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Chapter 18. Modulation of calcium entry by mitochondria

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Abbreviations

$[\text{Ca}^{2+}]_{\text{M}}$, mitochondrial matrix free $[\text{Ca}^{2+}]$

CRAC channels, Ca^{2+} -release activated Ca^{2+} channels

ER, Endoplasmic reticulum

CICR, Ca^{2+} -induced Ca^{2+} release

GDAP1, Ganglioside-induced differentiation-associated protein 1

IP_3 , inositol 1,4,5-trisphosphate

IS, Immunological synapse or Immune synapse

JPH1, junctophilin 1

MCU, Mitochondrial calcium uniporter

NFAT, Nuclear factor of activated T-cells

SERCA, Sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase

SOCE, Store-operated Ca^{2+} entry

STIM, Stromal interaction molecule

TRP, Transient receptor potential

Abstract

The role of mitochondria in intracellular Ca^{2+} signaling relies mainly in its capacity to take up Ca^{2+} from the cytosol and thus modulate the cytosolic $[\text{Ca}^{2+}]$. Because of the low Ca^{2+} -affinity of the mitochondrial Ca^{2+} -uptake system, this organelle appears specially adapted to take up Ca^{2+} from local high- Ca^{2+} microdomains and not from the bulk cytosol. Mitochondria would then act as local Ca^{2+} buffers in cellular regions where high- Ca^{2+} microdomains form, that is, mainly close to the cytosolic mouth of Ca^{2+} channels, both in the plasma membrane and in the endoplasmic reticulum (ER). One of the first targets proposed already in the 90s to be regulated in this way by mitochondria were the store-operated Ca^{2+} channels (SOCE). Mitochondria, by taking up Ca^{2+} from the region around the cytosolic mouth of the SOCE channels, would prevent its slow Ca^{2+} -dependent inactivation, thus keeping them active for longer. Since then, evidence for this mechanism has accumulated mainly in immunitary cells, where mitochondria actually move towards the immune synapse during T cell activation. However, in many other cell types the available data indicate that the close apposition between plasma and ER membranes occurring during SOCE activation precludes mitochondria from getting close to the Ca^{2+} -entry sites. Alternative pathways for mitochondrial modulation of SOCE, both Ca^{2+} -dependent and Ca^{2+} -independent, have also been proposed, but further work will be required to elucidate the actual mechanisms at work. Hopefully, the recent knowledge of the molecular nature of the mitochondrial Ca^{2+} uniporter will allow soon more precise studies on this matter.

18.1 Mitochondrial Ca^{2+} transport

Ca^{2+} is an intracellular second messenger able to fulfill a variety of different functions in all cell types with no exception, both excitable and non-excitable cells. Let's just mention muscle contraction, neurotransmitter secretion, fertilization, proliferation, development, learning, memory or cell death [1]. Most of these functions are triggered by changes in cytosolic Ca^{2+} , but several intracellular organelles also play a very important role in cellular Ca^{2+} homeostasis, either because Ca^{2+} plays specific roles inside the organelle or because Ca^{2+} -fluxes in and out the organelle are able to regulate cytosolic $[\text{Ca}^{2+}]$. The first organelle that was known to have an important role in Ca^{2+} homeostasis was the endoplasmic reticulum (ER), that was shown to release Ca^{2+} in the presence of IP_3 [2]. This organelle behaves as a Ca^{2+} store and in steady-state conditions has a very high $[\text{Ca}^{2+}]$ in its lumen, around 1-2 mM, as we have recently reported using a new double-mutated aequorin with very low Ca^{2+} affinity [3]. Different kinds of cell stimulation can induce a rapid release of the stored Ca^{2+} to the cytosol through several Ca^{2+} channels present in its membrane (IP_3 and ryanodine receptors, mainly).

Another organelle with an important role in cell Ca^{2+} homeostasis is mitochondria, the organelle responsible for aerobic energy production in the cell. The ability of mitochondria to take up large amounts of Ca^{2+} was known since the 60's [4]. In fact, until the end of the 70's, mitochondria were thought to be the main cellular Ca^{2+} store. Several findings changed then the view on this point. First, it was shown that Ca^{2+} uptake by mitochondria was carried out by a system (the uniporter) which had a very low Ca^{2+} -affinity (K_M above $10\mu\text{M}$) [5, 6]. In the same dates, the first measurements of cytosolic $[\text{Ca}^{2+}]$ in intact cells obtained with loaded dyes showed that cytosolic $[\text{Ca}^{2+}]$ under resting conditions was very low, around 100nM, and that even after cell stimulation, it did not usually rise above 1-2 μM , that is, still 10-fold below the K_M of the uniporter. Moreover, the finding that the endoplasmic reticulum had Ca^{2+} channels activated by IP_3 [2] suggested that the main responsible for intracellular Ca^{2+} release was the endoplasmic reticulum. As a consequence, mitochondria were thought not to have a significant role in Ca^{2+} homeostasis, at least under physiological conditions (although perhaps under pathological conditions). Then, in the beginning of the 90's, the general view on this point changed again when experiments performed in the laboratory of T. Pozzan and R. Rizzuto using recombinant aequorin targeted to the mitochondria showed that mitochondrial $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_M$) was able to undergo rapid changes during cell activation [7]. That was the origin of a series of works by several research groups, demonstrating that mitochondria are very active players indeed in the control of the global cell Ca^{2+} homeostasis.

The rationale developed in those years to explain the apparent contradiction among the low Ca^{2+} -affinity of the uniporter and the fast mitochondrial Ca^{2+} uptake observed in intact cells was based in the concept of high- Ca^{2+} microdomains. So, it is right that

mitochondria would be unable to take up much Ca^{2+} when exposed to the mean $[\text{Ca}^{2+}]$ of the cytosol, around $1\mu\text{M}$. However, during cell stimulation, some mitochondria may be placed very close to the Ca^{2+} channels responsible for either Ca^{2+} release from the ER or Ca^{2+} entry from the extracellular medium. These particular mitochondria would be exposed to a much higher $[\text{Ca}^{2+}]$ (a hot-spot or high- Ca^{2+} microdomain), enough to activate the uniporter and trigger fast mitochondrial Ca^{2+} uptake. A corollary of this hypothesis is that Ca^{2+} uptake by the mitochondrial network is heterogeneous, because only mitochondria close enough to those channels will take up significant amounts of Ca^{2+} , while the rest will remain with low $[\text{Ca}^{2+}]$. And, of course, the precise intracellular localization of mitochondria with respect to the Ca^{2+} channels becomes critical to determine the rate and amount of Ca^{2+} uptake. This point will be particularly important with regards to the interaction between mitochondria and capacitative Ca^{2+} entry.

Evidence for the multiple roles played by mitochondria in cell Ca^{2+} homeostasis has been continuously growing in the last years. First, it was already known that Ca^{2+} activates several Krebs cycle enzymes: pyruvate dehydrogenase, α -ketoglutarate dehydrogenase and isocitrate dehydrogenase [8], and it has been conclusively shown that an increase in $[\text{Ca}^{2+}]_{\text{M}}$ immediately leads to an increase in ATP production [9, 10]. This mechanism constitutes a basic homeostatic response that associates cell activation to energy production, in order to cover the energy requirements of the response to the stimulus. In addition, it has been recently shown that mitochondrial metabolism is also modulated by extramitochondrial $[\text{Ca}^{2+}]$, thanks to the activity of several Ca^{2+} -dependent mitochondrial carriers, including both the aspartate/glutamate carriers from the malate/aspartate shuttle and the ATP-Mg/Pi carriers [11-13]. These mitochondrial carriers are in the inner mitochondrial membrane but sense cytosolic Ca^{2+} through EF-hand motifs placed in the intermembrane space. Another Ca^{2+} -dependent mitochondrial dehydrogenase is the FAD-glycerol phosphate dehydrogenase, a key component of the glycerol-3-phosphate shuttle, which is also placed in the inner mitochondrial membrane and senses cytosolic Ca^{2+} in the intermembrane space [14]. This dehydrogenase has been recently shown to be the target of metformin, one of the most effective therapeutics for treating type 2 diabetes [15].

On the other hand, the fast mitochondrial Ca^{2+} -accumulation that occurs during cell stimulation constitutes in fact a mechanism of transient Ca^{2+} buffering (see [16] for a review). Because of the low- Ca^{2+} -affinity of their Ca^{2+} uptake mechanism, mitochondria are very well adapted to take up Ca^{2+} from local high- Ca^{2+} microdomains, such as those formed after the activation of plasma membrane or ER Ca^{2+} channels. These high- Ca^{2+} cytosolic microdomains are responsible for a variety of Ca^{2+} -dependent phenomena, such as neurotransmitter secretion or cardiac cell contraction. Mitochondria can therefore modulate these important cellular functions by acting as local Ca^{2+} sinks. For example, we have shown that mitochondria can modulate local cytosolic Ca^{2+} concentrations and Ca^{2+} -dependent catecholamine secretion in chromaffin cells [17, 18]. In these cells, a large fraction of the Ca^{2+} entering the cell during stimulation appears to be transiently buffered by mitochondria [18-20]. In fact, during chromaffin cell

stimulation, mitochondria can undergo reversible and repetitive near millimolar $[Ca^{2+}]_M$ transients [17], as a consequence of the generation of local high Ca^{2+} microdomains of up to 20-40 μM , compared with a mean cytosolic $[Ca^{2+}]$ of 2 μM . The ability of mitochondria to buffer local high- Ca^{2+} microdomains has also been used to explain the modulation by mitochondria of the activity of several Ca^{2+} channels showing Ca^{2+} -dependent inactivation. The role of mitochondria in these cases would be to control the size of the local $[Ca^{2+}]$ microdomain close to the cytosolic mouth of the channel. Capacitative Ca^{2+} entry channels were the first ones shown to be modulated in this way by mitochondria [21]. It was proposed that mitochondrial Ca^{2+} uptake in the vicinity of the channels in the plasma membrane reduced the local $[Ca^{2+}]$ and avoided their slow Ca^{2+} -dependent inactivation, thus keeping the channels active for longer. The same idea was then used to explain the modulation by mitochondria of plasma membrane voltage-dependent Ca^{2+} channels [22, 23] and ER IP_3 receptor channels [24, 25] (see [26] for a review).

Largely in parallel with the history of the capacitative calcium entry, most of the mitochondrial Ca^{2+} transport systems have resisted for many years all the attempts to characterize them molecularly, in spite of the efforts of many research groups. However, the last five years have been very fruitful at this respect and we have now a much clearer picture of the molecules involved. First the mitochondrial Na^+/Ca^{2+} exchanger [27] and then the mitochondrial Ca^{2+} uniporter [28, 29] and all its different regulatory components have been described in these years (for comprehensive reviews, see [30, 31]). Among the unexpected findings obtained, let us mention two main points. First, the large complexity of the mitochondrial Ca^{2+} uniporter, which has not only a pore subunit (the MCU protein, that most probably forms tetramers in the membrane), but also a paralog with dominant negative function (MCUb), and at least 5 additional regulatory subunits: MICU1, MICU2, MICU3, EMRE and MICUR1, all of them with different and in some cases opposite functions, which are still not completely clarified. The second surprise came about one year ago, when the group of T. Finkel developed a transgenic mouse with a knockout of the pore subunit (MCU), and it was not only viable, but in fact showed a very mild phenotype [32]. It is true that these mice are indeed only viable if they are maintained on a mixed genetic background, and that even in this case there appears to be still a significant amount of embryonic lethality [33]. But the survivors, which had no detectable protein expression of MCU and showed a complete abrogation of any fast mitochondrial calcium uptake in isolated mitochondria, were nevertheless nearly normal.

18.2 Capacitative Ca^{2+} entry

Capacitative Ca^{2+} entry was first described nearly 30 years ago [34, 35] as a mechanism able to activate Ca^{2+} entry from the extracellular medium after the depletion of Ca^{2+} of the intracellular stores, namely, the endoplasmic reticulum. The pathway was later renamed store-operated Ca^{2+} entry (SOCE, [36]) and also Ca^{2+} -release activated Ca^{2+} channels (CRAC channels or I_{CRAC} , [37]) when referred to the Ca^{2+} current

measured in the plasma membrane of immunitary cells after depletion of the intracellular Ca^{2+} stores. A large series of works has showed that SOCE is not only the predominant Ca^{2+} -entry pathway in non-excitable cells, but it is also present and very important in excitable cells, where it coexists with many different types of voltage-dependent and receptor-operated Ca^{2+} channels in the plasma membrane.

In spite of the long history of the SOCE channels, the molecular mechanism responsible for Ca^{2+} entry and the mechanism of signal transduction from the emptying of the Ca^{2+} stores to the activation of Ca^{2+} entry has been only elucidated in the last ten years. The discovery in 2005 and 2006 of the main constituents of the CRAC channels, the family of STIM proteins as sensors of the ER $[\text{Ca}^{2+}]$ and the family of Orai proteins as Ca^{2+} channels in the plasma membrane, finally provided the molecular tools required to explore the importance of the SOCE mechanism (for reviews, see [38-43]).

Co-expression of STIM1 and Orai1 generates Ca^{2+} currents identical to those of native I_{CRAC} , store-operated, with very high Ca^{2+} selectivity, inward rectifying and with extremely small single-channel conductance. However, although I_{CRAC} is certainly the best characterized Ca^{2+} current responsible for SOCE, it may not be the only one. Store-operated Ca^{2+} currents with different properties (particularly with lower Ca^{2+} selectivity or non-selective) have also been reported and named I_{SOC} to differentiate them from I_{CRAC} [44]. TRP channels, and particularly the TRPC subfamily, could play an important role in the I_{SOC} currents. Most of the members of the TRPC subfamily have been reported to generate store-operated Ca^{2+} currents and some authors sustain that these currents might be carried through homo or heteromeric combinations of different channel subunits involving TRPCs and/or Orai. Interaction of these plasma membrane homo/heteromeric channels with STIM1 in the ER would confer them store-operated properties. STIM1 has in fact been reported to interact physically both with Orai1 and TRPC channels [45-47].

18.3 Effect of mitochondrial Ca^{2+} buffering on SOCE activation

The classical mechanism proposed for regulation by mitochondria of the activity of SOCE channels relies in the ability of mitochondria to reduce the size of the local Ca^{2+} microdomain around the inner mouth of the channel and thus avoid their Ca^{2+} -dependent inactivation. CRAC channels have two types of Ca^{2+} -dependent inactivation. Fast inactivation occurs with time constants of 10-100ms and is due to Ca^{2+} binding at a site within less than 10nm of the pore. The molecular determinants of this fast Ca^{2+} -dependent inactivation have been found both in Orai1 and STIM1, and a role of calmodulin has also been suggested [39, 48]. Because of the extremely small spatio-temporal scale of this phenomenon, mitochondria are not considered to be able to modulate the Ca^{2+} microdomain responsible for this fast inactivation. However, there is also a slow Ca^{2+} -dependent inactivation of CRAC channels that develops with a time course of tens of seconds and involves a larger high- Ca^{2+} spatial domain. It is this slow inactivation mechanism the one that could be modulated by mitochondria. The

mechanism and molecular determinant of this slow Ca^{2+} -dependent inactivation is unknown, but it seems to be placed at around 100nm distance from the CRAC channel pore [44]. In the case of TRPC1 channels, which also have Ca^{2+} -dependent inactivation, there is evidence that it may be mediated by calmodulin [49].

The first evidences for the modulation of SOCE by mitochondria through this mechanism were obtained in 1997 [21]. Both mitochondrial depolarization and inhibition of the electron transport chain enhanced the slow inactivation of the CRAC channel current in Jurkat T lymphocytes. Therefore, energized mitochondria were necessary to prevent the slow inactivation of the channels and facilitate a sustained Ca^{2+} entry through CRAC channels. T cell activation after exposure to antigen or other stimuli requires this prolonged Ca^{2+} entry in order to activate the Ca^{2+} -dependent protein phosphatase calcineurin. Then, dephosphorylation of the transcription factor NFAT by calcineurin unmasks the nuclear localization sequence of NFAT, which then translocates to the nucleus to activate gene transcription of T-cell activation genes [50]. In addition, mitochondria could also have a direct role in the activation of the CRAC current. Removal of cytosolic Ca^{2+} by mitochondria was proposed to compete with store refilling by SERCA pumps, increasing IP_3 -induced ER depletion and leading to faster CRAC activation [51]. Thus, mitochondrial Ca^{2+} uptake would be essential both to activate and to sustain CRAC activity [52]. On the other hand, inhibition of mitochondrial Ca^{2+} efflux also diminished SOCE, suggesting that both uptake and release of Ca^{2+} from mitochondria were important to control SOCE [53].

In any case, some uncertainties remain on the role of mitochondria to control SOCE and the possible mechanism. The main mechanism proposed for mitochondrial modulation of SOCE involves local Ca^{2+} buffering to prevent the slow Ca^{2+} -inactivation of the channels. However, local Ca^{2+} microdomains in the SOCE Ca^{2+} -entry sites in T lymphocytes have been reported to be also essential to activate ryanodine receptors via Ca^{2+} -induced Ca^{2+} release (CICR), thus inducing further Ca^{2+} -release to avoid store refilling and keep SOCE active [54]. Coupling of ryanodine receptors and CRAC channels through this Ca^{2+} microdomain would therefore be critical to keep SOCE active in T cells. A similar mechanism has been described for the selective activation of adenylate cyclase by SOCE in defined microdomains. Ca^{2+} -sensitive adenylate cyclase would interact with Orai1 in order to be activated by the local Ca^{2+} microdomain created by channel activation [55]. In addition, local Ca^{2+} microdomains around Orai1 channels are also essential for translocation of NFAT to the nucleus, probably because NFAT dephosphorylation depends on the local recruitment of calcineurin in the microdomain region [56-58]. Thus, the local $[\text{Ca}^{2+}]$ near CRAC channels has to rise above a certain threshold in order to trigger NFAT migration. It is therefore difficult to envision the role of mitochondria controlling the architecture of this local Ca^{2+} microdomain, which would be at the same time responsible for SOCE inactivation and essential to trigger CICR and calcineurin activation.

Other mechanisms for Ca^{2+} -dependent mitochondrial regulation of SOCE have also been proposed, among them local ATP production, which could chelate Ca^{2+} close to mitochondria, competition for Ca^{2+} uptake with the plasma membrane Ca^{2+} ATPase, which could contribute to sustain intracellular $[\text{Ca}^{2+}]$ increase and direct modulation of ER Ca^{2+} -release through the Ca^{2+} -dependence of IP_3 receptors [59-61]. In addition, Ca^{2+} -independent mechanisms for mitochondrial regulation of SOCE may also be present. In RBL-1 cells and mouse embryonic fibroblasts, store depletion did not induce any change in the pattern of mitochondrial distribution, and most mitochondria were far from the plasma membrane, between 500nm and 1 μm . However, mitochondrial depolarization impaired the trafficking of STIM1 oligomers to ER-plasma membrane junctions and correspondingly inhibited SOCE [62]. This effect depended on the mitochondrion-shaping protein mitofusin-2. In the absence of mitofusin-2, STIM1 traffic and SOCE were no longer affected by mitochondrial depolarization. Therefore, mitochondria may act on SOCE by mechanisms unrelated to Ca^{2+} fluxes.

18.4 Mitochondrial subcellular localization and SOCE.

In order for mitochondria to be able to control the local $[\text{Ca}^{2+}]$ around the SOCE channels in the plasma membrane, they should be placed very close to them. As mentioned above, the low Ca^{2+} -affinity of the mitochondrial Ca^{2+} uniporter puts a lower limit to the cytosolic $[\text{Ca}^{2+}]$ around mitochondria required to trigger fast Ca^{2+} uptake by the organelle. Thus, although mitochondria have been shown to take up Ca^{2+} at submicromolar $[\text{Ca}^{2+}]$ [63, 64], high rates of Ca^{2+} uptake are only obtained when extramitochondrial $[\text{Ca}^{2+}]$ rises above 2-3 μM [17, 64]. Given that most cellular stimuli induce under physiological conditions mean cytosolic $[\text{Ca}^{2+}]$ increases below 1 μM , mitochondria should be quite close to the Ca^{2+} -entry sites in order to activate fast Ca^{2+} uptake through the MCU. In chromaffin cells, for example, Ca^{2+} entry through voltage-dependent Ca^{2+} channels generates local Ca^{2+} microdomains of 20-40 μM that trigger fast mitochondrial Ca^{2+} uptake in a mitochondrial population. However, more than 50% of the mitochondria in each cell, those placed far away from the channels, hardly take up any Ca^{2+} [17, 18].

In immunitary cells, considerable evidence suggests that mitochondria actually move towards the SOCE sites during cell activation. In the Jurkat T cell line, stimulation of Ca^{2+} entry facilitated translocation along microtubules of the complete mitochondrial network towards the plasma membrane, in a Ca^{2+} and kinesin-dependent manner [65]. In T cells, the interface between an antigen-presenting cell or target cell and the lymphocyte is known as the immunological synapse or immune synapse (IS). The junction between T cells and antigen-presenting cells is characterized by close membrane appositions, separated from one another by 'synaptic' regions. Inside the T cell, membrane microdomains containing signaling proteins reorganize and several organelles redistribute toward the IS, including mitochondria and endoplasmic reticulum [66]. Thus, T cell stimulation causes a redistribution of the mitochondrial network towards the plasma membrane and the immunological synapse (IS), and some

mitochondria approach to distances of 200nm of the SOCE channels. In addition, mitochondria close to the IS took up more Ca^{2+} than those farther away, and redistribution of mitochondria to the IS was necessary to maintain Ca^{2+} influx across the plasma membrane and Ca^{2+} -dependent T-cell activation [67-69]. In fact, once formed the IS, mitochondria move towards them in a Ca^{2+} -dependent manner, and maintain SOCE active specifically at the IS, even in the absence of Orai1 accumulation there. Mitochondrial localization would therefore determine where in the plasma membrane should Ca^{2+} entry be active [70] (for reviews see [60, 71]).

However, there are still some findings awaiting explanation. Redistribution of mitochondria to the IS was impaired by knockdown of the mitochondrial fission factor dynamin-related protein 1 (Drp1). Moreover, Drp1 knockdown induced mitochondrial depolarization. However, Drp1 knockdown increased T-cell receptor signal strength and produced a larger and more durable intracellular $[\text{Ca}^{2+}]$ increase, in spite of the absence of mitochondria close to the IS [72]. On the other hand, silencing of the outer mitochondrial membrane protein GDAP1, mutations of which produce Charcot-Marie-Tooth neuropathy, induced abnormal distribution of the mitochondrial network in SH-SY5Y human neuroblastoma cells and decreased Ca^{2+} influx through SOCE [73]. Interestingly, junctophilin 1 (JPH1) overexpression was able to restore the SOCE activity in GDAP1-silenced cells [74]. JPH1 is a protein placed in the ER within the mitochondria-associated membrane (MAM) complexes (as well as GDAP1 from the mitochondrial side), but relocates during SOCE activation to co-localize with STIM1 in ER-plasma membrane junctions. Surprisingly, although mitochondrial blockers produced severe inactivation of SOCE in control cells, SOCE activity in JPH1-overexpressing cells was no longer inhibited by mitochondrial blockers, indicating that the restoration of SOCE activity by JPH1 in GDAP1 deficient cells was not related to the recovery of mitochondrial distribution or function [74].

In other non-excitable cell types, the evidence for the regulation of SOCE by mitochondria is weaker. In HeLa cells, expression of the mitochondrial fission promoter protein hFis1 induced a rapid and complete fragmentation of mitochondria, which redistributed away from the plasma membrane and clustered around the nucleus, even though they maintained a normal transmembrane potential and pH, and took up normally the Ca^{2+} released from intracellular stores upon agonist stimulation. Despite the dramatic morphological alteration, SOCE was only marginally reduced. However, disruption of mitochondrial potential with uncouplers or oligomycin/rotenone still reduced SOCE by 35% [75]. Therefore, either close contact of mitochondria and Ca^{2+} influx channels is not required for SOCE modulation in these cells or mitochondrial inhibitors have additional effects directly on the channels. Also in HeLa cells, activation of SOCE has been shown not to produce significant local $[\text{Ca}^{2+}]$ microdomains. While release of Ca^{2+} from the ER generated $[\text{Ca}^{2+}]_{\text{M}}$ levels 5-10 fold higher in neighboring mitochondria than in the bulk cytosol, Ca^{2+} entry through SOCE generated similar $[\text{Ca}^{2+}]$ levels in plasma membrane mitochondria and in deep mitochondria [76, 77]. These results suggest that mitochondria are excluded in these cells from the regions

where SOCE occurs. In fact, in COS-7 cells, mitochondria were located either in the gaps between STIM1 puncta or in remote, STIM1-free regions, and the increase in $[Ca^{2+}]_M$ after Ca^{2+} addition was independent of the mitochondrion–STIM1 distance [78]. Similarly, in the cell lines RBL-2H3 and H9c2 cells, all mitochondria were shown to have contacts with the ER, but contacts between mitochondria and the plasma membrane were less frequent due to the presence of interleaving ER stacks [79, 80]. In fact, these findings provide structural basis for the observation that in some conditions Ca^{2+} reaches mitochondria only after crossing the ER [81].

These observations indicate that in these cells mitochondria are only exposed to Ca^{2+} diffusing laterally from the SOCE Ca^{2+} -entry sites, suggesting that the close apposition among ER and plasma membrane in the sites of STIM-Orai interaction represents indeed a physical barrier for access of mitochondria. These STIM-Orai interacting sites have also been shown to co-localize with SERCA pumps in non-excitabile cells [82, 83]. For example, in HEK293 cells, SOCE activation generates subplasmalemmal high Ca^{2+} microdomains, but most of the entering Ca^{2+} is taken by the ER and not by mitochondria. This may also be due to the small size of the microdomains generated by SOCE, as SERCA pumps have a much higher Ca^{2+} affinity than mitochondrial uniporters [83].

In summary, mitochondrial localization with respect to SOCE appears to change considerably among different cell types [48]. In cells such as HeLa, RBL or HEK293, mitochondria are far away from the SOCE Ca^{2+} entry sites, while in T cells or Jurkat T lymphocytes cell activation triggers a fast movement of mitochondria toward the IS. Regarding excitable cells, there is also a significant variability. In vascular smooth muscle A10 cells, mitochondrial Ca^{2+} uptake was triggered by SOCE activation, but not by activation of voltage-dependent Ca^{2+} channels [84]. In other excitable cells, instead, mitochondrial Ca^{2+} uptake was much larger after voltage-dependent Ca^{2+} channels activation [17, 76].

18.5 Effect of MCU knockout on SOCE activation

The recent discovery of the main constituents of the mitochondrial Ca^{2+} uniporter provides a much better tool to assay the effects of mitochondrial Ca^{2+} uptake on Ca^{2+} entry through SOCE. Knockout of the pore component of the uniporter, the MCU, has been recently used to study the effect on SOCE. In RBL-1 cells, knockout of MCU considerably reduced Ca^{2+} entry after store depletion with thapsigargin [85]. MCU silencing by siRNA in MDA-MB-231 breast cancer cells also reduced serum- or thapsigargin-induced SOCE [86]. Similarly, in HeLa cells, MCU knockout reduced the rate of Ca^{2+} entry through SOCE and favored the development of slow inactivation [87].

We have also studied this point by using specific shRNA to silence the MCU protein in HeLa cell clones. However, our data do not show any inhibition of SOCE in the MCU-silenced clones with respect to scrambled clones. Fig. 1 shows studies carried

out using aequorin targeted to mitochondria or endoplasmic reticulum. The histamine-induced $[Ca^{2+}]_M$ peak was nearly abolished in the MCU-silenced cells (Fig. 1a), and addition of Ca^{2+} to permeabilized cells produced no increase in $[Ca^{2+}]_M$ in the MCU-silenced cells (Fig. 1b), showing that MCU activity was almost fully abolished in the silenced clone. In Ca^{2+} -depleted cells, refilling with Ca^{2+} of the ER was in fact faster in the silenced cells (Fig. 1c-d), suggesting that Ca^{2+} -entry through SOCE channels was not reduced in the MCU-silenced cells. Regarding Ca^{2+} -release through IP_3 receptors induced by the agonist histamine, it was somewhat slower in the MCU –silenced cells (Fig. 1e). This observation may be due to the increased feed-back inhibition by Ca^{2+} of IP_3 receptors in the absence of mitochondrial Ca^{2+} uptake.

Imaging studies of cytosolic $[Ca^{2+}]$ (Fig. 2) showed that the histamine induced cytosolic $[Ca^{2+}]_c$ increase was significantly larger in the MCU-silenced cells (Fig. 2a), probably because of the lack of mitochondrial Ca^{2+} uptake. When SOCE was explored directly by looking at the cytosolic $[Ca^{2+}]$ increase induced by Ca^{2+} addition to Ca^{2+} -depleted cells, Ca^{2+} entry through SOCE was found to be significantly increased in the MCU-silenced cells (Fig. 2b). Consistently, the rate of Mn^{2+} entry in Ca^{2+} -depleted cells (Fig. 2c) was also significantly increased in the MCU-silenced cells. Therefore, our data indicate that the absence of mitochondrial Ca^{2+} uptake did not inhibit SOCE in HeLa cells. In fact, a significant stimulation was observed. The reasons for the discrepancy with previously reported data [87] are unknown.

18.6 Conclusions

Intracellular Ca^{2+} signaling is mainly controlled by ER and mitochondria, two organelles with opposite roles in terms of Ca^{2+} homeostasis. The ER is the main intracellular Ca^{2+} -store, releases Ca^{2+} to the cytosol during cell activation and then helps to end the Ca^{2+} transient by taking up Ca^{2+} back after the stimulation. In contrast, mitochondria have little $[Ca^{2+}]$ under resting conditions, but have a large capacity to take up Ca^{2+} during cell stimulation and then release it again to the cytosol at the end of the Ca^{2+} transient. In addition, because of the low- Ca^{2+} affinity of the mitochondrial Ca^{2+} uptake mechanism, this organelle is specially adapted to buffer Ca^{2+} from high- Ca^{2+} microdomains such as those formed around the cytosolic mouth of open Ca^{2+} channels. The classical mechanism proposed for mitochondrial regulation of SOCE activity relies in the effect of mitochondrial Ca^{2+} uptake controlling the local Ca^{2+} microdomain around the SOCE channels, in order to prevent its Ca^{2+} -dependent inactivation. To be effective, this mechanism requires very close contacts between mitochondria and SOCE channels. However, mitochondrial subcellular localization with respect to SOCE channels appears to be highly variable among different cell types. While in immunitary cells mitochondria move to the IS during cell activation, in other cells there is little evidence for that. Fig. 3 shows diagrams summarizing these mechanisms. In addition, activation of SOCE requires a close interaction among Orai (or TRPs) channels in the plasma membrane and STIM sensors in the ER. The steric hindrance due to the close apposition of plasma and ER membranes in the SOCE sites

constitutes a barrier that limits the approach of mitochondria. Even in immune cells, this makes the distance of mitochondria to the SOCE sites to be at least 200nm. In other cells the distance is even larger. On the other hand, the local Ca^{2+} microdomain created in the SOCE sites in immune cells seems to be also essential for T cell activation and NFAT dephosphorylation. Therefore, many uncertainties still remain on the role of mitochondria to control SOCE and the possible mechanism. Alternative pathways for mitochondrial modulation of SOCE, both Ca^{2+} -dependent and Ca^{2+} -independent, have also been proposed, but clear evidence for a specific mechanism is still lacking. The recent advances in the knowledge of the molecular nature of the mitochondrial Ca^{2+} uptake mechanism open new ways to approach this problem directly, avoiding the use of mitochondrial inhibitors or protonophors and all their large side-effects.

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Figure Legends

Fig. 1. Effect of MCU knockout on mitochondrial and ER $[Ca^{2+}]$ dynamics. HeLa cell clones expressing either shRNA for the MCU protein or scrambled shRNA were transfected with either mitochondrially targeted mutated (a) or double mutated (b) aequorin or ER-targeted double-mutated aequorin (c). a) Effect of stimulation with 100 μ M histamine on $[Ca^{2+}]_M$. b) Effect of perfusion of a 7 μ M $[Ca^{2+}]$ buffer in permeabilized cells (see [88] for other details). c) Refilling of the ER with Ca^{2+} and Ca^{2+} release induced by 100 μ M histamine (see de la Fuente et al., 2013 for other details). d) Increase in the steady-state ER $[Ca^{2+}]$ in MCU-silenced cells. e) Decrease in the Ca^{2+} release rate in MCU-silenced cells.

Fig. 2. Effect of MCU knockout on cytosolic $[Ca^{2+}]$ dynamics and Mn^{2+} entry. The shMCU and scrambled HeLa cell clones were loaded with fura-2 and prepared for single-cell imaging experiments as described previously [25]. a) Cells were stimulated with 100 μ M histamine. Data are mean $\pm$ s.e. from 5 experiments in each condition (221 cells scrambled, 191 cells shMCU). b) Cells were depleted of Ca^{2+} with 10 μ M benzohydroquinone and then SOCE was induced with 1mM extracellular $[Ca^{2+}]$. Data are mean $\pm$ s.e. from 9 experiments in each condition (700 cells scrambled, 602 cells shMCU). c) Cells were depleted of Ca^{2+} in Ca^{2+} -free medium and then Mn^{2+} entry was monitored at 360nm fluorescence excitation. Data are mean $\pm$ s.e. from 5 experiments in each condition (611 cells scrambled, 768 cells shMCU).

Figure 3. A model of the role of mitochondria in the regulation of SOCE. a) In immune cells, mitochondria move to the plasma membrane during cell activation and considerable evidence indicates that, after SOCE activation, mitochondrial Ca^{2+} uptake reduces the size of the local $[Ca^{2+}]$ microdomain around nearby Orai-STIM complexes and protects Orai1 channels from Ca^{2+} -dependent inactivation. b) In many other cell types, the available evidence suggests that mitochondria remain too far from the Orai-STIM complexes to be able to modulate directly the local Ca^{2+} microdomain. Mitochondria could anyway take up some of the incoming Ca^{2+} after passing through the ER, but the possible modulation of Ca^{2+} entry by mitochondria remains unclear. Alternative mechanisms of modulation based on mitochondrial ATP production, ER depletion induced by mitochondrial Ca^{2+} uptake, or others, have also been proposed. PM, plasma membrane; ERM, ER membrane; SERCA, ER Ca^{2+} ATPase.

Fig. 1

Fig. 2

Fig. 3