Mitochondrial calcium signalling: message of life and death

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Abstract. Upon physiological stimulation, mitochondria undergo a major rise in mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) in a wide variety of cell types. Here, particular attention will be focused on the mechanism that allows the low-affinity transporters of mitochondria to rapidly accumulate Ca^{2+}, despite the low amplitude of the cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) rises, i.e. the close apposition of mitochondria to the Endoplasmic Reticulum (ER), the main pool of agonist-releasable Ca^{2+}. Upon opening of IP3-gated channels, mitochondria are able to sense not the average $[Ca^{2+}]_c$ rise, but rather the much higher concentration occurring in the proximity of the open channels. We will then address the functional significance of this process, that spans from the activation of organelle metabolism to the alteration of organelle morphology, and consequent release of pro-apoptotic factors during apoptosis.

Key words: Apoptosis, Ca^{2+} , Bcl-2, Endoplasmic reticulum, ATP.

INTRODUCTION

Cells have an ubiquitous intracellular signalling element which controls many cellular functions (Fig.1): the divalent cation Calcium (Ca^{2+}), From the 1960s, Ca^{2+} is recognized to be a fundamental second messenger involved in learning, memory, fertilization, proliferation, development, muscle contraction and secretion (1). As second messenger, Ca^{2+} exerts its role inside the cell, where its concentration is highly controlled. In resting conditions (Fig.2), $[Ca^{2+}]_c$ is around 100nM, 4 order of magnitude lower than in the extracellular medium, where it reaches about 1mM. This condition is maintained in every moment by a dynamic equilibrium across the plasma membrane, more precisely is due to its low perme-

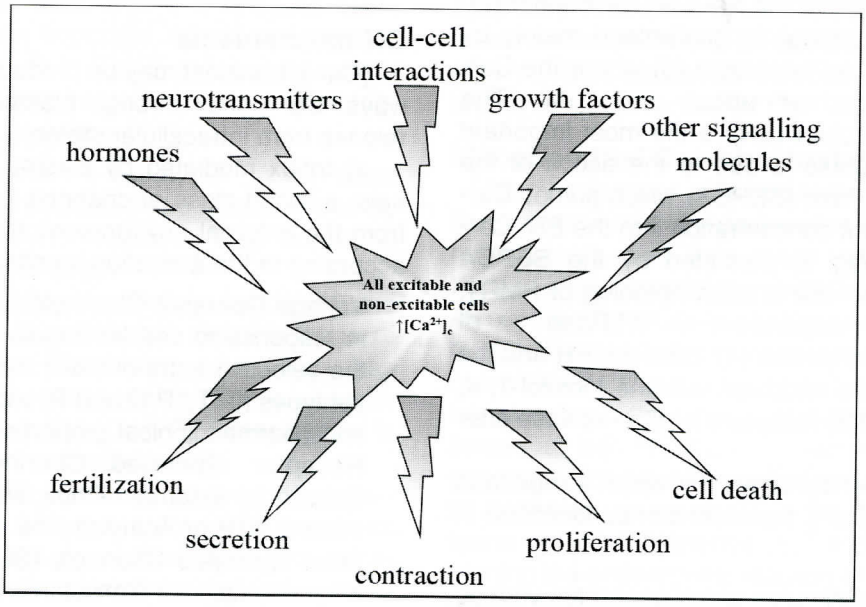

Figure 1
Central role of Ca^{2+} in the regulation of several cellular functions

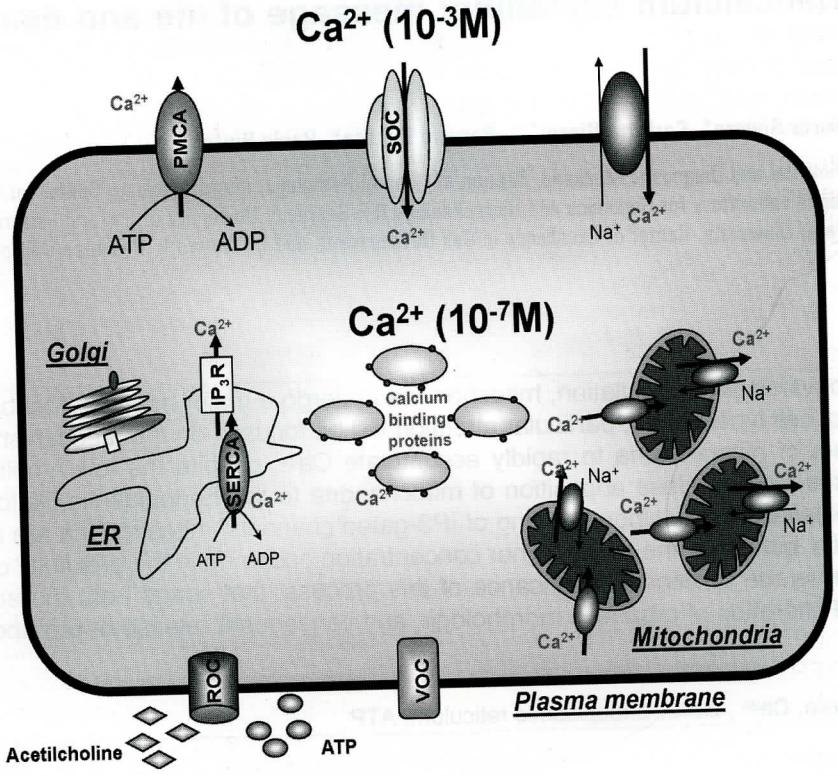

Figure 2

Scheme of a mammalian cell with the localization of the main players of Ca^{2+} homeostasis. PMCA: Plasma Membrane Calcium ATPase; SOC: Store Operate Channels; IP3R: Inositole-1-4-5-trisphosphate Receptor; VOC: Voltage Operated Channels; ROC: Receptor Operated Channel.

ability to ions and to the activity of plasma membrane Ca^{2+} ATPases (PMCAs), which pump Ca^{2+} outside the cells and to the Na^{+}/Ca^{2+} exchanger (2). Intracellular Ca^{2+} storage is guaranteed mainly by the ER and the Golgi apparatus (3) where the Ca^{2+} concentration reaches about 500 μM. The Endoplasmic Reticulum (ER) is the most important store and Ca^{2+} uptake is due to the action of the sarco/ER Ca^{2+} ATPase (SERCA) which pumps Ca^{2+} against the gradient concentration into the ER. Ca^{2+} uptake in the Golgi is mediated by the SERCA pumps and by the mammalian homolog of PMR1, the Mn^{2+}/Ca^{2+}-dependent ATPase of Saccharomyces cerevisiae (4). Both the ER and the Golgi apparatus are endowed with the Inositol-1, 4, 5-trisphosphate (IP3) receptors for Ca^{2+} release after IP3 generation.

The intracellular dynamics by which exogenous stimuli induce a $[Ca^{2+}]_c$ transient can be identified in four steps:

- Reception of a stimulus which generates various Ca^{2+} mobilizing signals;
- Activation of ON mechanisms to increase $[Ca^{2+}]_c$;
- Activation of Ca^{2+}-sensitive processes;
- Restoration of the resting conditions with OFF mechanisms;

ON mechanisms

$[Ca^{2+}]_c$ transient may be produced in two different ways: Ca^{2+} influx through plasma membrane and release from intracellular stores.

a) Influx mediated by plasma membrane channels: a broad class of channels controls Ca^{2+} entry from the external environment; they are subdivided according to the activation mechanism:

- Voltage Operated Channels (VOCs) are opened in response to cellular depolarization to mediate the selective entry of Ca^{2+}; they exist in different subtypes (L, T, P/Q and R) with different kinetics and pharmacological properties.
- Receptor Operated Channels (ROCs) are opened by external stimuli, such as neurotransmitters, ATP or Acetylcholine.
- Store Operated Channels (SOCs) are activated when intracellular Ca^{2+} stores are depleted.
- Second Messenger Operated Ca^{2+} Channels (SMOCs) are activated by G protein after the activation of G coupled receptors through the binding

of a specific agonist.

b) Intracellular stores release:

- The IP3 receptor consists in a tetrameric transmembrane protein, which exposes on the external site the binding site for IP3 while the transmembrane domain forms a Ca^{2+} channel which opens after the agonist has bound the receptor. IP3 signalling begins when an extracellular agonist interacts with a seven transmembrane domain receptor coupled with G_q proteins able to interact with a phospholipase C isoform (PLC). The PLC generates IP3 that diffuses to ER and Golgi apparatus to interact with the cited receptor (Fig.3a).
- Ryanodine receptor (RyR) is a transmembrane protein located on the SR/ER membrane, originally described in skeletal muscle, its activation is dependent on Ryanodine (a vegetal alkaloid that blocks the channels in their open conformations) caffeine and Ca^{2+} itself.
- Sphingolipid Ca^{2+} release-mediating protein of the ER (SCaMPER). The activation of this protein depends on sphingosine-1-phosphate.

OFF mechanisms

Once Ca^{2+} has exerted its function, it has to be rapidly removed by the OFF mechanisms which consist in various systems that remove Ca^{2+} from cytosol and reintegrate the intracellular Ca^{2+} stores:

- Plasma membrane Ca^{2+}-ATPase (PMCA) is embedded in the plasma membrane and it switches from the E1 state, with high affinity for Ca^{2+}, exposed on the intracellular side, to the E2 phosphorylated state with low affinity for Ca^{2+} and which is exposed on the extracellular side, where it releases Ca^{2+}. Calmodulin plays an important role in this mechanism: a rapid increase in $[Ca^{2+}]_c$ induces its activation, and the following interaction with PMCA triggers the extrusion expelling Ca^{2+}.
- Sarco-endoplasmic reticulum ATPases (SERCAs) can translocate two divalent ions by consuming one ATP molecule. Their activity is calmodulin independent.
- Na^+/Ca^{2+} exchanger. In resting conditions the activity of PMCA is sufficient to maintain the ionic equilibrium; after stimulation, when Ca^{2+} has reached high concentration, Na^+/Ca^{2+} exchanger activity is required. This Ca^{2+} OFF mechanism is mainly expressed in excitable cells.
- Ca^{2+} buffers. Cells contain large amount of Ca^{2+} binding proteins, both in cytosol and in the stores (ER/SR and Golgi apparatus) such as calsequestrin, calreticulin, calmodulin, calbindin, calretinin and parvalbumin. Approximately the 90% of the Ca^{2+} entering cells is normally bound to one of these buffering systems. Thus, a small percentage of free cytosolic Ca^{2+} is the responsible for the activation of Ca^{2+} sensors evoked by the stimulus.

Signalling functions of the Ca^{2+} machinery

The $[Ca^{2+}]_c$ transient regulates a large variety of cellular processes (Fig.1). Here, we will summarize some of the most important:

Cell proliferation. Ca^{2+} has a fundamental role in controlling both fertilization and cell proliferation. It has been demonstrated that at the very moment of the fecundation Ca^{2+} spiking stimulates CAMKII. This protein can dephosphorylate cyclin-dependent kinase 1, resulting in cyclin B activation and completion of meiosis. Then the periodic release of Ca^{2+} from the stores mediates the activation of specific mitotic events (5).

Skeletal muscle contraction: Membrane depolarization induces a conformational change in L-type VOC channels on the plasma membrane, these channels are directly connect to RyR1 embedded in the SR, which are responsible for the Ca^{2+} release and skeletal muscle contraction (6).

Neurons excitability: Ca^{2+} channels are located in different parts of neurons, N and P/Q type VOCs are mainly located at the synaptic ending and control the release of neurotransmitters. L-type VOCs in the neuronal body are involved in Ca^{2+} signal gene activation (7).

Secretion: RyRs are located also in non excitable tissue such as the pancreas; during secretion Ca^{2+} acts either in the release of enzymes or in the fluid secretion (8).

Apoptosis: After different stimuli, Ca^{2+} induces apoptosis mediated by an interplay between the mitochondria and the ER. For instance, the proapoptotic stimulus ceramide induces ER Ca^{2+} release and in turn an overload of Ca^{2+} in mitochondria (9).

Mitochondrial Ca^{2+} homeostasis

Many evidences collected in the last years underline a central role of mitochondria in regulating Ca^{2+} homeostasis, and thus many cellular processes, thanks to the capacity of this organelle to move Ca^{2+} across its membranes. On the basis of this ability, there is the principle of energy conservation in mitochondria (chemosmotic principle) represented by the translocation, through protein complexes, of H^+ across an ion-impermeable inner membrane, which generates an electrochemical gradient that, under physiological condition, is in the form of a membrane potential difference ($\Delta\psi$) and is employed for running the endoergonic reaction of ADP phosphorylation, but also for accumulating cations into the matrix. In resting conditions, the values of $[Ca^{2+}]_c$ oscillate between about 0.1-1 μM and the mitochon-

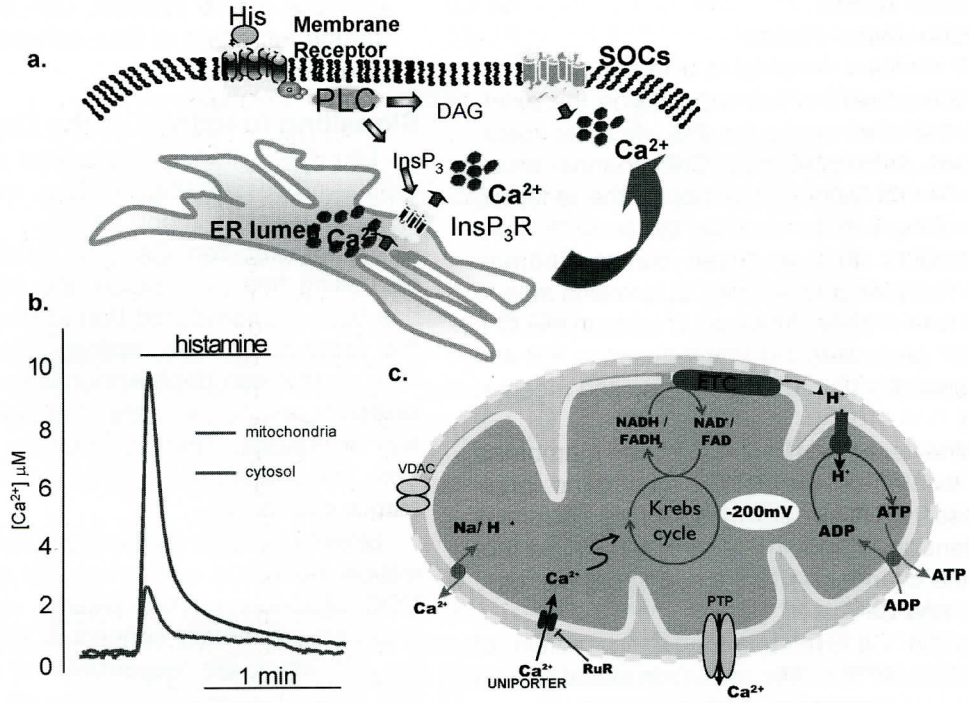

Figure 3

a. Mechanism of histamine-induced Ca^{2+} release from Endoplasmic Reticulum (ER). Through the interaction with a G-coupled receptor, Histamine activates Phospholipase C (PLC) which in turn hydrolyses the plasma membrane lipid phosphatidylinositol 4,5 bisphosphate into inositol 1,4,5 trisphosphate (IP3) and diacylglycerol. IP3 interacts with Ca^{2+} channels localised in the ER and Golgi apparatus). Ca^{2+} release from intracellular stores is most often accompanied by Ca^{2+} influx through Store-Operated Ca^{2+} Channels (SOCs), the opening mechanism of which is still debate. b. Representative traces of mitochondrial (red) and cytosolic (green) Ca^{2+} transient. c. Mechanisms regulating Ca^{2+} homeostasis in mitochondria. ETC: Electron Transport Chain, VDAC: Voltage-Dependent Anion Channel, PTP: Permeability Transition Pore, RuR: Ruthenium Red.

drial membrane potential is -180mV (negative inside), so, according to these thermodynamic parameters, we should expect a free Ca^{2+} concentration into the matrix ($[Ca^{2+}]_m$) of 0.1-1M. But, 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 parameters rather then thermodynamics (Fig.3c).

Pathways for Ca^{2+} transport

Mitochondrial Ca^{2+} uniporter is a channel which transports Ca^{2+}, Sr^{2+}, but not Mg^{2+} with different selectivity at very low affinity (10). The molecular structure of this uniporter is still unknown, but Trenker and co-workers, in a recent work, demonstrate an important role of UCP2 and UCP3 in mitochondrial Ca^{2+} uptake (11).

Na^+-dependent Ca^{2+} efflux (Na^+ - Ca^{2+} exchanger). The stoichiometry of this exchange is $3Na^+$:$2Ca^{2+}$(12).

Na^+-independent Ca^{2+} efflux (H^+-Ca^{2+}exchanger). This route extrudes Ca^{2+} against a gradient that is much higher than what thermodynamic parameters permit for an electroneutral H^+-Ca^{2+} exchanger (12).

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 inhibits it. The physiological role of mitochondrial Ca^{2+} induced permeability is still unclear, but some hypothesis have been suggested. For example, it has been proposed that it could 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 (13).

Mitochondrial Ca^{2+} signal in living cells: how mitochondria can accumulate Ca^{2+}

The thermodynamic and kinetic features of Ca^{2+} transport pathway suggest that, under physiological condition, mitochondrial Ca^{2+} uptake should not be

significant. The very low affinity of the uniporter (at physiological Mg^{2+} concentrations the Kd is >10 μM), while preventing a futile cycling of Ca^{2+} across the mitochondrial membrane, led to the prediction that mitochondrial Ca^{2+} uptake would be negligible not only at rest, but also during the transient increases to ~ 1-2 μM that occur in the cytoplasm of a stimulated cell. Thus, it was generally assumed that mitochondria, although capable of accumulating Ca^{2+}, would not significantly participate in physiological conditions, but would rather act as low affinity buffer in cases of Ca^{2+} overload (i.e. in a variety of pathophysiological conditions). On the contrary, a more important role of mitochondria in Ca^{2+} homeostasis has emerged thanks to the aequorin technology (14). Indeed, direct measurement of Ca^{2+} uptake using mitochondria-targeted aequorin (15), demonstrate that, the stimulation with G_q-coupled receptor agonists, which induce IP3 production and consequent release of Ca^{2+} from ER, causes a rapid rise in $[Ca^{2+}]_m$ (Fig.3b) (16). This effect has been detected in many cells type: HeLa cells, fibroblasts, endothelial and epithelial cells, cardiac and skeletal muscle cells, neurons and pancreatic β cells (17;18).

As to the mechanism allowing the fast mitochondrial response, the high sensitivity of mitochondria to the release of Ca^{2+} from the ER suggests that close contacts with the ER could be responsible for generating domains of high Ca^{2+} next to mitochondria. In support of this hypothesis, by using an ultrafast imaging system, which allows a high-resolution solving of the 3D structure of intracellular organelles we have provided a high-resolution view of mitochondria and ER in living cells, each forming an interconnected network, with a restricted number of close contacts (<80 nm apart). The existence of close appositions between the ER and the mitochondria is consistent with 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 clearly does not prove it. Direct evidence for the existence of such microdomains on the outer surface of the inner mitochondrial membrane is provided by the functional results obtained with an aequorin strategically located to the mitochondrial intermembrane space. The apparent mean $[Ca^{2+}]$ values monitored with this aequorin have been found to be consistently higher than in the bulk cytosol (19).

Moreover, different mechanisms can finely tune amplitude and kinetics of the mitochondrial Ca^{2+} responses. For example, Ca^{2+} uptake can be increased or decreased by protein kinases, such as protein kinase C (20) or p38 MAP kinases (21).

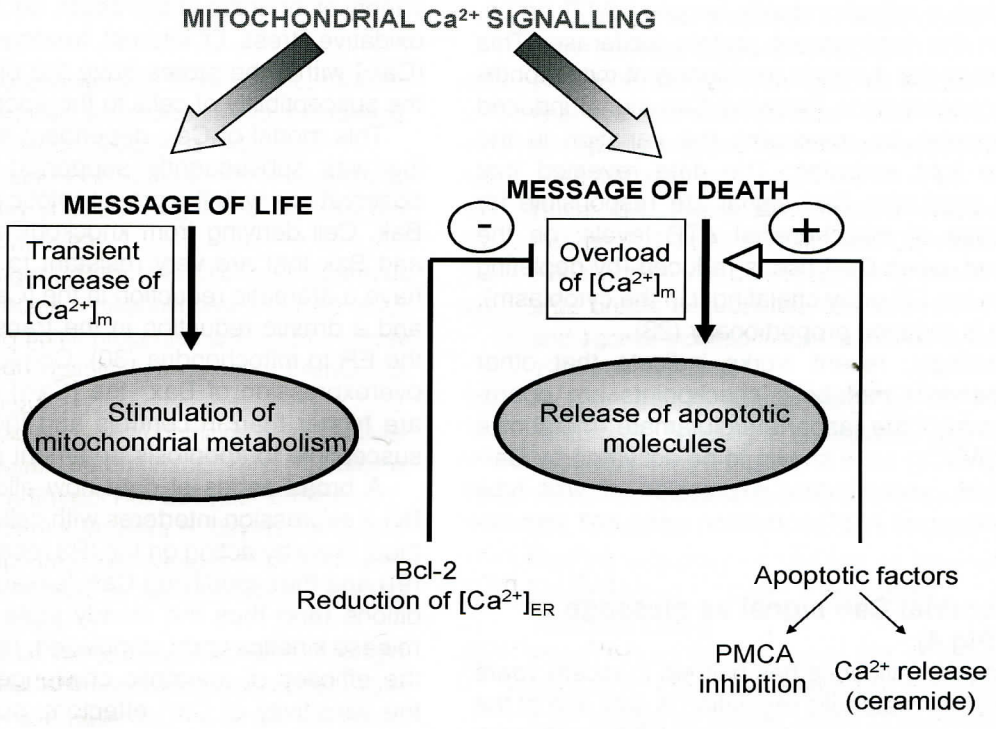

Figure 4

Overview on Ca^{2+} role on the regulation of cell fate.

Mitochondrial Ca^{2+} signal as message of life (Fig.4)

One of the basic pillars of biochemistry is that the main function of mitochondria is oxidative phosphorylation. An early hypothesis was that ATP production was controlled by the rate of ADP and inorganic phosphate (Pi), as supported by the observation that, isolated mitochondria in suspension could increase their ATP rate following addition of ADP and Pi supported only by substrate and oxygen. Today we know that this process, as demonstrated by Denton, McCormack and Hansford, is regulated by Ca^{2+}, in fact Ca^{2+} acts as an allosteric regulator for isocitrate dehydrogenase and oxoglutarate dehydrogenase, and it activates a Ca^{2+}-dependent phosphatase, which converts pyruvate dehydrogenase into an active form. Stimuli which induce a $[Ca^{2+}]_m$ rise are responsible for an increased activity of Krebs cycle and this increase lasts longer then the effect on $[Ca^{2+}]_m$, as demonstrated by the prolonged rise of NADH levels (22). By this way, the cell can decode a rapid signal (as the spike in cytoplasmic Ca^{2+} 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 Krebs cycle, NADH, is employed by the mitochondrial respiratory chain, which increases the activity of H^+ pump, creating an electrochemical gradient used to produce ATP. To confirm the relationship between $[Ca^{2+}{}_m]$ rise and ATP production, we constructed a mitochondrially targeted ATP probe, based on the luminescent protein luciferase. This probe allows the dynamic monitoring of mitochondrial ATP concentration following Ca^{2+}-signal induced by an agonist, by measuring the variation in the luciferase light emission. The data revealed that agonists producing Ca^{2+}-signal are responsible for an increase of mitochondrial ATP levels; on the other hand, when Ca^{2+} rise is reduced (by depleting Ca^{2+} from the ER or by chelating it in the cytoplasm), ATP rise is reduced proportionally (23).

Interestingly, recent works indicate that other Ca^{2+}-dependent metabolic checkpoints are operative. Namely, the aspartate/glutamate metabolite carriers (AGCs) were shown to be activated by Ca^{2+} and in turn, recombinant expression of wild type AGCs enhanced ATP production upon cell stimulation (24).

Mitochondrial Ca^{2+} signal as message of death (Fig.4)

Substantial evidence has built up in recent years indicating that metabolic regulation is only one of the roles of the mitochondrial Ca^{2+} signal. It now appears evident the role of mitochondrial Ca^{2+}in the control of cell death(9;25).

Early and pivotal events in apoptosis are now known to occur in mitochondria (and the ER), and the release of elements acting as caspase cofactor, such as cytochrome c, (from mitochondria) and Ca^{2+} (from the ER) into the cytosol are requisites for cell death in many cases (9;26).

Regarding apoptosis, the large interest is promoted by findings that apoptosis is involved in a wide range of pathological conditions. In cancer and autoimmune disorders the apoptotic processes are impaired, resulting in an inadequate removal of damaged or unwanted cells. In contrast, in several diseases, including degenerative and ischemic-related pathologies, an inappropriate increase in apoptosis is part of the disease progression (27).

The apoptotic mitochondrial pathway is regulated by members of the Bcl-2 protein family, subdivided into two groups, one with anti-apoptotic (Bcl-2) and one with pro-apoptotic (Bax, Bak) properties. Thus the relative concentrations of the pro- and anti-apoptotic members would decide whether a cell is entering or not in apoptosis.

What attracted the attention of many scientists to Ca^{2+} was the discovery of the effects of Bcl-2 on Ca^{2+} homeostasis. It has been shown that the Bcl-2 (localized in several intracellular membranes, including mitochondria and ER) reduces the steady state Ca^{2+} levels in the ER (and thus dampen the pro-apoptotic Ca^{2+} signal)(28;29). The reduction of $[Ca^{2+}]$ within Ca^{2+} stores has a protective effects toward a variety of inducers of cell death, such as ceramide or oxidative stress. Of interest, treatments that increase $[Ca^{2+}]$ within the stores have the opposite effect on the susceptibility of cells to the apoptotic stimulus.

This model of Ca^{2+}-dependent apoptosis triggering was subsequently supported by a series of observations with the pro-apoptotic proteins Bax and Bak. Cell 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 (30). Conversely, early after overexpression of Bax the $[Ca^{2+}]$ of the ER levels are higher than in controls and the cells are more susceptible to apoptosis treatment (31).

A broad series of data now allows to state that Bcl-2 expression interferes with cellular Ca^{2+} signals, most likely by acting on the IP3 receptor (reviewed in (9)) and thus modifying Ca^{2+} leakage in resting conditions (and thus the steady state $[Ca^{2+}]_{er}$) and its release kinetics upon stimulation. In turn, this affects the efficacy of apoptotic challenges by influencing the sensitivity of Ca^{2+} effectors, such as mitochondria.

Interestingly, the link between Ca^{2+} signalling and cell death has been reinforced by the study of a pro-

apoptotic protein not related to 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. 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 alteration (the opposite effect of Bcl-2) was not due to an alteration in ER Ca^{2+} handling (both the steady state levels and the release kinetics were the same in HBx-transfected and control cells) but rather to the caspase-dependent cleavage of PMCA, the most effective molecular route for rapidly returning $[Ca^{2+}]_c$ to basal values (32).

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