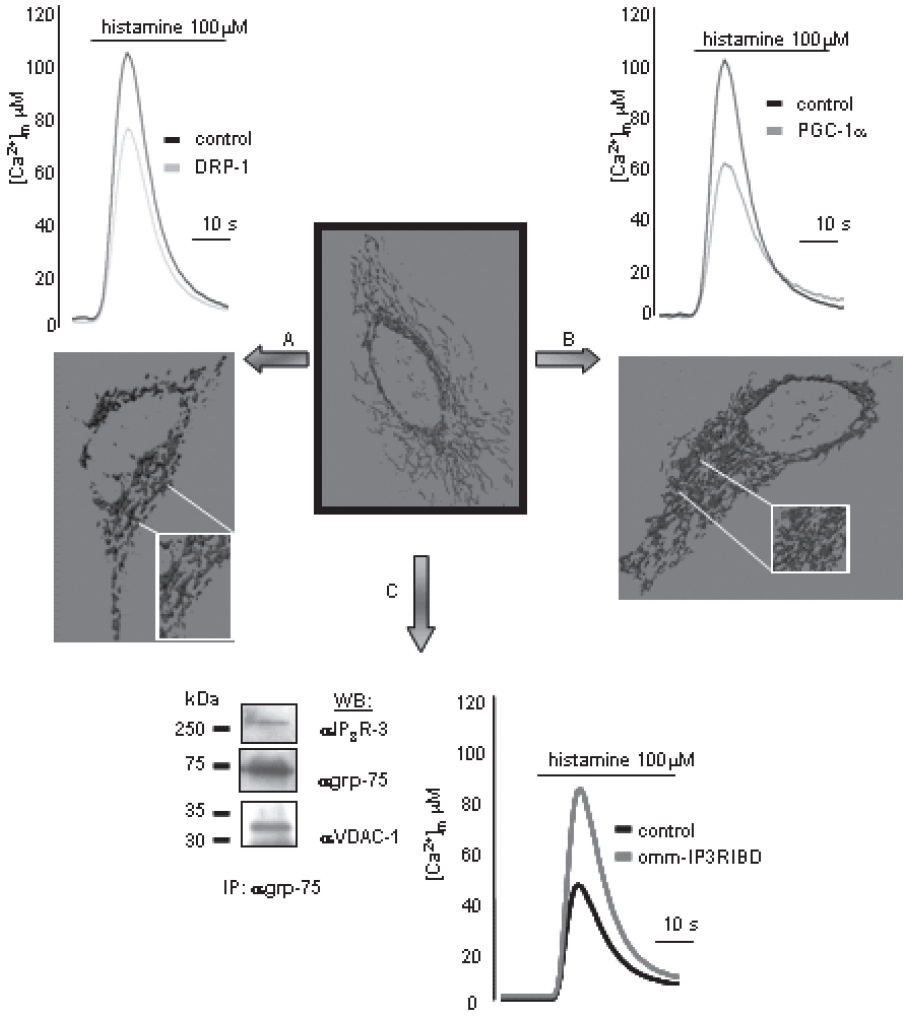

FIG. 2. Three mechanisms regulating the Ca^{2+} responsiveness of mitochondria: Different physio/pathological conditions can directly modulate mitochondrial Ca^{2+} uptake with different mechanisms. (A) Mitochondrial network fragmentation induced by the fission factor DRP1 (lower panel) reduces $[Ca^{2+}]_m$ in response to histamine stimulation (upper panel). (B) The reduction in $[Ca^{2+}]_m$ value after agonist stimulation (upper panel) is also observed in cells overexpressing PGC1α due to increasing in mitochondrial volume (lower panel). (C) Mitochondrial Ca^{2+} uptake can also be modulated by protein–protein interactions: ER and mitochondrial Ca^{2+} channels are coupled through the molecular chaperone Grp75 at ER–mitochondria contact sites (as demonstrated by immunoprecipitation on the left panel); moreover, expression of the ligand binding domain of the IP_3R1 tethered to the outer mitochondrial membrane (omm-IP3R1BD) can enhance mitochondrial Ca^{2+} uptake (see traces on the right panel). The 3D reconstructions were obtained from HeLa cells transfected with mtRFP for control (cell in the middle) and cotransfected with DRP1 or PGC1α (on the left and right, respectively). $[Ca^{2+}]_m$ traces were carried out measuring aequorin luminescence in a population of transfected HeLa cells with mtAEQmut for control and cotransfected with DRP1 or PGC1α.

Pinton et al 2004). We have investigated the effect of the complex mitochondrial changes triggered by the genomic program activated by the transcriptional co-activator peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC1α). By integrating the action of several transcription factors (Puigserver & Spiegelman 2003) PGC1α orchestrates the response to several physiological and pathological stimuli, such as cold exposure, fasting and muscle exercise. Its main effect on mitochondria is the stimulation of mitochondrial biogenesis in concert with the increased expression of electron transport chain (ETC) components and uncoupling proteins (UCP1/2, (Nakatani et al 2002, Puigserver et al 1998). This compound effect increases both the respiratory capacity of mitochondria and the leakiness of the inner membrane (therefore reducing mitochondrial 'efficiency'), thus accounting for the thermogenic response of brown fat and skeletal muscle. Conversely, the functional relevance in non-thermogenic tissues (and thus the role in other physiological conditions) is less clear. By using aequorin and GFP-based probes, we demonstrated that the $[Ca^{2+}]_m$ increases evoked by cell stimulation are markedly reduced in PGC1α expressing cells, As the number of ER/mitochondrial contacts appears unchanged, this effect is at least in part due to mitochondrial biogenesis, and thus redistribution of the Ca^{2+} load in a larger volume (Bianchi et al 2006). However, it is likely that the proteomic changes triggered by PGC1α overexpression also directly involve the still undefined machinery for mitochondrial Ca^{2+} uptake. Based on this intriguing possibility, we are searching *in silico*, among the genes up- or down-regulated by PGC1α, for potential candidates for the role of mitochondrial Ca^{2+} transporters and/or regulators. Finally, the reduction of mitochondrial Ca^{2+} responses is paralleled by reduced sensitivity to apoptotic challenges, a result that nicely matches the observation that PGC1α$^{-/-}$ shows alterations in cell death pathways (see the lesions in the striatal region leading to hyperactivity and thus an unexpected lean phenotype) (Lin et al 2004).

Protein–protein interactions between ER and mitochondrial Ca^{2+} channels

As already explained, our lack of molecular understanding of mitochondrial Ca^{2+} homeostasis is almost complete. Indeed, neither the MCU nor the exchangers have been identified. The only transporter with an established role is the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane (OMM) (Rapizzi et al 2002). Indeed, although it is located in the ion-permeable OMM, data obtained by our group demonstrated that it plays an important role in allowing the Ca^{2+} microdomain generated close to the IP3R to rapidly diffuse to the MCU, and thus maximize mitochondrial Ca^{2+} uptake. Indeed, the VDAC repertoire proved to be a key determinant of mitochondrial Ca^{2+} responsiveness. Based on these results, we hypothesized that VDAC could be part of larger signalling complex with ER and/or IMM proteins. We thus initiated a two-hybrid search of

VDAC interactors, with the aim of identifying other proteins playing a role in mitochondrial Ca^{2+} homeostasis. Among the proteins that were identified, the chaperone Grp75 could be shown to interact with the IP3R and VDAC itself, placing the two channel in close molecular proximity. Interestingly, this molecular proximity allowed the IP3BD of the IP3R to directly stimulate mitochondrial Ca^{2+} uptake, not only when Ca^{2+} is released from the ER but also in conditions in which the Ca^{2+} rise originates from the plasma membrane. Thus, the conclusion could be drawn that the direct interaction of the Ca^{2+} channels of the two organelles, in turn controlled by a partly cysotolic chaperone, is an additional checkpoint controlling the Ca^{2+} cross-talk between the two organelles.

Conclusions

The sites of close interaction between the ER and mitochondria appear to play a role in the participation of mitochondria in Ca^{2+} signalling. On the one hand, both organelles participate in the control of the $[Ca^{2+}]$ of this restricted space: the ER releases Ca^{2+} that reaches there a concentration allowing the low-affinity MCU to rapidly accumulate Ca^{2+} in the matrix, and mitochondria clear part of the Ca^{2+}, thus modulating the feedback control of the cation on the IP3R. In addition, the IP3R and VDAC are scaffolded in a macromolecular complex, in which their direct interaction alters the efficiency of mitochondrial Ca^{2+} uptake. Finally, a number of signalling pathways, as well as the fusion/fission state of mitochondria, also participate in regulating mitochondrial responsiveness to cytoplasmic Ca^{2+} signals. Much more work will be needed to clarify the mechanisms, as well as the relative importance, of all these effects, but certainly when completed this analysis will allow us to understand how mitochondria can translate Ca^{2+} signals into very different cellular functions, and which molecules can be designed to correct the dysfunctions occurring in pathophysiological conditions.

Acknowledgements

Experimental work in the authors' laboratory was supported by grants from the Italian University Ministry (PRIN,FIRB), Telethon-Italy (grant #GGP05284), the Italian Association for Cancer Research (AIRC), the Italian Space Agency (ASI), the EU NeuroNE network, the PRRIITT program of the Emilia-Romagna region and 'Obiettivo 2' funds from the Ferrara province.

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DISCUSSION

Nicholls: It has worried me for a long time that studies on mitochondrial Ca^{2+} transport seem to have been going along two independent but parallel lines. There is the area with non-excitable cells where we are dealing with the interaction between ER and mitochondria. But what we neuronal people find is that when we measure Ca^{2+} uptake by, for example, isolated brain mitochondria and calculate the activity of the Ca^{2+} uniporter, we do not see a 'low-affinity' transporter. The activity of the uniporter activity increases as the 2.5 power of the cytoplasmic Ca^{2+}. When the extramitochondrial Ca^{2+} rises above 0.5 μM, uptake exceeds efflux, as can be seen by repetitive Ca^{2+} addition experiments with isolated mitochondria. By the time levels of 3 μM are reached, all the respiratory capacity is devoted to Ca^{2+} uptake.

Experiments that Michael Duchen's group and others have done with mitochondrial transport in intact neurons and neural cells shows a precise correlation between what was shown with isolated mitochondria and what occurs in intact neurons. We need to accept that cell type has an enormous influence over what is actually happening. The ER–mitochondrial collaboration plays a major role in some cells, and in neuronal cells with mitochondrial moving on these microtubular tracks the transport properties fit closely to those of isolated mitochondria.

Rizzuto: I agree. To account for the rapid responsiveness in non-excitable cells, with these pulsatile changes, you have to account for a microdomain. This does Q1

not rule out the fact that in neurons and neuronal dendrites secretion is exactly as you say. Also, in a non-excitable cell which undergoes a sustained rise, such as Spat's data on long-term steroid production in luteal cells, there is a small Ca^{2+} increase. We don't need high responsiveness to Ca^{2+}. Your point is well taken: we are probably dealing with the most common signalling method in non-excitable cells. But we have to account for complexity, and recognize that in other cells the cells can simply respond to the bulk cytosolic rise.

Spiegelman: To what extent is the Ca^{2+} uptake by mitochondria a kinetic effect—that is, an equilibrium or steady-state effect influenced by membrane potential of the mitochondria? You have presented this as though it is controlled by uptake via the pores, and there is basically infinite capacity. To what extent is the uptake limited, not just by VDAC or by the uniporter, but also by the membrane potential of the mitochondrion itself?

Rizzuto: There are some nice data from Ole Peterson and his group in Liverpool. They have made a correlation between spiking and the changes in membrane potential in a pancreatic acinar cell. He sees minimal drops in the membrane potential for every Ca^{2+} spike, and this is physiological and so there is a relatively small Ca^{2+} pulse. In physiological signalling the net amount of Ca^{2+} that goes in does not change $\Delta\Psi$ as significantly as to make this a regulatory element.

Spiegelman: Are you saying that it is so negatively charged on the inside of the mitochondria it is a sink?

Lemasters: What about the converse: is a decrease in $\Delta\Psi$ going to slow Ca^{2+} up?

Rizzuto: We haven't looked in detail. We have done some work on cybrids harbouring mitochondrial mutations showing that this is the case.

Nicholls: The uptake is rather independent of membrane potential; it is not a thermodynamic equilibrium. This is another point where there is controversy. Work originated by Richard Hansford, Richard Denton and the Bristol group came up with the idea that free matrix Ca^{2+} concentration hovers around the 1 μM level. This is what is needed to activate the matrix dehydrogenases. We confirmed this a couple of years ago with brain mitochondria. Thus there is no significant free Ca^{2+} concentration gradient across the inner mitochondrial membrane, although vast amounts of *total* Ca^{2+} are accumulated as Ca^{2+} phosphate inside the matrix. It is not like TMRM which is being accumulated to equilibrium with the membrane potential.

Rizzuto: It is fair to conclude this in physiological conditions, but we have mitochondrial diseases and other pathological conditions in which this can vary. Making a titration of the severity of the respiratory deficiency could allow us to correlate it with the alteration in Ca^{2+} signalling. We haven't done this in detail apart from demonstrating that the respiratory deficiency decreases net uptake.

Jacobs: You showed one nice experiment, which in the light of what we heard yesterday supports the idea that the membrane potential is important. When you fragmented the mitochondrial network and prevented the fusion–fission cycle, you

interpreted that to indicate that there is some kind of alteration in the propagation of the signal. To me it seemed that there were mitochondria dotted around the cell that simply can't respond.

Rizzuto: We interpreted it as a lack of diffusion, but you may be right: there could be also some bioenergetic explanation.

Larsson: In your paper you mainly looked at acute responses induced by various types of manipulations. We have looked in animal models with decreased oxidative phosphorylation capacity, both in the heart and skeletal muscle. In collaboration with a group from the Karolinska institute we have examined Ca^{2+} signalling in isolated cardiomyocytes and skeletal muscle fibres. There are massive changes with smaller Ca^{2+} transients of shorter duration in respiratory chain deficient cardiomyocytes. We find a large secondary down-regulation of expression of many nuclear genes that encode proteins involved in Ca^{2+} handling, such as calsequestrin or SERCA2. These results suggest that reprogramming of gene expression causes aberrant Ca^{2+} metabolism.

Rizzuto: It is a difficult area because of the convergence of so many inputs. We don't know the molecular nature of the uniporter nor a reliable cell-permeant inhibitor, so we don't have a way to specifically affect the ability to take up Ca^{2+}. Any disease or bioenergetic manoeuvre we apply results in tens of changes in the cell. It is difficult to sort out specific Ca^{2+} signalling effects. We are now constructing a Ca^{2+} sponge located in mitochondria, so that we will be able to dampen significantly only the mitochondrial Ca^{2+} transients. We hope this will be a useful tool.

O'Rourke: You have to be careful in your conclusion that your PKC is modulating the uptake apparatus per se. It will modulate other things as well. For example, we have recently published a paper looking at fast mitochondrial Ca^{2+} uptake in heart cells (Maack et al 2006). It is highly Na^+ dependent. By modulating the efflux rate you affect the uptake rate, as well. PKC is known to alter Na^+/H^+ exchanger in the sarcolemma, among other effects.

Rizzuto: I am cautious by definition. We are forced to be reductionist. No doubt there are many other functions that have compensatory effects in the cell.

Nicholls: Can we focus on PKC and p66 for a moment?

Bernardi: This is an interesting observation. As you know the pore has been put online with p66 in the paper by Giorgio et al (2006). There is an additional piece of evidence which might be interesting here in a paper by Baldari and coworkers in Jurkat T cells (Pellegrini et al 2007). Ca^{2+}-dependent cell death in this system is strictly p66-dependent. She found that p66 phosphorylation itself is strictly Ca^{2+}-dependent. Pretreating with thapsigargin in Ca^{2+}-depleted cells prevents p66 phosphorylation. I see a lot of feedback going on here. If you need PKC to activate p66 you also need Ca^{2+} signalling in the cytosol. This rules out several Ca^{2+}-independent kinases such as ERK. Q1

Rizzuto: You are right. We need to apply some protocols. Of course, we demonstrate that oxidative stress can activate PKCβ, but the key activator of PKCβ is Ca^{2+}.

Martinou: Is p66 is phosphorylated at the plasma membrane?

Rizzuto: No, in the cytosol.

Martinou: Once it is phosphorylated, it enters the mitochondrion. So it must have a mitochondrial-targeting sequence.

Rizzuto: We are talking about just a fraction of p66. The majority remains in the cytosol where it plays a different role, i.e. it acts as a (relatively inefficient, compared to the other isoforms) growth factor adaptor.

Orrenius: Isn't it thought that once in the mitochondria, p66 is bound either to the TOM complex or to a heatshock protein, where it stays until it is released by a pro-apoptotic signal and can interact with cytochrome *c*?

Rizzuto: That is one scheme. We see that there is more p66 in mitochondria after PKC activation. The net amount of p66 in mitochondria is double when PKC is activated.

Jacobs: Given this nice hypothesis, and the benign mouse phenotype, wouldn't you expect p66 to be a tumour suppressor gene?

Rizzuto: It is not, which is surprising.

Jacobs: What about oxidative damage in the nuclear genome? Are the cells rendered susceptible to oxidative damage of DNA?

Rizzuto: This is the question that Pelicci is repeatedly asked since he published the *Nature* paper (Migliaccio et al 1999). The first question is why isn't this a tumour suppressor gene? He doesn't have the answer and neither do I. We need to understand how this apoptotic mechanism relates to immunosuppression of cancer cells. The other question is, why do we have it? Why hasn't it been selected against by evolution since we only see deleterious effects? The only piece of evidence for a negative effect of this is that the knockout mouse is less fertile. We don't understand why, but this could account for maintaining a potentially deleterious gene.

Martinou: I heard recently from Dr Pelicci at a meeting on apoptosis that they found these mice have problems in the brown adipose tissue. They are leaner. Dr Pelicci invoked the possibility that these mice eat less, and the lifespan extension could be consequent to caloric restriction.

Jacobs: Apoptosis is clearly important in many aspects of immune system function. One would also want to ask questions about whether the absence of this function made the mice susceptible to particular kinds of infectious challenge. It might be a specific effect.

Scorrano: The response of the immune system is very complicated, particularly if you are studying it at the whole animal level. Asking whether you have an immunological phenotype is too broad a question.

Jacobs: There are many ways you would want to ask this question. There is also the question of self-tolerance. If this balance were altered you would need specific assays to detect it.

Scorrano: You need to ask specific questions. For example, if Bcl-2 is expressed in T cells there are complex phenotypes, which depend on the dual effect of Bcl-2 on proliferation and apoptosis. If proliferation is suppressed on one side and apoptosis is suppressed on the other side, the net effect is that Bcl-2 doesn't have a major T cell phenotype. If we go to complex phenotypes such as immune system function, we should be extremely cautious and ask questions that are specific. I have a question: we know that hydrogen peroxide at 1 mM causes Ca^{2+} release from the ER and Ca^{2+} influx from the plasma membrane. Have you tried blocking calcineurin to see whether these changes still occur?

Rizzuto: We haven't, but we have done experiments replicating these effects with lower H_2O_2 concentrations. We still see the reduction of the Ca^{2+} transient.

Giulivi: You are studying this close interaction between IP_3 and mitochondria. The conclusion is that this close interaction results in Ca^{2+} that activates the dehydrogenases, produces ATP and favours the clearance of Ca^{2+} by the Ca^{2+}-dependent ATPases of the ER. You are looking at this interaction because it forces Ca^{2+} back to the ER. Is that what you are looking for?

Rizzuto: An important concept in Ca^{2+} signalling is that every time we deal with a channel we must consider that these channels are scaffolded in the plasma membrane as a signalling domain. The idea is that channels are not lost in the membrane, but are held together with the targets. This is an emerging theme in global Ca^{2+} signalling. This implies that regulators can be recruited to this domain and further participate in the regulation. The fact that this stimulates aerobic metabolism and activates the SERCA has been proven. What you say is right but it isn't directly related to this model. We find the SERCA in a different macromolecular complex. We still find it in these mitochondria-associated mitochondrial fractions, but not held together with IP_3 receptor and VDAC.

Giulivi: Do you think that this close interaction triggers the clearance of Ca^{2+}?

Rizzuto: This is a way to stimulate aerobic metabolism. Mitochondria produce ATP and this ATP is relevant for Ca^{2+} reuptake.

Nicholls: I want to ask a question for both of the last two talks. We have seen images in two dimensions but in reality we must think in three dimensions, with the complete 'sausage' of the mitochondrion. Gyorgy shows us specific, almost tubular contacts between the ER and the mitochondria. Because we don't know anything about the Ca^{2+} uniporter, if we start with the observation that this is uniformly dotted around the inner membrane, then only a small proportion of the Ca^{2+} uniporters will be opposed to the ER. Many will be out looking at naked cytoplasm. What proportion of mitochondrial Ca^{2+} uptake in your different model Q1

systems occurs directly from release from the ER, or occurs from global bulk uptake from the cytoplasm?

Hajnóczky: In RBL-2H3 cells, the IP_3 receptor-mediated Ca^{2+} release appears to maximally activate the mitochondrial Ca^{2+} uptake. In the context of the local Ca^{2+} control model this result suggest that the distribution of the mitochondrial uptake sites (uniporter and VDACs) allows all of them to interact with an IP_3 receptor. The IP_3 receptors form clusters in the ER membrane. We speculate that the Ca^{2+} uptake sites may show an inhomogeneous distribution in the mitochondrial membranes. In the RBL-2H3 cells, the mitochondria can take up 40% of the Ca^{2+} released from the ER. This seems to be the high end. In some other cell types, mitochondria would accumulate only a few percent of the Ca^{2+} released through the IP_3 receptors.

Rizzuto: Some VDAC is there and some is diffused. The same is true for the IP_3 receptor. For the uniporter it is difficult to understand how much is clustered, since its molecular identity is still elusive. In principle, however, given that its Ca^{2+} response is steep, the coupled uniporter is the relevant one, even if it is the only a fraction. There is evidence indicating that the macromolecular complexes bring together a signalling domain, through the work of Hajnóczky and Duchen. It is clear that mitochondrial Ca^{2+} uptake has a fundamental role in deciding the opening probability of the IP_3 receptor. If these IP_3 receptors were scattered through the whole ER, it would be irrelevant if a fraction of the IP_3 receptors is modulated by mitochondria. Their data show that the IP_3 receptors are modulated by mitochondria, which implies that the signalling proteins come together. If the IP_3 receptor comes together it makes a lot of sense that the uniporter is there.

Hajnóczky: We have talked about the IP_3 receptor, but in other cell types, the ryanodine receptors also form local interactions with the mitochondria to support the Ca^{2+} signal propagation from the sarcoplasmic reticulum to the mitochondria.

Orrenius: Importantly, VDAC is the most abundant protein in the outer mitochondrial membrane. Further, formation of a complex between VDAC and the IP_3 receptor may also be of interest in view of the observation by Snyder and colleagues (Boehning et al 2003) that cytochrome *c* can interact with the IP_3 receptor to delay closing of the receptor by the increasing cytosolic Ca^{2+} concentration during apoptosis. This might also provide a direct mechanism for filling the mitochondria with Ca^{2+} under apoptotic conditions.

Parekh: We and others have been patch clamping mitoplasts. It is hard to find a single uniporter channel: the density seems quite low. But when we find one we discover that they have a relatively high single channel Ca^{2+} conductance and a high open probability. We don't need many of them to get rapid mitochondrial Ca^{2+} uptake. In the model of Rizzuto, judicious location of the uniporter and IP_3 receptor by VDAC would result in effective coupling. Q1

Nicholls: Can you make an estimate of how many uniporters there are per mitochondrion?

Parekh: The open probability seems quite high, so perhaps 50 per mitoplast, which is around 1 μm in diameter.

Nicholls: Which could explain why we have looked for 40 years and not found any.

O'Rourke: I wanted to follow up on the IP_3 receptor binding domain. Have you looked at the K_m for ADP stimulation of respiration when you overexpress? This would give an idea of whether the VDAC conductance to nucleotides is altered.

Rizzuto: That's a good point. We haven't.

Shirihai: According to what you showed, the mitochondrion that falls into a pool of Ca^{2+} is doomed to stay there forever, and perhaps this would contribute to the initiation of apoptosis.

Nicholls: So you could get Ca^{2+} overload in specific mitochondria.

Hajnóczky: In the normal situation the recruitment of more mitochondria would work the opposite way. Mitochondria will provide the ATP and help the ER to reaccumulate the Ca^{2+}. They will also contribute to the buffering of the Ca^{2+}. If the resident mitochondria are not enough to support the Ca^{2+} handling, more mitochondria will be retained in the high Ca^{2+} zone to help out. Under excessive Ca^{2+} release or Ca^{2+} load conditions, the capacity may be worn down. But the Ca^{2+}-dependent control of the mitochondrial distribution seems to decrease the chance for the overloading.

Shirihai: Would you expect the Ca^{2+} to reduce the fusion capacity of these mitochondria?

Hajnóczky: For the period the mitochondria spend in the vicinity of the Ca^{2+} release, the fusion activity is probably reduced.

Rizzuto: With regard to the contacts, I am not discussing what creates the contacts. Once the contacts between the two organelles are made, this assures that the channels are put together and can talk to each other. But I don't think that the number of contacts is determined by the chaperone. This is a modulator of the function. Once you have created this domain, it allows the two systems to talk to each other.

Nicholls: You are uniformly increasing Ca^{2+} in your cells, and the mitochondria stop. Can one devise an experiment where there is focal stimulation of one pole of a mitochondrion?

Reynolds: Peter Hollenbeck published an interesting experiment in which he was looking at mitochondria travelling up and down a DRG axon. He took a bead of NGF, put a spot on the axon and then looked at the probability that mitochondria would stop. They pause on the way down. It is not quite the same thing as attraction, but they have a tendency to linger when they get there. This addresses the issue of activating the process that promotes docking. You raised the possibility Q1

of a hypothetical docking protein. You say you don't think the adaptor proteins are making the mitochondria stop, but perhaps they do.

Rizzuto: Not this adaptor protein. There may be others that do.

Shirihai: Is that what would keep mitochondria in active synapses.

Nicholls: That would be the hope. We always talk about an axon with a synapse at one end, which would be the neuromuscular junction, but real CNS synapses have hundreds of varicosities along them. Do the mitochondria derail themselves at particular synapses that are being active, saying 'hey we are needed here' because the Ca^{2+} is elevated?

Scorrano: There is a paper by Antonella Viola's group (Campello et al 2006). We collaborated with them. She showed that mitochondria were recruited at the uropods of migrating lymphocytes. This recruitment is separated from the recruitment of the ER and depends on the activation of the fission machinery. If you block fragmentation of the mitochondria, you block polarization of the T cell. If you induce fragmentation of the mitochondria by itself, this drives polarization of the T cell. This is Ca^{2+} independent.

Nicholls: Is there any relationship between the fission/fusion state and where the mitochondria will end up? Why are presynaptic mitochondria smaller than somatic mitochondria?

Youle: The inherited diseases of mitochondrial fission and fusion proteins are neuronal, such as CMT2A.

Nicholls: You showed some nice fission and fusion data. Does anyone want to comment?

Shirihai: These data are nice. I would like to comment on the use of the term 'kiss and run' here. Usually we use the term kiss and run where there is a sharing of solution content but not where sharing of membranes is found. Here there is clearly a sharing of membrane components; although the kinetics is slower. If this is a kiss and run event, it is a definitely a slow French kiss followed by a slow-motion run.

Duchen: One of the things you showed was that the more depolarized mitochondria were less likely to fuse later. Is this because they might be less likely to move, or could it be because they are Ca^{2+} loaded?

Shirihai: It is possible that the reason the depolarized mitochondria are less likely to fuse is their association with the cytoskeleton. We need to examine this.

Hajnóczky: With regard to the Ca^{2+} dependence, we have detected fusion events in Ca^{2+}-depleted cells. Thus a Ca^{2+} elevation in the cytosol or organelles does not seem to be required for the fusion event. Regarding the membrane potential dependence of the fusion events, the view has been that the membrane potential is critical, at least for the inner membrane fusion. Recording of the membrane potential simultaneously with the fusion events gives a hint that the fusion capacity is not lost at the same time when the membrane potential falls. If the membrane

potential is collapsed by the addition of FCCP, there are still fusion events in the next few minutes. Perhaps it is not the membrane potential per se that is needed for the mitochondrial fusion, but the membrane potential loss is probably converted to a chemical signal such as a cleavage of a protein, which prevents further fusion events. Perhaps this mechanism contributes to the selection of the pool that then undergoes autophagy.

Halestrap: How much is exchanged in a kiss-and-run? We know a lot about the structure of a mitochondrion. Many of the proteins are in a sort of matrix gel. They are hardly free. When you add an indicator like an extraneous protein, this may well move much better. But how many of the normal proteins move?

Schon: An experiment was done by Nonaka in which he fused ρ^0 cells with wild-types and looked for a recovery of cytochrome oxidase in the fusion product. It is remarkably fast.

Halestrap: In each kiss-and-run it may be a relatively small amount that is exchanged.

Reynolds: In terms of the magnitude of the structures we are looking at here, how much DNA is there in these pieces of mitochondria that are 2 μm long when they go into fission or fusion? Is every daughter going to have a piece of DNA?

Schon: Yes. It's amazing. If cells are stained with MitoTracker and you look for nucleoids, no matter what the size of the MitoTracker-positive object is, it will have at least one nucleoid in it. We have to be careful because it could be that only nucleoid-containing mitochondria become MitoTracker positive, but it is a remarkable correlation.

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