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Calcium and apoptosis: facts and hypotheses

Rosario Rizzuto*[,1], Paolo Pinton[1], Davide Ferrari[1], Mounia Chami[1], György Szabadkai[1], Paulo J Magalhães[2], Francesco Di Virgilio[1] and Tullio Pozzan[2]

[1]*Department of Experimental and Diagnostic Medicine and Center for the Study of Inflammation, University of Ferrara, Italy;* [2]*Department of Biomedical Sciences, University of Padova, Italy*

Although longstanding experimental evidence has associated alterations of calcium homeostasis to cell death, only in the past few years the role of calcium in the signaling of apoptosis has been extensively investigated. In this review, we will summarize the current knowledge, focusing on (i) the effect of the proteins of the Bcl-2 family on ER Ca^{2+} levels, (ii) the action of the proteolytic enzymes of apoptosis on the Ca^{2+} signaling machinery, (iii) the ensuing alterations on the signaling patterns of extracellular stimuli, and (iv) the intracellular targets of 'apoptotic' Ca^{2+} signals, with special emphasis on the mitochondria and cytosolic Ca^{2+}-dependent enzymes.
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Introduction

The term 'apoptosis' was first coined in 1972 by John Kerr, an Australian pathologist, who observed that certain dying cells shared a number of common morphologic features. All the criteria used to describe apoptotic cells were morphological and included condensation and margination of chromatin, cytoplasmic vacuolization, cellular shrinkage, increase in cellular density, nuclear fragmentation, and apoptotic body formation (Kerr *et al*., 1972). Several changes in cell surface molecules also occur in apoptotic cells, rendering them immediately recognizable by neighboring cells; the latter quickly phagocyte these flagged cells, ensuring their prompt elimination from tissues. It is often overlooked that apoptosis is essential for normal tissue development and homeostasis, in both vertebrate and invertebrate species. In parallel, however, it can also contribute to many forms of pathological cell loss – apoptosis probably plays a role in many chronic degenerative processes, including Alzheimer's and Parkinson's disease and heart failure, while inhibition of apoptosis can be at the basis of the abnormal cell growth in tumors. It should also be stressed that apoptosis is a highly regulated process that can be induced by a variety of physiologic and pharmacological stimuli. Given the multiplicity of the signals leading to apoptosis, it is not surprising that this process is regulated by distinct and highly complex pathways. In this review, we will focus on the role that Ca^{2+} plays in the choreography of apoptotic cell death.

The involvement of Ca^{2+} in cell death has been recognized very early in the history of programmed cell death, with the demonstration *in vitro* that Ca^{2+} ionophores, that is, molecules capable of transporting Ca^{2+} across membranes down its electrochemical gradient, are highly toxic to cells. The interest in the involvement of Ca^{2+} in apoptosis, however, grew enormously when it was recognized that the neurotransmitter glutamate, or related compounds, have the ability to induce neuronal death due to receptor overstimulation. Calcium ions play a critical role in this process, and intracellular Ca^{2+} overload appears to mediate the lethal effects of receptor overactivation (Choi, 1992). Ca^{2+} overload has even been suggested to be the final common pathway of all types of cell death. Indeed, over the last few years, several studies have shown that increases of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) occur, both at early and late stages of the apoptotic pathway (Martikainen *et al*., 1991; Kruman *et al*., 1998; Zirpel *et al*., 1998; Tombal *et al*., 1999; Lynch *et al*., 2000). More specifically, it has been suggested that both Ca^{2+} release from the ER and capacitative Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels are apoptogenic (Jiang *et al*., 1994; Wertz and Dixit, 2000; Pinton *et al*., 2001). There are also data suggesting that very high intracellular Ca^{2+} levels can promote cell death through necrosis, whereas lower intracellular Ca^{2+} increases induced by milder insults promote cell death through apoptosis (Choi, 1995; Nicotera and Orrenius, 1998).

The aim of this contribution is to present and discuss the available data that link alterations in intracellular Ca^{2+} homeostasis to various stages of the normal or altered apoptotic signaling cascade. There are many aspects of apoptosis itself that will not be broached here; interested readers are directed to excellent recent works (Green and Evan, 2002) and other chapters in this special issue.

The Bcl-2 company plays at different venues

Although the interest in the role of Ca^{2+} in programmed cell death dates back to over 20 years ago, the study of

*Correspondence: R Rizzuto, Department of Experimental and Diagnostic Medicine, Section of General Pathology, Via Borsari 46, 44100 Ferrara, Italy; E-mail: r.rizzuto@unife.it

the role of this second messenger in apoptosis gained further impetus from the discovery that important regulators of apoptosis, the proteins of the Bcl-2 family, are localized in organelles deeply involved in Ca^{2+} handling (the mitochondria and the ER), and may act as ion channels.

Let us first summarize a few key characteristics of the prototype of this family, the oncogene Bcl-2. Bcl-2 (from B-cell lymphoma/leukemia-2) was discovered almost 20 years ago in B-cell malignancies, but only several years later it was demonstrated that this protein has the unique capacity of inhibiting apoptosis (Tsujimoto *et al*., 1985a, b; Reed *et al*., 1987; Korsmeyer *et al*., 1990; Reed, 1994). Bcl-2 is in fact the mammalian homologue of the *C. elegans* protein Ced9, the first described endogenous inhibitor of programmed cell death. The inhibition of apoptosis by Bcl-2 is not limited to hematopoietic cells and the oncogene inhibits apoptosis triggered by a variety of death signals (Sentman *et al*., 1991). A major, still partially unsolved, puzzle is the subcellular distribution of Bcl-2 and the role, if any, of its heterogeneous localization. Bcl-2 has been detected in association with the outer mitochondrial membrane, with the ER, and with the nucleus, and a cytoplasmic form of Bcl-2 is also known to exist. Two distinct Bcl-2 proteins have been identified, p26-Bcl-2-alpha and p22-Bcl-2-beta. The former associates with cellular membranes, while the latter, lacking the membrane association domain, does not (Tanaka *et al*., 1993). It has been suggested that the distribution of p26-Bcl-2-alpha in membranes renders it capable of participating in the formation of multiprotein complexes (Krajewski *et al*., 1993) and, accordingly, inhibition of apoptosis by Bcl-2 has been suggested to be due to its capacity to interact with other anti- or proapoptotic proteins such as Bcl-X_L, Bax, procaspase-8, and p28-Bap31 (Oltvai *et al*., 1993; Antonsson *et al*., 1997; Nomura *et al*., 1999; Mikhailov *et al*., 2001). Although most investigators concur with the idea that only Bcl-2 bound to membranes is involved in inhibiting cell death, the mechanism, importance, and role of Bcl-2 in different cellular locations is still a matter of controversy. Bcl-2 mutants exclusively sorted to the ER are able to inhibit apoptosis in some experimental models, but not in others. Serum starvation induces apoptosis in MDCK cells expressing Bcl-2 targeted to the ER but not to the mitochondria, while, on the contrary, the protein located to the ER is more efficient in protecting from apoptosis Rat-1/myc cells. In Rat-1 fibroblasts, Bcl-2 targeted to the ER inhibits Myc- but not etoposide-induced apoptosis (Zhu *et al*., 1996; Lee *et al*., 1999). Overall, it seems fair to conclude at this stage that the presence of Bcl-2 in organelles as different as the mitochondria, ER, and nucleus (Lithgow *et al*., 1994) suggests that this oncogene may possess different mechanisms of action, possibly including the perturbation of Ca^{2+} homeostasis, the topic of this review. An effect on cellular ion homeostasis is suggested by the observation that Bcl-2 forms ion channels of limited cation selectivity when added to lipid bilayers, thus potentially interfering with ion fluxes in organelles (Minn *et al*., 1997; Schendel *et al*., 1998).

To further complicate the matter, in the last decade, it has been demonstrated that Bcl-2 is the prototype of a large protein family. Some members (Bcl-X_L, McL-1, Bfl-1, Bcl-w, A1) have an inhibitory effect on apoptosis, while others (Bax, Bad, Bid, Bak, Bok, Bik/NBK, Hrk), on the contrary, promote cell death (Farrow and Brown, 1996; Kroemer, 1997; Rinkenberger and Korsmeyer, 1997; for a review see Borner, 2003). Similar to Bcl-2, also the other pro- and antiapoptotic proteins of this family have a heterogeneous and complex subcellular distribution that varies after activation of the apoptotic pathways. Although most of the available evidence associates Bcl-2 to Ca^{2+} homeostasis, recent work has also highlighted a role for other members of the protein family, and it will be covered in this review.

The Ca^{2+} ballet

Given the importance of Ca^{2+} signaling in controlling so many different cellular functions, including cell death, it is not surprising that, once the role of Bcl-2 as a key antiapoptotic protein was established, many groups started to investigate whether the overexpression of this oncogene was affecting Ca^{2+} handling. A first clear indication of an effect of Bcl-2 on ER Ca^{2+} homeostasis was obtained by Distelhorst and co-workers, who showed a reduction in the thapsigargin-induced efflux of Ca^{2+} from the ER in stably expressing clones of WEHI7.2 lymphoma cells (Lam *et al*., 1994). The complete scenario was unraveled by the groups of Rizzuto and Krause, who using different experimental tools (targeted aequorins and GFP-based Ca^{2+} indicators) directly measured the Ca^{2+} concentration in the ER of cells transiently expressing Bcl-2. These investigators could show that Bcl-2 overexpression in HeLa (Pinton *et al*., 2000) or HEK-293 cells (Foyouzi-Youssefi *et al*., 2000) causes a reduction of the $[Ca^{2+}]_{ER}$, and thus of the amount of agonist-releasable Ca^{2+} pool (Figure 1). This effect was not due to a direct effect on the level or activity of the SERCA pump, or to a reduction of the resting $[Ca^{2+}]_c$, but rather to an increase in the Ca^{2+} leak across the ER membrane (Pinton *et al*.,

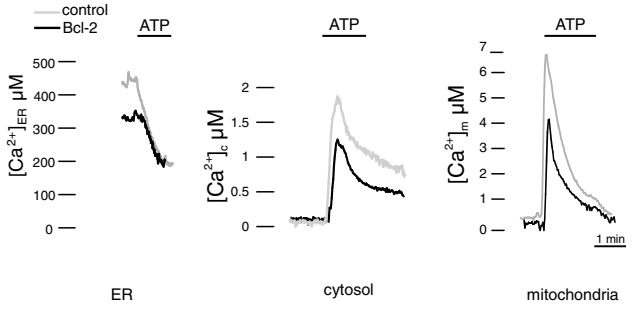

Figure 1 Effect of Bcl-2 overexpression on ER, cytosolic, and mitochondrial Ca^{2+} homeostasis in HeLa cells. Where indicated, the cells were stimulated with the IP3-generating agonist ATP. Gray and black traces correspond to control and Bcl-2 transfected cells, respectively

2000). In transiently Bcl-2-overexpressing HeLa cells, the steady-state $[Ca^{2+}]_{ER}$ is reduced by approx. 30%, and this in turn modified stimulus-dependent $[Ca^{2+}]$ increases both in the cytoplasm and mitochondria.

Further work proved that this alteration of intracellular Ca^{2+} homeostasis was not a side effect of Bcl-2 overexpression, but rather plays a key role in the antiapoptotic activity of the oncoprotein. The rationale of these experiments was that if the above-mentioned changes in the $[Ca^{2+}]_{ER}$ are important for the antiapoptotic function of the Bcl-2 protein, any maneuver that replicated them in the absence of Bcl-2 overexpression should reduce the sensitivity to apoptotic stimuli. In particular, it was predicted that agents acting through perturbation of mitochondrial structure and function should be inhibited by ER Ca^{2+} depletion, while, conversely, stimuli acting through other pathways should be insensitive to this parameter. A corollarium of this hypothesis was the prediction that an increased filling of the intracellular Ca^{2+} stores should potentiate the apoptotic effect. Indeed, stimulation with C_2-ceramide of HeLa cells having normal $[Ca^{2+}]_{ER}$ levels induced a prolonged increase in $[Ca^{2+}]_m$ that was maintained much longer than for a typical physiological challenge. This was followed by dramatic alterations in the morphology of mitochondria, that is, swelling of the organelle and rupture of the mitochondrial network. If, however, $[Ca^{2+}]_{ER}$ was reduced by a variety of experimental approaches (incubation at lower extracellular $[Ca^{2+}]$, partial inhibition of the ER Ca^{2+} ATPase or overexpression of the plasma membrane Ca^{2+} ATPase), there was a marked delay in the apoptotic death and no mitochondrial modifications occurred (Pinton *et al*., 2001). On the contrary, a stimulus such as CD95/Fas/APO-1 ligand, that in the cell model employed (HeLa cells) utilizes another signaling pathway, was not affected either by depletion of ER Ca^{2+} or Bcl-2 overexpression (Ferrari *et al*., manuscript in preparation).

This observation is in line with the idea (see below) that mitochondria are key players in apoptosis triggered by some, but not all, stimuli (Green and Reed, 1998; Bernardi, 1999; Vieira and Kroemer, 1999; Duchen, 2000). Indeed, according to some authors, the apoptotic process is preceded by collapse of the mitochondrial potential, opening of a multiprotein structure named permeability transition pore (PTP), swelling of the matrix and rupture of the outer membrane with ensuing changes in the permeability of the outer mitochondrial membrane, and release of apoptogenic factors from mitochondria (Petronilli *et al*., 1994; Skulachev, 1996; Bernardi *et al*., 1998; Petit *et al*., 1998); see below.

Scorrano and co-workers very recently provided further evidence in favor of the hypothesis that Ca^{2+} movement from the ER to mitochondria is a key process in the activation of apoptosis by some stimuli. These authors showed that mouse embryonic fibroblasts deficient of the two proapoptotic proteins Bax and Bak (double knockout, DKO, cells) are, as expected, markedly resistant to a variety of apoptotic stimuli, and have a much reduced Ca^{2+} concentration in the ER. If the ER Ca^{2+} levels are restored by recombinantly overexpressing the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), not only mitochondrial Ca^{2+} uptake in response to stimulation is re-established, but also the cells regain sensitivity to apoptotic stimuli such as arachidonic acid, C_2-ceramide, and oxidative stress. These results are in keeping with previous work by Ma and co-workers, demonstrating that SERCA overexpression in Cos cells causes ER Ca^{2+} overload and increases spontaneous apoptosis (Ma *et al*., 1999). In their work, Scorrano and co-workers also showed that re-expression of Bax induced an almost complete recovery of $[Ca^{2+}]_{ER}$ levels.

The work of Scorrano and co-workers further demonstrated that another group of apoptotic stimuli (staurosporine and etoposide) are partially insensitive to these alterations in the levels of ER Ca^{2+}, while they require the presence of proapoptotic proteins on the mitochondrial membrane. Thus, Bax specifically targeted to the outer mitochondrial membrane did not induce any change in $[Ca^{2+}]_{ER}$, but it made cells sensitive to apoptosis induced by the BH3-only protein tBID (Scorrano *et al*., 2003).

The overall picture that emerges from these contributions is that Ca^{2+} release from the ER and its uptake into mitochondria is pivotal in triggering apoptotic signals, and that one of the mechanisms through which overexpression of antiapoptotic proteins (or ablation of proapoptotic ones) counteracts cell death is the reduction in the amount of available Ca^{2+} in the ER. The amount of releasable Ca^{2+} – rather than the Ca^{2+} concentration of the ER – seems to be the relevant parameter for the transduction of the death signal, as it controls the 'amplitude' of the signal reaching mitochondria. In agreement with this notion, overexpression of calreticulin (an abundant luminal ER Ca^{2+} buffer) does not raise $[Ca^{2+}]_{ER}$, but increases the amount of releasable Ca^{2+}; in such a case, cell survival is drastically reduced upon C_2-ceramide treatment (Pinton *et al*., 2001). This result well matches the observation that cell lines derived from calreticulin knockouts are more resistant to apoptosis, indicating that the crucial requirement is the amount of Ca^{2+} released and not $[Ca^{2+}]_{ER}$ (Nakamura *et al*., 2000).

A recent paper, however, contrasts with this general scheme. Nutt *et al*. showed that, in PC-3 human prostatic adenocarcinoma cells, adenoviral gene delivery of Bax or Bak induced a strong reduction in the $[Ca^{2+}]_{ER}$ and that overexpression of Bcl-2 prevented this decrease. Surprisingly, Nutt *et al*. also showed that, in spite of the $[Ca^{2+}]_{ER}$ reduction, mitochondrial Ca^{2+} accumulation was enhanced in Bax and, more limitedly, in Bak-overexpressing cells. Mitochondrial Ca^{2+} accumulation was blocked by treating the cells with an inhibitor of mitochondrial Ca^{2+} uptake RU-360 that also reduced cytochrome *c* release and DNA fragmentation. It is difficult to reconcile these two sets of observations, especially the effects on $[Ca^{2+}]_{ER}$ (Nutt *et al*., 2002). At the moment, we can only speculate about the reasons for these discrepancies. First of all,

the experiments were carried out in different cell types, and, in the case of Nutt and co-workers, the subcellular localization of the overexpressed proteins was not verified. For example, it can be argued that a high expression level of the proteins in mitochondria could significantly alter their function, and thus reduce the ATP supply to the SERCA and hence ER Ca^{2+} accumulation. In our opinion, another way to reconcile the data of Nutt *et al*. with those of the previously mentioned groups will be that the effect of Bax and Bak overexpression on ER $[Ca^{2+}]$ is the consequence and not the cause of their activation of cell death. In other words, once Bax and Bak are overexpressed, they initiate the apoptosis signaling pathway, and the ER depletion is only a late event in the process leading to cell death.

Mitochondrial crossroads

The discovery that mitochondria, in addition to their well-characterized life-supporting processes, are also involved in death-promoting actions inevitably caught the attention of several investigators. This has led to a flood of observations that place this organelle either in the limelight of cell death events or merely as a minor contributor to the complex apoptosis play (see the review by Green in this volume). The key event in mitochondrial apoptotic signaling is the release of normal constituents (such as cytochrome *c* and AIF) into the cytosol, where they act as caspase cofactors and potentiate the apoptotic proteolytic cascade. The mechanism of this release is still controversial, as both permeabilization of the outer mitochondrial membrane by extramitochondrial factors and the opening of the PTP, with ensuing organelle swelling, have been proposed to take place. In these phenomena, mitochondrial Ca^{2+} signals have been proposed to intervene at different steps that will now be discussed.

A critical difference between the 'apoptotic' Ca^{2+} signals and the physiological responses to the numerous agonists coupled to Ca^{2+} is in the impact on mitochondrial structure and function. This became particularly evident in the elegant work of Hajnoczky and coworkers, who showed that, in hepatocytes, a subthreshold dose of the lipid mediator of apoptosis ceramide becomes effective when applied together with a physiological IP_3-coupled stimulus. In this case, the typical, transient mitochondrial Ca^{2+} response is converted into a sustained rise that causes the opening of PTP, the large-amplitude swelling of mitochondria, and the release of cytochrome *c* (Szalai *et al*., 1999). Along these lines, ceramide was shown in HeLa cells to cause ER Ca^{2+} release, mitochondrial Ca^{2+} loading and fragmentation, and swelling of the mitochondrial network (Pinton *et al*., 2001). Overexpression not only of VDAC, the voltage-dependent anion channel of the outer mitochondrial membrane (Rapizzi *et al*., 2002), but also of proapoptotic members of the Bcl-2 family, such as tBid (Csordas *et al*., 2002), enhances mitochondrial Ca^{2+} uptake and in turn sensitizes the cells to apoptotic stimuli. Thus, the expression level of this mitochondrial outer membrane protein (that, interestingly, is upregulated in apoptosis-sensitive cells) (Voehringer *et al*., 2000), as well as mitochondrial translocation of the proapoptotic Bcl-2 family member may allow to tune organelle Ca^{2+} signaling and control the final cellular outcome.

As to the molecular machinery decoding the Ca^{2+} rise into organelle structural alteration, much information has been acquired in the past few years. The first, obvious route proposed in many papers is the thoroughly investigated process of mitochondrial permeability transition. This process is characterized by massive swelling of the organelle (described in isolated mitochondria (Crompton *et al*., 2002), but see also De Giorgi *et al*., 2002) and, importantly, the rupture of the OMM (Petit *et al*., 1998). The presumed basis for this process is a multiprotein complex that transverses the intermembrane space; the putative essential components of this complex are VDAC in the OMM, the adenine nucleotide translocase (ANT) in the IMM, and cyclophilin-D in the mitochondrial matrix. The partial and transient PTP opening has been suggested to drain the proton gradient in order to protect mitochondria from hyperpolarization and increased ROS production (Skarka and Ostadal, 2002). In contrast, complete and irreversible PTP opening (induced, for example, by Ca^{2+} or ROS) renders the mitochondria permeable to larger molecules, leading to a complete loss of potential and respiratory function of the mitochondrial membrane (for a review, see Crompton *et al*., 2002). However, the active role of PTP opening in inducing apoptosis has been questioned by the fact that Ca^{2+}-induced permeability transition can occur in Bax/Bak-deficient, nonapoptotic cells (Scorrano *et al*., 2002). An alternative, interesting possibility is that PTP serves as an anchor to target these molecules to the OMM, close to contact sites where OMM permeabilization occurs (De Giorgi *et al*., 2002).

The elegant work of Youle and co-workers has revealed an alternative mechanism for the structural alterations of mitochondria, which directly impinges on the recently identified molecules that coordinately control the three-dimensional structure of mitochondria. They include membrane-bound GFPases (homologous to the fzo protein of Drosophila) that induce mitochondrial fusion (now identified in mammals and denominated mitofusins) and molecules inducing fission of the network, the dynamin-related proteins (drp) (Yoon and McNiven, 2001). Youle and coworkers showed that translocation of drp1 to mitochondria during apoptosis causes fragmentation of the mitochondrial network into punctate organelles, loss of membrane potential, and release of cytochrome *c* (Frank *et al*., 2001). Inhibition of this process (e.g. by dominant-negative mutants of drp1) blocks cell death. As to the trigger, the same authors showed that, early in apoptosis, Bax translocates to 'fission' sites where elements of the fission/fusion machinery are clustered (Karbowski *et al*., 2002) and initiates the fragmentation process. Regarding the role of Ca^{2+} signals, Shore and co-workers showed that Ca^{2+} release from the ER and

subsequent mitochondrial Ca^{2+} accumulation precede mitochondrial fragmentation, and the translocation of drp1 to mitochondria, that is, the induction of mitochondrial fragmentation, is strictly Ca^{2+} dependent (Breckenridge *et al*., 2003). In the model proposed, this mitochondrial pathway serves as an amplification loop to augment caspase-8-mediated apoptosis.

Other lines of evidence support alternative players on the apoptotic scene, that are also involved in mitochondrial physiological functions. The ATP dependency of apoptosis is a well-known paradigm; thus, it is perhaps not surprising that both the respiratory chain (McClintock *et al*., 2002) and the ATP synthase itself (Shchepina *et al*., 2002) have been given central roles in the apoptotic process. More importantly, the incidental lack of an electron acceptor in the respiratory chain leads to the formation of oxygen radicals and other ROS (Liu *et al*., 2002; Staniek *et al*., 2002), the accumulation of which has been shown to precede or coincide with cell death. One important effect of ROS is the peroxidation of lipids, including cardiolipin which is a fundamental constituent of the IMM. Interestingly, it has been shown that cardiolipin can also be present in the OMM, near IMM–OMM contact sites; its presence and peroxidation could affect the pore-forming ability of Bax (reviewed in Newmeyer and Ferguson-Miller, 2003). Since these processes are Ca^{2+} activated, it should again be emphasized that mitochondrial Ca^{2+} accumulation may amplify the apoptotic process in this way.

In summary, there is accumulating evidence that mitochondrial Ca^{2+} uptake promotes apoptosis in different ways, making this organelle a key component of the Ca^{2+}-regulated amplification loop of apoptosis.

Not so minor supporting players

Important as mitochondria may be, the role of Ca^{2+} in the control of the apoptotic process is by no means limited to these organelles. Indeed, the cytoplasm possesses effectors that can efficiently decode an extracellular signal into the induction of apoptosis in a Ca^{2+}-dependent manner. Multiple signaling cascades – critical for cell survival, differentiation, or degeneration – are mediated by $[Ca^{2+}]_c$ (Pozzan *et al*., 1994; Berridge *et al*., 2000). The signaling process in all these phenomena is dependent on the concerted activities of many intracellular factors, including protein kinases, phospholipases, proteases and endonucleases, often occurring in multiple isoforms, and the coordinate regulation of these factors plays a fundamental role in decoding the extracellular signal into the ultimate cellular event. This molecular machinery exhibits a large complexity and partial redundancy (most of the elements occur in different isoforms, with specific recruitment routes and substrate specificities), and the overall picture is far from being clarified. Thus, a detailed evaluation of the role of the various cytosolic Ca^{2+} effectors in apoptosis would be too lengthy to be included in a short review and, at the same time, largely incomplete. We will just focus on highlighting the possible mechanisms of action of cytosolic Ca^{2+} effectors, by reviewing a few possible checkpoints that received much attention in the recent years.

The signaling cascade: kinases and phosphatases

Among the various kinases directly or indirectly activated by Ca^{2+} signals, the protein kinase C (PKC) family has been proposed to play an important role in the Ca^{2+}-mediated signaling of apoptosis. Indeed, these proteins form a large group of related kinases, which differ in biochemical properties (the 'classical' are activated by Ca^{2+} and DAG, whereas the other subtypes, the 'novel' and 'atypical' PKCs, are Ca^{2+}-insensitive, and are activated by either DAG or other lipid mediators) and cellular distribution (Mellor and Parker, 1998). Thus, they could both 'sense' the alteration in Ca^{2+} signals occurring in apoptosis, and/or the coincidence of other lipid-mediated signaling pathways. The large experimental work carried out on this topic supports a role for PKCs in apoptosis, although the picture is by no means univocal: experiments carried out in different tissues or cell types, where the signaling machinery is different, provide apparently contradictory results, with the same isoform exerting opposite effects, for example, potentiating or counteracting the efficacy of the same apoptotic stimulus (Liu *et al*., 2002).

In most experimental systems, the 'classical' isoforms (and, in particular, the alpha and betaII isozymes) have been proposed to be antiapoptotic. In particular, PKCα exhibits an antiapoptotic function and may function as a survival factor in some types of cells (Dempsey *et al*., 2000). Indirect support to this notion is provided by the observation that PKCα is often blocked during apoptotic processes (e.g. undergoing dephosphorylation after treatment with ceramide; Lee *et al*., 1996), and in some cases degraded by calpains, caspases, or both. Also, the 'atypical' PKCs ζ and λ have been suggested to inhibit apoptosis (Huang *et al*., 1996; Jamieson *et al*., 1999), but their recruitment pathway remains ill defined.

At the opposite end of the spectrum, the 'novel' PKCδ has been suggested to participate in the mitochondria-dependent apoptotic pathway triggered by different physico-chemical stimuli (Dempsey *et al*., 2000). A catalytically active fragment of PKC is generated by proteolysis in cells undergoing apoptosis in response to ionizing radiation, DNA-damaging drugs, and anti-Fas antibody (Emoto *et al*., 1995; Mizuno *et al*., 1997). As to the site of action, PKC was shown to translocate during apoptosis to mitochondria (Majumder *et al*., 2000), as well as to the Golgi complex (Emoto *et al*., 1995; Konishi *et al*., 1999), where it is activated through a tyrosine-phosphorylation step.

In the signaling routes of apoptosis, the Ca^{2+}-dependent phosphatases also appear to play an important role. In particular, various apoptotic routes share the activation of the Ca^{2+}-dependent serine–threonine phosphatase calcineurin through a process blocked by Bcl-2 (Shibasaki and McKeon, 1995). In this

case, defined intracellular targets of utmost importance in apoptosis have been identified: calcineurin dephosphorylates and activates the BH3-only protein BAD (a proapoptotic member of the Bcl-2 family), thus enhancing BAD heterodimerization with Bcl-X_L and promoting apoptosis (Wang *et al*., 1999).

The intracellular proteases

The most obvious direct link between $[Ca^{2+}]_c$ elevations and the proteolysis of cellular targets (the paradigm of apoptosis) is through the activation of the family of Ca^{2+}-dependent proteases, known as calpains. Calpains are cysteine proteases that are synthesized as inactive proenzymes, and are activated by autocatalytic cleavage triggered by Ca^{2+}. The family of proteins include isozymes with different distribution (including ubiquitous and tissue-specific isoforms) and Ca^{2+} affinity (ranging from micromolar, for the μ calpains, to millimolar, for the m-calpains, levels (Carafoli and Molinari, 1998)). Activation of calpains that can be triggered by various pathophysiological stimuli has a direct impact on the execution of apoptosis, as calpains have been shown to cleave key elements in the apoptotic machinery, such as members of the Bcl-2 family, for example, Bcl-X_L (Nakagawa and Yuan, 2000) or Bid (Mandic *et al*., 2002), caspase-12 (Nakagawa and Yuan, 2000), and the X-linked inhibitor of apoptosis (XIAP) (Kobayashi *et al*., 2002). Parenthetically, in monocyte/macrophage cells, Ca^{2+} signaling is involved in NF-κB activation through the activation of calpain. Calpain inhibitors may thus be effective in inhibiting the activation of latently infected HIV (Teranishi *et al*., 2003). An important role for calpains in the apoptotic process is also provided by human genetic disorders of skeletal muscle. The concentration of ubiquitous calpains increases in Duchenne muscular dystrophy, and null mutations of muscle-specific calpain (calpain 3) cause a form of limb-girdle muscular dystrophy (Tidball and Spencer, 2000), thus highlighting both the importance of these proteins in muscle cell death and their complex interplay.

At the same time, the main actors of the apoptotic proteolytic cascades, the caspases, have been recently drawn to the Ca^{2+} field. This relates to two different mechanisms. The first is the recent inclusion of components of the Ca^{2+} signaling machinery among the caspase targets. As a consequence, Ca^{2+} signaling patterns are altered early after caspase activation and contribute to either the control or the amplification/commitment of the apoptotic process. This novel, direct modulation of Ca^{2+} signals will be discussed more in detail later in this chapter. The second link between caspases and Ca^{2+} homeostasis has been provided by the demonstration of the Ca^{2+} sensitivity of a member of the caspase protease family, caspase-12. Caspase-12 is localized in the ER (Nakagawa *et al*., 2000) and has been reported to be activated when the ER undergoes stress (including disruption of ER Ca^{2+} homeostasis and accumulation of excess proteins in ER), but not by membrane- or mitochondrial-targeted apoptotic signals. Caspase-12 thus participates in the ER stress-induced apoptosis pathway (Yoneda *et al*., 2001). Mice deficient for caspase-12 do not undergo ER stress-induced apoptosis, but their cells are capable of undergoing apoptosis induced by other stimuli. Based on these observations, caspase-12 has been proposed to be involved in the degenerative disorders in which ER stress and dysregulation of Ca^{2+} homeostasis is more likely to occur, for example, Alzheimer's disease. Two key questions remain open. The first relates to the route for caspase-12 recruitment and its site of action. Recent data supports the concept that caspase-12 is located on the cytosolic side of the ER, and links to Ca^{2+} signaling through the activity of calpains that act as the real Ca^{2+} sensors and have caspase-12 among their primary targets (Nakagawa and Yuan, 2000). This interesting notion highlights a direct crosstalk between the two classes of cytosolic proteases (calpains and caspases) that could occur in a variety of apoptotic routes, and strengthens the possibility that Ca^{2+} signaling represents a pharmacological target of broad relevance in apoptosis. The second open issue refers to the relative importance of this 'Ca^{2+}-associated' caspase within the family, and requires the exact assessment of expression level and tissue distribution of the protein. In this respect, the recent report of null mutations in a wide variety of human genotypes raises the possibility that, in our species, caspase-12 plays little or no role (Fischer *et al*., 2002), and thus most of information inferred from murine studies cannot be extrapolated to humans.

Proteolysing the Ca^{2+} signaling machinery: an apoptotic strategy

Along with nuclear and cytoskeletal damage, disruption of cell signaling and ion homeostasis could warrant irreversibility of the cell's commitment to death. In this respect, it is not surprising that amplification loops also involve a pleiotropic signaling route, such as that mediated by Ca^{2+} ions, although only very recent work has clarified molecular targets and cellular consequences.

Various components of the Ca^{2+} signaling machinery have been described to be cleaved by caspases, with potentially different cellular consequences. IP_3 receptor type 1 (IP_3R1) has been identified as a caspase-3 substrate. Caspase-3-dependent IP_3R1 cleavage results in the inhibition of IP_3-induced Ca^{2+} release activity. Given that Ca^{2+} release may act as a potentiation loop of apoptosis (Hirota *et al*., 1999), such an effect could represent a negative feedback mechanism. Along the same lines, Ca^{2+}-permeable glutamate receptors of the AMPA subtype have also been described to be a target of caspase in neuronal apoptosis and Alzheimer's disease (Chan and Mattson, 1999). Their inactivation would avoid excitotoxicity and Ca^{2+} overload in neurons destined to apoptosis (Glazner *et al*., 2000).

More recently, caspase-dependent cleavage of plasma membrane Ca^{2+} ATPase (PMCA), the most effective

route allowing the rapid return of $[Ca^{2+}]_c$ to basal levels (Camello *et al*., 1996; Brini *et al*., 2000), has also been described (Schwab *et al*., 2002); both the neuron-specific PMCA2 and the ubiquitous PMCA4 isoforms are cleaved by caspases. While PMCA2 is cleaved *in vivo* following brain ischemia and in neurons undergoing apoptosis after excitotoxic stimulation, PMCA4 is cleaved in non-neuronal cells induced to apoptosis by staurosporine. As a consequence, PMCA cleavage results in loss of function and aberrant intracellular Ca^{2+} transients (Schwab *et al*., 2002). Along the same lines, preliminary results have shown that the Na^+/Ca^{2+} transporter (NCX) type 1 is also cleaved by caspase-3 in cerebellar granule neurons undergoing apoptosis (Daniele Bano and Pierluigi Nicotera, personal communication). Our own recent work revealed a similar mechanism in a radically different model of cell death, that is, that triggered in hepatic cells by the expression of Hepatitis B virus X protein (HBx). Elevations of $[Ca^{2+}]_c$ signals in cells overexpressing HBx trigger caspase-3-dependent PMCA4 cleavage and inactivation (Chami *et al*., manuscript in preparation). Amplification of the cytosolic Ca^{2+} signals through the impairment of Ca^{2+} pumps is not entirely surprising, if one takes into account the functional properties of the Ca^{2+} release and uptake mechanisms. Indeed, while an increased Ca^{2+} filling of intracellular stores does not enhance Ca^{2+} release, due to the Ca^{2+} feedback inhibition on the IP_3R, impairment of PMCA is highly effective at increasing $[Ca^{2+}]_c$ (Brini *et al*., 2000). As to the functional consequences, this alteration of Ca^{2+} signaling may represent a powerful potentiation loop, facilitating the rapid commitment of cells to death.

Kalzium, ist das alles?…

Up to now, cellular Ca^{2+} overload has been considered as a final common pathway of cell death. Recent findings suggest that alterations in Ca^{2+} could act as a relevant amplification loop of the death signal. An integrative view of the links between Ca^{2+} homeostasis and apoptosis is presented in Figure 2. On the left panel, a standard Ca^{2+} signaling pathway, through IP_3-generating stimuli, promotes $[Ca^{2+}]_c$ increases, and ultimately stimulates mitochondrial metabolism and energy production. Against this background, the role of Ca^{2+} in the apoptotic process is outlined on the right. The main parameter linking Ca^{2+} homeostasis to apoptosis is the increase of cytosolic levels of Ca^{2+}. The action of pro- and antiapoptotic Bcl-2 family members can play different roles in modulating this increase; specifically, by acting on the ER Ca^{2+} contents, Bax and Bcl-2 enhance or buffer, respectively, the strength of an apoptotic signal. The action of agents such as ceramide or staurosporine also modulates $[Ca^{2+}]_c$ but, as described in the preceding sections, may also act downstream, in particular on mitochondria, that seem to be the true crossroads of the signals that commit cells to inevitable death. In the nearby future, strict criteria in the definition of the apoptotic process, allied to emerging techniques that allow single-cell Ca^{2+} analyses, should help to elucidate and define more clearly the exact role that this ubiquitous ion plays in one of the cell's truly vital processes.

Abbreviations

AIF, apoptosis-inducing factor; $[Ca^{2+}]_c$, cytosolic calcium concentration; $[Ca^{2+}]_m$, mitochondrial calcium concentration; $[Ca^{2+}]_{ER}$, calcium concentration in the endoplasmic reticulum;

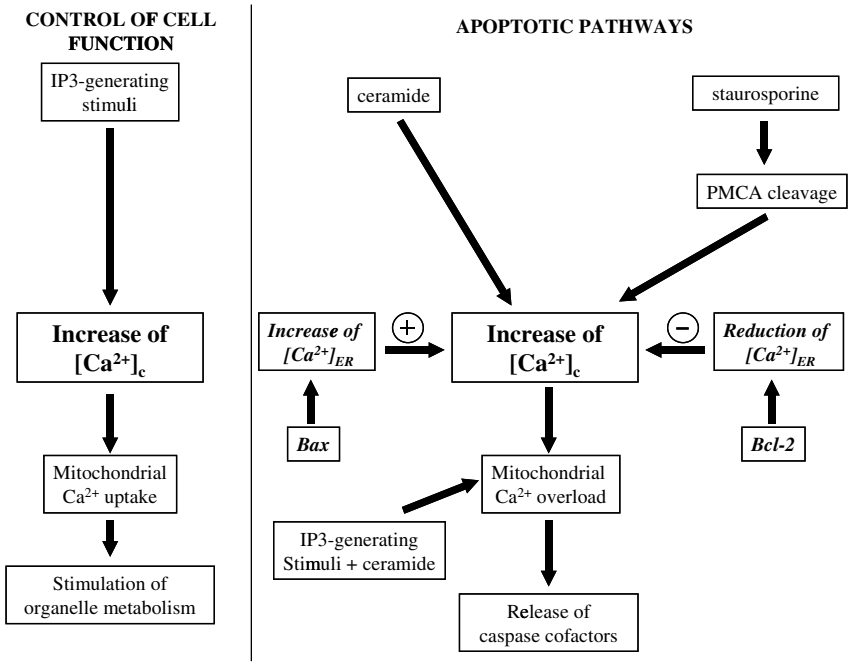

Figure 2 Differential decoding of Ca^{2+}-linked stimuli evoking cell activation or apoptosis

$[Ca^{2+}]_{Golgi}$, calcium concentration in the Golgi apparatus; DAG, diacylglycerol; drp, dynamin-like protein; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; IP_3, inositol 1,4,5 trisphosphate; IP_3R, inositol 1,4,5 trisphosphate receptor; OMM, outer mitochondrial membrane; PTP, permeability transition pore; ROS, reactive oxygen species; SERCA, sarco–endoplasmic reticulum Ca^{2+} adenosine triphosphatase.

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