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High and low calcium-dependent mechanisms of mitochondrial calcium signalling

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Abstract

The Ca^{2+} coupling between endoplasmic reticulum (ER) and mitochondria is central to multiple cell survival and cell death mechanisms. Cytoplasmic [Ca^{2+}] ([Ca^{2+}]$_c$) spikes and oscillations produced by ER Ca^{2+} release are effectively delivered to the mitochondria. Propagation of [Ca^{2+}]$_c$ signals to the mitochondria requires the passage of Ca^{2+} across 3 membranes, namely the ER membrane, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). Strategic positioning of the mitochondria by cytoskeletal transport and interorganellar tethers provides a means to promote the local transfer of Ca^{2+} between the ER membrane and OMM. In this setting, even >100μM [Ca^{2+}] may be attained to activate the low affinity mitochondrial Ca^{2+} uptake. However, a mitochondrial [Ca^{2+}] rise has also been documented during submicromolar [Ca^{2+}]$_c$ elevations. Evidence has been emerging that Ca^{2+} exerts allosteric control on the Ca^{2+} transport sites at each membrane, providing mechanisms that may facilitate the Ca^{2+} delivery to the mitochondria. Here we discuss the fundamental mechanisms of ER and mitochondrial Ca^{2+} transport, particularly the control of their activity by Ca^{2+} and evaluate both high and low [Ca^{2+}] activated mitochondrial calcium signals in the context of cell physiology.

Keywords

Ca^{2+}; endoplasmic reticulum; sarcoplasmic reticulum; mitochondria; IP_3 receptor; ryanodine receptor; VDAC; uniporter; mitochondrial dynamics

Mechanisms of ER-mitochondrial Ca^{2+} transport: Ca^{2+}-mediated feedforward and feedback pathways

Endoplasmic-and sarcoplasmic reticulum (ER/SR) Ca^{2+} release mediated by IP_3R/RyR

The ER forms a luminally interconnected network of tubules and cysternae throughout the cytoplasm and shows inhomogeneous distribution of the Ca^{2+} uptake and release sites. Based

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on these properties, different cytoplasmic signals may converge in the ER to form spatio-temporally controlled Ca^{2+} release patterns [1;2].

ER Ca^{2+} uptake—The resting [Ca^{2+}] in the lumen of the ER/SR ([Ca^{2+}]$_{ER}$) is in the range of 0.1–0.8 mM, ~4 orders of magnitude higher than [Ca^{2+}]$_c$. This steep [Ca^{2+}] gradient, which is comparable to the one across the plasma membrane, is built up primarily by means of Ca^{2+} pumps of the SERCA family at the expense of ATP hydrolysis (Fig1A). SERCA pumps are encoded by 3 genes that yield more than 10 different isotypes at the protein level due to alternative splicing. The expression of the different isotypes is tissue and developmental stage specific. SERCA pumps are controlled by a host of Ca^{2+}-dependent and independent cytosolic, intra-membrane as well as luminal factors [3]. Evidence has been presented that mitochondrial function e.g. ATP production and Ca^{2+} uptake may exert local control on SERCA activity in the adjacent ER [4–7].

ER luminal Ca^{2+} buffering—The ER Ca^{2+} storage capacity is dependent on the expression of the intra-ER Ca^{2+} binding proteins [8;9] that may also have a role in chaperoning Ca^{2+} release channel proteins [10]. In pancreatic acinar cells, the Ca^{2+}-binding capacity in the lumen of the ER was calculated to be ~100 times lower than in the cytosol (20 vs. ~1500–2000 [11]), suggesting greater mobility for the Ca^{2+} ions inside the store. The major luminal diffusible Ca^{2+} buffers are calreticulin (CRT) in the ER and calsequestrin (CSQ) in the SR. CRT may also bind to the SERCA [12] and CSQ forms a complex with the ryanodine receptor (RyR) [13] to effectively control [Ca^{2+}]$_{ER}$ close to the transport sites and to regulate their activity. Changes in the ER Ca^{2+} loading in association with CRT deficiency or overexpression were not paralleled by similar changes in [Ca^{2+}]$_{ER}$ [14;15], indicating that the steady-state [Ca^{2+}]$_{ER}$ is maintained under a wide range of store Ca^{2+} content. Interestingly, in CRT-overexpressing cells the IP_3-mediated [Ca^{2+}]$_m$ rise was suppressed and was transient probably due to the rapid reuptake of Ca^{2+} mediated by the SERCA [5].

ER Ca^{2+} release—Two major and phylogenetically related families of Ca^{2+} release channels in the reticular Ca^{2+} stores are: the inositol 1,4,5-trisphosphate receptors (IP_3Rs, types 1,2,3) that reside primarily in the ER (Fig1A) and the RyRs (types 1,2,3) predominantly in the SR of muscle cells. However, IP_3Rs are also present in the SR of some muscle cells and RyR in the ER of certain non-muscle cells. Different cell types contain various combinations of the 3 IP_3R isotypes, whereas in each tissue one RyR isotype is dominant (skeletal muscle, type 1; cardiac muscle, type 2; smooth muscle and brain, type 3). Recently, in a cell line that expresses all IP_3R isoforms, type 3 IP_3Rs were shown to preferentially transmit calcium signals to the mitochondria [16]. The IP_3Rs and RyRs encounter dozens of molecular interactions to receive input from different signalling systems (see [17–19] for recent reviews). One of the principal factors that determine the Ca^{2+} release signal output is the regulation of the release channels by Ca^{2+} itself. Both, IP_3R and RyR have been reported to be regulated by luminal and cytosolic Ca^{2+} as well. The control of Ca^{2+} release by luminal and cytosolic Ca^{2+} and other factors is expanded below.

The IP_3 sensitivity of the IP_3R is increased by luminal Ca^{2+} [20;21] but it was claimed that this co-agonistic effect had been established via a cytosolic Ca^{2+} binding site by Ca^{2+} exiting through the IP_3R [22]. On the other hand, single channel studies conducted at optimal [IP_3] levels indicated the presence of a luminal Ca^{2+} inhibitory site [23;24]. The inhibition attributed to this mechanism could be relieved by cytosolic ATP (500 μM) [25]. Recently, ERp44, an ER luminal protein has been shown to interact with the IP_3R1 in a Ca^{2+}, redox and pH dependent manner and to inhibit the Ca^{2+} release when the Ca^{2+} load falls [26]. This mechanism does not apply to other IP_3R isoforms and the luminal Ca^{2+} regulation of the IP_3R2&3s has not yet been studied. Similarly to the IP_3R, the activation of RyR is also regulated by the luminal [Ca^{2+}]. Multiple reports showed that increasing luminal [Ca^{2+}] causes a net increase in the

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channel activity [27–29]. A functionally relevant Ca^{2+} binding site has been localized on the RyR [30] but recent data shows that the stimulatory Ca^{2+} effect is mediated by the luminal CSQ that forms a regulatory complex with triadin and junction [13].

Overexpression of ER-targeted anti-apoptotic Bcl-2 protein caused increased Ca^{2+}-leak from the ER, suppressed the IP_3R-linked $[Ca^{2+}]_m$ responses and gave protection against ceramide-induced apoptosis [31;32]. Binding of Bcl-2 to the IP_3R may also be relevant for these effects [33;34]. To directly test the ER Ca^{2+} load dependence of the IP_3R-mediated Ca^{2+} release and the corresponding mitochondrial Ca^{2+} uptake, we preloaded permeabilized RBL-2H3 cells with small pulses of Ca^{2+}. As the ER Ca^{2+} load was augmented the Ca^{2+} release induced by optimal IP_3 increased in a linear fashion. Interestingly, the mitochondrial Ca^{2+} uptake associated with the IP_3-induced Ca^{2+} release displayed a supralinear relationship with increasing Ca^{2+} loading of the ER. This was also apparent when the $[Ca^{2+}]_c$ was clamped by EGTA at resting levels (Csordás& Hajnóczky, unpublished). Thus, the local Ca^{2+} signal propagation from IP_3R to the mitochondria is effectively controlled by the ER Ca^{2+} load.

IP_3R/RyRs show a bell-shaped $[Ca^{2+}]_c$ dependence for the activation of both receptors although some subtypes (RyR2 and in a study, IP_3R3) lack inactivation at physiologically relevant $[Ca^{2+}]_c$ ranges [reviewed in [17;18;35;36]]. Activation of the Ca^{2+} release channels by $[Ca^{2+}]_c$ is one of the principal mechanisms for coordination of individual release events with each other giving rise to regenerative propagation of the Ca^{2+} release [reviewed e.g. in [37]]. On the other hand, the bell-shaped $[Ca^{2+}]_c$ dependence of activation offers a mechanism for inhibiting release events by high $[Ca^{2+}]$ microdomains built at the mouth of the activated receptors. Besides the low-affinity inhibitory Ca^{2+}-binding site responsible for the inhibition by high $[Ca^{2+}]$ independent of $[IP_3]$, there is also a high-affinity site that inhibits the channel in the absence of IP_3 but with increasing ligand concentrations it gets converted to an activation site [17]. This latter mechanism may be important in increasing the fidelity of ligand-activated channel openings in groups of channels (see later).

Multiple lines of evidence have been presented that the local Ca^{2+} control of the IP_3R/RyR can be modulated by the Ca^{2+} uptake and metabolic activity of the mitochondria located in the immediate vicinity of the release channels at the close contacts of ER/SR and mitochondria. By this mechanism, mitochondria can contribute to the shaping of the global $[Ca^{2+}]_c$ signal. In permeabilized hepatocytes, IP_3-induced $[Ca^{2+}]_{ER}$ decreases were larger in low mitochondrial density regions of the cells as well as after pharmacological inhibition of the mitochondrial Ca^{2+} uptake [38], while enhancement of the mitochondrial Ca^{2+} accumulation after blocking the permeability transition pore suppressed the release activity [39]. On the other hand, $[Ca^{2+}]_c$ spikes generated by microinjection of a non-hydrolizable IP_3 analogue into Xenopus oocytes (IP_3R1) became enhanced and more synchronized after boosting mitochondrial Ca^{2+} uptake by the energizing substrate succinate [40]. Similarly, pharmacological stimulation of the mitochondrial Ca^{2+} uptake sites enhanced the histamine-induced $[Ca^{2+}]_c$ signals in HeLa and human fibroblast cells [41]. Besides the local Ca^{2+} clearance, mitochondria may also participate in the development of regenerative/oscillatory Ca^{2+} release patterns by returning Ca^{2+} in the inter-spike period via the mitochondrial Ca^{2+} extrusion pathways (Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers) to 'prime' the next release event [42]. However, in H295R cells uncoupling of the mitochondria enhanced the oscillatory pattern of the $[Ca^{2+}]_c$ signal and eliminated the amplitude difference between the $[Ca^{2+}]_c$ rise recorded in the perinuclear and in the subplasmalemmal regions [43]. The wide range of the mitochondrial feedback effects on the IP_3R-mediated $[Ca^{2+}]_c$ signal reflects the contribution of several factors, including the spatial relationship between ER and mitochondria and the amount of Ca^{2+} available for mobilization.

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The $[Ca^{2+}]_c$ signal generated by the activation of IP_3R/RyR ranges from greatly confined elementary release events produced by a single or a few channels through propagating repetitive waves of release to concerted simultaneous activation of the entire release channel population throughout the ER. The IP_3R/RyR channels are not distributed homogenously on the ER/SR surface but they form clusters where the inter-channel signalling is enhanced. These clustered channels may work as autonomous elementary release modules that can be activated independently of the rest of the channel population giving rise to isolated and spatially confined $[Ca^{2+}]_c$ transients called 'puffs' (IP_3R) or 'sparks' (RyR) [reviewed in [37]]. Depending on the channel density, the functional IP_3R release units may have different IP_3 sensitivities [44;45]. The release units formed by RyR in skeletal/cardiac muscle tissue show a regular pattern and subcellular distribution [46]. Dynamic reorganization of the IP_3R clusterization is induced in certain cell systems by sustained increases in $[Ca^{2+}]_c$ [47] or by prolonged openings of the IP_3R channels [48].

In muscle cell lines and myotubes, mitochondria can pick up Ca^{2+} mobilized from a single RyR release unit ($[Ca^{2+}]_c$ sparks) leading to the generation of single mitochondrial miniature $[Ca^{2+}]_m$ signals (Ca^{2+} marks, [49]) and to feedback control on the Ca^{2+} release [49;50]. The effect of $[Ca^{2+}]_c$ puffs on $[Ca^{2+}]_m$ has not been directly measured. However, in Xenopus oocytes $[Ca^{2+}]_c$ puffs were frequently colocalized with mitochondria but showed reduced frequency and intensities and never served as wave initiation sites, further indicating that mitochondria are involved in the control of local $[Ca^{2+}]_c$ signalling events [51]. Furthermore, recent modeling studies estimated that only 25–35 out of 40–70 IP_3Rs would contribute actively to the elementary release event Ca^{2+} puffs in Xenopus oocyte [45], which is likely due to local Ca^{2+}-inhibition of clusterized IP_3R-mediated release activity [52]. However, in response to optimal IP_3 every channel synchronously opens in the cluster. The need for synchronized opening of the neighboring IP_3Rs for activation of the mitochondrial Ca^{2+} uptake may be a reason why suboptimal IP_3-induced Ca^{2+} release is not very efficient in raising $[Ca^{2+}]_m$ [53].

Several factors originating from the mitochondria e.g. ATP, cytochrome c and reactive oxygen species (ROS) are important for the control of IP_3R/RyR activity. The mitochondrial output of these factors is commonly induced by a $[Ca^{2+}]_m$ signal. ATP (free of Mg^{2+}) has been reported to potentiate Ca^{2+}-activation of IP_3R1&3 and may be present at particularly high concentrations in the close appositions of mitochondria and ER, establishing progressive sensitization of IP_3R during oscillatory release activity [54–57]. Cytochrome c (cyto c) is normally confined to the mitochondrial intermembrane space and cristae, however pro-apoptotic conditions may cause permeabilization of the OMM and let cyto c escape to the cytosol. Boehning et al. found that cyto c specifically interacted in vitro and in vivo with IP_3R, enhanced its activity by relieving its Ca^{2+}-inhibition and was necessary for apoptosis in some paradigms based on the effects of a peptide specifically interfering with the cyto c binding to the IP_3R [58;59]. ROS are produced by the mitochondria under physiological, and in larger amounts under pathophysiological conditions. Both superoxide anion and hydrogen peroxide stimulate IP_3R/RyR-mediated $[Ca^{2+}]_c$ signalling [60–62].

ER-mitochondrial interface

Ca^{2+} uptake via the ruthenium-sensitive Ca^{2+} uniporter is driven by the 150–180 mV (inside negative) mitochondrial membrane potential, its half-maximal transport rate, as measured in mitochondrial suspensions, is attained in the range of 10^{-6}–10^{-4} M Ca^{2+} [63]. In an electrophysiological study on mitoplasts (mitochondria without the OMM) [64] the ruthenium red-sensitive Ca^{2+} transport was found to occur through highly selective ion channels. The inward rectifying Ca^{2+} current had half-saturation of the Ca^{2+} carrying capacity at 19 mM $[Ca^{2+}]_c$, orders of magnitude higher than that of Ca^{2+} uptake in mitochondrial suspension.

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(This difference may be attributed to the rapid dissipation of the membrane potential during Ca^{2+} entry, causing flux saturation at much lower $[Ca^{2+}]_c$ in mitochondrial suspension, as opposed to the voltage-clamped mitoplasts). In view of these transport characteristics it has been assumed that $[Ca^{2+}]_c$ far exceeding the micromolar range is required for net Ca^{2+} uptake, however, such $[Ca^{2+}]_c$ values have not been observed experimentally in over the whole (so-called global) cytoplasm.

Protein binding significantly limits Ca^{2+} diffusion in the cytoplasm [65]. Therefore Ca^{2+}, entering a cytosolic domain confined by neighbouring membranes, can generate local Ca^{2+} transients with amplitudes far exceeding those measured over the global cytoplasm. Mitochondria, forming a complex cytoplasmic tubulovesicular system [66;67], are frequently apposed to the smooth as well as the rough ER (Fig1B). The apposition, often termed as contact point, has been observed in several cell types by means of electron microscopy or tomography (e.g. [68]). Microdomains are thus formed and Ca^{2+} released through IP_3R into such a confined space, may induce supramicromolar, or even submillimolar Ca^{2+} signal (Fig1A). The possibility of such an event was indicated in 1993 by the experiments of Rizzuto and his coworkers in Pozzan's laboratory [69]. The mitochondrial response to a mean 600 nM elevation of $[Ca^{2+}]_c$ in histamine-stimulated HeLa cells could be reproduced by superfusing the cells with a cytosolic medium containing at least 5 μM Ca^{2+} after cell permeabilization. Similar results were obtained in cardiac (H9c2) cells where the RyR-mediated elevation of global $[Ca^{2+}]_c$ increases were in the submicromolar range but the rate of $[Ca^{2+}]_m$ increases was as large as that induced with 30 μM Ca^{2+} in permeabilized cardiomyocytes [70]. Also in cardiac and skeletal muscle, EGTA/BAPTA antagonized the RyR-mediated increase in $[Ca^{2+}]_c$ but had substantially less impact on $[Ca^{2+}]_m$ [70–72]. In permeabilized RBL-2H3 cells IP_3-induced Ca^{2+} elevation reached values more than 20-fold higher in the vicinity of mitochondria than those measured for the global cytosol [53]. In hepatocytes the long lag between $[Ca^{2+}]_c$ and NAD(P)H increases induced by Ca^{2+} influx, as opposed to IP_3 –induced Ca^{2+} release, was due to a delayed uptake of Ca^{2+} into the mitochondrial matrix [73]. The dependence of mitochondrial Ca^{2+} uptake on the distance between ER and mitochondria was examined in H295R adrenocortical cells. Following stimulation with angiotensin II both mitochondrial Ca^{2+} uptake rate and $[Ca^{2+}]_m$ peak showed a close correlation with the vicinity of ER vesicles, where the distance of ER from the mitochondria was assessed on basis of the fluorescence intensity of a GFP-tagged protein targeted to the ER [74].

Measurement of the mitochondrial redox state in adrenal glomerulosa cells [75] and hepatocytes [73] showed that despite comparable $[Ca^{2+}]_c$ increases, voltage and store-operated Ca^{2+} influx were much less effective than Ca^{2+} release from the IP_3 –sensitive store in activating Ca^{2+} -sensitive mitochondrial dehydrogenases and increasing mitochondrial NAD (P)H levels. These observations also supported the notion that mitochondrial Ca^{2+} uptake occurs preferentially from high-Ca^{2+} microdomains formed between the IP_3R and the mitochondrion.

Targeting aequorin to the outer surface of the IMM in HeLa cells made the measurement of $[Ca^{2+}]$ in the mitochondrial intermembrane space possible. After stimulation with histamine $[Ca^{2+}]$ rose in the intermembrane space to significantly higher values than in the global cytosol [76]. This observation has given a strong support to the concept that net mitochondrial Ca^{2+} uptake occurs from high-Ca^{2+} perimitochondrial microdomains.

The issue was raised whether the vicinity between ER and mitochondria is a stochastic event due to the dense packing of the organelles within the cell or specific interactions support their apposition. In HeLa cells repeatedly exposed to histamine, the comparison of nuclear and mitochondrial Ca^{2+} responses suggested the existence of specific interactions rather than stochastic approach of the two particles [77]. The existence of physical support for the ER-

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mitochondrial interface has been indicated by co-sedimentation of ER particles with mitochondria and electron microscopic observations of close associations between mitochondria and ER vesicles [68;78;79]. At these sites the shortest ER-OMM distance varies from 10nm to 100nM. Based on transmission electron micrographs of fixed H9c2, RBL-2H3 and DT40 cells, approx. 10% of the entire mitochondrial surface is at <100nm distance from the ER (Csordás& Hajnóczky, unpublished). In cells exposed to ER stress (serum starvation, tunicamycin) an increase in the ER-mitochondrial interface and enhanced ER-mitochondrial calcium signalling have been observed. Also, coupling of the two organelles with a fusion protein increased the ER-mitochondria interface area, reduced the ER-mitochondrial distance to about 6 nm and greatly facilitated the transfer of cytosolic Ca^{2+} signal into the mitochondria of RBL-2H3 cells [80].

Evidence has been presented for the contribution of three different components to the ER-mitochondrial scaffold (Fig1A): (a) anchorage of both ER and mitochondria to cytoskeletal structures; (b) tethers, forming direct links between ER membrane and OMM and (c) multimolecular complexes involving both IP_3Rs and voltage-dependent anion selective channels (VDACs).

(a) Anchorage of both ER and mitochondria to cytoskeletal structures: Both ER and mitochondria are anchored to microtubules and microfilaments [81;82]. Organization of ER and mitochondria along the microtubules is apparent in cell processes (see Fig1B). Actin and tubulin [83;84] as well as microtubule and microfilament associated proteins e.g. gelsolin, a Ca^{2+}-dependent actin regulatory protein and microtubule associated protein 2 (MAP2) [85; 86] have been shown to interact with OMM structures. In addition, the microtubular and microfilamental motor proteins are primarily involved in dynamic positioning of ER and mitochondrial domains.

(b) Tethers, forming direct links between ER membrane and OMM: Recently, electron tomography analysis revealed the presence of tethers directly linking the ER to the OMM and showing similar morphology in both isolated organelles and in intact cells and tissue [80;87]. The tethers appear as narrow linear densities ending on both smooth ER and rough ER, in the latter case on or near ER-bound ribosomes. The inter-membrane distances spanned by the tethers range from 6 to 15 nm for the smooth ER and 19–30 nm for the rough ER. Since the ion channels protrude from the membrane surface [88], the vicinity of the tethers may also allow close association and in turn, communication between ER and OMM Ca^{2+} channels.

Based on the sensitivity to proteases the tethers are made of proteins [80]. The knock-out of all IP_3R subtypes in DT40 cells still shows ER – mitochondrion tethers, indicating that IP_3R-independent linkage exists between ER and mitochondria [80]. Recently, many mitochondria- or ER-associated proteins have been shown to be important for maintaining the spatial relationship between ER and mitochondria and hence, have also been implicated as possible ER-mitochondrial linking elements (DLP-1/DRP1-1[89;90], tumor autocrine motility factor receptor (AMFR)[91], PACS-2 and BAP-31 [92]). A complication is that a change in the position of mitochondria relative to the ER can be caused by ER-independent mechanisms e.g. an impairment in the cytoskeletal transport of the mitochondria. Furthermore, sustained IP_3R-mitochondrial Ca^{2+} transfer has been observed even after experimentally induced redistribution of mitochondria [93]. However, it cannot be excluded that under these conditions the ER-mitochondrial associations may be sustained or reformed since the ER is present throughout the cell. A Ca^{2+}-regulated connection between ER and mitochondria seems to be mediated by AMFR in MDCK cells [91;94].

(c) Role of multimolecular complexes involving both IP3Rs and VDAC: Studies of the IP_3R-interacting proteins did not reveal direct interaction between IP_3R and the VDAC. However,

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both IP_3R and VDAC1 serves as a scaffold for many cytoplasmic proteins and both of these proteins may reside in a common multimolecular complex (Fig1A). Grp-75, a molecular chaperone is candidate for bringing together and for regulating the Ca^{2+} transfer between IP_3R and VDAC1 [95]. The IP_3R-VDAC complex is not likely to form the tethers described above since the tethers were also demonstrated in cells lacking any IP_3Rs [80]. The IP_3R-VDAC complex has multiple Ca^{2+} binding sites and is exposed to fluctuations in [Ca^{2+}], providing a hint that this form of linkage may be controlled by [Ca^{2+}].

Early immuno-electronmicroscopy studies indicated the concentration of IP_3Rs at the ER-mitochondrial interface [96;97]. In RBL-2H3 cells, the ER-mitochondrial coupling displays a “quasi-synaptic” organization that would involve enrichment of both IP_3Rs and the mitochondrial Ca^{2+} uptake sites at the interface [53]. While the IP_3R has not been visualized directly with transmission electron microscopy, the distribution of native RyRs has been established in muscle. RyRs decorate predominantly the SR surface of the terminal cysternae facing toward the T tubules, opposite to the mitochondria [66]. The shortest distance between RyR and the OMM is ~37nm in cardiac muscle [71] and more variable and usually longer in skeletal muscle, up to ~130 nm in mouse leg muscles [66]. Since microdomain formation requires the vicinity of membranes and not all the mitochondria are in juxtaposition to ER or the plasma membrane, only a fraction of the mitochondria is exposed to Ca^{2+} hotspots, therefore the Ca^{2+} signal of single mitochondria within the same cell is heterogenous. The fraction of mitochondria showing great responsiveness to $[Ca^{2+}]_c$ elevation [98–101] may reflect the ultrastructure of a given cell type.

Several recent results suggest that the ER-mitochondrial interface is dynamically controlled. In tracheal smooth muscle cells acetylcholine or depolarization-induced Ca^{2+} signal is followed by significant reduction of the proportion of mitochondria that was enveloped by sarcoplasmic reticulum [102]. In contrast, in permeabilized MDCK cells, the addition of rat liver cytosol stimulates the dissociation of smooth ER and mitochondria under conditions of low [Ca^{2+}] but [Ca^{2+}] above 1 μM favors their close association [91]. Narrowing of the ER-mitochondrial gap occurs in intact cells exposed to ER stress agents (serum starvation, tunicamycin treatment) and may be an important step in the execution of apoptosis [80]. Stability of the ER-mitochondrial association is also supported by the inhibition of mitochondrial movements in the presence of increased $[Ca^{2+}]_c$ [81]. The ER-mitochondrial interface may also be modified by ROS. ROS are well known to induce cytosolic and mitochondrial Ca^{2+} signal, followed by the deterioration of mitochondrial function [103]. In adrenal glomerulosa cells high-power UV light induces a high rate of superoxide formation and evokes Ca^{2+} signalling whereas low-power light induces a low rate of $O_2^{\cdot -}$ formation without eliciting Ca^{2+} signalling. Nevertheless, the latter impairs the transfer of Ca^{2+} signal from the cytosol into the mitochondrial matrix only in angiotensin-stimulated cells but not when $[Ca^{2+}]_c$ is elevated by voltage or store-operated Ca^{2+} influx [104], suggesting that the local ER-mitochondrial Ca^{2+} coupling has been specifically affected by the UV-treatment.

Ca^{2+} permeation through OMM

Early studies of isolated mitochondria and purified VDAC reconstituted in artificial membranes provided considerable support for the view that the OMM is freely permeable for Ca^{2+} due to the presence of VDAC. However, a series of recent experiments conducted in permeabilized or in intact cells indicate that the OMM permeability may limit the transport of ions and small molecules between cytoplasm and mitochondria e.g. ADP, H^+ [105–107] or Ca^{2+} [108;109]. It is possible that the OMM transport barrier is less apparent in isolated mitochondria because the preparation procedure led to some rearrangement or damage in the OMM structure. Indeed an increase in the OMM permeability of isolated cardiac mitochondria for 3nm nanoparticles has been documented [110]. Also, the studies of the purified VDAC

were usually conducted at supraphysiological ionic strength and [Ca^{2+}], which could have affected the channel function. Evaluation of this possibility by incubation of purified VDAC and isolated OMM incorporated to lipid bilayers or liposomes in physiological intracellular medium showed small subconductances of the VDAC and low Ca^{2+} permeability [111]. Higher subconductances and sustained opening of VDAC to maximal conductance occurred when [Ca^{2+}] was elevated, indicating that Ca^{2+} affects the gating of VDAC. Importantly, to attain an increase in VDAC Ca^{2+} permeability it was sufficient to increase [Ca^{2+}] from 20nM to 2μM [111], indicating a possible physiological relevance of the Ca^{2+} dependence of the VDAC. Most recently, Tan and Colombini have reported a lack of effect of Ca^{2+} on VDAC conductance but again the measurement was done in the presence of 1M KCl and [Ca^{2+}] was not tested in the low [Ca^{2+}] buffer (Figure 1 in [112]). A putative Ca^{2+} binding site of the VDAC has been identified by Shoshan-Barmatz and coworkers [113;114] but the Ca^{2+} effect can also be conferred to VDAC by an associated protein that is differently accessible in physiological and higher ionic strengths. Since the Ca^{2+} effect was documented in ATP free conditions, Ca^{2+}-dependent protein phosphorylation does not seem to be involved.

Importantly, the limitation of Ca^{2+} transport by the OMM appeared only when the response of mitochondria to short-lasting IP_3R-mediated $[Ca^{2+}]_c$ spikes was studied [108],[109]. Therefore, the Ca^{2+} permeability of the OMM seems to become a limiting factor when subregions of the mitochondrial surface have to allow the rapid equilibration of the intermembrane space [Ca^{2+}] with the cytosol. VDAC expression and distribution in the OMM is relevant for this process [109]. Furthermore, regulation of VDAC gating by Ca^{2+} may provide a supplementary mechanism to enhance the permeation of ions and solutes across the OMM during repetitive $[Ca^{2+}]_c$ elevations [111].

Ca^{2+} permeation through the IMM

Ca^{2+} influx occurs through a channel termed the uniporter (UP) and the rate of influx depends upon the driving force (Fig1A), the main component of which is the highly negative transmembrane potentials across the IMM generated by the electron transport in normally respiring mitochondria. Ca^{2+} efflux is mediated by separate pathways that are also coupled to the proton motive force developed by the respiratory chain. The IMM has a $Ca^{2+}/2H^+$ exchanger and/or a $Ca^{2+}/3Na^+$ exchanger analogous to that found in the plasma membrane (Fig1A). Ca^{2+} efflux along the concentration gradient may also occur through a mechanism referred as the permeability transition pore (PTP). The molecular structure of each of these IMM Ca^{2+} flux pathways remains to be solved. Therefore it is of significance that Graier and coworkers have shown that overexpression and silencing of uncoupling proteins 2 and 3 (UCP2 and UCP3) effectively increased and suppressed mitochondrial Ca^{2+} uptake, respectively. Furthermore, liver mitochondria isolated from UCP2−/− mice showed lack of the ruthenium red sensitive mitochondrial Ca^{2+} sequestration [115]. These data would indicate that UCP2 is either part of the UP or is an important controller of it. Since in UCP2 and UCP3 expression in hepatocytes has been under the limit of detection, a minuscule amount of UCP would need to be sufficient to support the robust mitochondrial Ca^{2+} uptake in these cells [116;117]. It is also relevant that a change in UCP2 and UCP3 expression may affect mitochondrial metabolism for example the production of ROS [118;119] that may also exert an effect on the UP activity. Furthermore, recent studies of UCP2 knockout and overexpression paradigms have also indicated that UCP2 is not required for mitochondrial Ca^{2+} uptake and ATP production in pancreatic β cells [119;120].

Another candidate for the UP activity in heart mitochondria has been the RyR [121]. Follow-up studies have indicated that the biochemical and pharmacological properties of the protein isolated from the mitochondrial preparation is similar to RyR1 [122;123], whereas electrophysiological studies indicate both common and distinctive properties of the SR and

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mitochondrial preparation derived channels [123]. The RyR1 knockout mice are expected to provide a powerful model to establish the role of RyR1 in Ca2+ transport by cardiac mitochondria.

Evidence has been accumulating that numerous signalling molecules and drugs affect the calcium signal propagation to the mitochondria. In many cases, no distinction has been made between targeting of the OMM and IMM components of the Ca2+ uptake and is unclear whether the driving force of the Ca2+ uptake was affected. Pinton et al. showed that various protein kinase C (PKC) isozymes exert differential control on the [Ca2+]c signal delivery to the mitochondria [124]. Szanda et al. have presented both pharmacological and genetic evidence that p38 MAPK serves as a negative modulator of the mitochondrial Ca^{2+} uptake [125]. p38 MAPK seems to act in concert with PKC epsilon, in inhibition of the $[Ca^{2+}]_m$ signal [125]. Notably, p38 MAPK phosphorylates Bcl-2 family proteins that may confer an effect to the UP [126]. Also, PKC epsilon may target the mitochondrial K(ATP) channels [124] inducing a change in matrix volume which may alter the mitochondrial Ca^{2+} uptake. A p38 MAPK inhibitor, SB202190 also caused augmented mitochondrial Ca^{2+} uptake in ATP-free conditions [127;128], indicating that interfering with protein phosphorylation is not the sole mechanism of the drug's effect.

Allosteric regulation of the mitochondrial Ca^{2+} uptake by Ca^{2+} has been established first in studies of isolated mitochondria [129]. In cell paradigms, both Ca^{2+}-induced desensitization [130–132] and Ca^{2+}-induced potentiation of the mitochondrial Ca^{2+} uptake [133;134] have been observed (for a recent review see [135]). A mechanism of the Ca^{2+} induced facilitation seems to be mediated through calmodulin to the UP [131;134] but other Ca^{2+} sensing molecules and multiple signalling pathways may also be involved. The regulatory pathways to the UP allow tuning of mitochondrial Ca^{2+} uptake activity to the changing needs of the stimulated cells.

Ca^{2+} buffers and effectors in the mitochondrial matrix

Calcium ions entering the mitochondrial matrix increase $[Ca^{2+}]_m$ but over 99.9% of the total matrix calcium content is in bound form. The calcium binding species includes cardiolipin and other anionic phospholipids that bind Ca^{2+} with high affinity. Another important Ca^{2+} binding species are the carboxy-anion-containing metabolites of the Krebs cycle (citrate, oxalo-acetate) and inorganic phosphate, which can also form poorly soluble salts with Ca^{2+}. There is no evidence for the presence of a specialized Ca^{2+} buffering protein in the mitochondrial matrix. Buffering of gradually accumulated Ca^{2+} is more efficient than that of a bolus of Ca^{2+} [136]. Ca^{2+} binding affinity of the anionic metabolites of the Krebs cycle is sensitive to matrix pH. Furthermore, the IMM pH gradient also affects the distribution of the carboxylated anions and phosphate between the mitochondria and cytosol. Thus, matrix Ca^{2+} buffering is dynamically regulated by mitochondrial metabolism primarily, through the changes in matrix pH.

Despite the robust Ca^{2+} buffering capacity in the mitochondrial matrix, $[Ca^{2+}]_c$ spikes yield rapid elevations in $[Ca^{2+}]_m$ from 100nM to at least micromolar and in some cases over hundred micromolar [101]. Mitochondrial matrix Ca^{2+} effectors are several key enzymes that enhance ATP production, providing an important mechanism for synchronizing energy production with the energy demands of Ca^{2+}-activated processes during cell stimulation (excitation–metabolism coupling). Another effector is the PTP that is activated during massive mitochondrial Ca^{2+} loading or by the combination of some forms of stress and Ca^{2+} loading and leads to mitochondrial membrane permeabilization and ensuing cell death, either apoptotic or necrotic.

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Dynamic changes in ER/mitochondrial morphology

The amount of ER and mitochondria, the distribution and connectedness of the organelles in the cell may affect both the bulk cytoplasm-mediated and the local ER-mitochondrial Ca^{2+} signalling but only few data have become available. The ER and mitochondrial fraction of cell volume shows large, cell type specific differences and undergoes changes during cell development and differentiation. Overexpression of a the peroxisome-proliferator-activated-receptor-c-coactivator -1α (PGC-1α) that triggers mitochondrial biogenesis resulted in selective suppression of the $[Ca^{2+}]_m$ signal by reducing the efficacy of mitochondrial Ca^{2+} uptake sites and increasing organelle volume [137]. The distribution of the ER and mitochondria are controlled by cytoskeletal transport and anchorage [82]. In certain cell types, subdomains of the ER and subsets of mitochondria have a stable position but the majority of organelles display permanent movement. Ca^{2+}-dependent control of the motility allows a homeostatic distribution of the mitochondria to the sites of ATP and Ca^{2+} buffering demand [81;138–140]. Importantly, Ca^{2+}-induced attenuation of mitochondrial motility occurs in the sub-micromolar range of $[Ca^{2+}]_c$ ($IC_{50} \approx 400nM$), indicating that mitochondrial motility is controlled in the physiological range of $[Ca^{2+}]_c$ [79]. Ca^{2+} uptake leads to the formation of Ca^{2+} hot-spots in the mitochondrial matrix and Ca^{2+} diffusion is limited to short segments of the mitochondrial network [141]. Szabadkai et al. have provided evidence that fragmentation of the mitochondria leads to suppression of the spreading of Ca^{2+} in the matrix of the mitochondrial network [142]. Ca^{2+} is a potent inducer of fragmentation of both ER [143]; [47] and mitochondria [144]. Ca^{2+} engages calcineurin to dephosphorylate and in turn, activate the mitochondrial fission protein, Drp1 [145].

Transfer of submicromolar $[Ca^{2+}]_c$ elevations into the mitochondrial matrix

There are reports on intact cells suggesting that Ca^{2+} is sequestered by mitochondria also when the organelle is exposed to Ca^{2+} at submicromolar concentration. In these cases Ca^{2+} enters the cytosol through the plasma membrane and it can be assumed that the formation of a high-Ca^{2+} microdomain between the plasma membrane and subplasmalemmal mitochondria was not responsible for the mitochondrial Ca^{2+} uptake. The limitation of these studies, similarly to those reporting on high-Ca^{2+} microdomains, is that perimitochondrial $[Ca^{2+}]$ cannot be directly measured but only assessed. In bullfrog sympathetic neurons increased mitochondrial calcium uptake could be detected when depolarization with K^+ raised $[Ca^{2+}]_c$ above 300–500 nM [146;147]. In a further study on the same cell type mitochondrial Ca^{2+} transport processes were analyzed on basis of the kinetics of depolarization-induced Ca^{2+} signal and the application of transport inhibitors. It was found that above 400 nM $[Ca^{2+}]_c$, net mitochondrial Ca^{2+} transport is dominated by uptake but net Ca^{2+} uptake occurs even at 200–300 nM [148]. Considering, however, that at least in one of these studies [147], the extent of Ca^{2+} accumulation depended on proximity of mitochondria to the plasma membrane, the formation of subplasmalemmal high-Ca^{2+} microdomains in these experiments may not be ruled out. In another excitable cell type, the arterial smooth muscle cells, $[Ca^{2+}]_c$ was raised by brief activations of voltage-operated Ca^{2+} channels. Even when $[Ca^{2+}]_c$ remained in the low submicromolar range, mitochondrial depolarisation slowed the rate of Ca^{2+} removal from the cytosol, indicating that polarized mitochondria can sequester Ca^{2+} also at low $[Ca^{2+}]_c$ values [149]. In HeLa cells, a $[Ca^{2+}]_c$ signal was evoked by histamine (IP_3–induced Ca^{2+} release), thapsigargin (Ca^{2+} leak from the ER) or readdition of Ca^{2+} following store depletion. The efficiency of mitochondrial Ca^{2+} uptake from high-Ca^{2+} perimitochondrial microdomains was clearly shown by the observation that for equivalent $[Ca^{2+}]_c$ increases, the rate of $[Ca^{2+}]_m$ rise was greater with IP_3 –induced signal than any other source. However, irrespective of the source of Ca^{2+}, already in the range of 100 to 400 nM $[Ca^{2+}]_c$, Ca^{2+} uptake varied nearly linearly with $[Ca^{2+}]_c$. This indicates the ability of mitochondria to take up Ca^{2+} in the low submicromolar range under

some situations. It is worth mentioning that the theshold value for Ca^{2+} uptake by individual mitochondria showed great variance [130].

There are observations also in endocrine cells indicating mitochondrial Ca^{2+} uptake in the low submicromolar $[Ca^{2+}]_c$ range. Aldosterone secretion by adrenal glomerulosa cells is rapidly stimulated by angiotensin II. Mitochondrial Ca^{2+} response to angiotensin is generated through the formation of high-Ca^{2+} perimitochondrial microdomain [150]. Physiological elevation of extracellular $[K^+]$ induces long-lasting but small elevation of $[Ca^{2+}]_c$. Rat glomerulosa cells respond to an elevation of $[K^+]$ as small as 0.5 mM with a slowly developing $[Ca^{2+}]_c$ signal that never attains 200 nM [151;151], yet this signal is followed by elevated $[Ca^{2+}]_m$, mitochondrial NAD(P)H level and aldosterone production [152]. Store-operated Ca^{2+} influx both in glomerulosa [75] and ovarian luteal cells [153] also results $[Ca^{2+}]_c$ signals never exceeding 200 nM and they are again associated with a mitochondrial NAD(P)H signal. Considering that in these cases the $[Ca^{2+}]_c$ signal is formed very slowly whereas its rapid formation would be needed for the generation of a high Ca^{2+} microdomain, mitochondrial Ca^{2+} uptake may not be attributed to the formation of such microdomains. In support of this assumption it should be recalled that in the related adrenocortical cell line (H295R) stimulated with K^+, Ca^{2+} uptake by subplasmalemmal mitochondria did not differ from that by perinuclear mitochondria [43]. These observations suggested that net mitochondrial Ca^{2+} uptake may take place without the formation of high-Ca^{2+} microdomains. The effect of $[Ca^{2+}]_c$ on $[Ca^{2+}]_m$ was examined in permeabilized cells, applying cytosol-like Ca^{2+} buffers. With such a protocol the formation of microdomains could be avoided. When extramitochondrial $[Ca^{2+}]$ was raised from 50 or 60 nM to less than 200 nM, reproducible increases in $[Ca^{2+}]_m$ were measured in glomerulosa [154]}[155], luteal [153] and insulin-producing INS-1cells [154]. The biological significance of the measured small increases in $[Ca^{2+}]_m$ was demonstrated by the detection of concomitant increase in NAD(P)H [154]. Further stepwise increases, still in the submicromolar range, of $[Ca^{2+}]_c$ resulted in stepwise increases of steady-state $[Ca^{2+}]_m$. The $[Ca^{2+}]_m$ elevations were ruthenium red-sensitive, developed slowly and were maintained as long as raised $[Ca^{2+}]_c$ was present. These data provided evidence that net mitochondrial Ca^{2+} uptake and Ca^{2+} sensitive dehydrogenase activation can be enhanced by low submicromolar increases in $[Ca^{2+}]_c$. In harmony with these observations, in permeabilized pancreatic β cells methyl succinate, a cell permeable substrate induced mitochondrial hyperpolarization and increased $[Ca^{2+}]_m$, without the formation of high-Ca^{2+} perimitochondrial microdomain [156]. Notably, a rapid mode of Ca^{2+} uptake has also been documented in isolated mitochondria exposed to $[Ca^{2+}]$ pulses which peak at 200nM [157].

Differing from the primary glomerulosa and luteal cells (v.s.), the $[Ca^{2+}]_c$ threshold for mitochondrial Ca^{2+} uptake was ~500 nM and 1 μM in H295R adrenocortical and HeLa cells, respectively [158]. This latter value is much higher than that observed by Collins et al. [159] but is lower than observed by Montero *et al.* [127]. Net Ca^{2+} uptake in T lymphocytes begins at ~400 nM [160], in ventricular myocytes at 200–500 nM [70;161] whereas supramicromolar threshold values were reported for RBL-2H3 [53] and chromaffin cells [101]. Mitochondrial Ca^{2+} uptake at the $[Ca^{2+}]_c$ threshold is relatively slow, new steady-state of $[Ca^{2+}]_m$ is attained after several tens of seconds.

The mechanisms underlying the great variance in the $[Ca^{2+}]_c$ threshold for mitochondrial Ca^{2+} uptake and the unexpectedly low threshold in some cell types are unknown. Ca^{2+}-induced potentiation of the mitochondrial Ca^{2+} uptake may be involved when the kinetics of the $[Ca^{2+}]_m$ rise is slow [133;134]. It has been known for three decades that Mg^{2+} modifies Ca^{2+} uptake of isolated mitochondria [162–165]. Yet, we are not aware of any publication which would have considered cytosolic $[Mg^{2+}]$ as a factor influencing the $[Ca^{2+}]_c$ threshold for net mitochondrial Ca^{2+} uptake. We have examined this issue in permeabilized HEK293T cells and found that in the physiological range of 0.25 – 2.5 mM Mg^{2+} this threshold inversely

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correlated with [Mg^{2+}]. In addition, in the submicromolar range of [Ca^{2+}]$_c$ mitochondrial Ca^{2+} uptake rate was also reduced by increase in [Mg^{2+}] (G. Szanda, A. Spät & J. Garcia-Sancho, manuscript in preparation). In view of these data it may be presumed that cell-dependent Mg^{2+} balance and unchecked Mg^{2+} concentrations in cytosol-like media may be a cause of the apparent cell-to-cell variance in the mitochondrial responsiveness to submicromolar cytosolic Ca^{2+}.

Future directions

The evidence on local communication between ER/SR and mitochondria presents a major challenge for microscopy. The spatial resolution required for tracking the interacting molecules both in vitro and in vivo exceeds the resolution of light microscopy therefore creative application of some current techniques (e.g. FRET) may be useful and establishing of novel means is necessary. For the in vitro studies, high-voltage electron tomography offers a promising approach. Also, labeling of the endogenous proteins needs novel technology. This direction also needs the long sought information on the molecular identity of the mitochondrial Ca^{2+} transport proteins. The varying affinity for Ca^{2+} of the mitochondrial Ca^{2+} uptake in different paradigms highlights the need for deciphering the mechanisms that control the Ca^{2+} sensitivity of the mitochondrial Ca^{2+} transporters in the OMM and IMM. An intriguing question remains regarding the subcellular distribution of the signalling proteins that target the IMM components. An impetus for these efforts is the growing support for the broad and fundamental biological significance of the ER-mitochondrial interactions.

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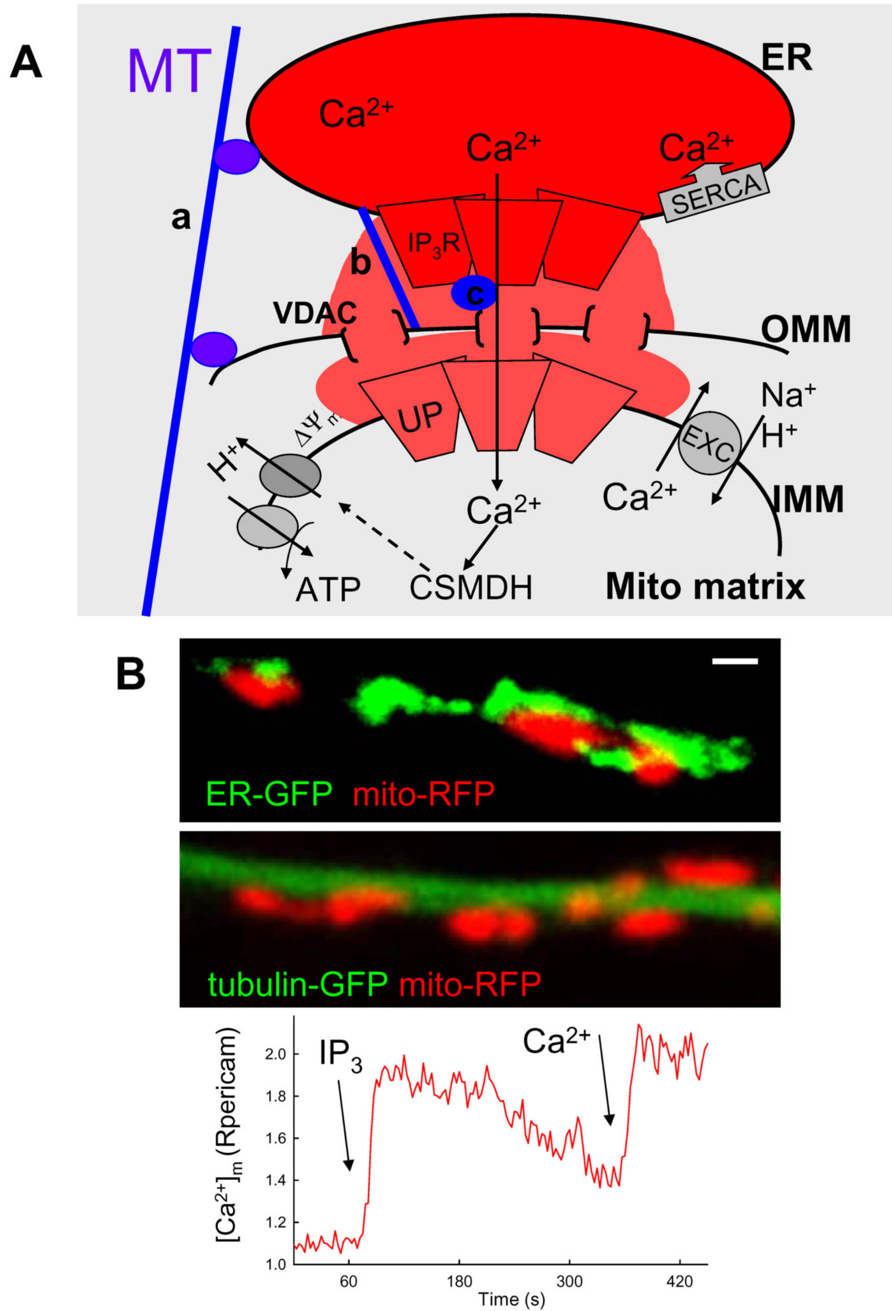

Fig1. Local coupling between ER and mitochondria

(A) Scheme depicting the mechanisms of local Ca^{2+} transport and physical linkage between an ER stack and an adjacent mitochondrion. Ca^{2+} stored in the ER lumen is released through the IP_3 receptors (IP_3R) giving rise to a high [Ca^{2+}] microdomain that exposes an adjacent mitochondrion. Ca^{2+} traverses the outer mitochondrial membrane (OMM) through the voltage dependent anion-selective channels (VDAC) and the inner mitochondrial membrane (IMM) via the uniporter (UP). In the mitochondrial matrix Ca^{2+} binds to the Ca^{2+} sensitive mitochondrial dehydrogenases (CSMDH) to stimulate energy metabolism. Ca^{2+} exits mitochondria through the Na+/Ca^{2+} or H+/Ca^{2+} exchanger (EXC) and is taken back to the ER by the Ca^{2+} pumps (SERCA). [Ca^{2+}] is indicated by shades of red.

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Components of the physical coupling between ER and mitochondria: a, binding of both ER and mitochondria to a microtubule (MT) or to other cytoskeletal fibers; b, protein tethering ER membrane directly to the mitochondria and c, multimolecular complexes involving both the IP_3Rs and VDACs.

(B) Visualization of mitochondria and ER (upper), mitochondria and a microtubule (middle) and the IP_3-induced mitochondrial calcium signal (lower) in a projection of an RBL-2H3 cell. Confocal imaging was performed in cells expressing either an ER-targeted enhanced green fluorescent protein (ER-GFP) and a mitochondrial matrix targeted red fluorescent protein (mito-RFP) (upper) or tubulin-targeted enhanced green fluorescent protein (tubulin-GFP) and mito-RFP (middle). The length of the scalebar is 1μm. Fluorescence imaging was conducted in mitochondrial matrix targeted ratiometric pericam expressing permeabilized cell sequentially stimulated with IP_3 (7.5μM) and Ca^{2+} (30μM). The graph shows the time course of the pericam ratio calculated for a mitochondrion located in a projection.