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Review

The versatility of mitochondrial calcium signals: From stimulation of cell metabolism to induction of cell death

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ABSTRACT

Both the contribution of mitochondria to intracellular calcium (Ca^{2+}) signalling and the role of mitochondrial Ca^{2+} uptake in shaping the cytoplasmic response and controlling mitochondrial function are areas of intense investigation. These studies rely on the appropriate use of emerging techniques coupled with judicious data interpretation to a large extent. The development of targeted probes based on the molecular engineering of luminescent proteins has allowed the specific measurement of Ca^{2+} concentration ($[\text{Ca}^{2+}]$) and adenosine triphosphate concentration ($[\text{ATP}]$) in intracellular organelles or cytoplasmic subdomains. This approach has given novel information on different aspects of mitochondrial homeostasis.

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1. Introduction

Virtually in all eukaryotic cells the dynamic regulation of cytosolic calcium concentration ($[\text{Ca}^{2+}]_c$) is fundamental for cell life and for controlling extremely diverse functions such as muscle contraction, hormone secretion, neuronal circuits, immune responses and gene expression [1,2]. The use of Ca^{2+} as a second messenger rests on the maintenance of a low cytosolic Ca^{2+} concentration, through the energy-consuming pumping activity of Ca^{2+} ATPases located in endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) (SERCA) or plasma membrane (PMCA). As to the triggering mechanism of the $[\text{Ca}^{2+}]_c$ rise, the route involves either a stimulation acting through G protein mediated activation of phospholipase C and consequent generation of inositol 1,4,5-trisphosphate (IP₃) from the hydrolysis of the lipid phosphatidyl-inositol 4,5-diphosphate or growth factors receptors (also causing the production of IP₃ through the activation of phospholipase C) [2]. An alternative route for raising $[\text{Ca}^{2+}]_c$ depends on the opening of various classes of plasma membrane Ca^{2+} channels [3]. The concerted action of channels with distinct spatial distribution and kinetics of opening determines a high spatio-temporal specificity of the signals elicited by different agonists, which in turn are decoded into radically different intracellular effects.

A broad repertoire of cytosolic Ca^{2+} effectors (i.e. enzymes, channels or structural proteins) modify their activity upon binding of Ca^{2+} . However, there are processes occurring within intracellular organelles (gene transcription, post-translational modification of

proteins and aerobic metabolism) that are also modulated by $[\text{Ca}^{2+}]$ changes [4,5].

Regarding mitochondria, it was possible to demonstrate that, upon physiological stimulation of cells, Ca^{2+} is rapidly accumulated in the matrix. Here, we will discuss the basic characteristics of this process and its role in modulating physiological and pathological events such as the regulation of aerobic metabolism and the induction of cell death.

The present article is focused on some aspects of Ca^{2+} signalling related to metabolic stimulation and apoptosis. It stems from the authors' own work on the topic. We apologize in advance for the bias and the omissions. Please refer to more extended reviews for the aspects that are overlooked.

2. Mitochondrial Ca^{2+} homeostasis

Mitochondria are delimited by two membranes. The outer mitochondrial membrane (OMM) is permeable to ions and small proteins (MW < 10 kDa) due to the abundance of a large conductance channel, known as mitochondrial porin or voltage-dependent anion channel (VDAC). It should be noted, however, that the channel appears to be gated *in vivo*, and permeability is controlled by ATP and other regulatory factors [6]. For these reasons, Ca^{2+} diffusion through the OMM was traditionally considered not to be a limiting factor in mitochondrial Ca^{2+} uptake. Recent data showed that the availability and selective placement of VDAC channels at ER/mitochondria contact sites facilitate mitochondrial Ca^{2+} accumulation, in keeping with the idea that the latter process requires the fast and efficient transfer of Ca^{2+} microdomains from the mouth of the Ca^{2+} channels located in neighbouring ER or plasma membranes to the transporters of the ion-impermeant inner membrane (IMM) [7,8]. The IMM is an ion-imper-

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meable membrane, with a much larger extension of the OMM (and consequent formation of foldings into the internal space, known as cristae [9]). The activity of respiratory chain complexes allows the translocation of H^+ in the space between the two membranes, which consequently generates an electrochemical gradient ($\Delta\mu H$) composed of a chemical (ΔpH) and electrical ($\Delta\psi H$) component. In mitochondria, most of the $\Delta\mu H$ established by the respiratory chain is supposed to be in the form of $\Delta\psi H$ (~ 180 mV), which provides a huge driving force for Ca^{2+} entry into the organelle. Indeed, collapse of the $\Delta\psi H$ by protonophores, such as *p*-[trifluoromethoxyl]-phenyl-hydrazine (FCCP), abolishes mitochondrial Ca^{2+} uptake.

Mitochondria maintain an electrical gradient ($\Delta\psi_m$) across their IMM virtually in all cells, independently of whether the cell performs aerobic or glycolytic metabolism [10] providing a huge driving force for accumulation of cations in the mitochondrial matrix.

Calcium mitochondrial traffic takes place essentially through two pathways: i) an electrophoretic uniporter, allowing the accumulation of Ca^{2+} down its electrochemical gradient, and activating in a cooperative manner by external $[Ca^{2+}]$ and ii) Ca^{2+} efflux through the Na^+/Ca^{2+} (mNCX) and H^+/Ca^{2+} (mHCX) exchangers that prevent the attainment of an electrochemical equilibrium (Fig. 1) [11]. Unfortunately, although the biochemical properties of these transport pathways have been known for over three decades, there is still limited knowledge at the molecular mechanisms regarding the regulation of this system and the actual protein(s) involved in this process are unknown. However in a recent study, Trenker and co-workers demonstrated that the uncoupling proteins 2 and 3 (UCP2 and UCP3) are essential for mitochondrial Ca^{2+} uptake. Using overexpression, knockdown (small interfering RNA) and mutagenesis experiments, they demonstrate that UCP2 and UCP3 are elementary for mitochondrial Ca^{2+} sequestration in response to cell stimulation under physiological conditions expanding our knowledge of the physiological role for mitochondrial Ca^{2+} sequestration [12].

3. Mitochondrial calcium measurements using aequorin

Aequorin is a Ca^{2+} -sensitive photoprotein naturally present in the medusa *Aequorea victoria* (Fig. 2A). Aequorin, as produced by various *Aequorea* species, includes an apoprotein and a covalently bound prosthetic group (coelenterazine). The Ca^{2+} -dependent luminous reaction requires both the protein and the prosthetic group (Fig. 2). Since recombinant expression yields only the polypeptide, the prosthetic group must be added exogenously. This process (the “reconstitution” of the active protein) is a critical step in the use of the photoprotein. It requires diffusion of the prosthetic group across the cell membrane and incorporation in the recombinant polypeptide. Both events occur quite easily in a wide variety of cell types, albeit relatively slowly. Thus, it is sufficient to add coelenterazine to the culture medium of the cells provided that aequorin is exposed to low Ca^{2+} . In this way, consumption is limited and enough active photoprotein is formed in 1–2 h to carry out the experiment [13].

When Ca^{2+} ions bind to three high-affinity sites (EF-hand type), aequorin undergoes an irreversible reaction, in which a photon is emitted. For $[Ca^{2+}]$ between 10^{-7} and 10^{-5} M, there is a relationship between the fractional rate of consumption (i.e. L/L_{max} , where L_{max} is the maximal rate of discharge at saturating $[Ca^{2+}]$) and $[Ca^{2+}]$. Due to the cooperativity between the three binding sites, light emission is proportional to the 2nd–3rd power of $[Ca^{2+}]$; this property on one hand accounts for the excellent signal to noise ratio of aequorin but on the other hand may significantly affect the measurements. Given that the probe (different from fluorescent indicators) is gradually consumed throughout the experiment, the signal tends to decrease, and the conversion into Ca^{2+} concentration is obtained only at the end of the experiment when the total aequorin content is estimated and L/L_{max} can be back-calculated for each data point.

In general, targeted aequorins [14] have proved to be extremely valuable, and allowed many new data and novel concepts in Ca^{2+} signalling to be obtained (Fig. 2B). The most important ones not covered in this review are the estimates of ER $[Ca^{2+}]$ in the near-

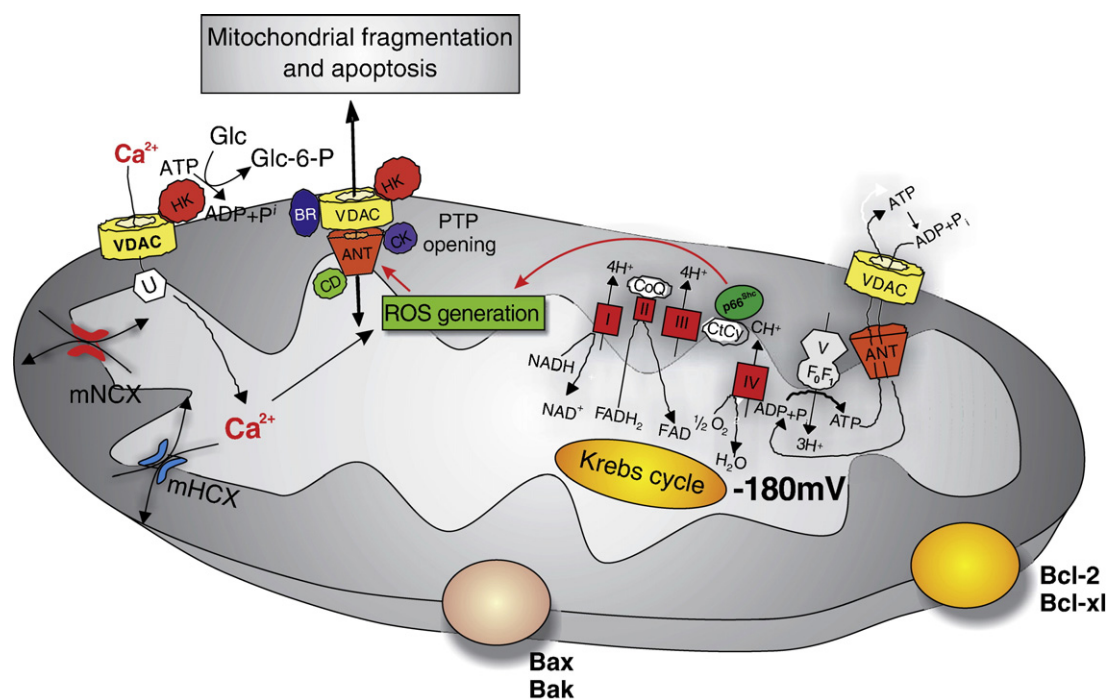


Fig. 1. Schematic representation of mitochondrial calcium homeostasis. Ca^{2+} entry takes place via a low affinity uniporter (U), due to the high electronegative potential (~ 180 mV) in the mitochondrial matrix. Extrusion of Ca^{2+} takes place through an electro neutral antiporter (in exchange with either Na^+ , mNCX or H^+ , mHCX). In the matrix, Ca^{2+} stimulates the activity of three Ca^{2+} -sensitive dehydrogenases of the Krebs cycle (NAD⁺-isocitrate-, 2-oxoglutarate-, and pyruvate-dehydrogenase) thus promoting electron flow through the electron transport chain. VDAC: voltage-dependent anion channel, ANT: adenosine nucleoside transporter, HK: hexokinase, CD: cyclophilin D, CK: creatine kinase, BR: Benzodiazepine Receptor.

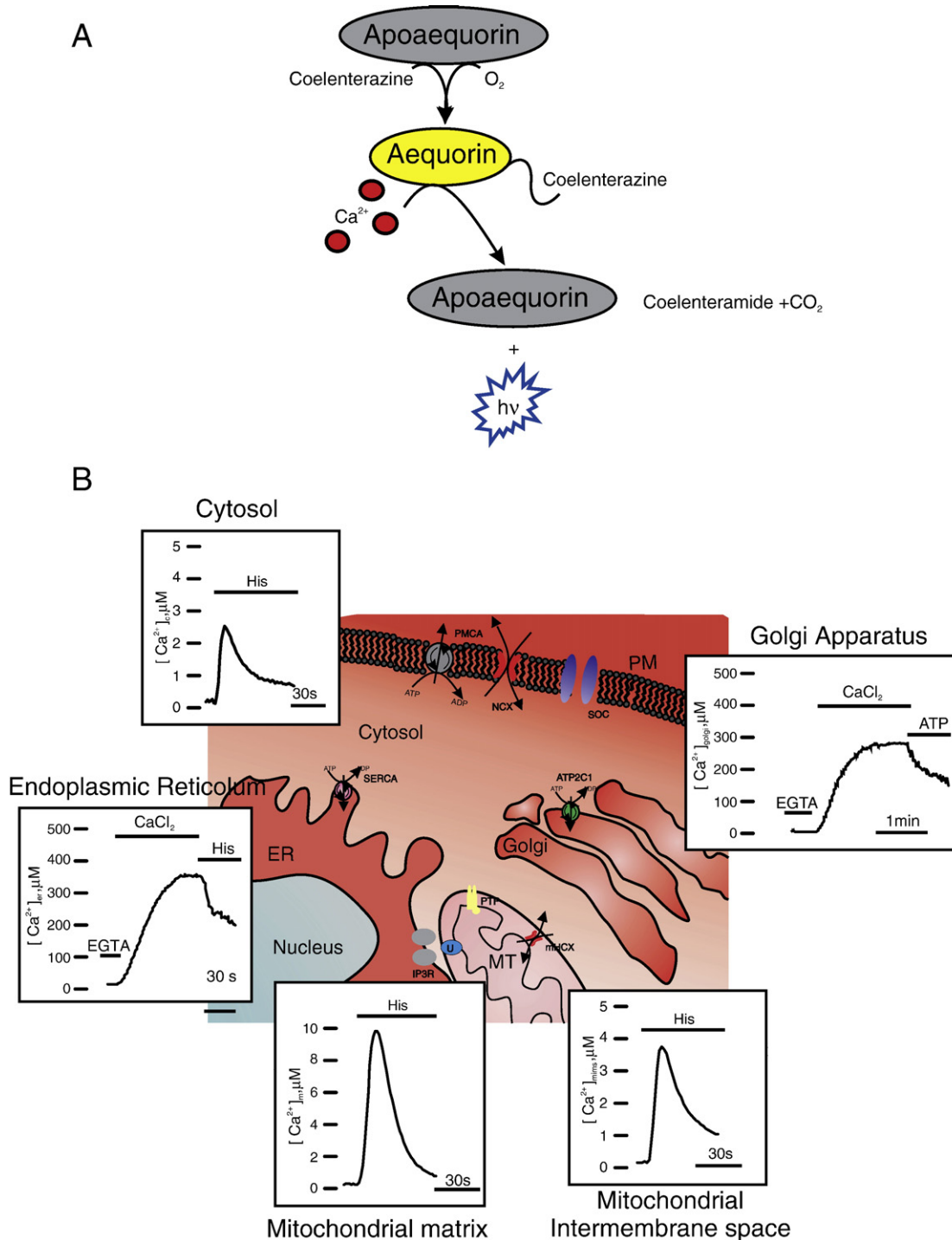


Fig. 2. (A) Schematic model of the irreversible reaction of aequorin. When Ca²⁺ ions bind to the EF-hand binding sites of the reconstituted aequorin (the prosthetic group, coelenterazine, is indicated) a photon is emitted and aequorin is irreversibly discharged. (B) Scheme of an idealised mammalian cell with the localization of the main players of Ca²⁺ homeostasis. Abbreviations: PM: plasma membrane; NCX, Na⁺/Ca²⁺ exchanger; PMCA, plasma membrane Ca²⁺ ATPase; ATP2C1, Golgi-resident Ca²⁺ ATPase; SERCA, sarco/endoplasmic reticulum Ca²⁺ ATPase; IP3R, IP3 receptor; mt, mitochondrion; ER, endoplasmic reticulum. In the insets the [Ca²⁺] measurements in different cellular compartments during agonist stimulation are represented.

millimolar range (~0.5 mM) [15], the role of the agonist-sensitive Ca²⁺ store played by the Golgi apparatus (endowed with a resting [Ca²⁺] of ~0.3 mM and rapidly emptying after agonist stimulation) [16], the rapid equilibration of cytosolic and nuclear [Ca²⁺] [17], and the estimates of resting and stimulated [Ca²⁺]_c under the plasma membrane, which is well above those of the bulk cytosol [18].

The present review focuses on mitochondria, as Ca²⁺ handling by these organelles was not only significantly different from that

expected but also identified them as critical checkpoints, in which radically different effects can be triggered by a rise in [Ca²⁺]_c.

As a result, in this section we will concentrate on the use of aequorin technology for specifically measuring mitochondrial Ca²⁺ levels in living mammalian cells.

Measurement of [Ca²⁺] in mitochondria requires the presence of aequorin in this compartment. The strategy followed to achieve the correct delivery of aequorin to mitochondria takes advantage of the

natural signal peptides present in nuclearly encoded mitochondrial proteins. The vast majority of mitochondrial proteins are encoded in nuclear DNA, synthesized in the cytosol, and imported into the organelle. Their correct targeting is due to a signal peptide, usually present at the N-terminus. Different proteins possess different signal peptides with no consensus sequence, which simply bear some common characteristics regarding their charge distribution [19].

Subunit VIII of human cytochrome *c* oxidase possesses a 25 amino acid signal peptide at its N-terminus, which is cleaved by a matrix protease upon import; it is presumed that the action of the protease is dependent on a motif present within the first few amino acids of the mature protein. We fused the initial 31 amino acids of this subunit, comprising the signal peptide and the first 6 amino acids of the mature protein, to the N-terminus of aequorin. In this way, the correct delivery of functional aequorin to mitochondrial matrix was achieved [20].

For the delivery of aequorin to the mitochondrial intermembrane space (MIMS), we exploited the characteristics of another mitochondrial protein. Glycerol phosphate dehydrogenase (GPD) is an enzyme present in the mitochondrial inner membrane with a C-terminal domain protruding into the MIMS. To target aequorin to this space, we fused the photoprotein to the C-terminal portion of GPD [21].

Cell stimulation with agonist, such as histamine that act on a G protein coupled-receptor, leads to the generation of IP₃, consequent release from the intracellular Ca²⁺ stores (ER and Golgi apparatus), and Ca²⁺ influx from extracellular medium, which causes a biphasic kinetics of [Ca²⁺]_c in HeLa cells as measured in cells transfected with wild-type aequorin (without any targeting sequences and thus cytosolic) as shown in Fig. 2B. The release of Ca²⁺ from the stores causes an initial rapid but transient increase of [Ca²⁺]_c (to values of approximately 2.5 μM), followed by a sustained increase of [Ca²⁺]_c above normal basal levels. This increase in [Ca²⁺]_c is maintained throughout the stimulation period (Fig. 2B).

The measurements of [Ca²⁺]_m obtained upon stimulation with histamine in HeLa cells transiently expressing mtAEQ reveal that, there is a rapid increase in [Ca²⁺]_m up to approximately 10 μM, followed by a return, to almost basal values within 2 min (Fig. 2B). Large [Ca²⁺]_m changes were observed in a variety of cell lines, as well as different primary cultures [22]. To ensure that our [Ca²⁺]_m measurements were indeed due to changes in the mitochondrial matrix, we analyzed the effect of uncouplers on the [Ca²⁺]_m values generated by our chimeric probe. Since Ca²⁺ entry into the mitochondrial matrix is made possible by the electrochemical gradient across the inner mitochondrial membrane, collapse of this gradient with an uncoupler should abolish the changes in [Ca²⁺]_m measured by our probe. Using FCCP that dissipates the proton gradient across the IMM, the mitochondrial Ca²⁺ response was drastically reduced to values which were unable to detect a significant response to the histamine stimulus. Further confirmation that our probe is indeed localized in the mitochondrial matrix and that calcium accumulation occurs via the known uptake mechanism (the Ca²⁺ uniporter) came from experiments with a potent inhibitor, Ruthenium red (RR). Given that cells are not permeable to RR, cells were first permeabilized and calcium uptake was initiated by supplementing the medium with known concentrations of Ca²⁺. Upon challenging with 2 μM free Ca²⁺, [Ca²⁺]_m uptake in presence of RR, was almost completely abolished.

The fact that alterations in [Ca²⁺]_m are much larger than those observed for [Ca²⁺]_c was originally unexpected given the low affinity of the mitochondrial uniporter for this ion. However, such an apparent contradiction could be explained if the ER and mitochondria have a close structural relationship, and mitochondria are thus capable of sensing microdomains of high [Ca²⁺]_c generated in close proximity of ER Ca²⁺ release channels [21].

To analyze the possible existence of such high Ca²⁺ microdomains, we used a different aequorin chimera targeted to the mitochondrial

intermembrane space (mimsAEQ). Since the OMM is freely permeable to ions and small molecules, aequorin molecules present between the two mitochondrial membranes are located in a region that is in rapid equilibrium with the cytosolic portion. Such a chimera is thus sensitive to changes in Ca²⁺ in the cytosolic region immediately adjacent to the mitochondria. HeLa cells transfected with mimsAEQ and stimulated with histamine show a biphasic response, as shown in the specific inset of Fig. 2B. An initial rise in [Ca²⁺]_{mims} to ~ 3.5 μM is followed by a rapid decrease that gradually levels out to values above the initial [Ca²⁺]_c (Fig. 2B). This type of response is due to two mechanisms of action of the agonist, namely the release of Ca²⁺ from intracellular stores and the entry of Ca²⁺ from the extracellular medium [22].

To determine the exact location of mimsAEQ, we performed the following experiments. We had previously determined that the collapse of the proton gradient drastically reduces the accumulation of Ca²⁺ in the matrix. If mimsAEQ were localized within the matrix, the rise in [Ca²⁺]_m should be abolished in the presence of uncouplers. Conversely, if mimsAEQ were localized in the space between the two mitochondrial membranes, the presence of uncouplers should not affect the [Ca²⁺]_m values obtained. HeLa cells transfected with mimsAEQ and treated with FCCP do not show a significant difference in [Ca²⁺]_m dynamics when compared to similar coupled cells, strongly suggesting that the aequorin moiety lies in the MIMS. The comparison of [Ca²⁺]_c and [Ca²⁺]_{mims} responses to histamine shows a clear difference only in the initial phase (which is due to the release of Ca²⁺ from intracellular stores). These data support the hypothesis that the opening of IP₃-sensitive channels in close proximity to mitochondria generates microdomains of high [Ca²⁺]_c. Indeed, in such microdomains the [Ca²⁺]_c is much higher than the average [Ca²⁺]_c and thus the low affinity Ca²⁺ uptake systems present in mitochondria are capable of efficiently accumulating Ca²⁺ in the matrix of the organelle.

Moreover, different mechanisms can fine tune the amplitude and kinetics of the mitochondrial Ca²⁺ responses. For example, the activity of defined PKC isoforms was shown to modulate mitochondrial responses, while leaving global Ca²⁺ signals unaffected. Mitochondria thus emerge as a “sink” of Ca²⁺ released from the ER or entered through plasma membrane channels endowed with unique properties. Indeed, on the one hand they participate in decoding Ca²⁺-linked agonist stimulations (through intramitochondrial and extramitochondrial effects), on the other hand they can vary their response based on the convergence of PKC-mediated (and possibly other) signalling pathways. While future work will address the molecular targets of this regulatory mechanism, these results may already highlight novel pharmacological routes for specifically modifying Ca²⁺-dependent cellular dysfunctions that occur in a variety of genetic and acquired human disorders [23]. Another interesting example is represented by the complex program activated by the transcriptional peroxisome-proliferator-activated-receptor- γ -coactivator-1 α (PGC-1 α), i.e. mitochondrial biogenesis and modification of the protein repertoire of the organelle, that modifies the responsiveness of mitochondria to cellular Ca²⁺ signals, and thus tunes Ca²⁺-dependent organelle functions, such as the sensitivity to apoptotic stimuli. This novel mechanism may represent the basis of some of the recently identified effects of PGC-1 α (e.g. neuro and cardiac protection) and the site of pharmacological intervention. The latter possibility, and the crosstalk with other signalling routes or pathophysiological events (e.g. ROS production) will be the subject of further studies [24].

4. Mitochondrial ATP homeostasis

In most normal aerobic cells, mitochondria are the source of the majority of cellular ATP. Thus, knowledge of intramitochondrial ATP concentration ([ATP]_m) is of central importance to understanding the bioenergetics of the living cell and its regulation by nutrients, hormones, and other stimuli. Of particular importance is the role of

changes in $[Ca^{2+}]_m$ (see above), which are likely to activate mitochondrial oxidative metabolism [25] through the stimulation of mitochondrial dehydrogenases [26]. Conversely, decrease in $[ATP]_m$ may be an important and possible event in programmed cell death (PCD). The inhibition of ATP production has been observed in both type I and type III of programmed cell death.

This phenomenon occurs relatively late in type I PCD (apoptosis) as the complete apoptotic program involves the energy-dependent formation of the apoptosome and hydrolysis of macromolecules, by contrast, type III PCD (necrosis) is characterized by an early loss of ATP synthesis [27,28].

Conversely, decreases in $[ATP]_m$ may be an important and possibly early event in programmed cell death, resulting from a catastrophic collapse of the mitochondrial membrane potential [29].

5. Mitochondrial ATP measurements using luciferase

At present the best available biosensor for ATP (i.e. one capable of binding the nucleotide and producing a readily detectable signal) has proved to be firefly luciferase. Although purified luciferase protein has been microinjected into cells and used to measure cytosolic free ATP concentration [30], this simple approach precludes targeting of the reporter to the lumen of cellular organelles or its attachment to intracellular membranes. Therefore, expression of the protein from introduced cDNA is necessary. Fortunately, a boost to achieve this goal was provided by the need to use a convenient probe for gene expression. Since cytosolic luciferase expressed downstream of regulatable promoters was expected to provide an excellent genetic reporter, the luciferase protein was used [31]. Firefly luciferase was thus cloned [32], and versions of the gene optimised for thermostability and expression in mammalian cells were then generated. The enzyme uses an oxidisable substrate, termed luciferin, which is converted to an AMP adduct before final oxidation with molecular oxygen and the release of a photon of light.

In the last years, we have extended this technology to the detection of changes in free intracellular ATP concentration [33,34]. Here, constantly high levels of luciferase are expressed from strong viral promoters so that small fluctuations in free ATP concentration can be monitored.

Like aequorin, luciferase cDNA can conveniently be fused 3' to cDNA encoding the mitochondrial import sequence of cytochrome c oxidase subunit VIII. This leads to exclusive mitochondrial localization of the probe. It should be stressed that luciferase displays a relatively high selectivity for ATP over ADP, so that the latter is only a weak inhibitor of activity. As a result, luciferase activity within the cell is likely to report largely $[ATP]$ and not $[ATP]/[ADP]$ ratio (or phosphorylation potential). Studies with recombinant targeted luciferases have allowed us to monitor changes in free cytosolic ATP concentration under a number of situations where this is perturbed either by changes in fuel supply to the cell or through cell stimulation with receptor agonists [33]. The latter included hormones, which mobilize intracellular Ca^{2+} and alter mitochondria metabolism via Ca^{2+} accumulation within the mitochondrial matrix and stimulation of intramitochondrial dehydrogenases [25,26]. These studies demonstrated that Ca^{2+} increases in the cytosol (and hence mitochondria) are able to activate mitochondrial ATP synthesis (Fig. 3B), elevating $[ATP]_c$. This effect lasts longer than the Ca^{2+} signal itself, highlighting a form of cellular "metabolic memory".

Interestingly, recent work indicates that other Ca^{2+} -dependent metabolic checkpoints are operative. Indeed, some metabolite transporters were shown to be regulated by Ca^{2+} and also participate in the enhancement of aerobic metabolism upon cell stimulation. Namely, the aspartate/glutamate metabolite carriers (AGCs) were shown to include EF-hand domains, and Ca^{2+} binding to these sites was shown to increase their activity. In turn, recombinant expression of wild type AGCs enhanced ATP production upon cell stimulation, an

effect that was not observed with truncated mutants lacking the Ca^{2+} -binding domain [35].

6. Mitochondrial homeostasis and cell death

The knowledge that excess Ca^{2+} within cells is highly toxic, causing massive activation of proteases and phospholipases, was known to cell biologists since the late 1960s. Electron micrographs of clearly damaged cells with swollen mitochondria full of Ca^{2+} phosphate precipitates and the toxicity of Ca^{2+} ionophores in cultured cells were one of the first effects of these molecules to be discovered. However, classically, this toxic role of Ca^{2+} has been associated to necrosis, i.e. the catastrophic derangement of cell integrity and function following exposure to different types of cell injury and leading to activation of Ca^{2+} -activated hydrolysing enzymes. Typical examples are the complement induced cell death and excitotoxicity, in which glutamate-dependent hyperstimulation leads neurons to necrotic death [36,37]. The link to apoptosis was only appreciated more recently. In this field the observation that turned the attention of many scientists to Ca^{2+} was the discovery that a classical antiapoptotic protein such as Bcl-2 was potentially capable of somehow affecting Ca^{2+} signalling.

The first of such observation dates back to 1993 where upon withdrawal of IL-3 in hemopoietic cell lines, the Bcl-2 overexpression was able to prevent the reduction in the Ca^{2+} concentration of the ER, $[Ca^{2+}]_{er}$ [38]. While this finding could be ascribed to the antiapoptotic effect of Bcl-2 (by preventing the loss of energy and thus of ER Ca^{2+} during apoptosis), surprisingly Bcl-2 overexpression alone was also reported to decrease the size of the ER Ca^{2+} pool [39]. In support of this possibility, we showed that $[Ca^{2+}]_{er}$ levels are higher than in controls early after overexpression of the BAX protein (i.e. the classical pro-apoptotic Bcl-2 family member) in HeLa cells, moreover at later stages (during progression into apoptosis), the difference in $[Ca^{2+}]_{er}$ with control cells becomes virtually undetectable [40]. A second totally independent hint suggesting a potential role of Bcl-2 in regulating Ca^{2+} signals came from the observation that the three-dimensional structure of the Bcl-2 homologue, Bcl-XL, bears a strong resemblance to some pore-forming bacterial toxins [41]. Bcl-2 itself was shown to form cation channels of low selectivity in artificial lipid bilayers [42], and later studies showed that these channels can conduct Ca^{2+} ions [43]. These findings, however, remained simply as correlations until a few novel discoveries were made. Although complex and hard to reconcile into a unifying mechanism, Bcl-2 and Ca^{2+} homeostasis were unquestionably associated. In particular: i) Hockenbery and colleagues demonstrated in a seminal study, the association of Bcl-2 with cellular membranes and in particular with mitochondrial membranes [44]. This observation was pursued by a number of further studies, and it was concluded that Bcl-2 shows a heterogeneous distribution to various other cell compartments including in particular the endoplasmic reticulum. In other words, Bcl-2 tends to accumulate on the membranes of the two organelles, the ER and mitochondria, playing a key role in Ca^{2+} homeostasis [3]; ii) it was demonstrated that the mitochondrial enzyme cytochrome c is released into the cytosol in response to several apoptotic stimuli and results in the activation of caspases. Because antiapoptotic members of the Bcl-2 family prevented this release and protected cells from various death insults, it has been assumed that Bcl-2 family members primarily regulate mitochondrial integrity. The precise nature of this protection is still partially unclear though [45].

Another line of research was carried out primarily in isolated mitochondria. In a series of elegant studies, the group of Bernardi and coworkers rejuvenated a relatively old observation, i.e. the activation by excess Ca^{2+} accumulation into mitochondria of a large conductance pore, also called the permeability transition pore, PTP. They showed that PTP, opened by an increase of $[Ca^{2+}]_m$ in the matrix above a given threshold and by a series of other toxic insults, including oxidative stress, could lead to mitochondrial swelling, breaking of the outer

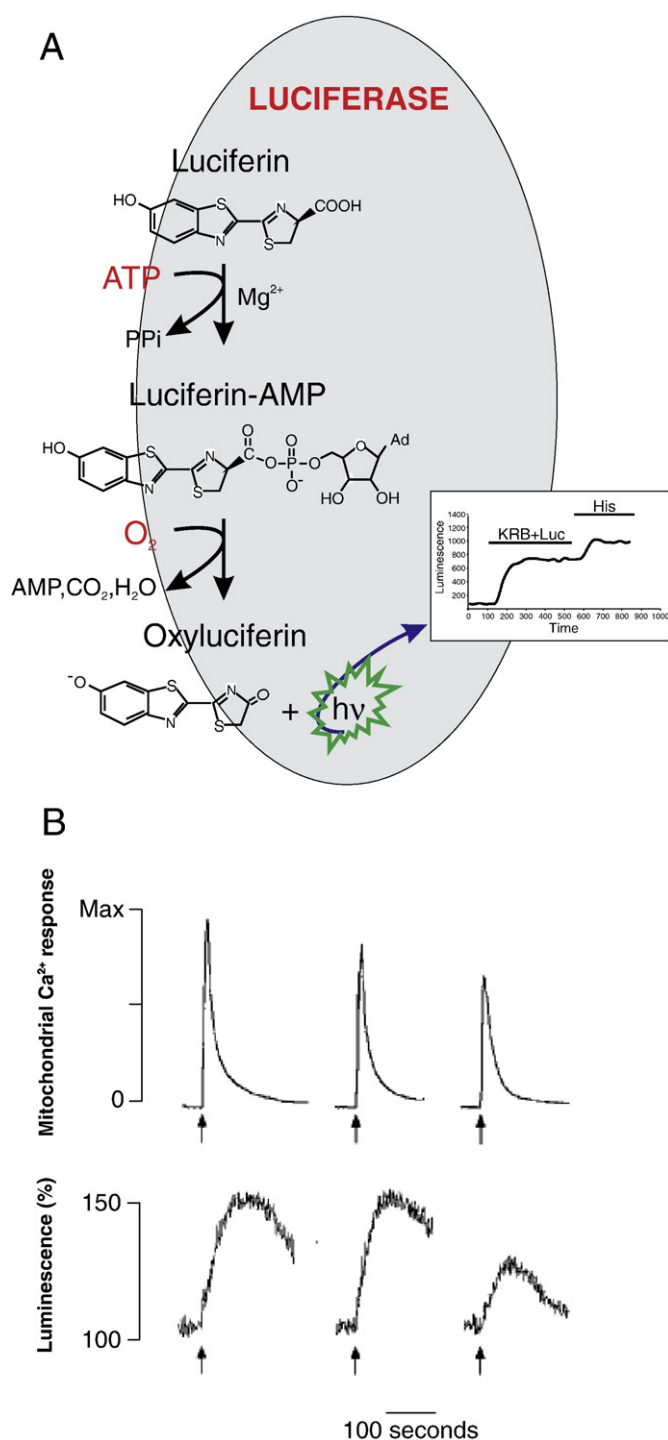


Fig. 3. (A) The ATP-dependent luminescence reaction of luciferase. Light is produced by the oxidation of a luciferin. The rates of this reaction between luciferin and oxygen are extremely slow until they are catalyzed by luciferase. The reaction is very energy efficient: nearly all of the energy input into the reaction is transformed into light. In this case, on the contrary of aequorin reaction, luciferase is not irreversibly discharged. (B) Correlation between $[Ca^{2+}]_m$ increase and $[ATP]_m$ production induced by histamine stimulation. The traces show agonist-dependent $[Ca^{2+}]_m$ and $[ATP]_m$ changes at different states of filling of the ER store. The agonist-dependent $[Ca^{2+}]_m$ increase is the trigger for the rise in $[ATP]_m$, the amplitude of which is controlled by the value of the $[Ca^{2+}]_m$ spike. Throughout the experiments shown in the figure, the cells were perfused in Krebs–Ringer buffer (KRB) and, where indicated (arrow), challenged with histamine (100 μ M). The traces show the effects of increased time of incubation with KRB supplemented with EGTA (1 mM) (control, 90 s, and 300 s before histamine stimulation) on $[Ca^{2+}]_m$ rise (mtAEQ), and $[ATP]_m$ rise (mtLuc).

membrane, and loss into the outer medium of proteins, such as cytochrome *c* contained in the intermembrane space [46].

Thus in a few years, independent lines of research led to the following seminal observations: i) mitochondria and ER are intimately involved in Ca^{2+} signalling; ii) Bcl-2 is located on the membranes of both organelles and can thus potentially modulate Ca^{2+} signalling; iii)

excess Ca^{2+} accumulation by mitochondria could lead to the release from mitochondria of pro-apoptotic signalling molecules.

The data reported above strongly support (despite the matter is not completely clarified) the notion that a reduction of $[Ca^{2+}]_{er}$ is correlated with the overexpression of antiapoptotic proteins, while overexpression of pro-apoptotic genes leads to an increase of $[Ca^{2+}]_{er}$,

but is this relevant for apoptosis or is it a side effect of these proteins? An indication that the reduction of $[Ca^{2+}]_{er}$ is a component of the antiapoptotic mechanism by Bcl-2 comes from the work of our group that was published in 2001. We showed that when mimicking the Bcl-2 effect on $[Ca^{2+}]_{er}$ by different pharmacological and molecular approaches (but in the absence of the oncoprotein), the cells were protected from the apoptotic stimulus of ceramide, which is also sensitive to Bcl-2 overexpression on the other hand, ceramide treatments that increased $[Ca^{2+}]_{er}$ had the opposite effect [47]. These results are in keeping with previous work by Ma et al., demonstrating that SERCA overexpression in COS cells causes ER Ca^{2+} overload and increases spontaneous apoptosis [48]. Last but not least, in calreticulin overexpressing cells, in which the amplitude and duration of Ca^{2+} signals from ER lumen toward cytosol are enhanced without changing $[Ca^{2+}]_{er}$, cell viability is drastically reduced upon ceramide treatment [47], yet cell lines derived from calreticulin knockouts, which show a marked decrease in ER Ca^{2+} release upon cell stimulation, are more resistant to apoptosis [49].

Along the same lines of reasoning, White et al. showed that DT40 cells, in which all three IP3R isoforms had been deleted, are more sensitive to apoptotic challenges than the wild-type cells. At the same time, re-expression of IP3R not only partially depletes the Ca^{2+} store but also provides a “survival” signal per se [50].

What is the role of mitochondria in this scenario? In response to different apoptotic stimuli, mitochondria release a few proteins, including cytochrome *c*, Smac/DIABLO and AIF, i.e. potent activators of effector caspases [51]. The mechanism of this release is probably complex (including the oligomerization on the outer mitochondrial membrane of Bax and Bak that form a channel permeable to cytochrome *c*), but a key role is played by the PTP mentioned above. The PTP in fact is activated by a variety of toxic challenges and cell signals (including increases in $[Ca^{2+}]_m$) which causes mitochondrial swelling and fragmentation. PTP opening thus appears a likely candidate for Ca^{2+} -dependent effector of apoptosis [52,53]. We and others thus verified whether apoptotic challenges led to mitochondrial alterations, and whether Ca^{2+} was involved in the process. In HeLa cells, we showed [47] that the apoptotic stimulus ceramide induced the release of Ca^{2+} from the ER and its uptake by mitochondria (at least in the first phase of the process). Eventually mitochondria swell and fragment and in parallel release cytochrome *c*. These changes were prevented by Bcl-2 expression as well as by experimental conditions that reduced $[Ca^{2+}]_{er}$ or prevented the rise in cytosolic Ca^{2+} [54]. The importance of the size of the releasable pool, i.e. the concept that the key determinant is the net amplitude of the cytosolic rise (as well as of the mitochondrial loading) was further reinforced by the study of a viral protein. The antiapoptotic Cocksackie viral protein 2B was indeed shown to reduce ER Ca^{2+} levels and in turn to protect the cells to different apoptotic stimuli [55]. Thus, the alteration of the Ca^{2+} signal reaching the mitochondria and/or the combined action of apoptotic agents or pathophysiological conditions (e.g. oxidative stress, Cadmium injury) can induce a profound alteration of organelle structure and function [47,56].

The observation that ceramide induced ER Ca^{2+} release and mitochondrial Ca^{2+} uptake results in a dramatic activation of the apoptotic process contrasts the fact that much larger Ca^{2+} releases and mitochondrial accumulations, elicited by a number of stimuli, do not trigger cell death. Rather, they are beneficial, for example, by increasing the cellular ATP levels (for review see [3]). This apparent contradiction is explained by the hypothesis of the double hit model proposed by Pinton et al. [47]. Accordingly, apoptotic stimuli such as ceramide have a dual target. On one hand, ceramide causes the release of Ca^{2+} from the mitochondria and on the other hand makes the mitochondria more sensitive to the potential Ca^{2+} damaging effects. An elegant study of Szalai et al. nicely confirms the model. In particular, the authors showed that ceramide facilitates PTP opening, thus transforming physiological IP3-mediated Ca^{2+} signals into

inducers of apoptosis [57]. In summary, Bcl-2 and other antiapoptotic 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 enhances the loading of the ER Ca^{2+} store, and in turn boosts the Ca^{2+} load to which the apoptotic effector systems (including mitochondria) are exposed upon physiological and/or pathological challenges. This effect of Bax coincides with gross perturbation of mitochondrial structure and function. In apoptotic progression, the development of an altered signalling phenotype, includes impaired ER Ca^{2+} release upon cell stimulation, and thus reduction of cellular Ca^{2+} signals [58].

The importance of the mitochondrial homeostasis for cell fate is supported by recent works on the role of p66Shc, the first mammalian protein whose mutation was demonstrated to increase resistance to oxidative stress and to prolong life span. Compared to the knowledge on the role of Ca^{2+} and mitochondria (for which we summarized above a broad body of data), the information on oxidative stress in mitochondrial signalling is very scant, and its mechanism of action and real significance in apoptosis are still largely unclear. Among the most important players of oxidative stress are ROS originating from exogenous sources such as ultraviolet and ionizing radiations, or from several intracellular sources. Mitochondria are quantitatively the most important source of intracellular ROS and leak from the electron transfer chain is supposed to be the main route [59]. Recently, a totally new, unexpected pathway has emerged, that involves p66Shc in mitochondria ROS production. In higher organisms about 20% of fibroblast p66Shc is localized to mitochondria and, in response to oxidative stress, part of the cytosolic pool of p66Shc translocates to mitochondria [60]. Within mitochondria p66Shc then binds cytochrome *c* and acts as an oxidoreductase, shuttling electrons from cytochrome *c* to molecular oxygen [61]. The redox activity of p66Shc is likely to be the explanation of the increase in ROS production caused by the recombinant expression of p66Shc, and the decrease in ROS levels typical of p66Shc knockout cells [62].

ROS production in mitochondria is regulated by metabolic activity, substrate supply, and mitochondrial membrane potential. Interestingly, p66Shc^{-/-} fibroblasts have altered mitochondrial bioenergetic properties. In particular, p66Shc^{-/-} cells have a reduction in both their resting and maximal oxidative capacity, suggesting that the ablation of p66Shc may extend life span by repartitioning metabolic energy conversion away from oxidative and toward glycolytic pathways [63]. Given the information summarized above, the mitochondrial effects of p66Shc in response to oxidative stress are of great interest and significance. Indeed, p66Shc is one of a handful of identified mammalian aging genes, and it is one of three with a small effect on body size [64]. One interpretation of the data is that p66Shc mediates deleterious triggering of apoptosis [65], and its ablation induces a significant expansion of the life span. The mitochondrial effects of p66Shc may represent the apoptotic “switch,” by controlling the increased mitochondrial production of ROS, and a variety of consequences on mitochondrial functions such as Ca^{2+} homeostasis and organelle morphology. Intriguingly, during oxidative stress PKC β is activated and induces p66Shc phosphorylation, thus allowing p66Shc to be recognized by Pin1, isomerised and imported into mitochondria after dephosphorylation by type 2 protein serine/threonine phosphatase (PP2A). At this point, the protein translocated into the appropriate cell domain, can exert the oxidoreductase activity, generating H₂O₂ and inducing the opening of PTP. This event in turn perturbs mitochondria structure and function (as revealed by the reduced Ca^{2+} responsiveness and the alteration of mitochondrial three-dimensional structure). Caspase cofactors, such as cytochrome *c*, are released and the cell progresses into apoptosis. By this way p66Shc may regulate the mitochondrial clock controlling the life span. Overall, these results thus identify and clarify a novel signalling mechanism, that is operative in the pathophysiological

condition of oxidative stress, and may open new possibilities for pharmacologically addressing the process of organ deterioration during aging [66].

All these observations lead to a really complex but fascinating picture, where mitochondria represent a sort of decoding station: they can sense different environmental conditions or receive diverse signals and integrate them all together, finally producing an outcome that can decide the fate of the cell. The understanding of these complex mechanisms is a hard and challenging task, but within a few years, it will provide new opportunities to comprehend the numerous yet still poorly understood human pathologies.

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References

- [1] D.E. Clapham, Calcium signaling, *Cell* 131 (2007) 1047–1058.
- [2] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, *Nat. Rev. Mol. Cell Biol.* 4 (2003) 517–529.
- [3] R. Rizzuto, T. Pozzan, Microdomains of intracellular Ca²⁺: molecular determinants and functional consequences, *Physiol. Rev.* 86 (2006) 369–408.
- [4] M.R. Duchen, Mitochondria and calcium: from cell signalling to cell death, *J. Physiol.* 529 (Pt 1) (2000) 57–68.
- [5] R. Dolmetsch, Excitation–Transcription coupling: signaling by ion channels to the nucleus, *Sci. STKE*. 2003 (2003) E4.
- [6] M. Colombini, VDAC: the channel at the interface between mitochondria and the cytosol, *Mol. Cell Biochem.* 256–257 (2004) 107–115.
- [7] E. Rapizzi, P. Pinton, G. Szabadkai, M.R. Wieckowski, G. Vandecasteele, G. Baird, R.A. Tuft, K.E. Fogarty, R. Rizzuto, Recombinant expression of the voltage-dependent anion channel enhances the transfer of Ca²⁺ microdomains to mitochondria, *J. Cell Biol.* 159 (2002) 613–624.
- [8] D. Gincel, H. Zaid, V. Shoshan-Barmatz, Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function, *Biochem. J.* 358 (2001) 147–155.
- [9] T.G. Frey, C.W. Renken, G.A. Perkins, Insight into mitochondrial structure and function from electron tomography, *Biochim. Biophys. Acta* 1555 (2002) 196–203.
- [10] M.R. Duchen, Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death, *J. Physiol.* 516 (Pt 1) (1999) 1–17.
- [11] T.E. Gunter, D.I. Yule, K.K. Gunter, R.A. Eliseev, J.D. Salter, Calcium and mitochondria, *FEBS Lett.* 567 (2004) 96–102.
- [12] M. Trenker, R. Malli, I. Fertschaj, S. Levak-Frank, W.F. Graier, Uncoupling proteins 2 and 3 are fundamental for mitochondrial Ca²⁺ uniport, *Nat. Cell Biol.* 9 (2007) 445–452.
- [13] P. Pinton, A. Rimessi, A. Romagnoli, A. Prandini, R. Rizzuto, Biosensors for the detection of calcium and pH, *Methods Cell Biol.* 80 (2007) 297–325.
- [14] F. De Giorgi, Z. Ahmed, C. Bastianutto, M. Brini, L.S. Jouaville, R. Marsault, M. Murgia, P. Pinton, T. Pozzan, R. Rizzuto, Targeting GFP to organelles, *Methods Cell Biol.* 58 (1999) 75–85.
- [15] M.T. Alonso, M.J. Barrero, P. Michelena, E. Carnicero, I. Cuchillo, A.G. Garcia, J. Garcia-Sancho, M. Montero, J. Alvarez, Ca²⁺-induced Ca²⁺ release in chromaffin cells seen from inside the ER with targeted aequorin, *J. Cell Biol.* 144 (1999) 241–254.
- [16] P. Pinton, T. Pozzan, R. Rizzuto, The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca²⁺ store, with functional properties distinct from those of the endoplasmic reticulum, *EMBO J.* 17 (1998) 5298–5308.
- [17] M. Brini, M. Murgia, L. Pasti, D. Picard, T. Pozzan, R. Rizzuto, Nuclear Ca²⁺ concentration measured with specifically targeted recombinant aequorin, *EMBO J.* 12 (1993) 4813–4819.
- [18] R. Marsault, M. Murgia, T. Pozzan, R. Rizzuto, Domains of high Ca²⁺ beneath the plasma membrane of living A7r5 cells, *EMBO J.* 16 (1997) 1575–1581.
- [19] Y. Gavel, G. von Heijne, Cleavage-site motifs in mitochondrial targeting peptides, *Protein Eng* 4 (1990) 33–37.
- [20] R. Rizzuto, A.W. Simpson, M. Brini, T. Pozzan, Rapid changes of mitochondrial Ca²⁺ revealed by specifically targeted recombinant aequorin, *Nature* 358 (1992) 325–327.
- [21] R. Rizzuto, P. Pinton, W. Carrington, F.S. Fay, K.E. Fogarty, L.M. Lifshitz, R.A. Tuft, T. Pozzan, Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca²⁺ responses, *Science* 280 (1998) 1763–1766.
- [22] P. Pinton, M. Brini, C. Bastianutto, R.A. Tuft, T. Pozzan, R. Rizzuto, New light on mitochondrial calcium, *Biofactors* 8 (1998) 243–253.
- [23] P. Pinton, S. Leo, M.R. Wieckowski, G. Di Benedetto, R. Rizzuto, Long-term modulation of mitochondrial Ca²⁺ signals by protein kinase C isozymes, *J. Cell Biol.* 165 (2004) 223–232.
- [24] K. Bianchi, G. Vandecasteele, C. Carli, A. Romagnoli, G. Szabadkai, R. Rizzuto, Regulation of Ca²⁺ signalling and Ca²⁺-mediated cell death by the transcriptional coactivator PGC-1 α , *Cell Death. Differ.* 13 (2006) 586–596.
- [25] R.M. Denton, J.G. McCormack, N.J. Edgell, Role of calcium ions in the regulation of intramitochondrial metabolism. Effects of Na⁺, Mg²⁺ and ruthenium red on the Ca²⁺-stimulated oxidation of oxoglutarate and on pyruvate dehydrogenase activity in intact rat heart mitochondria, *Biochem. J.* 190 (1980) 107–117.
- [26] G.A. Rutter, Ca²⁺-binding to citrate cycle dehydrogenases, *Int. J. Biochem.* 22 (1990) 1081–1088.
- [27] M. Leist, B. Single, A.F. Castoldi, S. Kuhnle, P. Nicotera, Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis, *J. Exp. Med.* 185 (1997) 1481–1486.
- [28] J.S. Kim, L. He, J.J. Lemasters, Mitochondrial permeability transition: a common pathway to necrosis and apoptosis, *Biochem. Biophys. Res. Commun.* 304 (2003) 463–470.
- [29] S. Orrenius, D.H. Burgess, M.B. Hampton, B. Zhivotovsky, Mitochondria as the focus of apoptosis research, *Cell Death. Differ.* 4 (1997) 427–428.
- [30] K.C. Bowers, A.P. Allshire, P.H. Cobbold, Bioluminescent measurement in single cardiomyocytes of sudden cytosolic ATP depletion coincident with rigor, *J. Mol. Cell Cardiol.* 24 (1992) 213–218.
- [31] G.A. Rutter, H.J. Kennedy, C.D. Wood, M.R. White, J.M. Tavares, Real-time imaging of gene expression in single living cells, *Chem. Biol.* 5 (1998) R285–R290.
- [32] J.R. de Wet, K.V. Wood, M. DeLuca, D.R. Helinski, S. Subramani, Firefly luciferase gene: structure and expression in mammalian cells, *Mol. Cell Biol.* 7 (1987) 725–737.
- [33] L.S. Jouaville, P. Pinton, C. Bastianutto, G.A. Rutter, R. Rizzuto, Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming, *Proc. Natl. Acad. Sci. U.S.A.* 96 (1999) 13807–13812.
- [34] H.J. Kennedy, A.E. Pouli, E.K. Ainscow, L.S. Jouaville, R. Rizzuto, G.A. Rutter, Glucose generates sub-plasma membrane ATP microdomains in single islet beta-cells. Potential role for strategically located mitochondria, *J. Biol. Chem.* 274 (1999) 13281–13291.
- [35] F.M. Lasorsa, P. Pinton, L. Palmieri, G. Fiermonte, R. Rizzuto, F. Palmieri, Recombinant expression of the Ca(2+)-sensitive aspartate/glutamate carrier increases mitochondrial ATP production in agonist-stimulated Chinese hamster ovary cells, *J. Biol. Chem.* 278 (2003) 38686–38692.
- [36] P. Nicotera, S. Orrenius, The role of calcium in apoptosis, *Cell Calcium* 23 (1998) 173–180.
- [37] S.L. Budd, D.G. Nicholls, Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells, *J. Neurochem.* 67 (1996) 2282–2291.
- [38] G. Baffy, T. Miyashita, J.R. Williamson, J.C. Reed, Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production, *J. Biol. Chem.* 268 (1993) 6511–6519.
- [39] P. Pinton, R. Rizzuto, Bcl-2 and Ca²⁺ homeostasis in the endoplasmic reticulum, *Cell Death. Differ.* 13 (2006) 1409–1418.
- [40] M. Chami, A. Prandini, M. Campanella, P. Pinton, G. Szabadkai, J.C. Reed, R. Rizzuto, Bcl-2 and Bax exert opposing effects on Ca²⁺ signalling, which do not depend on their putative pore-forming region, *J. Biol. Chem.* (2004).
- [41] S.L. Schendel, Z. Xie, M.O. Montal, S. Matsuyama, M. Montal, J.C. Reed, Channel formation by antiapoptotic protein Bcl-2, *Proc. Natl. Acad. Sci. U.S.A.* 94 (1997) 5113–5118.
- [42] A.J. Minn, P. Velez, S.L. Schendel, H. Liang, S.W. Muchmore, S.W. Fesik, M. Fill, C.B. Thompson, Bcl-x(L) forms an ion channel in synthetic lipid membranes, *Nature* 385 (1997) 353–357.
- [43] M. Lam, G. DUBYAK, L. Chen, G. Nunez, R.L. Miesfeld, C.W. Distelhorst, Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca²⁺ fluxes, *Proc. Natl. Acad. Sci. U.S.A.* 91 (1994) 6569–6573.
- [44] D. Hockenbery, G. Nunez, C. Millman, R.D. Schreiber, S.J. Korsmeyer, Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death, *Nature* 348 (1990) 334–336.
- [45] S. Lucken-Ardjomande, J.C. Martinou, Regulation of Bcl-2 proteins and of the permeability of the outer mitochondrial membrane, *C.R. Biol.* 328 (2005) 616–631.
- [46] P. Bernardi, Mitochondrial transport of cations: channels, exchangers, and permeability transition, *Physiol. Rev.* 79 (1999) 1127–1155.
- [47] P. Pinton, D. Ferrari, E. Rapizzi, F.D. Di Virgilio, T. Pozzan, R. Rizzuto, The Ca²⁺ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action, *EMBO J.* 20 (2001) 2690–2701.
- [48] T.S. Ma, D.L. Mann, J.H. Lee, G.J. Gallinghouse, SR compartment calcium and cell apoptosis in SERCA overexpression, *Cell Calcium* 26 (1999) 25–36.
- [49] K. Nakamura, E. Bossy-Wetzel, K. Burns, M.P. Fadel, M. Lozyk, I.S. Goping, M. Opas, R.C. Bleackley, D.R. Green, M. Michalak, Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis, *J. Cell Biol.* 150 (2000) 731–740.
- [50] C. White, C. Li, J. Yang, N.B. Petrenko, M. Madesh, C.B. Thompson, J.K. Foskett, The endoplasmic reticulum gateway to apoptosis by Bcl-x(L) modulation of the InsP3R, *Nat. Cell Biol.* 7 (2005) 1021–1028.
- [51] G. Kroemer, J.C. Reed, Mitochondrial control of cell death, *Nat. Med.* 6 (2000) 513–519.
- [52] A.P. Halestrap, E. Doran, J.P. Gillespie, A. O'Toole, Mitochondria and cell death, *Biochem. Soc. Trans.* 28 (2000) 170–177.

- [53] V. Petronilli, D. Penzo, L. Scorrano, P. Bernardi, F. Di Lisa, The mitochondrial permeability transition, release of cytochrome *c* and cell death. Correlation with the duration of pore openings in situ, *J. Biol. Chem.* 276 (2001) 12030–12034.
- [54] P. Pinton, D. Ferrari, E. Rapizzi, F. Di Virgilio, T. Pozzan, R. Rizzuto, A role for calcium in Bcl-2 action? *Biochimie* 84 (2002) 195–201.
- [55] M. Campanella, A.S. de Jong, K.W. Lanke, W.J. Melchers, P.H. Willems, P. Pinton, R. Rizzuto, F.J. van Kuppeveld, The coxsackievirus 2B protein suppresses apoptotic host cell responses by manipulating intracellular Ca²⁺ homeostasis, *J. Biol. Chem.* 279 (2004) 18440–18450.
- [56] M. Biagioli, S. Pifferi, M. Raghianti, S. Bucci, R. Rizzuto, P. Pinton, Endoplasmic reticulum stress and alteration in calcium homeostasis are involved in cadmium-induced apoptosis, *Cell Calcium* (2007).
- [57] G. Szalai, R. Krishnamurthy, G. Hajnoczky, Apoptosis driven by IP(3)-linked mitochondrial calcium signals, *EMBO J.* 18 (1999) 6349–6361.
- [58] C. Giorgi, A. Romagnoli, P. Pinton, R. Rizzuto, Ca²⁺ signaling, mitochondria and cell death, *Curr. Mol. Med.* 8 (2008) 119–130.
- [59] J.F. Turrens, Mitochondrial formation of reactive oxygen species, *J. Physiol* 552 (2003) 335–344.
- [60] F. Orsini, E. Migliaccio, M. Moroni, C. Contursi, V.A. Raker, D. Piccini, I. Martin-Padura, G. Pelliccia, M. Trinei, M. Bono, C. Puri, C. Tacchetti, M. Ferrini, R. Mannucci, I. Nicoletti, L. Lanfrancone, M. Giorgio, P.G. Pelicci, The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential, *J. Biol. Chem.* 279 (2004) 25689–25695.
- [61] M. Giorgio, E. Migliaccio, F. Orsini, D. Paolucci, M. Moroni, C. Contursi, G. Pelliccia, L. Luzi, S. Minucci, M. Marcaccio, P. Pinton, R. Rizzuto, P. Bernardi, F. Paolucci, P.G. Pelicci, Electron transfer between cytochrome *c* and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis, *Cell* 122 (2005) 221–233.
- [62] E. Migliaccio, M. Giorgio, P.G. Pelicci, Apoptosis and aging: role of p66Shc redox protein, *Antioxid. Redox. Signal.* 8 (2006) 600–608.
- [63] S. Nemoto, C.A. Combs, S. French, B.H. Ahn, M.M. Fergusson, R.S. Balaban, T. Finkel, The mammalian longevity-associated gene product p66shc regulates mitochondrial metabolism, *J. Biol. Chem.* 281 (2006) 10555–10560.
- [64] J.K. Quarrie, K.T. Riabowol, Murine models of life span extension, *Sci. Aging Knowledge Environ.* 2004 (2004) re5.
- [65] M. Pellegrini, S. Pacini, C.T. Baldari, p66SHC: the apoptotic side of Shc proteins, *Apoptosis*. 10 (2005) 13–18.
- [66] P. Pinton, A. Rimessi, S. Marchi, F. Orsini, E. Migliaccio, M. Giorgio, C. Contursi, S. Minucci, F. Mantovani, M.R. Wieckowski, G. Del Sal, P.G. Pelicci, R. Rizzuto, Protein kinase C beta and prolyl isomerase 1 regulate mitochondrial effects of the life-span determinant p66Shc, *Science* 315 (2007) 659–663.