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Endoplasmic reticulum, Bcl-2 and Ca^{2+} handling in apoptosis

D. Ferrari,[1,a] P. Pinton,[1,a] G. Szabadkai,[1] M. Chami,[1] M. Campanella,[1] T. Pozzan,[2] R. Rizzuto[1]

[1]Department of Experimental and Diagnostic Medicine, Section of General Pathology, Telethon Center for Cell Imaging (TCCI) and Interdisciplinary Center for the Study of Inflammation (ICSI), University of Ferrara, Via Borsari 46, I-44100 Ferrara, Italy
[2]Department of Biomedical Sciences and CNR Center for the Study of Biomembranes, University of Padova, Via Colombo 3, I-35121 Padova, Italy

Summary In the complex signalling interplay that allows extracellular signals to be decoded into activation of apoptotic cell death, Ca^{2+} plays a significant role. This is supported not only by evidence linking alterations in Ca^{2+} homeostasis to the triggering of apoptotic (and in some cases necrotic) cell death, but also by recent data indicating that a key anti-apoptotic protein, Bcl-2, has a direct effect on ER Ca^{2+} handling. We will briefly summarise the first aspect, and describe in more detail these new data, demonstrating that (i) Bcl-2 reduces the state of filling of the ER Ca^{2+} store and (ii) this Ca^{2+} signalling alteration renders the cells less sensitive to apoptotic stimuli. Overall, these results suggest that calcium homeostasis may represent a pharmacological target in the fundamental pathological process of apoptosis.

A ROLE FOR Ca^{2+} IN NECROTIC CELL DEATH

Ca^{2+} signalling is responsible for the regulation or modification of virtually all processes in healthy cells [1]. Thus, it is not surprising that changes in the Ca^{2+} concentration of the cytoplasm ($[Ca^{2+}]_c$) as well as in different organelles have a causal role in cell death induced by different means. A large $[Ca^{2+}]_c$ elevation initiates a number of self-destructive cellular pathways among which the most important are: (i) activation of catabolic enzymes, (ii) production of free radicals, and (iii) derangement of structure/impairment of function of different organelles. Given the complexity of Ca^{2+} signalling, in the last 30 years tremendous effort has been made to distinguish between Ca^{2+} signal disturbances secondary to cell injury and primary processes including perturbed Ca^{2+} signal leading to cell regression and death. The most intensively investigated models of necrosis, liver, heart and brain ischemia/reperfusion-induced cell injury [2], equally include Ca^{2+} as a fundamental player and there is now general consensus on the fact that cellular Ca^{2+} overload is responsible for further events leading to decomposition of cell integrity.

Ca^{2+} overload originates from (i) Ca^{2+} influx from the extracellular space, either through damaged plasma membrane such as for toxin-induced death of hepatocytes [2], or by sustained activation of ligand gated ion channels such as the acetylcholine (AChR) or *N*-methyl-D-aspartate (NMDA) type glutamate receptors [3,4]; (ii) release of Ca^{2+} from the intracellular stores; or (iii) impairment of Ca^{2+} extrusion through the plasma membrane.

The point of no return in cell necrosis appears to depend on the loss of function of mitochondria, most probably due to opening of the permeability transition pore (PTP). Mitochondrial Ca^{2+} accumulation leads to irreversible PTP opening followed by depolarisation [5], ATP loss and reactive oxygen intermediates (ROIs) generation [6]. Conditions such as collapse of the ATP levels favour release of stored Ca^{2+} and, in addition, impair extrusion of the ion from the cell, contributing to the increase of $[Ca^{2+}]_c$. Oxidative stress renders Ca^{2+} overload even larger, since it further increases Ca^{2+} influx and release from the ER and inhibits the Ca^{2+} extrusion mechanisms [7].

Several studies disclosed a number of intracellular Ca^{2+} targets. Ca^{2+}-mediated activation of the calpain cystein proteases, catalyses, for example, the proteolysis of cytoskeletal and membrane-associated proteins leading to

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[a]The first two authors equally contributed to this work.

Correspondence to: Dr Rosario Rizzuto, Department of Experimental and Diagnostic Medicine, Section of General Pathology, Telethon Center for Cell Imaging (TCCI) and Interdisciplinary Center for the Study of Inflammation (ICSI), University of Ferrara, Via Borsari 46, I-44100 Ferrara, Italy; e-mail: r.rizzuto@unife.it

severe cell damage [8]. The detrimental effect of calpains is strengthened by overproduction of ROIs. High $[Ca^{2+}]_c$ also activates different phospholipase A_2 isoforms that, in addition to direct damage of cell membranes, induce the production of arachidonic acid, the increased catabolism of which represents a significant source of ROIs [9]. Moreover, Ca^{2+}-calmodulin-dependent activation of nitric oxide synthase (NOS) induces production of NO that reacting with superoxide leads to the formation of the highly toxic compound peroxynitrate with an exacerbation of the damaging loop [10]. Ca^{2+} is also responsible for the activation of DNAses with consequent breakage of nuclear DNA [11].

A ROLE FOR Ca^{2+} IN APOPTOTIC CELL DEATH

Several lines of evidence support the view that alterations of the intracellular Ca^{2+} homeostasis are also important in apoptosis (for recent reviews on apoptosis see [12–14]). Increases of $[Ca^{2+}]_c$ can be observed during both the early and late phases of apoptosis in neurons, thymus and T cells, upon serum withdrawal, treatment with staurosporine or exposure to cadmium [15–17]. The action of Ca^{2+} on neuronal survival appears to be complex. Small and controlled increases in $[Ca^{2+}]_c$ have been shown to have beneficial effects on neurons, promoting survival in vitro [18]. A further demonstration of the positive effect of Ca^{2+} on cell survival comes from experiments in which inhibition by ethanol of calcium signalling through glutamatergic Ca^{2+} channels (that blocks NMDA receptor-dependent Ca^{2+} signalling) causes massive neuronal apoptosis during brain development [19]. On the other hand, progressive $[Ca^{2+}]_c$ waves can take part in the signalling cascade triggering or executing apoptotic cell death. In cortical neurons, apoptosis can be induced by activation of NMDA receptors by low agonist levels [20]. Ca^{2+} overload induced by ionophores has been shown to induce apoptosis in neurons as well as in prostatic cancer cells [21]. In HeLa cells, ceramide-induced apoptosis is also accompanied by progressive $[Ca^{2+}]_c$ increase [22]. Further support for a role of Ca^{2+} in apoptosis comes from experiments showing that chelation of cytosolic Ca^{2+} either by BAPTA [23] or by overexpressing the cytosolic Ca^{2+} buffering protein calbindin-D28K [24] protects from apoptosis.

Surprisingly, numerous targets that are well-known mediators of Ca^{2+} action in physiological conditions become, through cooperation with specific effectors, essential in the control of the apoptotic process. Mitochondrial Ca^{2+} uptake during cell stimulation by activating the Ca^{2+}-dependent dehydrogenases of the Krebs cycle [25] finely tunes mitochondrial ATP production according to cellular needs. However, if the increase of mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) triggers PTP opening [26], it results in swelling of mitochondria, rupture of the mitochondrial outer membrane and release of apoptotic factors, such as cytochrome *c*, apoptosis inducing factor (AIF), pro-casp-9, Smac/DIABLO as well as endonuclease G [13,27–30].

In this context, the main Ca^{2+}-regulated targets identified in the cytosol are: (i) calcineurin (PPA2 phosphatase), (ii) protein kinase C (PKC), (iii) caspases, and (iv) calpains (cysteine proteases).

(i) The Ca^{2+}-calmodulin-dependent serine-threonine phosphatase activity of calcineurin, was shown to be involved in apoptosis in several cell systems [31,32]. Calcineurin dephosphorylates BAD (a proapoptotic member of the Bcl-2 family, see below), enhancing its heterodimerisation with Bcl-X_L and promoting apoptosis [33]. However, it should be mentioned that in another experimental model activation of the Ca^{2+}-calmodulin-dependent protein kinase kinase (PKK) activates protein kinase B, which in turn phosphorylates BAD and protects cells from death [34].

(ii) The different Ca^{2+}-dependent PKC isoforms (i.e. the "classical" PKCα and β and the "novel" PKCδ) are also good examples of the ambiguous effects of Ca^{2+} in apoptosis, since each type may have pro- or anti-apoptotic effect (for review see [14]). It is worthy to note that during apoptosis both PKCα and PKCδ translocate to mitochondria, but while the δ isoform induces cytochrome *c* release and subsequent caspase activation [35], PKCα phosphorylates Bcl-2 in mitochondria and suppresses apoptosis [36].

(iii) Ca^{2+} changes can also modulate caspase activation and function. Casp-9 is released from mitochondria during Ca^{2+}-mediated permeability transition [13]. Moreover, Ca^{2+} induces casp-3 activation [37]. ER stress (e.g. after treatment with brefeldin-A, tunicamycin or thapsigargin) is directly connected to the activation of pro-casp-12 in a Ca^{2+}-dependent way [38].

(iv) Interestingly, the Ca^{2+}-activated family of cystein proteases, calpains, has been recognised not only to be involved in necrotic, but also in apoptotic cell death in neurons and immune cells, even if caspase activation is not required for the cell to die [8]. Calpains could confer Ca^{2+} sensitivity to caspases, e.g. leading to proteolytic activation of pro-casp-12 [38].

ER STRESS AND APOPTOSIS

Apart from the central role of the ER in cellular Ca^{2+} signalling [39] this compartment provides the site for folding and processing of newly synthesised membrane and secreted proteins. The importance of this compartment for proper cell function is indicated by the observation that under conditions associated with ER dysfunction (i.e.

disturbance of ER Ca^{2+} homeostasis or impairment of the folding and processing reactions), two highly conserved stress responses are activated, the ER-overload response (EOR) reviewed in [40], and the unfolded-protein response (UPR, reviewed in [41]). Prolonged ER stress leads to cell death and is linked to the pathogenesis of some neurodegenerative disorders including ischemia, Alzheimer's and Parkinson's diseases [42].

Major cross-talk exists between the UPR and Ca^{2+} signalling in the ER. Bip/GRP78 prevents apoptosis induced by the Ca^{2+} ionophore ionomycin by modulating the glutamate-triggered mobilisation of ER Ca^{2+} [43]. Conversely, suppression of Bip/GRP78 expression causes an increase in cell death induced by Ca^{2+} depletion in the ER [44]. The calcium-binding chaperone calreticulin is also induced by UPR and its overexpression leads to sensitisation to apoptosis [45].

The importance of ER in the modulation of apoptosis is not limited to the consequences of severe conditions of organelle stress. Indeed, early observations already pointed to the involvement of the ER Ca^{2+} pool in apoptosis, showing that alterations of this pool are sufficient to induce apoptosis [46]. It has been shown that Ca^{2+} released by the ER can sensitise cells to ceramide-induced apoptosis [47]. The involvement of inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) of the ER membrane in sensitising cells to apoptotic stimuli is also supported by work carried out in transgenic mice and by antisense techniques. $InsP_3R1$-deficient lymphocytes are resistant to a large panel of apoptosis inducers [48]. Moreover, antisense oligonucleotide-mediated downregulation of $InsP_3R3$ decreases cell death in glucocorticoid-treated T cells [49]. Similarly, pharmacological agents such as thapsigargin and cyclopiazonic acid which induce $[Ca^{2+}]_c$ increase by fully emptying the ER Ca^{2+} stores, have been shown to induce apoptosis in a wide variety of cell types [50,51].

It is now clear that changes in the steady-state ER Ca^{2+} level itself have a significant influence on the apoptotic pathways. However, this issue appears to be extremely complicated, owing to the complexity of processes connected to ER Ca^{2+} homeostasis. Modulation and deregulation of ER proteins is considered a powerful tool in the understanding of $[Ca^{2+}]_{er}$ participation in apoptosis, but still there is no consensus on the basic question, i.e. what is the relationship between $[Ca^{2+}]_{er}$ and cellular sensitivity to apoptogenic factors.

Bcl-2 AN ONCOGENE WITH MULTIPLE FUNCTIONS

The oncogene Bcl-2 (B-cell lymphoma/leukaemia-2) is translocated in most follicular non-Hodgkin's B-cell lymphomas, with consequent overexpression of the protein [52] which confers increased cell survival by blocking apoptosis [53]. Although the mechanism by which Bcl-2 inhibits apoptosis has been the focus of intense investigation, it is still not completely elucidated. Different findings suggest that Bcl-2 may not only inhibit the release of cytochrome *c* from mitochondria [54] but also, thank to its localisation to other intracellular organelles [55] act through different, but equally important, mechanisms. While the putative role in modulating ER Ca^{2+} levels will be discussed in greater detail in the following paragraph, we here briefly summarise its action on mitochondria. Susin et al. reported that in isolated mitochondria, overexpression of Bcl-2 prevented apoptosis by inhibiting the release of an apoptosis-inducing factor (AIF) [56]. Interaction of Bcl-2 with Bax seems to be important for its activity, and in fact Bcl-2 and Bax can be immunoprecipitated as a complex. Using genetic gain- and loss-of-function models for Bcl-2 and Bax, Knudson and Korsmeyer showed that apoptosis and thymic hypoplasia, that are characteristic features of Bcl-2-deficient mice, are largely absent in mice also deficient in Bax. These authors also suggested that, although an *in vivo* competition exists between Bax and Bcl-2, each factor is able to regulate apoptosis independently [57].

INVOLVEMENT OF Bcl-2 IN Ca^{2+} HOMEOSTASIS

It has been shown that Bcl-2 can act as an ion channel in isolated lipid bilayers [58]. Thus, Bcl-2 could alter ion homeostasis of the intracellular organelles where it putatively localises. This possibility is supported by the observation that recombinant expression of Bcl-2 reduces the state of filling of intracellular Ca^{2+} stores and alters the kinetics and amplitudes of cellular Ca^{2+} responses [59–62]. We directly investigated the role of Bcl-2 in Ca^{2+} homeostasis of different cell compartments: cytosol, ER, Golgi and mitochondria by using targeted aequorin chimeras [63].

Fig. 1 shows that in Bcl-2-overexpressing cells stimulated with ATP (which acts through P2Y purinergic receptors inducing the production of $InsP_3$ and thus the release of stored Ca^{2+}) the cytosolic and mitochondrial $[Ca^{2+}]$ rises were markedly smaller. This reduction was due to a lower $[Ca^{2+}]$ of the ER and Golgi lumina (i.e. the agonist-sensitive Ca^{2+} stores) as measured by the targeted aequorin probes. Krause et al. obtained very similar results by using a different approach, i.e. a GFP-based Ca^{2+} probe targeted to the ER [62]. On the other hand, Ca^{2+} overload in the ER causes apoptosis, as demonstrated in SERCA-overexpressing cells [64]. Bcl-2 affects the ER Ca^{2+} handling by increasing the passive leak of the ion across the ER membrane without changing the Ca^{2+} uptake capacity of SERCA pumps [61,62].

In principle, a reduction in the steady state $[Ca^{2+}]_{er}$ level due to Bcl-2 overexpression should activate capacitative

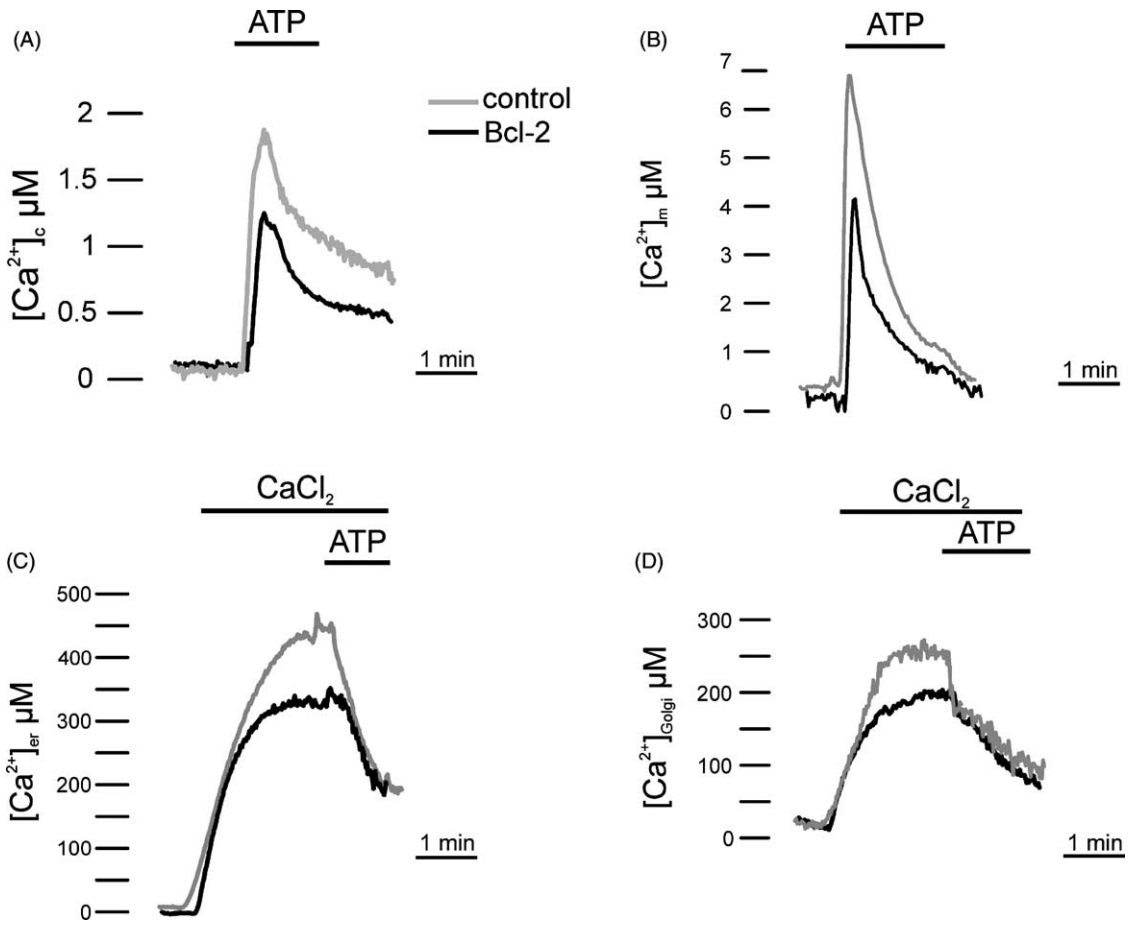

Fig. 1 Intracellular Ca^{2+} homeostasis in different intracellular compartments in control and Bcl-2-overexpressing HeLa cells. The traces show representative $[Ca^{2+}]$ measurements performed in the cell cytoplasm (A), mitochondria (B), endoplasmic reticulum (C), and Golgi apparatus (D). Grey: control cells; black: Bcl-2-overexpressing cells. HeLa cells were cotransfected with different aequorin chimeras (cytAEQ, mtAEQ, erAEQ or GoAEQ). Ca^{2+} measurements were carried out 36 h after transfection and calibrated into $[Ca^{2+}]$ values as elsewhere described [73]. Cells were perfused with Krebs–Ringer saline solution and challenged, where indicated, with 100 µM ATP.

Ca^{2+} influx [65]. In contrast, in our studies resting cytosolic Ca^{2+} levels were always indistinguishable in control and Bcl-2-overexpressing cells, as measured by the fluorescent indicator fura-2. This apparent contradiction was reconciled by the demonstration that Bcl-2 also caused a downregulation of the capacitative Ca^{2+} influx, possibly by an adaptive mechanism to the long lasting reduction in steady state $[Ca^{2+}]_{er}$. Indeed, the same phenomenon was observed after a long-term reduction of $[Ca^{2+}]_{er}$, obtained independently of Bcl-2 overexpression, i.e. by a prolonged incubation of cells in the presence of a low extracellular $[Ca^{2+}]$. The reduction of this ubiquitous Ca^{2+} influx pathway may prevent potentially dangerous Ca^{2+} overload. On the other hand, the pharmacological agent thapsigargin, a SERCA inhibitor that induces a drastic $[Ca^{2+}]_{er}$ depletion has been shown to activate programmed cell death [50]. This is not in conflict with our results because, in contrast to Bcl-2, which induces a small and long lasting drop in $[Ca^{2+}]_{er}$, thapsigargin produces a complete and acute Ca^{2+} depletion (interfering with the basic activity of ER chaperonins) and a strong activation of the capacitative Ca^{2+} pathway, which conversely is downregulated in Bcl-2 transfected cells. In other words, the treatment by thapsigargin may mimic cell stress, causing apoptosis, whereas Bcl-2 overexpression could cause a long-term anti-apoptotic adaptation of Ca^{2+} signalling.

Although these results are suggestive of a possible and interesting link between the alteration in Ca^{2+} signalling and the anti-apoptotic activity of Bcl-2, they had to be validated by checking whether Ca^{2+} modifications observed in Bcl-2-overexpressing cells were able to prevent cell death triggered by an apoptotic stimulus, such as ceramide, an endogenous lipid mediator of apoptosis, which is sensitive to Bcl-2 inhibition [22]. For this purpose we mimicked/antagonised the $[Ca^{2+}]$ changes caused by Bcl-2 using different experimental approaches and

Table 1

Experimental conditions	$[Ca^{2+}]_{er}$ (μM)	Percent of living cells after C_2 ceramide treatment
Normal extracellular $[Ca^{2+}]$ (1 mM)	310 ± 87	9 ± 4
Low extracellular $[Ca^{2+}]$ (40 μM)	91 ± 10	42 ± 9
10 μM tBuBHQ in 1 mM extracellular $[Ca^{2+}]$	80 ± 29	56 ± 8

verified that the $[Ca^{2+}]_{er}$ levels inversely correlated with the efficacy of this apoptotic stimulus. As shown in Table 1, in C_2 ceramide-treated HeLa cells, 16 h after the addition of ceramide, approximately 90% of the cell population died by apoptosis and all conditions that decreased the steady state $[Ca^{2+}]_{er}$ levels reduced the percentage of dead cells. To mimic the decrease in $[Ca^{2+}]_{er}$ due to Bcl-2 overexpression we used different experimental approaches. In the simplest one, HeLa cells were maintained in a saline solution supplemented with a lower Ca^{2+} concentration, a condition that is known to cause a reduction in steady state $[Ca^{2+}]_{er}$ levels. Interestingly, an extracellular $[Ca^{2+}]$ of ≈50 μM that caused a partial emptying of ER Ca^{2+} (i.e. an effect similar to that of Bcl-2) markedly increased cell survival upon ceramide treatment. A very similar result was obtained without altering the extracellular $[Ca^{2+}]$, by treating cells with different concentrations of a specific SERCA blocker, *tert*-butyl-benzohydroquinone (tBuBHQ) [66] which causes a reduction of $[Ca^{2+}]_{er}$ proportional to the concentration employed.

Table 1 shows that for reductions in $[Ca^{2+}]_{er}$, comparable to those caused by the "protective" extracellular $[Ca^{2+}]$ described above, that were obtained with the application of 10 μM tBuBHQ, cell survival upon ceramide treatment was significantly increased.

The same results were obtained by a "molecular approach", i.e. by recombinantly expressing Ca^{2+} transporters. Overexpression of the plasma membrane Ca^{2+} pump (PMCA), that causes a ≈20% reduction of $[Ca^{2+}]_{er}$ [67] was associated to reduced susceptibility to ceramide-induced death, thus confirming that a reduction in $[Ca^{2+}]_{er}$ levels under normal values decreases the cytotoxic effect of ceramide.

In agreement with this concept, $[Ca^{2+}]_{er}$ overload leads to enhanced sensitivity of cells to ceramide. Indeed, overexpression of the ER Ca^{2+} pump (SERCA), that causes about 25% increase in the $[Ca^{2+}]_{er}$ [67] was associated to a higher mortality induced by ceramide, thus indicating that an increase in $[Ca^{2+}]_{er}$ levels above normal values potentiates the effect of the pro-apoptotic mediator.

Finally, overexpression of calreticulin, i.e. the main Ca^{2+} buffering protein of the ER lumen, allowed us to establish whether reduction in $[Ca^{2+}]_{er}$ and protection from ceramide-induced cell death were due to events occurring in the ER environment or to changes in the amount of Ca^{2+} released toward the cytosol. Calreticulin overexpression enhanced the amplitude and duration of the cytosolic Ca^{2+} signals from the ER, without increasing $[Ca^{2+}]_{er}$ [68]. In calreticulin-overexpressing cells, viability was significantly reduced after ceramide addition. In agreement with this observation, it has been reported that cell lines derived from calreticulin knockouts are more resistant to apoptosis [69]. We thus concluded that protection by experimental manouvers acting on Ca^{2+} homeostasis (that we believe mimic the effect of Bcl-2) depends on the reduction of the releasable ER Ca^{2+} pool, rather than on Ca^{2+}-dependent luminal processes.

We then addressed the mechanisms that allow this signalling alteration to be protective, and observed that ceramide has direct effects on intracellular Ca^{2+} homeostasis. C_2 ceramide (but not its non apoptotic analogue di-hydroceramide) induced a $[Ca^{2+}]_c$ rise by releasing Ca^{2+} from intracellular stores and by activating capacitative Ca^{2+} entry pathway. These phenomena caused prolonged mitochondrial Ca^{2+} accumulation, in turn responsible for dramatic alterations in the organelle morphology, i.e. swelling and fragmentation (Fig. 2). We verified whether alterations in the mitochondrial structure could be prevented by mimicking Bcl-2 action on calcium homeostasis. To this purpose two different experimental approaches were used: (i) incubation of cells in solutions with lower $[Ca^{2+}]$ (as in the experiments described above) or (ii) loading of the Ca^{2+} chelator BAPTA into the cells. Both conditions, protecting mitochondria from Ca^{2+} overload avoided the structural mitochondrial damage.

A role of ER Ca^{2+} in the control of apoptosis is supported also by other lines of evidence. Kim et al. showed that in hepatoma cells, TNF-induced apoptosis is dependent on Ca^{2+} release from the ER. Moreover, Bcl-2 expression decreased Ca^{2+} release from the ER and blocked TNF-induced apoptosis [70]. The importance of $[Ca^{2+}]_{er}$ in determining cell fate is supported by the observation that calsenilin, a protein interacting with presenilin 1 and 2, which are located in the ER and Golgi apparatus, enhances cell death induced by thapsigargin by increasing the release of Ca^{2+} from the intracellular Ca^{2+} stores [71].

Overall, the currently available evidence strongly suggests that ER Ca^{2+} depletion caused by Bcl-2 overexpression is not a side effect of the oncoprotein, but has a fundamental role in its anti-apoptotic function. More

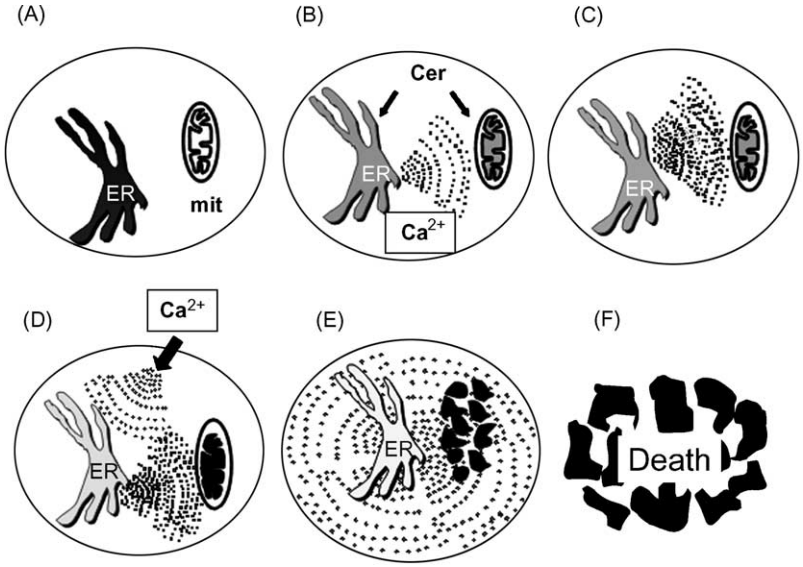

Fig. 2 Effect of ceramide on intracellular Ca^{2+} homeostasis. Ceramide acts on intracellular Ca^{2+} homeostasis by inducing a leak of stored Ca^{2+} from the ER (panel B and C). Depletion of the ER Ca^{2+} content activates capacitative calcium influx (panel D) with further $[Ca^{2+}]_c$ increase and mitochondrial Ca^{2+} overload (panel D). In turn this causes major morphological alterations of mitochondria, and thus release of pro-apoptotic factors to the cytoplasm (panel E). The cell eventually dies (panel F).

recently, the importance of mitochondrial Ca^{2+} uptake in apoptosis comes also from the demonstration that tcBid (a proapoptotic protein of the Bcl-2 family), increases the mitochondrial Ca^{2+} signal after $InsP_3$-associated stimulation, by a selective permeabilisation of the outer mitochondrial membrane [72].

CONCLUSIONS

Taken together, these findings indicate that ER, via specific components of its luminal environment or by interactions among ER, mitochondria, and other signalling pathways, may play an important role in the modulation of cell sensitivity toward apoptosis. The involvement of Ca^{2+} in cell life and death is highly complex and not yet completely understood. There is no doubt that dramatic derangements in cellular Ca^{2+} handling are incompatible with cell survival, but evidence has been accumulating in the last few years indicating that more subtle changes in the processes controlling Ca^{2+} homeostasis can have profound effects onto the decision between life and death. It is a challenge for the next future to understand the molecular mechanisms that are at the basis of these phenomena.

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