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A role for calcium in Bcl-2 action?

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Abstract

Changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) translate a variety of extracellular signals into widely diverse intracellular effects, ranging from secretion to movement, proliferation and also cell death. As regards the last one, it has long been known that large $[Ca^{2+}]_c$ increases lead cells to death. More recently, experimental evidence has been obtained that the oncogene Bcl-2 reduces the state of filling of intracellular Ca^{2+} stores and thus affects the Ca^{2+} responses induced by physiological and pathological stimuli. In this contribution, we will discuss this effect and its significance for the mechanism of action of Bcl-2, an important checkpoint of the apoptotic process.

Keywords: Bcl-2; Calcium; Apoptosis; Intracellular stores; Cell signaling

1. Calcium, mitochondria and apoptosis

Calcium is an important second messenger that can stimulate complex cellular functions, such as contraction, secretion, fertilization, proliferation and metabolism. Transient elevations of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) indeed activate a number of calcium-dependent enzymes including protein kinases, dehydrogenases, phospholipases, nitric oxide synthase, proteases and endonucleases [1,2].

As to cell death, Wyllie and colleagues demonstrated that treatment with the calcium–magnesium ionophore A23187 induced chromatin condensation in murine lymphoid cell lines and rat thymocytes [3]. Now, wide experimental evidence supports the notion that prolonged and unregulated $[Ca^{2+}]_c$ elevations are deleterious to a cell and can lead to apoptosis (or necrosis) [4].

Switch from the control of physiological functions to the death program may involve alterations in the spatio-temporal pattern of the calcium signal or changes at the level of effectors activated by calcium, such as cytosolic proteins (e.g. calpain, calcineurin) or organelles (e.g. mitochondria, endoplasmic reticulum).

In this review we will analyze: 1) the effect of the oncogene Bcl-2 on calcium signaling and its relevance for the antiapototic activity of the protein, and 2) the possible involvement of mitochondria in decoding a calcium-mediated apoptotic signal.

2. Bcl-2: an anti-apoptotic factor with multiple proposed mechanisms

The B-cell lymphoma/leukaemia-2 gene (Bcl-2) has been associated to B-cell malignancies [5,6] as in most follicular non-Hodgkin's B-cell lymphomas this oncogene is translocated from 18q21 to the immunoglobulin heavy-chain locus (14q32) with consequent enhanced production of *bcl-2* mRNA and overexpression of the protein [7–9]. The oncogenic potential of Bcl-2 was demonstrated by gene transfer experiments [10] and it became evident that Bcl-2 has the peculiar capacity of increasing cell survival. In 1988, it was shown that Bcl-2 was able to prolong cell survival in IL-3-dependent cells [11] by its ability to block apoptosis [12,13]. Studies performed on different cells showed that

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inhibition of apoptosis by Bcl-2 was not exclusively detected in cells of hemopoietic origin as Bcl-2 inhibited multiple forms of apoptosis [14].

Lichnovsky and coworkers reported that Bcl-2 is expressed in many organs of the human embryo, including the gastrointestinal tract, mesenchymal cells surrounding primitive bronchial epithelium, cells of the metanephronic blasteme and ureteric buds, pointing to a role of this gene in the development of human tissues [15]. Bcl-2 seems to be important also for turn-over of intestinal cells, as its expression exhibits in the gut a decreasing gradient from crypt to villus, in the ileum but not in the colon [16]. Bax/Bcl-2 ratio is significantly increased during development in nigral dopamine neurons undergoing apoptosis in rats [17] and Bcl-2 seems to play a role not only in the developing nervous system but also in protecting neurons from various insults [18].

Human *bcl-2* transcripts generate two proteins, p26 Bcl-2-alpha and p22 Bcl-2-beta, having in common the first 196 NH_2-terminal amino acids; p26 Bcl-2-alpha associates with cellular membranes while p22 lacks the transmembrane domain [19]. Despite some early observations on localization of Bcl-2 in the inner mitochondrial membrane [13], recent data showed that Bcl-2 is associated to the outer mitochondrial membrane, nuclear envelope and endoplasmic reticulum (ER) membrane, although it was also detected in the cytoplasm [20]. The non-uniform distribution of p26-Bcl-2-alpha in intracellular membranes suggested that Bcl-2 participates in protein complexes perhaps involved in transport [21].

It was originally suggested that Bcl-2 protected cells from apoptosis by interfering with the respiratory chain but Jacobson and coworkers reported that cells devoid of mitochondrial DNA and thus lacking a functional respiratory chain, were still susceptible to apoptosis, and overexpression of Bcl-2 still inhibited the process [22]. Susin and coworkers reported that in isolated mitochondria, overexpression of Bcl-2 prevented apoptosis by inhibiting the release of an apoptosis-inducing factor (AIF) [23]. Overexpression of Bcl-2 also prevents release of cytochrome *c* from the mitochondrial intermembrane space during apoptosis [24,25]. Inhibition of cytochrome *c* translocation to the cytoplasm blocks 'apoptosome' formation and caspase activation, although it has also been shown that upon down-regulation of *bcl-2* by antisense oligonucleotides, Fas-induced cytocrome *c* release and caspase activation are still possible [26]. According to several observations, inhibition of apoptosis by Bcl-2 seems to be related to its capacity to interact with other anti- or pro-apoptotic proteins such as Bcl-X_L, Bax, procasp-8 and p28 Bap31. Interaction within mitochondria between Bcl-2 and the proapoptotic factor Bax has also been shown in living cells [27,28]. Nomura and coworkers found that in isolated mitochondria, Bax translocation was triggered by cytosolic extracts from apoptotic cells and inhibited by the presence of Bcl-2 [29], while Mikhailov and coworkers found that the protective effects of Bcl-2 did not require Bcl-2/Bax association. Therefore, they suggested that Bcl-2 might protect cells from apoptosis by preventing Bax oligomerization in the outer mitochondrial membrane [30] and this would be in agreement with the capacity of Bcl-2 to block Bax channel formation in lipid membranes [31].

Apoptotic agents can decrease Bcl-2 expression, Sawada and coworkers reported that exposure of cells to etoposide or C_2 and C_6 ceramides induced Bcl-2 down-regulation, leading to an increase in the Bax/Bcl-2 ratio and subsequent activation of caspases -9 and -3 [32].

Involvement of Bcl-2 in apoptosis-induced acidification has also been hypothesized. Thangaraju and coworkers reported that Bcl-2 did not inhibit apoptosis triggered by direct acidification but it was effective in counteracting the cytoplasmic protein tyrosine phosphatase SHP-1, which is required for acidification-dependent apoptosis consequent for example to Fas ligation [33].

It has also been hypothesized that Bcl-2 protects cells from apoptosis by inhibiting the import of the apoptogenic protein p53 into the nucleus. Froesh and coworkers showed that Bcl-2 inhibited apoptosis mediated by p53 and also interfered with p53 target genes and this effect required the presence of the Bcl-2 anchoring domain [34]. However, Bcl-2 could also prevent apoptosis by acting on the ER function. Serum starvation induced 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 was more efficient in protecting from apoptosis Rat-1/myc cells. Lee and coworkers reported that in Rat-1 fibroblasts, Bcl-2 targeted to the ER was able to inhibit Myc- but not etoposide-induced apoptosis [35].

Overexpression of Bcl-2 protein has been reported to increase glutathione concentration and protect cells from death induced by oxidative stress. Schor and collegues reported that the importance of Bcl-2 on glutathione metabolism is cell line-dependent. Overexpression of Bcl-2 in PC12 but not in MCF-7 cells is associated with glutathione accumulation after treatment with *N*-acetylcysteine [36]. McCullough and coworkers reported that overexpression of gadd153/chop sensitizes cells to ER stress, with the ensuing down-regulation of Bcl-2 expression, depletion of cellular glutathione, and enhanced production of reactive oxygen species, while restoration of Bcl-2 expression in gadd153-overexpressing cells led to replenishment of glutathione and reduced reactive oxygen levels, thus protecting cells from ER stress-induced cell death [37].

3. Bcl-2 and calcium

The specific localization of Bcl-2 in the membranes of ER and mitochondria [38], organelles playing a key role in intracellular Ca^{2+} homeostasis [39,40], suggests that the latter process could be a target of the action of this oncogene. Several experimental observations support this possibility.

First, Bcl-2 family members have been shown to insert in lipid bilayer and act as low selectivity ion channels [41,42]. Second, the ER localization of Bcl-2 appears necessary for its activity. Indeed, the importance of the ER localization of Bcl-2 is confirmed by the observation that a Bcl-2 mutant exclusively sorted to the ER, is able to inhibit apoptosis in some experimental models [35,43]. Third, the measurement of cytosolic calcium concentration ($[Ca^{2+}]_c$) and/or release of $^{45}Ca^{2+}$ from the ER allowed a Bcl-2-dependent alteration of ER Ca^{2+} handling to be demonstrated [44–46].

To obtain further insight into these events we have utilized the recombinant aequorin approach developed in our laboratory [47–50] that allows us to measure specifically the $[Ca^{2+}]$ within defined intracellular compartments (e.g. ER, mitochondria and cytosol). In transiently transfected HeLa cells, we have observed that Bcl-2 overexpression reduced by about 30% the state of filling of intracellular Ca^{2+} stores (ER and Golgi apparatus) and consequently the stimulus-dependent $[Ca^{2+}]$ increases in the cytoplasm and in the mitochondria, potential targets for apoptogenic effects of Ca^{2+}. The decrease in the steady state $[Ca^{2+}]_{er}$ was not due to a decreased rate of Ca^{2+} accumulation (due to either a direct effect on the activity of the SERCA pump or a reduction of the resting $[Ca^{2+}]_c$) but rather to an increase in the Ca^{2+} leak across the ER membrane [51]. Interestingly, similar results (partial ER depletion and increased Ca^{2+} leak in Bcl-2 overexpressing cells) were obtained by Krause and coworkers using an ER-targeted Ca^{2+}-sensitive GFP in HEK-293 cells [52].

In agreement with these results, it has been demonstrated that SERCA overexpression and the subsequent ER Ca^{2+} overload increases spontaneous apoptosis [53]. Bcl-2 overexpression also down-regulates capacitative Ca^{2+} influx, probably as an adaptive consequence of the prolonged reduction in the $[Ca^{2+}]_{er}$ steady state [51], avoiding the deleterious effects of a prolonged $[Ca^{2+}]_c$ increase.

These data are only apparently in contrast with previous reports showing that inhibition of SERCA by thapsigargin, tBuBHQ or cyclopiazonic acid is followed by apoptosis [54]. Ca^{2+} depletion following these treatments is complete and rapid, while in Bcl-2 transfected cells, the drop in $[Ca^{2+}]_{er}$ is modest and develops slowly. A drastic reduction in the level of $[Ca^{2+}]_{er}$ might interfere with a number of processes occurring in the ER and activate a cellular stress response affecting, for example, the regulation of ER protein folding and chaperone interactions. Conversely, a partial $[Ca^{2+}]_{er}$ reduction can significantly affect Ca^{2+} signaling with minor effects on ER structure and function [55].

Is the Ca^{2+} depletion induced by Bcl-2 a side effect of Bcl-2 overexpression or is it part of its mechanism of action? We have investigated this issue in a more recent work in which we demonstrated that $[Ca^{2+}]_{er}$ is a key determinant of the susceptibility to ceramide-induced apoptosis. Indeed we have mimicked the Bcl-2 effect on $[Ca^{2+}]_{er}$ by different pharmacological and molecular approaches, and observed that all conditions that lowered $[Ca^{2+}]_{er}$ (at values similar to those of Bcl-2 overexpressing cells) protected cells from ceramide, a Bcl-2-sensitive apoptotic stimulus, while treatments that increased $[Ca^{2+}]_{er}$ had the opposite effect [56].

But does the correlation between $[Ca^{2+}]_{er}$ and susceptibility to apoptotic cell death depend on events occurring in the ER lumen, given that $[Ca^{2+}]_{er}$ regulates processes occurring in the organelle, including activation of resident caspases [57], or does a lower $[Ca^{2+}]_{er}$ (by reducing the Ca^{2+} flow across the channels of the ER membrane), reduce the amplitude of the Ca^{2+} responses elicited by physiological and/or pathological stimuli? In other words, is the relevant parameter the luminal Ca^{2+} concentration or rather the amount of Ca^{2+} that can be released toward the cytosol (and mitochondria)?

Several observations indicate that the latter is the case. We have observed that overexpression of calreticulin, the luminal ER Ca^{2+} buffer (which does not raise $[Ca^{2+}]_{er}$ but increases the amount of releasable Ca^{2+}), drastically reduces cell survival upon ceramide treatment [56]. This result is in agreement with the observation that cell lines derived from calreticulin knock-outs are more resistant to apoptosis [58], indicating that the crucial requirement is the amount of released Ca^{2+} and not $[Ca^{2+}]_{er}$ [59–61].

We also observed that ceramide causes a progressive Ca^{2+} release from intracellular stores, with a rise in $[Ca^{2+}]_c$ that was maintained by activation of the capacitative Ca^{2+} influx [56]. Bcl-2 reduced both the release of Ca^{2+} from the ER (by lowering $[Ca^{2+}]_{er}$) and Ca^{2+} influx (by down-regulating the capacitative current), and this could contribute to protecting cells from deleterious $[Ca^{2+}]_c$ increases as schematically depicted in the model of Fig. 1.

The targets of a Ca^{2+}-mediated apoptotic signal could be various [62]; they include Ca^{2+}-activated cytosolic proteins such as calpains, protein kinase C or calcineurin. However, obvious candidates are also mitochondria, given that key events occurring in the mitochondrial matrix, such as the stimulation of ATP production [63] and possibly the opening of the permeability transition pore (PTP) [64], are regulated by variations of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$. Interestingly, ceramide treatment induced a prolonged increase in $[Ca^{2+}]_m$ that was maintained for much longer than for a typical physiological challenge, and this was followed by dramatic alterations in mitochondrial morphology [56].

All these results indicate that ER Ca^{2+} depletion caused by Bcl-2 overexpression is an integral part of the anti-apoptotic program set in action by this oncoprotein and suggest that mitochondria could be the target of this Ca^{2+}-mediated apoptotic signal.

4. Mitochondrial Ca^{2+} accumulation and apoptosis

The demonstration that mitochondria, early in the death pathway, release procaspases (2, 3 and 9) and caspase co-factors, such as cytochrome *c* and AIF, has highlighted

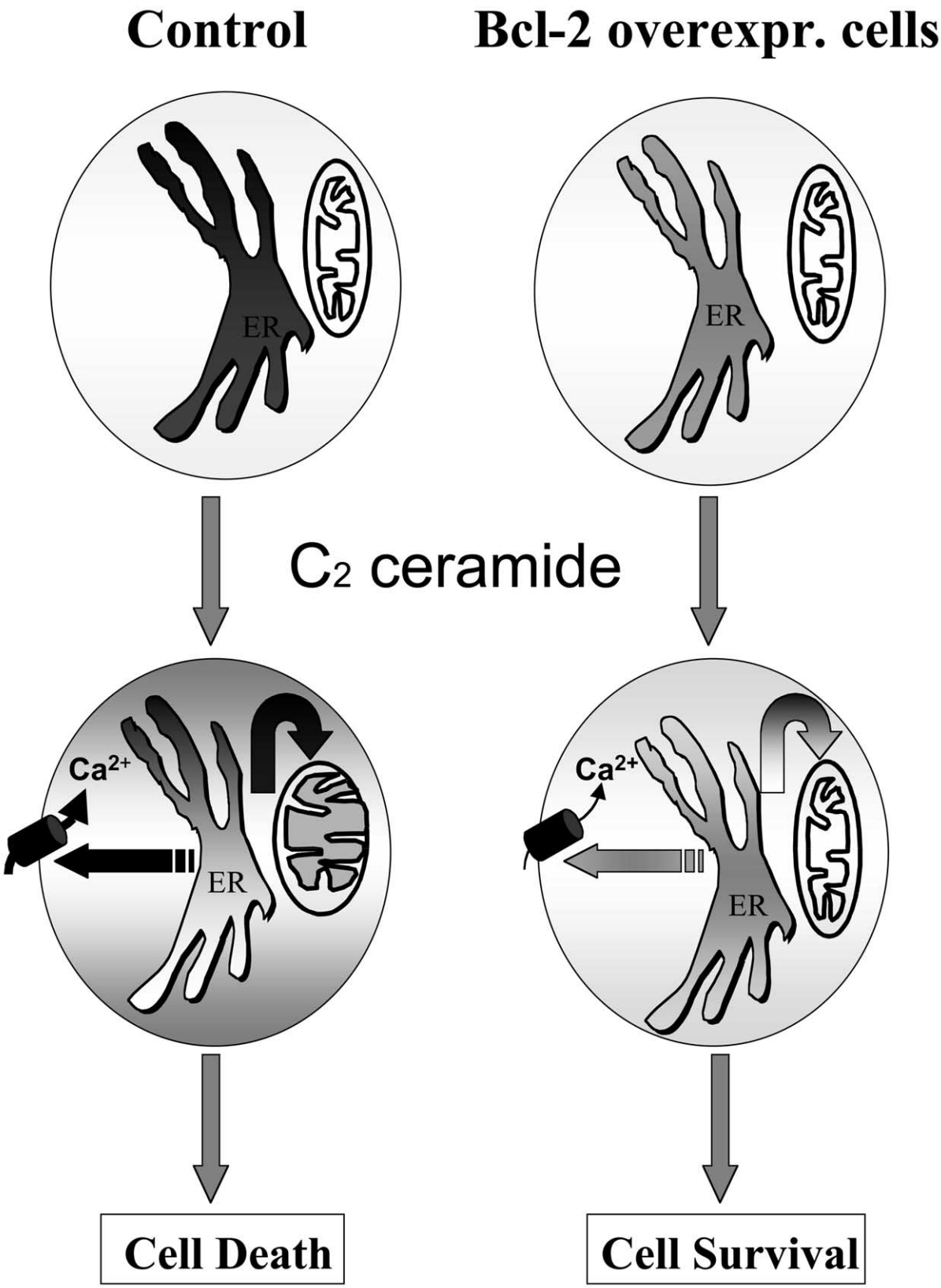

Fig. 1. Effects of Bcl-2 on the ER Ca^{2+} levels and C_2 ceramide-mediated toxicity. Overexpression of Bcl-2 induces a decrease in the [Ca^{2+}] of the ER. Stimulation with C_2 ceramide triggers in Bcl-2-overexpressing cells a smaller release of calcium from ER and a reduced capacitative Ca^{2+} influx, with low repercussion on mitochondrial physiology and intracellular Ca^{2+}-modulated processes. On the contrary C_2 ceramide induces a large Ca^{2+} release from the ER of control cells with a dramatic effect on mitochondria that might induce the release of caspase cofactors. This effect, possibly combined to the activation of cytoplasmic effectors, could be an important trigger of the apoptotic program.

the central role played by these organelles in apoptosis. As to the mechanism of this release, it is still controversial, since both a regulated transport across the outer membrane [65] and a major organelle swelling, with ensuing rupture of the outer membrane [66], have been proposed to occur. In the latter case, a key role is supposed to be played by the PTP, a high-conductance unselective channel, the molecular nature and regulatory mechanisms of which are still undefined [66,67]. Its opening/closing characteristics are influenced by multiple parameters, such as the redox state of critical matrix thiols, the $NAD(P)H_2/NAD(P)$ ratio, the matrix pH, and the concentration of Ca^{2+} in the mitochondrial matrix ($[Ca^{2+}]_m$) [68,69]. There is also evidence that PTP might be a target for the regulatory effects of the Bcl-2 family proteins and that release of proapoptotic factors in the cytosol may occur without the rupture of the mitochondrial outer membrane [65,70].

In this scenario, it is thus conceivable that mitochondrial Ca^{2+} uptake during the apoptotic process could trigger the opening of PTP, and thus the release of pro-apototic factors. On the other hand, it is largely accepted today that mitochondrial Ca^{2+} accumulation is triggered by a broad variety of physiological stimuli, and regulates in turn organelle (e.g. activation of metabolism) and cytosolic (e.g. the diffusion of Ca^{2+} waves) functions [71–73]. Thus, not differently from the mitochondrial pro-apoptotic factors (e.g. cytochrome *c*), Ca^{2+} uptake in the organelle may serve a dual function, i.e. either activate essential processes in the life of the cell or trigger its own death. The narrow line between these radically different effects is directly demonstrated by a series of experiments by Hajnoczky and coworkers, showing that in cells pretreated with pro-apoptotic stimuli, inositol-1,4,5-triphosphate (IP_3) receptor-mediated mitochondrial Ca^{2+} uptake caused depolarization of the inner membrane due to the opening of PTP [56]. As a consequence cytochrome *c* is released leading to activation of caspases. In these experiments cells are briefly exposed to pro-apoptotic agents, and apoptosis occurs only if rises in $[Ca^{2+}]_m$ are coincident with the exposure. The absence of necrotic cell death is probably due to the fact that PTP opening is transient and followed by restoration of mitochondrial ATP production. Thus, IP_3-mediated $[Ca^{2+}]$ signals can serve as an efficient and selective activator of apoptosis.

Numerous other pieces of evidence support a role for mitochondrial Ca^{2+} accumulation in triggering apoptosis. In the central nervous system, massive Ca^{2+} influx occurs in response to a number of pathophysiological events, such as ischemia/reperfusion injury, exposure to some toxins, excessive stimulation of *N*-methyl-*D*-aspartate (NMDA) receptors in glutamate-induced neurotoxicity [74–77]. Although some aspects of the cell death signaling process may be related to cytosolic events directly stimulated by Ca^{2+}, there is a growing appreciation that toxicity in certain circumstances is highly dependent upon Ca^{2+}-mediated alteration of mitochondrial function. Recent studies have implicated mitochondrial Ca^{2+} uptake as a necessary event in glutamate toxicity in primary cultures of neurons. Budd and Nicholls have blocked neuronal cell death by preventing mitochondrial Ca^{2+} sequestration. The authors induced mitochondria depolarization in cerebellar granule cells using rotenone, an inhibitor of complex I of the mitochondrial respiratory chain, and oligomycin. When the cells under these conditions are exposed to glutamate, they cannot sequester Ca^{2+} in the mitochondria, because the driving force for Ca^{2+} uptake is removed, and they are protected from cell death [76]. Stout and coworkers, who have investigated the role of mitochondrial calcium buffering in excitotoxic cell death of cultured rat forebrain neurons, used a similar approach. They prevented mitochondrial Ca^{2+} uptake by transient treatment of the cells with uncouplers during glutamate exposure. Although inhibition of mitochondrial Ca^{2+} sequestration enhances the $[Ca^{2+}]_c$ increase, it significantly reduces glutamate-stimulated neuronal cell death [78].

Kruman and Mattson suggested that mitochondrial Ca^{2+} uptake is a pivotal event that leads to collapse of mitochondrial membrane potential in neuronal cell apoptosis and necrosis. In their experiments PC12 cells were exposed to either staurosporine (STS) or aldehyde 4-hydroxynonenal (HNE), an apoptotic and necrotic insult, respectively. STS and HNE induced an early increase of $[Ca^{2+}]_c$ followed by increase of $[Ca^{2+}]_m$. Overexpression of Bcl-2 blocked the elevation of $[Ca^{2+}]_m$ in cells exposed to STS, whereas the cytoplasmic calcium chelator, BAPTA-AM, and the inhibitor of mitochondrial Ca^{2+} uptake, ruthenium red, prevented both apoptosis and necrosis. Moreover, the addition of cyclosporin A prevented both forms of cell death, indicating the critical role for membrane depolarization in both circumstances [79]. Recently, Schild and colleagues showed that, in contrast to other tissues, an increase in $[Ca^{2+}]_c$ into the low micromolar range is sufficient to induce the release of cytochrome *c* from brain mitochondria, while the opening of PTP requires relatively high micromolar $[Ca^{2+}]_c$. Furthermore, they provided evidence that this event is reversible and is caused by influx of Ca^{2+} into the mitochondrial matrix [80].

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