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MINIREVIEW

The renaissance of mitochondrial calcium transport

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Although the capacity of mitochondria for accumulating Ca^{2+} down the electrical gradient generated by the respiratory chain has been known for over three decades, the physiological significance of this phenomenon has been re-evaluated only recently. Indeed, it was long believed that the low affinity of the mitochondrial Ca^{2+} transporters would allow significant uptake only in conditions of cellular Ca^{2+} overload. Conversely, the direct measurement of $[Ca^{2+}]$ in the mitochondrial matrix revealed major $[Ca^{2+}]$ increases upon agonist stimulation. In this review, we will summarize: (a) the mechanisms that allow this large response, reconciling the biochemical properties of the transporters and the large amplitude of the mitochondrial $[Ca^{2+}]$ rises, and (b) the biological role of mitochondrial Ca^{2+} signalling, that encompasses the regulation of mitochondrial function and the modulation of the spatio-temporal pattern of cytosolic $[Ca^{2+}]$ increases.

Keywords: calcium; mitochondria; signalling; channel; metabolism.

MITOCHONDRIAL Ca^{2+} TRANSPORT: A HISTORICAL VIEW

In the reviews on intracellular Ca^{2+} homeostasis written in the 1970s one of the longest chapters was that concerning mitochondria. In fact, except for the sarcoplasmic reticulum, SR, of striated muscle fibres, mitochondria were, in those years, the only organelle known to actively accumulate calcium ions and thus they were considered the main and most dynamic Ca^{2+} storage compartment in cells other than skeletal or cardiac muscles. This scenario changed suddenly at the beginning of the 1980s, when it became clear that the ubiquitous Ca^{2+} mobilizing second messenger inositol 1,4,5-trisphosphate ($InsP_3$) was acting not on mitochondria, but on the endoplasmic reticulum (ER) or on some of its specialized subcompartments (reviewed in [1]). In addition, the direct measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) with fluorescent indicators [2] demonstrated that no, or marginal, increases in $[Ca^{2+}]_c$ were induced by promoting Ca^{2+} release from mitochondria. The final blow to the idea that mitochondria were important players in the cellular Ca^{2+} game was the demonstration that in situ the mitochondrial Ca^{2+} content was orders of magnitude lower than that of the ER [1].

Throughout most of the 1980s the general view was that, even if mitochondria had the potential to accumulate and release vast amounts of Ca^{2+}, they only did it when cells were injured and a massive increase of Ca^{2+} occurred in the cytosol. Though this concept was generally accepted, it remained to be explained why (a) the sophisticated system of uptake and release of Ca^{2+} has been highly conserved during evolution, suggesting a key role of this function in cell physiology, and (b) two enzymes of the Krebs cycle (isocitrate- and oxoglutarate-dehydrogenase) and pyruvate dehydrogenase are regulated by matrix $[Ca^{2+}]$ [3]. At the beginning of the '90 s the role of mitochondria Ca^{2+} transport began again to attract the interest of a wider audience and today it has become clear that numerous cellular events directly or indirectly depend on this process. It is the purpose of this review to critically analyse the most recent findings that put mitochondrial Ca^{2+} handling into a new context of an integrated signalling pathway.

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Abbreviations: ER, endoplasmic reticulum; $InsP_3$, inositol 3-phosphate; PTP, permeability transition pore;
Enzymes: put enzyme names here if there are any

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PROPERTIES OF MITOCHONDRIAL Ca^{2+} HANDLING

Before discussing in detail the basis for this renewed interest, let's first summarize the main established features of mitochondrial Ca^{2+} transport, as firmly established from *in vitro* experiments since the 1970s. While the mitochondrial outer membrane is believed to be freely permeable to ions and molecules up to 1000 Da (see below), the inner membrane is tightly sealed to all ions, but for the presence of specific transporters. The uptake of Ca^{2+} into the mitochondrial matrix under physiological conditions does not depend on ATP hydrolysis, but rather on the presence of a so called 'Ca^{2+} uniporter' (presumably a gated channel) and the driving force is provided by the negative (in the matrix) membrane potential generated by the respiratory chain. When the respiratory chain is inhibited, the membrane potential can be maintained by a reversal of the H^+ ATPase. Accordingly, to block mitochondrial Ca^{2+} uptake in living cells endowed with an active glycolysis, both the respiratory chain and the H^+ ATPase must be inhibited. If mitochondria accumulate large amounts of Ca^{2+} (in the presence of phosphate) they store it in the matrix as insoluble phosphate salts and the release occurs very slowly. If accumulation is limited, the release is faster and depends on two pathways. The first, and more efficient, exchanges Ca^{2+} for Na^+ [4], the second exchanges Ca^{2+} for H^+ (reviewed in [5]). The stoichiometry of the exchangers, i.e. whether they are strictly electroneutral and/or whether they are modulated, at

least in part, by membrane potential, has not been established with certainty yet (reviewed in [1,5]). Under steady state conditions the Ca^{2+} concentration in the matrix ($[Ca^{2+}]_m$) thus depends on a futile cycle of Ca^{2+} uptake via the uniporter and release via the antiporters, the mitochondrial 'Ca^{2+} cycle' [6]. The recycling of Ca^{2+} across the mitochondrial inner membrane would be deleterious in terms of energy drain, but for the fact that the affinity for Ca^{2+} of the uniporter is very low (under physiological Mg^{2+} concentrations the K_d is > 10 μM). Thus at the resting $[Ca^{2+}]_c$-values (≈ 100 nM) the uptake rate is very slow and the consumption of energy reduced to a minimum. The low Ca^{2+} affinity of the uniporter and the existence of electroneutral exchangers are also essential to prevent the attainment of electrochemical equilibrium that for a membrane potential of ≈180 mV would predict a $[Ca^{2+}]_m$-value higher than 0.1 M.

MITOCHONDRIAL Ca^{2+} UPTAKE IN LIVING CELLS

The low affinity of the uniporter, in theory, should prevent significant accumulation of Ca^{2+} by mitochondria even upon cell stimulation. The $[Ca^{2+}]_c$ increases induced by physiological stimuli hardly ever exceed 1–2 μM, values at which the uniporter is still very inefficient at transporting Ca^{2+}. Scattered data were published, particularly in neurons, which opposed this view. For example Thayer and Miller [7] showed that in cultured neurons activation of glutamate ionotropic receptors resulted in different changes of cytosolic $[Ca^{2+}]$ rises depending on whether or not mitochondria were allowed to accumulate Ca^{2+}. Other indirect evidence, based primarily on the use of mitochondrial inhibitors, suggested that significant mitochondrial Ca^{2+} accumulation occurs also under physiological conditions, but these observations failed to change the general view that mitochondria participate in Ca^{2+} handling primarily or solely under pathological conditions. It was thus quite surprisingly that selective monitoring of $[Ca^{2+}]_m$ revealed that the $[Ca^{2+}]_c$ increases triggered by agonist-dependent InsP_3 generation were paralleled by rapid, transient elevations of $[Ca^{2+}]_m$, the amplitude of which largely exceeded the $[Ca^{2+}]_c$ rises [8]. Using either targeted aequorin [8] or fluorescent indicators [9] this result was confirmed in a wide variety of mammalian cell types. The reasons for the discrepancy between *in vitro* and *in vivo* data could be twofold: (a) the existence of factors in the cytosol that increase the affinity for Ca^{2+} of the uniporter or (b) the capacity of mitochondria to sense not the bulk $[Ca^{2+}]_c$ changes, but rather microdomains of high Ca^{2+} in their vicinity. Pieces of indirect evidence favoured the latter possibility. In particular: (a) in permeabilized cells the perfusion with buffers containing $[Ca^{2+}]$ in the low micromolar range results in sluggish Ca^{2+} accumulations, while similar or smaller $[Ca^{2+}]$ increases following the release of Ca^{2+} from the ER via the InsP_3 receptors causes a very rapid Ca^{2+} accumulation, similar in amplitudes and kinetics to that induced by receptor stimulation of intact cells [8,10,11] (b) small increases in $[Ca^{2+}]_c$, such as those observed during agonist induced oscillations in hepatocytes, result in more rapid and efficient activation of the Ca^{2+} dependent dehydrogenases than more ample and sustained increases in $[Ca^{2+}]_c$, elicited however, by non specific Ca^{2+} leak and/or activation of Ca^{2+} influx through the plasma membrane [9].

The hypothesis was thus proposed that mitochondria are somehow located close to InsP_3 gated channels and transiently exposed to local high $[Ca^{2+}]$ microdomains induced by release from the ER, rather than the bulk changes in $[Ca^{2+}]_c$.Given that in the ER the $[Ca^{2+}]$ is around 0.5–1 mM, it is likely that in the close proximity of those channels the local Ca^{2+} concentration would be much higher than in the rest of the cytoplasm, i.e. in a range adequate to activate the low affinity mitochondrial Ca^{2+} uniporter. By comparing the rates of mitochondrial Ca^{2+} accumulation in intact cells with those observed after plasma membrane permeabilization and perfusion with Ca^{2+} buffers of known concentration it has been calculated that the $[Ca^{2+}]$ in the localized hot spots should be around 20–30 μM [11,12]. In physiological terms this hypothesis has important implications: on the one hand, phenomenologically speaking, the mitochondria appear to sense the derivative of $[Ca^{2+}]_c$, i.e. they accumulate Ca^{2+} efficiently during the upstroke of a Ca^{2+} rise and not during a sustained increase, on the other the microdomain hypothesis assures that mitochondria do not become overloaded with Ca^{2+} during prolonged stimulation. In fact the local high $[Ca^{2+}]$ at the mouth of the channels is rapidly dissipated by diffusion that thus serves the function of an automatic shut off mechanism for the rapid mitochondrial Ca^{2+} uptake. A number of recent data have provided convincing evidence in favour of this model. Using an ultra-fast image acquisition system and high resolution 3D reconstruction algorithms, close appositions between the two organelles (less than 100 nm distance) could be directly visualized in intact living cells expressing organelle-targeted mutants of GFP [10]. A serendipitous bonus of these morphological studies has been the direct demonstration of a concept repeatedly proposed by cytologists in the past, i.e. that, rather than individual sausage-like organelles, mitochondria *in vivo* are a largely interconnected network, with budding and fusion occurring at its periphery. The contacts between the ER and mitochondria may imply specific interactions between the two membranes, e.g. at clusters of InsP_3 receptors. Indeed, cisternae of ER particularly enriched in InsP_3 receptors, such as those of Purkinje neurons, are very often apposed to the mitochondria [13]. Conversely, in cells characterized by a relative sparse ER with very few contacts with mitochondria the uptake of Ca^{2+} in the latter organelle is slow and limited, as expected of mitochondria exposed to the bulk increases in $[Ca^{2+}]_c$ [14]. Close appositions of mitochondria and clusters of InsP_3 receptors have been also observed in oligodendrocytic cells in culture, albeit after fixation and immunostaining with specific antibodies [15]. These close contacts could be the morphological locus of microdomains generated at the mouth of InsP_3 gated channels. Indeed, a Ca^{2+} sensitive photoprotein localized selectively on the outer surface of the inner mitochondrial membrane reveals Ca^{2+} increases that are significantly larger than those of the bulk cytoplasm, but only when Ca^{2+} is released from the ER via the InsP_3 receptor [10]. The emerging scenario is that of two reticular organelles, the ER and the mitochondria, closely connected at some selective sites. The release of Ca^{2+} from the ER occurs at clusters of InsP_3 receptors facing a mitochondrial loop located at close distance. The rapid Ca^{2+} uptake in the latter thus occurs at discrete sites, and Ca^{2+} then diffuses within the mitochondrial matrix intralumenally to induce the activation of Ca^{2+} dependent enzymes therein localized.

The question then arises as to whether rapid mitochondrial Ca^{2+} accumulation occurs only, and if, Ca^{2+} is released from InsP_3 gated channels. This appears not to be the case. In fact rapid Ca^{2+} accumulation in mitochondria has been observed upon opening of another type of intracellular Ca^{2+} release channel, the ryanodine receptor, in striated muscle and in chromaffin cells [12,17] and upon activation of plasma membrane channels [18]. The microdomain hypothesis predicts that also in these cell types mitochondria should be in close

proximity of Ca^{2+} hotspots. Although the matter has not been investigated in detail, at least morphological evidence exists in striated muscle and neurons indicating close apposition of mitochondria to either ryanodine receptors in the SR terminal cisternae and to the plasma membrane, particularly in the dendritic tree and synaptic terminals. In cardiac cells, in particular, the distance between the terminal cisternae and closely apposed mitochondrial membranes is less than 10 nm and the increases in $[Ca^{2+}]_m$ elicited by caffeine are almost unaffected by the introduction in the cytoplasm of high concentrations of the Ca^{2+} chelator BAPTA (up to 3 mM), that almost completely suppress the increases in bulk $[Ca^{2+}]_c$ [19]. Along the same line, using electron microscopic analysis of total Ca^{2+} content in rapidly (msec) frozen frog neuromuscular junctions, Pezzati *et al*. showed that, both in the muscle and in the presynaptic terminal, some mitochondria are heavily loaded with Ca^{2+} after a brief stimulation of the nerve [20].

Rapid accumulation of Ca^{2+} is not the only feature of the system. In fact, $[Ca^{2+}]_m$, after the agonist-dependent increase, rapidly returns towards basal level, also if a sustained cytosolic plateau is maintained. This decrease is due on the one hand to the dramatic drop in the uptake rate (because of the dissipation of the high Ca^{2+} microdomains), on the other to Ca^{2+} extrusion via the antiporters, activated by the rise in $[Ca^{2+}]_m$. Indeed, specific inhibitors of mitochondrial antiporters drastically delay the return of $[Ca^{2+}]_m$ to basal. Conversely, no role in this release phase can be attributed to the permeability transition pore (PTP), an unspecific mitochondrial channel that is attracting a large interest [5–21], as the release process is not affected by the PTP inhibitor cyclosporin A.

THE PHYSIOLOGICAL ROLE OF MITOCHONDRIAL Ca^{2+} TRANSPORT

What are the functional implications of this sophisticated arrangement of mitochondria and ER (or other organelles) and of the transient Ca^{2+} accumulation occurring within the mitochondrial matrix?

Based on the Ca^{2+}-sensitivity of the matrix dehydrogenases, the first consequence of the $[Ca^{2+}]_m$ rise is the activation of mitochondrial metabolism. Ample experimental evidence now supports this notion, by directly demonstrating the correlation between $[Ca^{2+}]_m$ increases and enzyme activation, increases in NADH levels, mitochondrial ATP production and O_2 consumption [9,22] (reviewed in [1]). Interestingly, this Ca^{2+}-dependent control mechanism works as an integrator of the cytosolic Ca^{2+} signal. In fact, Hajnoczky *et al*. demonstrated that in oscillating hepatocytes, when the frequency of the Ca^{2+} spikes is low, the $[Ca^{2+}]_m$ is also oscillatory and so is the reduction of NADH, though the kinetics of the latter are somewhat slower [9]. However, when the oscillations in cytosolic and mitochondrial Ca^{2+} concentration become more frequent, the NADH remains largely reduced, indicating that the deactivation of the dehydrogenases did not cope with the rapid kinetics of $[Ca^{2+}]_m$ and, as a consequence, the enzymes remained activated also during the interspike period. By this means mitochondria can decode the frequency of $[Ca^{2+}]_c$ into different levels of activation of the dehydrogenases, ensuing a continuous supply of reducing equivalents to the respiratory chain during intense stimulation.

In terms of cell energy balance, the Ca^{2+} microdomain model is highly advantageous. In particular it is well known that during Ca^{2+} accumulation the mitochondrial membrane potential drops and ATP synthesis is abolished. Thus mitochondrial ATP production come to a stop, as a consequence of Ca^{2+} uptake, but only for a very short period of time. Because of the relatively slow decay of $[Ca^{2+}]_m$ the overall balance of ATP production will be thus largely positive.

Recent data clearly demonstrate that mitochondrial Ca^{2+} uptake not only serves the function of controlling organelle function, but plays an unexpected role in the control of important, often apparently unrelated events which occur in the cytosol of a living cell.

The first of these events is the complex spatio-temporal patterning of Ca^{2+} signals. In an elegant series of experiments performed in *Xenopus* oocytes, Jouaville *et al*. demonstrated that mitochondrial Ca^{2+} uptake can modulate the shape and velocity of InsP_3 induced Ca^{2+} waves [23]. The interpretation of the authors, that is consistent with the microdomain model described above, was that the local Ca^{2+} buffering effect of mitochondria can modulate the kinetics of InsP_3 channel gating with ensuing modification of the cytoplasmic Ca^{2+} waves. Recently, the speed and extent of Ca^{2+} release from the ER has been shown to be modulated by the local Ca^{2+} buffering of mitochondria also in mammalian cells [24]. The role of mitochondria in modulating the activity of Ca^{2+} channels is not limited, however, to the ER. Evidence has recently been provided for a role of mitochondria in modulating the activity of plasma membrane channels, such as store-operated Ca^{2+} channels [25]. Finally, mitochondrial Ca^{2+} handling appears to play a major role in shaping the kinetics of $[Ca^{2+}]_c$ in neurons and neuroendocrine cells [18,27]. In particular, not only mitochondrial Ca^{2+} uptake reduces the amplitude of the $[Ca^{2+}]_c$ peak induced by strong depolarizing stimuli, but the release from mitochondria that follows is largely responsible for the long-lasting $[Ca^{2+}]_c$ plateau.

Finally, Tinel *et al*. recently showed that in pancreatic acinar cells mitochondrial Ca^{2+} accumulation can prevent the spreading of a Ca^{2+} wave from its site of generation close to the secretory pole towards the basolateral region of the cell [28]. This phenomenon depends on the strategic location of mitochondria beneath the granular region.

OTHER ROLES OF MITOCHONDRIAL Ca^{2+} TRANSPORT IN CELL FUNCTION

Mitochondrial Ca^{2+} handling plays a major role in controlling hormone synthesis and/or release by endocrine cells. In aldosterone producing cells, mitochondrial Ca^{2+} accumulation is a key step driving hormone biosynthesis within the mitochondrial matrix. The role of mitochondrial Ca^{2+} accumulation in the secretion of insulin by pancreatic β cells appear to more complex. In particular, up until recently the attention was largely concentrated on ATP synthesis by the organelle that in turn controls the gating of ATP sensitive K^+ channels and thus the initiation of the secretion process. In this context mitochondrial Ca^{2+} accumulation serves mainly the purpose of activating the dehydrogenases and thus of providing reducing equivalents to the respiratory chain to fuel ATP synthesis. Recent evidence indicates that the scenario may be even more complex, with the generation by mitochondria of a Ca^{2+} activated coupling factor that is essential in the secretion of the hormone [29]. In agreement with these observations, β cell lines deficient in mitochondrial DNA are selectively deficient in glucose induced insulin release, while the action of other secretagogues is not impaired. Maechler *et al*. have very recently shown that the Ca^{2+} activated coupling factor generated by mitochondria is glutamate [30].

A similarly fascinating role of mitochondria in the secretion of catecholamines by chromaffin cells has been recently proposed by Montero *et al*. [12]. The authors demonstrated

not only that the changes in $[Ca^{2+}]_m$ during stimulation of these cells is much higher than previously estimated (they can reach values as high as 5–800 μM), but also that inhibition of mitochondrial Ca^{2+} uptake can lead to a massive increase in hormone release (> fivefold the controls).

Apart from Ca^{2+} signalling, mitochondria have been recently proposed to play a central role in programmed cell death. In fact, Bcl-2, and related gene products, appear to be located and interact with the proapoptotic members of the gene family (e.g. *Bax*) on the outer mitochondrial membrane [31]. Moreover, ample evidence suggests that a key event in the apoptotic process is the release of a mitochondrial protein, cytochrome *c* [32,33], which is a potent *in vitro* activator of caspases, the key executors of apoptosis. It has been suggested that cytochrome *c* release depends on the opening of the mitochondrial PTP, which in turn is known to be activated by massive accumulation of Ca^{2+} by the organelles (reviewed in [21]). Although attractive, the possible involvement of mitochondrial Ca^{2+} changes in controlling apoptosis via the opening of PTP is, in our opinion, still an attractive hypothesis awaiting direct experimental confirmation.

CONCLUSIONS

In the 1980s mitochondrial Ca^{2+} accumulation has been looked at as an interesting *in vitro* phenomenon, with little or no physiological relevance. In the last few years, thanks to a set of complementary technical developments and conceptual new information this feature of the mitochondria has triggered the interest of a number of investigators concerned with different experimental problems. The clearest conclusion from these different approaches is that not only mitochondrial Ca^{2+} handling plays a pivotal role in diverse phenomena such as energy production, control of ion channels and, possibly, programmed cell death, but that the spatio-temporal features and the supramolecular organization of the organelle are key determinants in shaping its role in living cells. However, nearly 50 years after the discovery of mitochondrial Ca^{2+} uptake [34], none of the mitochondrial proteins involved in this process have been purified and cloned. Similarly, the molecular basis of the control of mitochondrial structure, as well as of the interactions with the ER, is still obscure. Given the renaissance of mitochondrial Ca^{2+} handling in cell biology, the understanding in molecular terms of the phenomena described in this review is an exciting challenge for the near future.

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