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Review

Mitochondrial Ca^{2+} as a key regulator of cell life and death

M Giacomello[1], I Drago[1], P Pizzo[1] and T Pozzan*[,1,2]

Mitochondrial Ca^{2+} homeostasis is today at the center of wide interest in the scientific community because of its role both in the modulation of numerous physiological responses and because of its involvement in cell death. In this review, we briefly summarize a few basic features of mitochondrial Ca^{2+} handling *in vitro* and within living cells, and its involvement in the modulation of Ca^{2+}-dependent signaling. We then discuss the role of mitochondrial Ca^{2+} in the control of apoptotic death, focusing in particular on the effects of pro- and anti-apoptotic proteins of the Bcl-2 family. Finally, the potential involvement of Ca^{2+} and mitochondria in the development of two diseases, Ullrich muscular dystrophy and familial Alzheimer's disease, is briefly discussed.
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General Features of Ca^{2+} Handling by Mitochondria

The ability of isolated mitochondria to take up Ca^{2+} from the medium and to accumulate it in their matrix in an energy dependent way was first described over 40 years ago.[1] At that time, the chemiosmotic hypothesis was still not widely accepted by the scientific community. It was thought that the driving force for Ca^{2+} accumulation was provided by an unknown high-energy phosphorylated intermediate, $X\sim P$, generated by the electron flow through the respiratory chain or by the hydrolysis of ATP. Later, in the 1970s, the idea that the energy derived from substrate oxidation or by ATP hydrolysis is transformed in a H^+ gradient across the inner membrane became widely accepted and was honored by a Nobel Prize to Peter Mitchell in 1978. The concept that Ca^{2+} uptake by energized mitochondria is driven by the membrane potential across the inner membrane (negative inside) was thus clarified. Acceptance of this idea led to the prediction that, at equilibrium, the accumulation ratio between matrix and extracellular $[Ca^{2+}]$ should only be dictated by the Nernst equation. An inevitable corollary was that with a membrane potential of 180 mV, a divalent cation should accumulate within the matrix with a concentration 1 million fold that of the cytosol, that is for a cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) of 100 nM, a matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) of 0.1 M would be expected. This was clearly contrary to all experimental data and unquestionably incompatible with cell physiology. The paradox was eventually solved when it was demonstrated that the accumulation of Ca^{2+} within the mitochondrial matrix depends not only on the existence of an electrogenic uniporter (that tends to bring Ca^{2+} to an electrochemical equilibrium), but also on antiporters (Na^+ or H^+/Ca^{2+}), driven by the ion gradients, that extrude Ca^{2+} from the matrix.[2] A steady-state $[Ca^{2+}]_m$ is reached, far from electrochemical equilibrium, when the rate of Ca^{2+} influx through the uniporter equals the rate of Ca^{2+} efflux through the antiporters. The futile Ca^{2+} cycle occurring continuously across the mitochondrial inner membrane represents a small energy drain, but it is reduced to a minimum under physiological conditions because the 'affinity' for Ca^{2+} of the antiporter is low. In particular, the dependence of the uptake rate on the $[Ca^{2+}]$, at physiological Mg^{2+} concentration, is highly cooperative and the apparent K_M under these conditions is around 20 μM Ca^{2+}. Accordingly, the rate of Ca^{2+} influx at resting cytosolic $[Ca^{2+}]$ is $\sim$0.1% of the maximal uptake rate. The functional consequences of this kinetic model are valid to date; any change in the speed of the influx or efflux pathways must lead to changes in the $[Ca^{2+}]$ within the matrix. It is often erroneously assumed that microdomains of high Ca^{2+} in the cytosol are necessary for driving Ca^{2+} accumulation by mitochondria in living cells (see below). This is clearly a misinterpretation of the microdomain hypothesis that was formulated to explain the *fast* Ca^{2+} uptake by mitochondria in living cells. In fact, no matter how small the rise of $[Ca^{2+}]_c$, an increase should occur also in the mitochondrial matrix (for a review, see Rizzuto and Pozzan[3]).

Regarding the factors that modulate Ca^{2+} influx (V_{max} or K_M) through the uniporter, by far the most important is the extramitochondrial $[Ca^{2+}]$. Changes in the $[Ca^{2+}]_c$ in a living cell occur both under physiological or pathological conditions upon opening of plasma membrane or intracellular Ca^{2+} channels. Polyamines, such as spermine or cadaverine, slightly increase the K_M, but this is observed only at Ca^{2+}

[1]Department of Biomedical Sciences and CNR Institute of Neurosciences, University of Padua, Viale G Colombo 3, Padua, Italy and [2]Venetian Institute of Molecular Medicine, Padua, Italy
*Corresponding author: T Pozzan, Department of Biomedical Sciences and CNR Institute of Neurosciences, University of Padua, Viale G Colombo 3, 35121, Padua, Italy. Tel: +39 049 827 6070; Fax: +39 049 827 6049; E-mail: tullio.pozzan@unipd.it
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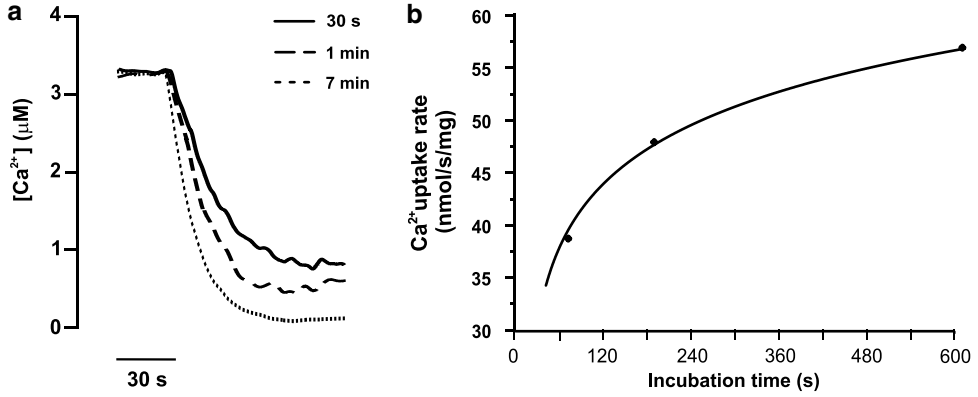

Figure 1 Time dependence of the Ca^{2+} activation of mitochondrial Ca^{2+} uptake. The experiment was carried out in isolated mitochondria incubated in sucrose medium, essentially as described in Pozzan *et al*.[2] Extramitochondrial Ca^{2+} was measured with the fluorescent Ca^{2+} indicator Calcium Green-5N. (**a**) Non-respiring mitochondria (treated with rotenone and oligomycin) were incubated in medium containing 3 μM $CaCl_2$ for different periods of time (30 s, 1 and 7 min) before adding 2 mM succinate to initiate Ca^{2+} uptake. Typical traces are shown. (**b**) Initial rate of mitochondrial Ca^{2+} uptake as a function of the preincubation time in 3 μM $CaCl_2$; each point is the mean of two experiments carried out in the same mitochondrial preparation. Similar results were obtained in at least six independent trials

concentrations well above the physiological ones. Recently (see also below), it has been suggested that the uniporter rate (or better, its affinity for Ca^{2+}) can be modulated (increased or decreased) by protein kinases, in particular by p38 MAP kinases (inactivation),[4] or protein kinase C (the ζ isoform activates, whereas the β/δ isoforms inactivate it[5]), although this modulation still requires further clarification. A highly interesting, yet largely neglected phenomenon, is the Ca^{2+}-induced activation of Ca^{2+} uptake, a phenomenon initially described 20 years ago by Kroner[6] and recently re-visited by Moreau *et al*.[7] It consists in a substantial acceleration of the Ca^{2+} uptake rate if non-energized mitochondria are first exposed for different periods of time to micromolar Ca^{2+} concentrations. A simple experiment demonstrating this phenomenon in isolated mitochondria is shown in Figure 1. Non-respiring mitochondria are exposed to 3 μM Ca^{2+} for 30 s, 1 or 7 min before Ca^{2+} uptake is triggered by the addition of succinate. The longer the incubation in 3 μM Ca^{2+}, the faster the initial rate of Ca^{2+} uptake. This phenomenon is inhibited by the calmodulin inhibitor calmidazolium, but it is insensitive to CAM kinase II inhibitors.[7] The molecular mechanisms involved in this phenomenon remain mysterious, but it should be taken into account to explain some of the kinetic behavior of mitochondria in living cells.

The Ca^{2+} efflux mechanisms appear to be regulated by the Ca^{2+}, Na^+ (or H^+) concentrations outside and/or inside the mitochondria and by *in vivo* treatment with glucagon and β-adrenergic agonists (for a review, see Rizzuto *et al*.[8]). The Na^+/Ca^{2+} antiporters are most likely electrogenic[9] (three Na^+/H^+ ions are exchanged for one Ca^{2+}) and, accordingly, a negative membrane potential inside favors Ca^{2+} extrusion. Ca^{2+} efflux can also occur through the uniporter if the membrane potential is collapsed. Of interest, the classical inhibitor of the uniporter, Ruthenium Red (RR), poorly inhibits the Ca^{2+} release, which was largely incomprehensible until it was elucidated by the channel nature of the uniporter[10] and see below: RR, a cation with six positive charges, is attracted into the Ca^{2+} channel at negative membrane potential in the matrix (probably occluding it), but is released when the membrane potential is collapsed. Another Ca^{2+} efflux pathway is the so-called permeability transition pore, PTP (see below). Indeed, as a consequence of the collapse of membrane potential caused by the PTP activation, Ca^{2+} can be lost both through the open PTP itself and through reversal of the uniporter.

All the functional characteristics of mitochondrial Ca^{2+} handling machinery described so far were largely known by the end of the 1970s. At the time, mitochondria were believed to represent an important Ca^{2+} storage organelle, from where Ca^{2+} could be mobilized upon cell activation. The demonstration that mitochondria in healthy living cells contain trace amounts of Ca^{2+} and the characterization of the uniporter kinetics mentioned above (low influx rate at the physiological Ca^{2+} concentrations not only of resting cells, but also upon activation), convinced most investigators that mitochondrial Ca^{2+} uptake played a marginal role in the overall control of Ca^{2+} homeostasis under physiological conditions and that it becomes important only under frank pathological situations.[11] As to the latter (see below), it was in fact already well known that necrotic cells had often electron-dense material within mitochondria, represented by precipitates of Ca^{2+} phosphate.[11] As a consequence, during the decade that followed, the scientific community paid little attention to mitochondrial Ca^{2+} handling; episodically, however, papers were published that indirectly suggested that this organelle could have a more important role in the cell physiology of Ca^{2+} handling (for a review, see Pozzan *et al*.[12]).

A major change happened in the early 1990s, when we pioneered the use of recombinant targeted aequorins to monitor directly $[Ca^{2+}]_m$.[13] With this novel methodology we could show that a rise in $[Ca^{2+}]_m$ occurred for any, even very small, change in $[Ca^{2+}]_c$ (as predicted by the model discussed above); at the same time, and unexpectedly, we also observed that in a large number of cells a physiological rise of $[Ca^{2+}]_c$ was accompanied by very fast and large increases in $[Ca^{2+}]_m$ – much faster and much larger than the prediction based on the low affinity of the uniporter *in vitro*.[14,15] This new paradox was unraveled through the hypothesis of local microdomains. The fast Ca^{2+} uptake observed depends on the generation of very high $[Ca^{2+}]$, close to mitochondria,

where the organelles happen to be closely apposed to Ca^{2+} channels (plasma membrane or ER/SR); it is these microdomains of high [Ca^{2+}] that induce a very fast, but transient accumulation of Ca^{2+} in the mitochondria[14] (for a recent review, see Rizzuto and Pozzan[3]). The hypothesis has received broad experimental support, leading to a renewed interest in the mechanisms and role of mitochondrial Ca^{2+} handling. The observation that really brought mitochondria back into the limelight was published only a few years later: the demonstration that programmed cell death could depend in many instances on the release from mitochondria of a small protein, cytochrome *c*, located between the inner and the outer mitochondrial membranes, and that this release could in turn lead to the activation of effector caspases (for a review, see Ferri and Kroemer[16]). The link between cytochrome *c* release, apoptosis and mitochondrial Ca^{2+} was immediately clear: overaccumulation of Ca^{2+} by mitochondria was already known to activate the PTP (in a cyclosporine A sensitive mechanisms, see below) and this large membrane pore in the inner mitochondrial membrane can lead to matrix swelling, causing the rupture of the outer mitochondrial membrane and release of cytochrome *c*.[16–18]

Mitochondrial Ca^{2+} handling thus became the center of a very important biological problem, with key pathophysiological consequences for a number of human pathologies, from cell death due to ischemia, to excitotoxicity in the CNS, to degenerative diseases. Add to this the finding that pro- and anti-apoptotic genes can modulate mitochondrial Ca^{2+} handling (see below), and the importance of mitochondrial Ca^{2+} handling properties becomes immediately clear, with excursions even into problems related to cell growth and cancer development.

It should be stressed that, as far as mitochondrial Ca^{2+} handling is concerned, extreme care should be taken in comparing results obtained in isolated organelles with those within intact cells. The isolation of the organelles disrupts the anatomical connections with other cellular structures, in particular, the plasma membrane and ER/SR Ca^{2+} channels, that are vital for the generation of Ca^{2+} microdomains.[3] Within living cells, mitochondria are exposed both to oxidizable substrates and to a high concentration of ATP, generated by the organelles themselves and by glycolysis. This latter, trivial consideration, is often overlooked by many investigators. Thus, if isolated mitochondria are incubated with a respiratory substrate (e.g. glutamate) the inhibition of the respiratory chain (by rotenone or antimycin) prevents mitochondrial Ca^{2+} accumulation because the membrane potential generated by the respiratory chain is rapidly collapsed. On the contrary, in the majority of living cells, Ca^{2+} accumulation in response to a stimulus is practically unaffected by the same drugs, because the membrane potential can be regenerated by reversal of the mitochondrial ATPase, using ATP produced by glycolysis. To block efficiently mitochondrial Ca^{2+} uptake in a living cell one must use either an uncoupler (but other side effects need to be taken into consideration) or a combination of a respiratory chain blocker and oligomycin, to block the ATPase as well. Effects on Ca^{2+} handling, often reported, of respiratory chain inhibitors only (or oligomycin alone) most likely reflect the consequences of these drugs on functions that are not linked to mitochondrial Ca^{2+} uptake.

Ironically, in spite of the enormous interest in mitochondrial Ca^{2+} homeostasis, the molecular identification of the players involved is still missing. The search for the mitochondrial Ca^{2+} uniporter, for example, has been a frustrating issue for the last 40 years. Several groups claimed to have isolated this molecular complex, but none of these claims has been ultimately confirmed. At one stage, the best known candidate was the so-called 'P30 glycoprotein', a peripheral glycosylated membrane protein that the authors thought could transport Ca^{2+} across the inner membrane with a mechanism similar to that of classical antibiotic ionophores, such as valinomycin.[19,20] In 1979, based on a series of indirect data, we proposed that the uniporter was a gated ion channel[21] that is an integral protein of the inner mitochondrial membrane. This proposal was never followed up until Clapham and coworkers, by patch clamping the inner mitochondrial membrane, very elegantly showed that the uniporter is indeed a selective divalent cation channel.[10]

The search for the antiporters has been less intense, and we are unaware of any conclusive study leading to their identification. To our knowledge, *in silico* searches for potential candidate genes, have fared no better – in spite of the fact that the identified members of the mitochondrial proteome continue to grow steadily. In summary, over 40 years after the initial functional identification of the mitochondrial Ca^{2+} uniporter (and over 30 after that of the antiporters) the molecular identities of these key regulators of the mitochondrial Ca^{2+} handling machinery remain unknown.

Physiological Roles of Mitochondrial Ca^{2+} Accumulation

What is the function of mitochondrial Ca^{2+} uptake? It is beyond the focus of this review to discuss the physiological consequences of mitochondrial Ca^{2+} uptake in detail. A few key physiological processes – directly or indirectly modulated by mitochondrial Ca^{2+} uptake and release – must, however, be addressed briefly.

One of the best characterized functions of mitochondrial Ca^{2+} uptake is the control of organelle metabolic activity. Three crucial metabolic enzymes within the matrix (pyruvate, α-ketoglutarate and isocitrate dehydrogenases) are activated by Ca^{2+}, using two distinct mechanisms. In the case of pyruvate dehydrogenase, a Ca^{2+}-dependent dephosphorylation step is involved; in the other two cases, the activation is through direct binding of Ca^{2+} to the enzyme complex (for a recent review, see Rizzuto and Pozzan[3]). Given that these enzymes represent the rate limiting step for feeding electrons into the respiratory chain, Ca^{2+} within the matrix is ultimately the positive modulator of mitochondrial ATP synthesis; this aspect was directly addressed by Rizzuto's group a few years ago using targeted recombinant luciferase to monitor, in living cells, the ATP concentration within the cytoplasm and the mitochondrial matrix.[22] Recently, also some metabolite transporters have been shown to be regulated by Ca^{2+} and to participate in the enhancement of aerobic metabolism upon cell activation.[3] One issue remains to be clarified: given that the matrix dehydrogenases are activated by [Ca^{2+}] in the low micromolar range, why do $[Ca^{2+}]_m$ rises reach values up to several tens or hundreds of μM? Several explanations could

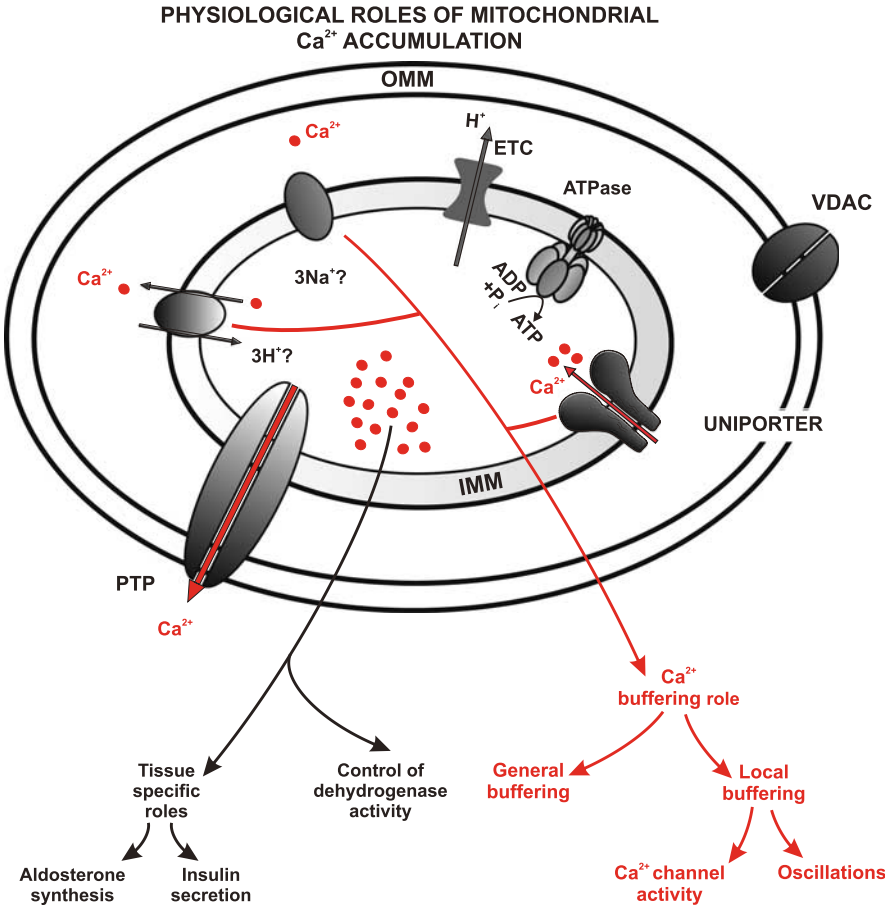

Figure 2 Schematic view of the process of Ca^{2+} homeostasis in mitochondria. The key molecular players in the process of Ca^{2+} accumulation/release are schematically drawn with their supposed intramitochondrial localization. ETC, electron transport chain, VDAC, voltage-dependent anion channel, OMM: outer mitochondrial membrane, IMM, inner mitochondrial membrane, PTP, permeability transition pore

be offered. The first is that most $[Ca^{2+}]_m$ estimates were obtained in prolonged, supramaximal stimulations with Ca^{2+}-mobilizing agonists, and it is likely that these conditions do not fully mimic the response to physiological challenges (that give rise to smaller and more transient $[Ca^{2+}]_c$ increases). The second is that a large $[Ca^{2+}]_m$ response (together with other mechanisms, such as the enzymatic delay in the rephosphorylation of the PDH complex) could be a route to extend the metabolic activation well beyond the duration of the $[Ca^{2+}]_c$ rise. The latter explanation may be particularly relevant in heart cells, where $[Ca^{2+}]_m$ oscillates on a beat-to-beat basis, while the activity of the dehydrogenases must be constantly elevated.

A second process that depends on mitochondrial Ca^{2+} handling regards the kinetics of cytosolic Ca^{2+} changes in response to agonist stimulation. Specific examples in this case are too numerous to be addressed in detail here, but the following aspects provide a general framework. Mitochondria, by buffering local $[Ca^{2+}]$ (generated by Ca^{2+} channels on the plasma membrane or the ER/SR), can augment or decrease the release/influx of Ca^{2+} and modulate the frequency of Ca^{2+} oscillations; this phenomenon depends on the cell type and the channel involved (see, e.g., Rizzuto *et al.*,[23] Collins *et al.*,[24] Hajnóczky *et al.*,[25] Landolfi *et al.*,[26] Vay *et al.*[27]). In addition, mitochondria can also exert a more classical buffering role. One example is the cluster of mitochondria that isolate functionally distinct domains in polarized cells: a mitochondrial 'firewall' was shown to prevent the spread of Ca^{2+} signals from the apical region of pancreatic acinar cell to the basolateral region.[28] Another example, this time in neurons, sees mitochondria buffering $[Ca^{2+}]$ increases in defined cellular regions, that is the presynaptic motoneuron ending.[29] The mitochondrial Ca^{2+} efflux mechanisms have also been involved in shaping the cytoplasmic Ca^{2+} kinetics in neurons in response to intense electrical stimulation (by slowly releasing the accumulated Ca^{2+}),[30] or by allowing the efficient refilling of the ER and thus modulating Ca^{2+} oscillations.[27,31] While the above mentioned roles are more or less similar in every cell type, there are also other roles of mitochondrial Ca^{2+} that appear to be tissue specific. The two examples best studied are the endocrine pancreas, where mitochondrial Ca^{2+} modulates insulin secretion,[32] and the granulosa cells of the adrenal gland, where it controls a key step in aldosterone synthesis.[33] A schematic view of mitochondrial Ca^{2+} regulation and of the physiological processes that are modulated is presented in Figure 2.

Currently, the most studied role of mitochondrial Ca^{2+} handling is, by far, the control of apoptosis/necrosis. Regard-

ing apoptosis, it is probably a semantic problem to include it in the physiology or pathology of the cell. For reasons of simplicity we have decided to discuss apoptosis in the next chapter.

Mitochondrial Ca^{2+} Homeostasis and Cell Death by Necrosis and Apoptosis

Necrosis is the catastrophic derangement of cell integrity and function that follows exposure to different types of cell injury, leading to the activation of Ca^{2+}-activated hydrolysing enzymes.[34,35] The fact that major increases in $[Ca^{2+}]_c$ are capable of causing necrosis has been known for a long time. Similarly, it is well established that mitochondria represent a major target of Ca^{2+}-dependent necrosis, as demonstrated by the formation of the Ca^{2+}-phosphate deposits in necrotic cells, mitochondrial membrane potential collapse, rapid drop in ATP levels and production of ROS (for a review, see Rizzuto *et al.*[36]). More recently, it has been shown that Ca^{2+} can also play a central role in triggering some forms of a more subtle and controlled pathway of cell death, *i.e.* the apoptotic program. In this case, what attracted the attention of many scientists to Ca^{2+} was the discovery of the effects of Bcl-2 on Ca^{2+} signaling. The first of such observations dates back to 1993, when it was found that Bcl-2 overexpression decreases the amount of Ca^{2+} capable of being mobilized from the ER.[37] In 2000, an additional seminal observation was made contemporarily by our group and by that of K.H. Krause:[38,39] briefly, it was not only found that cells overexpressing Bcl-2 display an enhanced survival upon treatment with some apoptogenic drugs (as expected), but also, surprisingly, that such cells display an $\sim$30% reduction in the $[Ca^{2+}]$ levels within the lumen of the ER (and of the Golgi apparatus). Both groups also showed that the major consequence of this reduction in stored Ca^{2+} was a large decrease of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ increases elicited in these cells by stimuli coupled to IP_3 generation.[38,39] The question then arose as to whether this reduction of $[Ca^{2+}]$ within the stores, due to overexpression of anti-apoptotic proteins, is relevant for apoptosis or whether is it a side effect of the overexpression of these proteins. Indirect support for the first hypothesis came from the work of Pinton *et al.*[40] which showed that the reduction of $[Ca^{2+}]$ within the stores plays an important role in the anti-apoptotic mechanism of Bcl-2: mimicking the Bcl-2 effect on $[Ca^{2+}]$ by different pharmacological and molecular approaches (but in the absence of the oncoprotein) the cells were protected from the apoptotic stimulus ceramide. Of interest, treatments that increased $[Ca^{2+}]$ within the stores had the opposite effect on the susceptibility of cells to the apoptotic stimulus.[40]

These observations fitted nicely with other data published in the same period, totally independently, by other groups, such as: (i) the association of Bcl-2 with mitochondrial and ER membranes;[41] (ii) the demonstration, mentioned above, that excess Ca^{2+} accumulation by mitochondria leads to opening of the PTP and to massive mitochondrial swelling;[17,18,42] and (iii) that cytochrome *c* is released into the cytosol in response to several apoptotic stimuli. Taken together these data allowed the formulation of a rational working hypothesis: (i) an apoptotic insult causes the release of Ca^{2+} from the ER; (ii) the amount of Ca^{2+} content of the ER determines the amount of Ca^{2+} taken up by mitochondria; (iii) when the latter organelles accumulate an excess of Ca^{2+} the PTP is activated, release of cytochrome *c* and other pro-apoptotic factors occur and eventually executor caspases are irreversibly activated. It could be argued that this model is in contradiction with the well established fact that similarly large (or even larger) Ca^{2+} releases and mitochondrial accumulations, elicited by a number of stimuli, do not trigger cell death, but rather are beneficial, for example by increasing the cellular ATP levels. Pinton *et al.*,[40] to solve this contradiction, proposed the 'double hit' hypothesis; that is that apoptotic stimuli, such as ceramide, have a dual target: on the one hand cause the release of Ca^{2+} from the ER and its uptake by mitochondria, on the other make the mitochondria more sensitive to the potential Ca^{2+} damaging effects. This hypothesis fits nicely with elegant studies by Hajnoczky and coworkers. In particular, they showed that ceramide facilitates PTP opening, thus transforming physiological IP_3-mediated Ca^{2+} signals into inducers of apoptosis.[43] Finally, unpublished results by our group show that activators of mitochondrial Ca^{2+} uptake potently synergize with sub-threshold doses of apoptotic agents.

This model of Ca^{2+}-dependent apoptosis triggering was subsequently supported by a series of observations, the most important of which can be briefly described as follows. Scorrano *et al.* demonstrated not only that embryonic fibroblasts from knockout mice lacking the pro-apoptotic proteins Bax and Bak are very resistant to apoptotic death, but also that they have a dramatic reduction in the $[Ca^{2+}]$ within the ER and a drastic reduction in the transfer of Ca^{2+} from the ER to mitochondria.[44] Furthermore, silencing Bcl-2 in Bax/Bak knockout cells partially restores $[Ca^{2+}]$ values within the ER to control levels[44] and normalizes the mitochondrial Ca^{2+} responses. In the Bax/Bak knockout cells, when the ER Ca^{2+} levels were restored by recombinantly over-expressing the ER Ca^{2+} ATPase (SERCA2b), not only was mitochondrial Ca^{2+} uptake in response to stimulation re-established, but the cells regained sensitivity to apoptotic stimuli such as arachidonic acid, C_2-ceramide and oxidative stress.[44] Along the same line of reasoning, calreticulin over-expressing cells, that have an augmented ER Ca^{2+} content, are more susceptible to apoptosis induced by ceramide treatment,[40] while calreticulin knockout cell lines, that show a marked decrease in ER Ca^{2+} release upon cell stimulation, are more resistant to apoptosis.[45] Chami *et al.*[46] also showed that early after overexpression of Bax in HeLa cells the $[Ca^{2+}]$ of the ER is higher than in controls. Finally, Tsien and coworkers not only confirmed that Bcl-2 overexpression leads to decreased ER Ca^{2+} levels, but also showed that the green tea compound epigallocatechin gallate, known to bind and inactivate Bcl-2, restored $[Ca^{2+}]$ of the ER to that of normal cells.[47] We are aware that not all experts in the field concur with these conclusions – see, for example, Chen *et al.*,[48] He *et al.*,[49] Lam *et al.*,[50] Wang *et al.*,[51,52] Zhong *et al.*,[53] Ichimiya *et al.*,[54] Wei *et al.*,[55] Kuo *et al.*,[56] Zhu *et al.*[57] But it is our biased opinion that in many of these studies the different conclusions depend on the specific experimental approach (indirect methods of monitoring Ca^{2+} in the ER, use of clones, etc.). A very interesting possibility would be that the

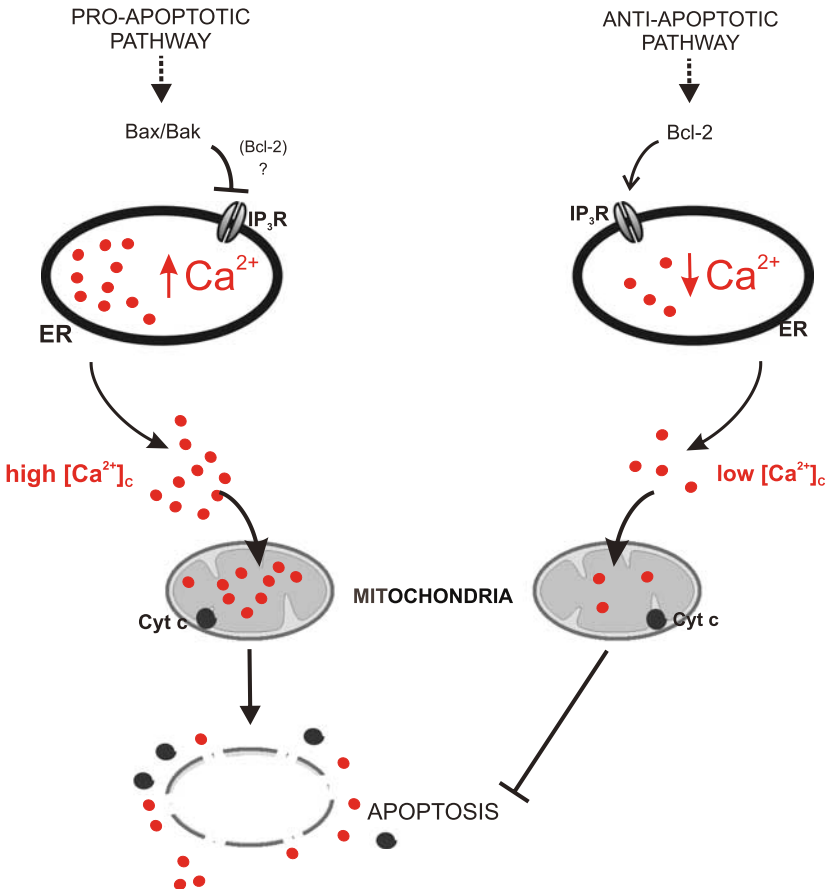

Figure 3 Proposed model of the interplay among mitochondria, ER and pro- or anti-apoptotic Bcl-2 family members in triggering Ca^{2+}-modulated apoptosis

modulation of Ca^{2+} handling by pro- and anti-apoptotic proteins is exerted because they can alter the gating properties of specific isoform of the IP_3 receptor, IP_3R. If this is the case the effects of the pro- and anti-apoptotic proteins on Ca^{2+} handling should depend on the specific cell model employed and the expression profile of IP_3Rs. Recent evidence indicates that indeed the last hypothesis may be true.[58–60] An obvious corollary to this hypothesis is that the effect of pro- and anti-apoptotic proteins is only one of the mechanisms through which these proteins control cell death, as indeed clearly shown by Scorrano *et al.*[44]

In summary, Bcl-2 and other anti-apoptotic proteins reduce ER Ca^{2+} levels, and consequently moderate the efficacy of apoptotic mediators that use Ca^{2+} signals (and the involvement of mitochondria as downstream effectors) as a potentiation/commitment factor. Conversely, Bax (and other pro-apoptotic proteins of the family) enhances the loading of the ER Ca^{2+} store, and thus boosts the Ca^{2+} load to which the apoptotic effector systems (including mitochondria) are exposed upon physiological and/or pathological challenges. The model is schematically described in Figure 3.

Ca^{2+} and Human Genetic Diseases

The number of clinically relevant pathological events in which the proposed model of Ca^{2+} activated cell death may play a central role are numerous: for example, ischemic death in the heart and other tissues, glutamate dependent excitotoxicity in the CNS, Parkinson's disease, Alzheimer's disease, and even Ullrich muscular dystrophy (UMD) and Duchenne muscular dystrophy.

In this last chapter we will briefly discuss the involvement of Ca^{2+} and mitochondria in UMD and Alzheimer's disease, given that in these latter two pathologies the role of Ca^{2+} and of mitochondria is less obvious and/or still a matter of some controversy.

UMD is a severe muscular dystrophy, with different age of onset and gravity. The involvement of mitochondria in the pathogenesis of the fiber degeneration in UMD was a very surprising and unexpected observation. UMD, and the less severe Bethlem myopathy, are muscle diseases caused by defects in collagen VI. Bernardi and coworkers,[61] showed that in muscle fibers of knockout mice for collagen VI, mitochondria have a latent defect, that is a strong susceptibility of the PTP to open for mild mitochondrial insults; mitochondria isolated from knockout animals *in vitro* have a very modest capacity to accumulate Ca^{2+} in response to Ca^{2+} challenges, due to very rapid PTP opening (sensitive to cyclosporine A). The molecular link between the collagen VI defect and the mitochondrial instability is still largely mysterious. From the therapeutical point of view, the important fact is that injection in the knockout mouse of the PTP inhibitor

cyclosporine A led to a dramatic recovery of the muscle lesions. Even more important, the same group very recently showed in biopsies from human patients that chelation of intracellular Ca^{2+}, addition of collagen VI or treatment with cyclosporins that inhibit the PTP could, *in vitro*, ameliorate mitochondrial dysfunctions of myoblasts from UMD patients,[62] thus offering for the first time the possibility of a pharmacological therapy for this incurable and devastating genetic disease.

Recent evidence suggests that Ca^{2+} may play a key role also in Alzheimer's disease (AD), or at least in the genetic forms of the disease. AD is the most common neurodegenerative disorder[63] and it accounts for about 50% of the cases of senile dementia. This pathology, first described by Alois Alzheimer in 1906, is characterized by cortical atrophy, accumulation of abnormal fibres in neuronal cell bodies, and the presence, in the extracellular space, of senile plaques, whose main component is the so-called Aβ peptide. The latter derives from the transmembrane protein amyloid precursor protein (APP), which can be alternatively processed by three different enzymes, named α, β, and γ secretases. The combined action of β and γ secretases leads to the formation of a soluble fragment (sAPPβ) and of the Aβ peptide, together with its cytosolic counterpart AICD (APP IntraCellular Domain).

Mutations in the genes encoding for APP, Presenilin1 and 2 (PS1 and PS2), two proteins belonging to the γ-secretase enzymatic complex, have been linked to the familial form of AD (FAD; for a recent review, see St George-Hyslop and Petit[64]). Since the common phenotype of all these mutations is an increased Aβ production, Hardy *et al.*[65] proposed the amyloid cascade hypothesis, stating that accumulation of Aβ is the chief molecular event that causes the onset of the disease (but see also De Strooper[66]).

About 10 years ago it has been proposed that an alteration in intracellular Ca^{2+} homeostasis could contribute to the development of FAD. The majority of published data, obtained mainly by analyzing PS1 mutations, report that mutated presenilins increase the ER Ca^{2+} content (for a recent review, see Smith *et al.*[67]). The hypothesis was thus proposed[68] that a Ca^{2+} overload could either lead to an increased Aβ production or, alternatively, that the overload of Ca^{2+} stores caused by presenilin mutations could exacerbate the Aβ toxicity by favoring Ca^{2+}-dependent cell death. A strong support for this hypothesis came recently from the demonstration that wild type presenilins, but not the mutated forms, can form leak channels in the ER (they also form divalent cation permeable channels in lipid bilayers[69]). According to the above discussed model of ER-mitochondria crosstalk in triggering apoptosis, it could be speculated that in neurons expressing the mutated PS isoforms a reduction in the ER Ca^{2+} leak leads to over-accumulation of Ca^{2+} in the ER and thus can favor its transfer to mitochondria, leading to neuronal apoptosis. Although this hypothesis has gained popularity, there are data that are difficult to reconcile with it. In particular, Zatti *et al.* [70–72] showed that some FAD-linked PS2 mutations caused a reduction, yet not an increase, in ER Ca^{2+} levels.

A possible alternative hypothesis could be suggested for hereditary AD due to presenilin mutations: by increasing Aβ formation (and in particular of the 42 AA form), the mutated enzymes lead to neuronal degeneration. At the same time, by modulating Ca^{2+} within the ER they can exacerbate or reduce the toxicity of Aβ. Presenilin mutations (PS1 in particular) that cause an increase in the ER Ca^{2+} exacerbate cell death, while mutations (in particular of PS2) that decrease the ER Ca^{2+} levels partially protect cells from Ca^{2+}-dependent cell death. This hypothesis would be consistent with the afore mentioned role of ER and mitochondrial Ca^{2+} relationship, and with the clinical observation that FAD-linked PS2 mutations have been associated to milder phenotypes.[71,72]

In conclusion, the capacity of mitochondria to accumulate and release Ca^{2+} appears intimately linked to the multiple roles of these organelles within cells: on the one hand, Ca^{2+} accumulation/release is instrumental in modulating the key bioenergetic role of mitochondria (e.g., respiratory substrate oxidation and ATP synthesis) and in modulating the kinetics and amplitude of the $[Ca^{2+}]_c$ signal (and thus the cell functions that depend on this second messenger); on the other, massive accumulation of Ca^{2+} in the mitochondria leads to necrotic cells death; finally more modest increases in $[Ca^{2+}]_m$, but in the presence of other toxic insults, trigger the mitochondrial gateway to apoptosis.

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