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Review article

Controlling metabolism and cell death: At the heart of mitochondrial calcium signalling

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ABSTRACT

Transient increases in intracellular calcium concentration activate and coordinate a wide variety of cellular processes in virtually every cell type. This review describes the main homeostatic mechanisms that control Ca^{2+} transients, focusing on the mitochondrial checkpoint. We subsequently extend this paradigm to the cardiomyocyte and to the interplay between cytosol, endoplasmic reticulum and mitochondria that occurs beat-to-beat in excitation–contraction coupling. The mechanisms whereby mitochondria decode fast cytosolic calcium spikes are discussed in the light of the results obtained with recombinant photoproteins targeted to the mitochondrial matrix of contracting cardiomyocytes. Mitochondrial calcium homeostasis is then highlighted as a crucial point of convergence of the environmental signals that mediate cardiac cell death, both by necrosis and by apoptosis. Altogether we point to a role of the mitochondrion as an integrator of calcium signalling and a fundamental decision maker in cardiomyocyte metabolism and survival.

Contents

1. Introduction

Ca^{2+} has long been recognized as a fundamental second messenger involved in learning, memory, fertilization, proliferation, development, muscle contraction and secretion [1]. Its concentration in the cytosol ($[Ca^{2+}]_c$) varies dynamically under the tight control of coordinated homeostatic mechanisms. In resting conditions, $[Ca^{2+}]_c$ is around 100 nM, approximately 4 orders of magnitude lower than in the extracellular medium, thanks to the activity of active systems pumping Ca^{2+} outside the cells, the plasma membrane Ca^{2+} ATPases and the Na^+/Ca^{2+} exchanger (reviewed in [2]). This concentration gradient allows rapid influx of Ca^{2+} into the cell upon opening of a broad variety of plasma membrane channels. Within the cell, Ca^{2+} is stored in large amounts in specialized compartments, mainly the ER/SR and the Golgi apparatus [3]. The endoplasmic reticulum (ER), and its specialized counterpart of muscle cells, the sarcoplasmic reticulum

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(SR), are quantitatively the most important stores, and actively participate in generating the precise spatio-temporal patterns of the $[Ca^{2+}]_c$ increases. Due to the activity of the sarco/ER Ca^{2+} ATPase (SERCA), the $[Ca^{2+}]$ of the ER/SR is maintained at approximately 100–400 μM, thus allowing the rapid release of Ca^{2+} upon opening of Ca^{2+} channels residing in the organelle membrane. These include the ubiquitously expressed inositol 1,4,5 trisphosphate receptors (IP3R), the most important route for $[Ca^{2+}]_c$ increase in non-excitable cells, and the ryanodine receptors (RyR), mostly expressed in muscle cells and neurons. Specifically, the RyR isoforms 1 and 2 sense the opening of plasma membrane Ca^{2+} channels and promote the larger $[Ca^{2+}]_c$ increase responsible for muscle contraction in skeletal and cardiac muscle, respectively. RyR 1 and RyR2 differ in the activation mechanism, direct coupling to the plasma membrane channel for RyR1 and opening in response to the priming $[Ca^{2+}]_c$ influx (calcium-induced Ca^{2+} release) for RyR2 (Fig. 1). We refer to more detailed reviews for an accurate coverage of the mechanistic and molecular aspects of ER/SR Ca^{2+} handling [4,5]. We only anticipate here, and discuss in more detail later, the importance of these highly dynamic Ca^{2+} stores, that generate in their proximity microdomains of high $[Ca^{2+}]$, for mitochondrial Ca^{2+} homeostasis. Ca^{2+} uptake in the Golgi is mediated both by the SERCA pumps and by the Secretory Pathway Ca^{2+} ATPase (SPCA), the mammalian homolog of PMR1, the Mn^{2+}/Ca^{2+}-dependent ATPase of *Saccharomyces cerevisiae* [6]. Ca^{2+} release from the Golgi apparatus appears also mediated by the IP3R, and the dynamic changes appear relevant both for the modulation of intraluminal processes (e.g. protein processing and trafficking) and for the generation of local signalling events in proximity of the organelle.

Finally, it should be remembered that while the absolute resting values of the $[Ca^{2+}]$ in the cytosol and in the stores are determined by the balance of the activities of pumps and channels, the dynamic changes are strongly influenced by the presence of buffering systems, dampening the increase (or decrease) till their saturation (or complete uncharging). Organelles themselves (mitochondria, but also peroxisomes and secretory vesicles) accumulate part of the Ca^{2+} released from stores, thereby acting as fixed buffers. In addition, the Ca^{2+} signalling apparatus encompasses a large number of Ca^{2+} binding proteins, both in the cytosol and in the ER/SR and Golgi stores. The former include proteins containing high affinity EF-hands Ca^{2+} binding sites (calbindin D 28K, parvalbumin, calreticulin, calmodulin) that act as mobile buffers preventing the rapid spread of high Ca^{2+} microdomains from the site where they were generated (the mouth of an open channel) to the rest of the cytoplasm. The luminal Ca^{2+} binding proteins of the stores act as low affinity, high capacity buffers that increase the amount of releasable Ca^{2+}, without affecting the $[Ca^{2+}]_{er/sr}$, thus not imposing a high energetic cost.

2. Mitochondrial Ca^{2+} homeostasis

The main driving force for Ca^{2+} accumulation across the inner mitochondrial membrane is the electrochemical gradient ($\Delta\mu H$), established and maintained by the respiratory chain. Indeed, respiring mitochondria maintain a membrane potential of 180–200 mV, which constitutes a strong driving force for Ca^{2+} influx into the matrix. Applying the Nernst equation, thermodynamic equilibrium for Ca^{2+} ($\Delta\mu Ca = 0$) would be reached at Ca^{2+} concentrations 10^6 higher than in the cytoplasm, i.e. giving an average resting $[Ca^{2+}]_c$ of 10^{-7} M, around 100 mM. Conversely, experimental evidence demonstrates that, in resting conditions, $[Ca^{2+}]_m$ is similar to $[Ca^{2+}]_c$, reinforcing the idea that mitochondria do not act as a Ca^{2+} store, and that Ca^{2+} distribution is modulated by kinetic rather than simply thermodynamic parameters [7-9]. In other words, Ca^{2+} concentrations in the mitochondrial matrix are controlled by low affinity uptake pathways and by efflux mechanisms that re-extrude Ca^{2+}, at the cost of a significant futile cycle across the inner membrane.

This paragraph describes the main control systems for $[Ca^{2+}]_m$. Influx is mainly supported by a Ca^{2+} electrophoretic mechanism ("uniporter") whereas the Na^+–Ca^{2+} and Na^+–H^+ exchangers are responsible for Ca^{2+} efflux. While the activity of the exchangers tend to saturate when $[Ca^{2+}]_m$ increases, the uniport is a channel and does not saturate. A direct consequence of this transport system is that, when $[Ca^{2+}]_m$ increases above a certain threshold, mitochondria have to cope with the risk of Ca^{2+} overload. Avoiding mitochondrial Ca^{2+} overload is energetically demanding for the cell, which has evolved a number of transport mechanisms to rationalize the system and adapt it to varying conditions of energy expenditure.

The mitochondrial Ca^{2+} uniporter (MCU) is a channel, which transports Ca^{2+}, Sr^{2+}, but not Mg^{2+} with different selectivity and very low affinity [10]. Several modulators of the MCU have been identified, including ruthenium red, an allosteric inhibitor, and divalent cations that do not permeate through the channel, such as Mn^{2+}, Ba^{2+} and lanthanides. Several estrogen receptors ligands have also recently been shown to modulate the activity of the uniporter [11]. The molecular structure of this transporter remains elusive but various hypotheses have been proposed over the years, starting in the 1970′s when purification was first attempted. A highly selective Ca^{2+} channel activity with kinetic and pharmacological characteristics of the mitochondrial uniporter has been identified and characterized from the electrophysiological point of view [10]. Trenker and co-workers, in a recent work, proposed an important role of UCP2 and UCP3 in mitochondrial Ca^{2+} uptake [12].

The mitochondrial Ca^{2+} uniporter is activated by external, i.e. cytosolic Ca^{2+} and it has also been shown to be controlled allosterically by external adenine nucleotides [13]. The calculated kinetic parameters, however, would indicate that at physiological $[Ca^{2+}]$ values measured in the cytosol even during cell stimulation (low micromolar range), the activity of the uniporter is extremely low.

2.1. Rapid mode of uptake (RaM)

If energised mitochondria are exposed to repeated calcium pulses at physiological levels they are more efficient at taking up calcium than when simply exposed to a steady state level corresponding to the peak value for the same stimulation time. Based on this observation a Rapid Mode of uptake (RaM) was proposed to take place, that is highly conductive at the beginning of a Ca^{2+} pulse and inhibited by the rising Ca^{2+} concentration of the pulse itself, with a long recovery phase. Such a property would allow the sequestration of significant amounts of calcium at the beginning of each sequence of pulses. The identity, and even the existence, of such an influx pathway, different from the MCU, is controversial. Indeed, the putative Ca^{2+} transport system responsible for this effect shares the sensitivity to Ruthenium Red with the MCU but has a considerably higher IC_{50} and is Mg^{2+}-insensitive. The possibility is thus open that, under specific conditions and/or differences in molecular composition, the properties of the MCU may vary and thus encompass all the reported properties of this influx pathway.

2.2. Na^+-dependent Ca^{2+} efflux (Na^+–Ca^+ exchanger)

Na-dependent Ca efflux is characteristic of excitable cells, but a small component can also be detected in non-excitable cells. Recent observations indicate that the mitochondrial Na^+–Ca^+ exchanger (mNCX) plays and important role in the control of $[Ca^{2+}]_c$ in non-excitable cells, since the mNCX inhibitor CGP37157 blunts oscillations induced by G-protein coupled agonists [14]. Located in the inner mitochondrial membrane, the mNCX mediates the efflux of Ca^{2+} from the mitochondria coupled to the influx of Na^+, with a stoichiometry of $3Na^+$:$2Ca^{2+}$ [8,9]. This implies that the exchanger is electrogenic because it transports an excess of positive charge into the matrix; in respiring mitochondria, the relative membrane depolarization caused

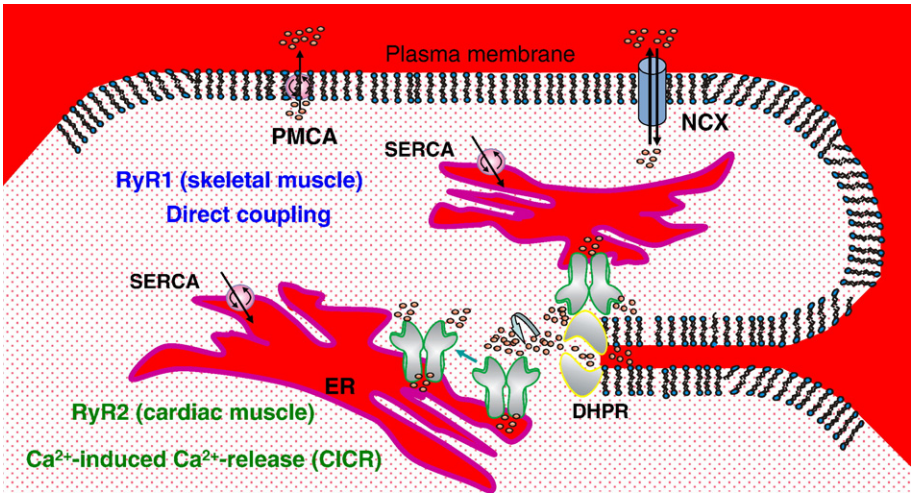

Fig. 1. Mechanisms of Ca^{2+} release from intracellular stores in cardiac and skeletal muscle. Schematic representation of the main Ca^{2+} release channel of the sarcoplasmic reticulum, the ryanodine receptor (RYR) and of the different mechanisms of activation of skeletal RYR1 and cardiac RYR2 by the L-type VOC/dihydropyridine receptor (DHPR). The main players in Ca^{2+} homeostasis are also represented. PMCA, plasma membrane Ca^{2+} ATPase, NCX, Na^+/Ca^{2+} exchanger, SERCA, sarco-endoplasmic reticulum ATPase. Red indicates high $[Ca^{2+}]$.

by the activity of the exchanger is compensated by proton pumping through the respiratory chain. As for most ion transporters of the inner mitochondrial membrane, the molecular identity of the mNCX is still uncertain but it has been proposed that the mitochondrial transporter could be identical to the plasma membrane Na^+–Ca^+ exchanger, i.e. represent an alternative localization of the protein [15]. Previous observations however, pointed to a different molecular identity of mitochondrial and plasma membrane NCX, based on pharmacological evidence in lipid bilayers and lack of common immunoreactive bands in mitochondrial and plasma membrane fractions [16].

2.3. Na^+-independent Ca^{2+} efflux (H^+–Ca^{2+} exchanger)

This is the main Ca^+ efflux system in non-excitable cells and since no specific cations have been found to be exchanged with Ca^{2+} it is believed to be a Ca^{2+}–H^+ exchanger [9]. This transport mechanism requires transmembrane potential, since it is not observed in non-energized mitochondria, which indicates that it is not an electro-neutral passive $1Ca^{2+}$–$2H^+$ exchanger [17]. Indeed, this system is able to extrude Ca^{2+} against a gradient that is much higher than predicted from thermodynamics for an electroneutral H^+/Ca^{2+} exchanger, which indicates that it uses a component of the electrochemical gradient for its activity. A characteristic of this transporter is that it saturates at low calcium loads and its kinetics are extremely slow [18].

This emphasizes a feature of the mitochondrial Ca^{2+} machinery, i.e. that it is equipped with high V_{max} uptake transport systems coupled to slow and easily saturable release systems, increasing the risk of Ca^{2+} overload.

2.4. Permeability transition pore (PTP)

The mitochondrial Ca^{2+}-induced permeability transition is caused by the opening of a large pore in the mitochondrial inner membrane named permeability transition pore (PTP). PTP opening can be induced by intramitochondrial Ca^{2+}, while ATP, ADP, Mg^{2+} and cyclosporin A inhibit it. The identity of the PTP channel components remains elusive but several lines of evidence indicate a role for cyclophilin D and the adenine nucleotide translocase [19,20]. The physiological role of mitochondrial Ca^{2+} induced permeability is still unclear; indeed, opening of a large pore in the inner mitochondrial membrane would allow maximal Ca^{2+} flux, due to the collapse of the membrane potential and guarantee fast Ca^{2+} release even for very small $[Ca^{2+}]$ gradients. The PTP has also been proposed to represent a way of clearing the mitochondrial matrix of damaged or unneeded molecules; permeability transition could also provide an important pathway for inducing apoptosis or for removing damaged mitochondria [18].

3. Mitochondrial Ca^{2+} dynamics in living cells

The thermodynamic and kinetic features of Ca^{2+} transport predict that mitochondrial Ca^{2+} uptake should not be significant under physiological condition. Indeed, the uniporter has a very low affinity for Ca^{2+}, since at physiological Mg^{2+} concentrations the Kd is >10 μM, i.e. higher than $[Ca^{2+}]_c$ measured even after stimulation. This implies that mitochondrial Ca^{2+} uptake should be negligible, both at rest and during the transient increases of $[Ca^{2+}]_c$ to ~1–2 μM that occur under physiological conditions. Thus, it was generally assumed that mitochondria, although capable of accumulating Ca^{2+}, would not significantly participate to Ca^{2+} dynamics under physiological conditions, but rather act as a low affinity, fixed buffer in case of Ca^{2+} overload, such as that occurring in a variety of pathological conditions. Conversely, a novel and prominent role of mitochondria in Ca^{2+} homeostasis has emerged when $[Ca^{2+}]_m$ was measured directly in living cells using the Ca^{2+}-sensitive photoprotein aequorin specifically targeted to the mitochondria by recombinant DNA technology [21,22]. Indeed, mitochondria respond rapidly to physiological increases in $[Ca^{2+}]_c$ and stimulation with G_q-coupled receptor agonists, which induce IP_3 production and consequent release of Ca^{2+} from ER, causes a rapid rise in $[Ca^{2+}]_m$ (Fig. 2) [23]. This effect has been detected in many cells types: HeLa cells, fibroblasts, endothelial and epithelial cells, cardiac and skeletal muscle cells, neurons and pancreatic β cells [24,25].

From the mechanistic point of view, however, it was still difficult to reconcile the rapid mitochondrial Ca^{2+} uptake observed with the low affinity of the Ca^{2+} transport systems. The high sensitivity of mitochondria to the release of Ca^{2+} from the ER led to the hypothesis that close contacts between mitochondria and the ER could be responsible for generating domains of high $[Ca^{2+}]$ sensed by neighbouring mitochondria. We have provided a structural basis for this hypothesis, by showing that mitochondria and ER form an interconnected network in living cells, with a restricted number of close contacts (<80 nm apart) [26]. The existence of tight structural relationships between the ER and the mitochondria does strengthen the hypothesis that the efficiency of Ca^{2+} accumulation by the latter organelles in vivo depends on their capacity to sense the

microdomains of high $[Ca^{2+}]$ generated at the mouth of the IP3 gated channels, but does not prove it. The existence of these microdomains on the outer surface of the inner mitochondrial membrane has been demonstrated using an aequorin strategically targeted to the mitochondrial intermembrane space. Indeed, the $[Ca^{2+}]$ detected by this aequorin is consistently higher than those measured in the bulk cytosol [27].

Ca^{2+} uptake into the mitochondria is also controlled by a number of cellular signals that cooperate in the fine tuning of amplitude and kinetics of the mitochondrial Ca^{2+} responses. It has been shown that Ca^{2+} uptake *in vivo* can be modulated by protein kinases, such as protein kinase C [27] or p38 MAP kinases [28]. The regulatory effects of the cross talk between mitochondrial Ca^{2+} and kinases can be exerted i) by modulating the activity of the mitochondrial Ca^{2+} transport systems [29]; ii) through regulation of Ca^{2+} release from the ER [30,31] and by grading the co-localization of ER and mitochondria [32].

4. Mitochondrial Ca^{2+} and cell metabolism

Although it is correct to state that ATP synthesis is the prime function of mitochondria, this process is certainly one of many in which mitochondria play an active role. Fundamental biosynthetic reactions occur in the mitochondrial matrix which lead to the synthesis of heme, urea, nucleotide precursors and fatty acids. In addition, mitochondria are at the same time players of calcium homeostasis and downstream effectors that directly link calcium transients to variations in energy metabolism.

Seminal work by Denton, McCormack and Hansford demonstrated that Ca^{2+} directly controls oxidative metabolism through allosteric regulation of isocitrate dehydrogenase and oxoglutarate dehydrogenase, and through activation of a Ca^{2+}-dependent phosphatase, which converts pyruvate dehydrogenase into an active form [33,34]. Stimuli which induce a $[Ca^{2+}]_m$ rise thus cause an increase in the activity of the Krebs cycle. The effects last longer than the $[Ca^{2+}]_m$ transient itself, as demonstrated by the prolonged rise of NADH levels observed after an oscillatory $[Ca^{2+}]_c$ signal [35]. This allows the cell to decode a transient signal (i.e. the cytsolic Ca^{2+} spike and the following $[Ca^{2+}]_m$ increase) into a long-term activation of mitochondrial metabolism, through a prolonged activation of dehydrogenases. The final product of the Krebs cycle, NADH, is utilized by the mitochondrial respiratory chain for energizing the translocation of H^+ and thus the generation of the electrochemical gradient utilized for producing ATP. To confirm the relationship between $[Ca^{2+}]_m$ rise and ATP production, we constructed an ATP-sensitive probe, based on the luminescent protein luciferase, and specifically directed to the mitochondria by fusion to a mitochondrial targeting sequence. This probe allows the dynamic monitoring of mitochondrial ATP concentration by measuring the variation in the luciferase light emission following Ca^+-signals induced by an agonist. Our results show that mitochondrial ATP levels increase following stimulation with agonists coupled to Ca^{2+} transients; on the other hand, when Ca^{2+} transients are dampened by depleting Ca^{2+} from the ER or by chelating it in the cytoplasm, the ATP rise is proportionally reduced [36]. Interestingly, it has recently been shown that additional Ca^{2+}-dependent metabolic checkpoints are operative in mitochondria. The aspartate/glutamate metabolite carriers (AGCs) were shown to be activated by Ca^{2+} and AGCs enhanced ATP production upon cell stimulation when expressed in cells in a wild type form [37].

In summary, $[Ca^{2+}]_m$ is a fundamental segment of the signalling route that receives and integrates environmental stimuli and endogenous requests, namely the effector arm that translates them into an appropriate energetic and biosynthetic output. Different cells facing the challenge of diverse functional demands exploit the mitochondrial Ca^+ signalling machinery in the appropriate mode of operation.

5. Calcium signal and mitochondria in cardiac cells

The specific metabolic demand of the heart is very extreme and at every beat the heart needs to precisely match workload and energy production. It has been estimated that every heartbeat uses up approximately 2% of cellular ATP [38] and the mitochondrial oxidative metabolism has to be finely synchronized in time and intensity to the contractile activity of the cardiomyocytes.

During excitation–contraction coupling in cardiac cells, voltage-gated Ca^{2+} entry through sarcolemmal L-type Ca^{2+} channels is amplified by additional Ca^{2+} release from the sarcoplasmic reticulum (SR). The resulting rise in $[Ca^{2+}]_c$ acts on the troponin complex causing the sliding of the acto-myosin contractile apparatus.

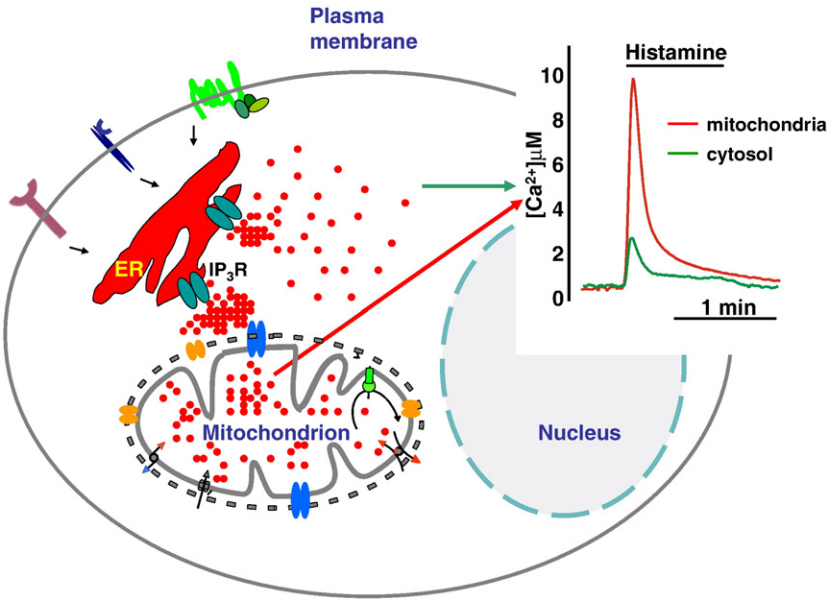

Fig. 2. Microdomains of high $[Ca^{2+}]_c$ close to the mouth of the Ca^{2+} release channels are sensed by mitochondria. Schematic representation of ER–mitochondria contact sites and of their functional consequences. Ca^{2+} transients following IP3 receptor activation yield $[Ca^{2+}]_m$ increases which are higher than those measured in the bulk cytosol. A similar scenario can be envisaged for RYRs.

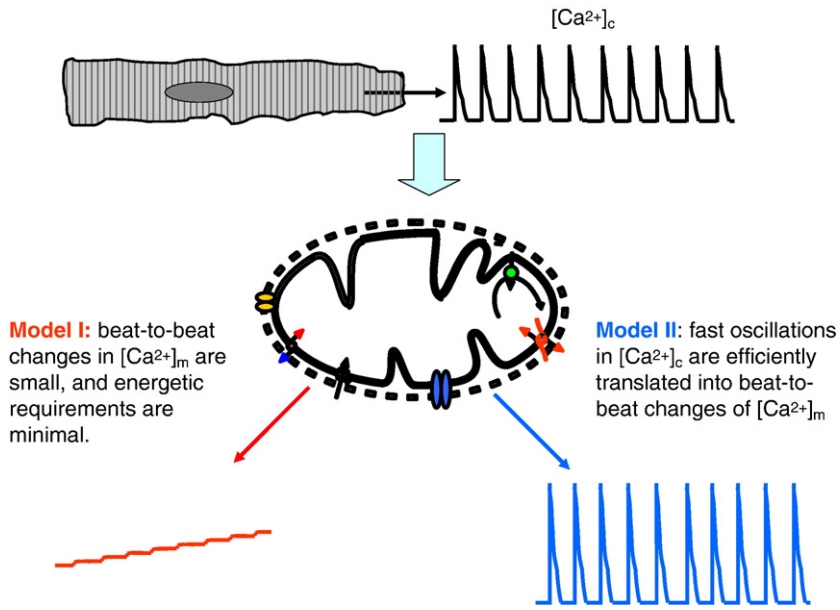

Fig. 3. Functional hypotheses for the transduction of periodic cytosolic Ca^{2+} transients into changes of $[Ca^{2+}]_m$. Data obtained in beating neonatal cardiomyocytes in culture show large beat-to-beat increases in $[Ca^{2+}]_m$, thus favoring hypothesis II.

Relaxation is brought about by Ca^{2+} reuptake into the intracellular stores catalyzed by SERCA pumps and Ca^{2+} extrusion by sarcolemmal Na^+/Ca^{2+} exchange. Thus, cardiac myocytes display periodic $[Ca^{2+}]_c$ transients, which reach peak concentrations of 1 to 2 μM within 50 ms and decline subsequently within less than 500 ms [39].

The principle of mitochondrial Ca^{2+} handling in the heart and the mechanism of metabolic coupling remain controversial. Indeed, there is no doubt that in cardiomyocytes, as in virtually all cells, mitochondria accumulate Ca^{2+} ions during $[Ca^{2+}]_c$ elevations [40]. However, an open issue remains as to whether the fast $[Ca^{2+}]_c$ transients underlying excitation–contraction coupling in beating cardiomyocytes are rapidly transmitted into the mitochondrial matrix compartment or, rather, slowly integrated by the mitochondrial Ca^{2+} transporters. This controversy has essentially two causes: i) the low affinity of the mitochondrial Ca^{2+} uptake mechanism is difficult to reconcile with the relatively small amplitude of $[Ca^{2+}]_c$ changes occurring in physiological conditions [41]. It has been demonstrated, however, that in many cells, including cardiomyocytes, the mitochondria are exposed to micro-domains of very high $[Ca^{2+}]_c$ close to the mouth of Ca^{2+} release channels. As a consequence, comparably modest average changes in $[Ca^{2+}]_c$ elicit large transients in $[Ca^{2+}]_m$. In the case of cardiac cells stimulated with caffeine, it has been shown that mitochondria sense microdomains of high $[Ca^{2+}]_c$ even in the presence of elevated concentations of a cytosolic Ca^{2+} buffer [42-45]. ii) The uniport/antiport system was thought to be too slow to respond in the 150–200 ms range that characterizes $[Ca^{2+}]_c$ changes [46]. As mentioned before, the Rapid Mode of Ca^{2+} uptake (RaM), has a relative slow "resetting time" after a Ca^{2+} pulse [47]. Therefore, the question whether $[Ca^{2+}]_m$ can respond to the rapid $[Ca^{2+}]_c$ changes occurring during bouts of contraction remained long unsolved [48,49].

Two different theories were developed on how mitochondria decode rapid cardiac $[Ca^{2+}]_c$ transients (Fig. 3). Model I, proposed by Crompton [50], states that Ca^{2+} influx into the mitochondria is a slow process coupled to an even slower release of accumulated Ca^{2+} ions. $[Ca^{2+}]_c$ oscillations are thus integrated by the Ca^{2+} transport machinery of the inner mitochondrial membrane in such a way that increasing the frequency or the amplitude of the $[Ca^{2+}]_c$ spikes yields a Ca^{2+} influx into the mitochondrial matrix which reaches a new steady state when Ca^{2+} uptake during a single cycle equals Ca^{2+} efflux. Consequently, beat-to-beat changes in $[Ca^{2+}]_m$ are small, and energetic requirements of integrated mitochondrial Ca^{2+} transport are minimal. Model II is largely different, in that it predicts that fast oscillations in $[Ca^{2+}]_c$ result in beat-to-beat changes of $[Ca^{2+}]_m$. A prerequisite for this model to work is that uptake and efflux of Ca^{2+} are extremely fast and Ca^{2+} uptake is large enough to overcome the buffering capacity of cytosol and mitochondria.

The major obstacle to reaching a definitive conclusion depended essentially on the methodologies that were available for a long time to measure $[Ca^{2+}]_m$. Most studies were performed with fluorescent indicators loaded into cells and within mitochondria via their membrane-permeable AM esters [51–55. The partition of the dyes between cytosol and mitochondria varies considerably for the different indicators and they are invariably also present in the cytosol; their selective removal from the cytosolic compartment is technically difficult. In addition, the dyes accumulate in organelles other than mitochondria, confusing the interpretation of the fluorescence changes.

The issue of whether the rapid cytosolic Ca^{2+} changes are transmitted as slow tonic increases or conversely trigger repetitive synchronous oscillations of $[Ca^{2+}]_m$ was clarified using a novel methodological approach, targeted recombinant Ca^{2+} probes (i.e. Ca^{2+}-sensitive photoprotein aequorin and green fluorescent protein-based Ca^{2+} indicator pericam), specifically directed to the mitochondria of spontaneously contracting neonatal cardiomyocytes in culture [43]. The main conclusions of this work were that in cardiac cells $[Ca^{2+}]_m$ oscillates synchronously with $[Ca^{2+}]_c$ and that modulation of the amplitude and frequency of $[Ca^{2+}]_c$ changes also influences mitochondrial Ca^{2+} dynamics and metabolism. As elucidated in great detail in other cell systems (for a review, see [56]), the existence of domains of close contact between ER and mitochondria may provide also in this highly specialized cell system, the structural basis for rapid coupling between $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, specifically the transfer of the signal beat-to-beat.

6. Mitochondrial Ca^{2+} signal as message of death

Substantial evidence has built up in recent years indicating that metabolic regulation is only one of the roles of the mitochondrial Ca^{2+} signal. In particular, the role of mitochondrial Ca^{2+} in the control of cell death is now well established [57,58]. Early and critical events in apoptosis occur in mitochondria and in the ER, and the release of elements acting as caspase cofactors, such as cytochrome c (from

mitochondria) and Ca^{2+} (from the ER) into the cytosol, are requisites for cell death in many cases [59,60].

The mitochondrial pathway of apoptosis is regulated by members of the Bcl-2 protein family, subdivided into two groups, endowed with anti-apoptotic (Bcl-2) and pro-apoptotic (Bax, Bak) properties respectively. The link between Bcl-2 (localized in several intracellular membranes, including mitochondria and ER) and Ca^{2+} homeostasis has been established by showing that Bcl-2 reduces the steady state Ca^{2+} levels in the ER thereby dampening the apoptotic signal [61,62]. The reduction of $[Ca^{2+}]$ within Ca^{2+} stores has protective effects toward a variety of inducers of cell death, such as ceramide and oxidative stress.

This model of Ca^{2+}-dependence of apoptosis was subsequently supported by a series of observations with the pro-apoptotic Bcl-2 family members Bax and Bak. Cells deriving from knockout mice lacking Bax and Bak, that are very resistant to apoptotic death, 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 [63]. Conversely, early after overexpression of Bax, the $[Ca^{2+}]$ levels of the ER are higher than in controls and the cells are more susceptible to apoptotic treatments [64].

Taken together, these results indicate that Bcl-2 influences cellular Ca^{2+} signals, most likely by acting on the IP3 receptor (reviewed in [65]) and thus modifying Ca^{2+} leakage under resting conditions (and accordingly the steady state $[Ca^{2+}]_{er}$) and the kinetics of release upon stimulation. This, in turn, affects the efficacy of apoptotic challenges by influencing the sensitivity of Ca^{2+} effectors, such as mitochondria.

Ca^{2+} has been shown to act as an inter-organellar messenger which coordinates the activities of mitochondria and ER during apoptosis. Apoptotic stimuli induce a permeability transition in the mitochondrial membrane triggering the release of cytochrome c from a subset of mitochondria. Cytochrome c then binds the IP3 receptor and increases its conductance, causing a stronger Ca^{2+} transient and massive release of cytochrome C from the bulk of mitochondria [66]. As a consequence, apoptosome formation and caspase activation take place, leading to cell death.

Interestingly, the link between Ca^{2+} signalling and cell death has been reinforced by the study of a pro-apoptotic protein not related to the Bcl-2 family, the x-protein of the hepatitis B virus (HBx), which also conceptually extended the molecular mechanisms through which the Ca^{2+} effect can be tuned by modulators of apoptosis. In this case, when cells were transfected with HBx, a marked enhancement of the cytosolic Ca^{2+} responses evoked by cell stimulation and an increase in spontaneous apoptosis was detected. This effect is opposite to that of of Bcl-2 and is not due to an alteration in ER Ca^{2+} handling, since both the steady state levels and the release kinetics are the same in HBx-transfected and control cells. Indeed, the cause for the enhanced Ca^{2+} responses observed with HBx resides in the caspase-dependent cleavage of PMCA, the most effective molecular route for rapidly returning $[Ca^{2+}]_c$ to basal values [67].

7. Mitochondria and cardiac apoptosis

A decrease in the contractile mass of the heart ultimately leads to heart failure. Understanding the molecular mechanisms of cardiomyocyte loss and the subsequent cellular adaptations of the failing myocardium is thus of pivotal importance for target discovery and drug design as well as to identify prognostic factors in heart disease [68]. Cardiomyocytes have long been thought to die only by necrosis, undergoing a bioenergetic crisis due to impaired mitochondrial function and, ultimately, an energy-independent lysis, which causes massive release of intracellular content into the surrounding tissue. This, in turn, elicits an inflammatory response and eventually collagen deposition leading to fibrosis, which further impairs contractility of the organ. Recent results, however, have challenged this paradigm and it has become evident that apoptosis significantly contributes not only to heart development [69] but also to cardiomyocyte loss and pathologic remodeling in adult heart [70,71].

Mitochondria play a fundamental role in cardiomyocyte cell death and are directly involved in the control of both necrosis and apoptosis. How mitochondria can orient the cell death process towards necrosis or apoptosis is however an open issue in this field of research [72]. The mitochondrial permeability transition pore is thought to be an important checkpoint in cell death [73]. Opening of the PTP leads to increased proton permeability, which dissipates the two components of the proton motive force, the pH gradient and the membrane potential. This is a potentially lethal event because it also triggers a vicious circle, since oxidative phosphorylation is not only inhibited under these conditions but also reversed, and it starts hydrolyzing ATP. Given the elevated energetic requirement of myocytes, PTP opening in a significant percentage of mitochondria sets an irreversibility threshold towards cell death. The importance of the PTP in heart disease has been recently highlighted using knockout mice for cyclophilin D, a regulator of the PTP. These mice are protected from heart failure induced by Ca^{2+} overload though transgenic overexpression of a sarcolemmal calcium channel combined with stimulation of beta-adrenergic receptors [74]. Bcl-2 overexpression does not exert a protective effect in this model system, indicating that this is a *bona fide* necrotic cell death [75].

In other cases, however, a causal connection between PTP opening and triggering of apoptosis was established. An inhibitor of the Na^+–H^+ exchanger-1 (NHE-1), an important regulator of cellular pH, has been shown to have a protective effect on the myocardium after ischemia/reperfusion (I/R). The protective effects on myocyte apoptosis *in vivo* (AIF and cytochrome c release) correlate with the ability of the inhibitor to reduce PTP opening *in vitro*, thus suggesting a causal relationship between PTP opening and apoptosis in cardiac cells [76]. This, however, has not been confirmed in intact cells to date.

One could envisage that opening of the pore can be transient and induce primarily a swelling of the matrix compartment due to water influx following the collapse of ionic gradients. An important consequence of this event is mitochondrial outer membrane permeability (MOMP), which leads to the release of proteins normally located in the intermembrane space. Key players of apoptosis, such as cytochrome c, and apoptosis-inducing factor (AIF) are then released in the cytosol where they promote caspase activation. AIF is extremely important in cardiomyocytes, where it regulates the activity of mitochondrial complex I [77] and it has been observed that harlequin mutant mice, which express a reduced amount of AIF, are more sensitive to cell damage induced by I/R. Indeed, it has been shown that AIF is a mitochondrial free radical scavenger in the heart and that cardiomyocytes from harlequin mice are defective in the catabolism of H_2O_2 [78]. AIF is found in the cytosol and nuclei of cardiomyocytes after I/R and is involved in their apoptotic cell death [79].

The importance of apoptosis in cardiomyocytes is confirmed by the prominent role of Bcl-2 family proteins in cardiac cell survival [80]. Transgenic mice with cardiac-specific overexpression of Bcl-2 are less sensitive to I/R and display a significant degree of protection, detectable both at the cellular level and in terms of recovery of cardiac performance. Bcl-2 protects cardiac cells through different parallel mechanisms. First, it counteracts the action of the Bax and Bak on outer membrane permeability. Second, Bcl-2 decreases the open probability of the PTP by increasing the Ca^{2+} threshold, an important permissive factor sensitizing the PTP to a variety of inducing factors.

This notion is also reinforced by the analysis of the pro-apoptotic Bcl-2 family members in cardiac cells. Treatments that induce mitochondrial dysfunction and cell death induce an increased expression of Bax and a concomitant reduction in Bcl-2 indicating that the fate of cardiomyocytes is also controlled by the ratio of pro- and anti-apoptotic Bcl-2 family members [81]. Indeed, Bax-deficient mice are resistant to I/R damage [82] and Bax is therefore a potential target for cardioprotective drug design.

Taken together, these data implicate a complex signalling network at the crossroad of energy metabolism and survival. The molecular determinants of the protean role of Ca^{2+} in cardiomyocyte life and death await further mechanistic definition.

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