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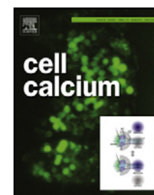

Review

Calcium and cell death mechanisms: A perspective from the cell death community

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ABSTRACT

Research during the past several decades has provided convincing evidence for a crucial role of the Ca^{2+} ion in cell signaling. Hence, intracellular Ca^{2+} transients have been implicated in most aspects of cell physiology, including gene transcription, cell cycle regulation and cell proliferation. Further, the Ca^{2+} ion has been found to also play an important role in cell death regulation. Thus, necrotic cell death was early associated with intracellular Ca^{2+} overload, and multiple functions in the apoptotic process have subsequently been found to be governed by Ca^{2+} signaling. More recently, other modes of cell death, notably anoikis and autophagic cell death, have been demonstrated to also be modulated by Ca^{2+} transients. Characteristics, interrelationship and mechanisms involved in Ca^{2+} regulation of these cell death modalities are discussed in this review.

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1. Introduction

Starting with the pioneering work by Kerr and colleagues [1], cell death studies have become an increasingly important area of biomedical research. In addition to apoptosis and necrosis, several other modes of cell death have now been described and characterized based on various morphological and biochemical criteria. In 2009, the Nomenclature Committee on Cell Death proposed unified criteria for the definition of twelve cell death modalities (four typical and eight atypical) [2]. Among the best characterized of these cell death modes are apoptosis, autophagy, cornification and necrosis. Until recently, a requirement for gene expression was documented only for apoptotic and autophagic cell death. Cornification is a special form of programmed cell death in the epidermis. To some extent it represents an example of terminal differentiation, similar to the maturation of red blood cells or lens epithelium; however, at the biochemical level there are significant differences between them. For many years, necrosis was regarded as the result of an accidental and uncontrolled process. However, accumulating evidence suggests that necrotic cell death might also be mediated by a specific set of signal transduction pathways and degradative mechanisms. Similar to apoptosis, cell death with a necrotic appearance can contribute to embryonic development as well as tissue homeostasis in the adult organism. Moreover, there is cross-talk between these two cell death modalities. Recently, the term necroptosis has been introduced to designate a special type of programmed necrosis that depends on serine/threonine kinase RIP1 activity [3].

Ca^{2+} signaling has long been known to be critically involved in both the initiation and effectuation of cell death. Hence, necrosis was early found to be associated with a perturbation of intracellular Ca^{2+} homeostasis, and key events in the apoptotic process are known to be triggered by Ca^{2+} signals [4]. More recently, also some forms of autophagic cell death and anoikis have been shown to be Ca^{2+}-dependent (Fig. 1). In this review, we shall discuss current knowledge about the role of Ca^{2+} in both the initiation and effectuation of various modes of cell death with a focus on the apoptotic process.

2. Apoptosis: morphological and biochemical characteristics

Apoptosis has been described as a form of cellular suicide, since death appears to result from induction of active processes within the cell itself. The dying cell then undergoes rapid

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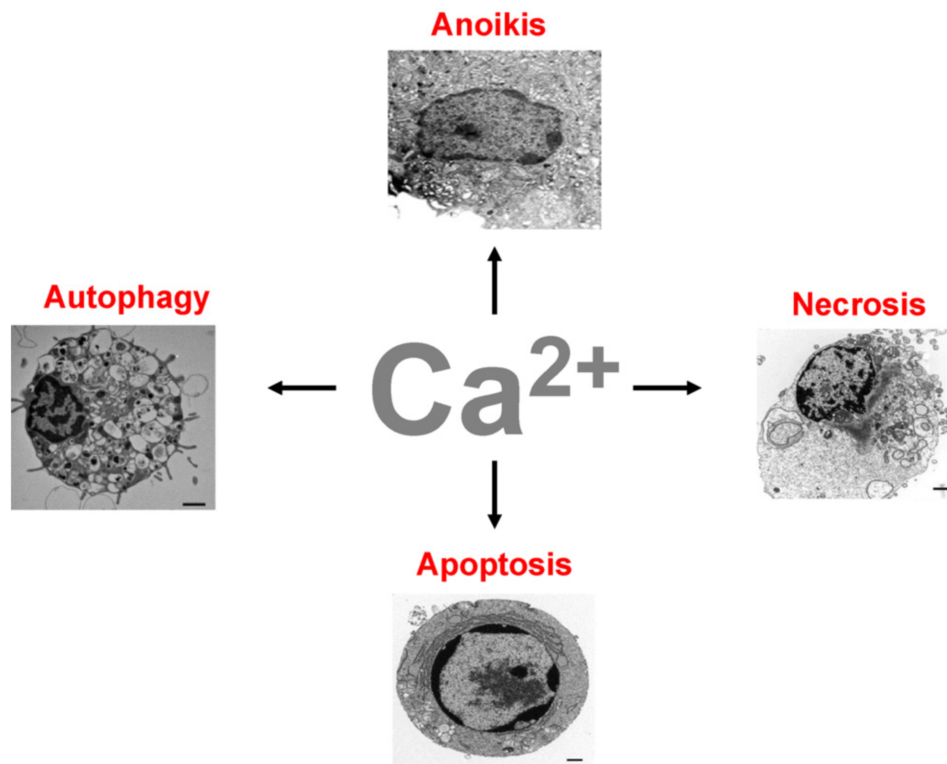

Fig. 1. Calcium involvement in various cell death modalities.

changes, which are reflected in both its structure and biochemistry. Morphologically, apoptosis is characterized by margination and condensation of nuclear chromatin (pyknosis), cytoplasmic shrinkage, nuclear fragmentation, and blebbing of the plasma membrane. The cell subsequently breaks up into membrane-enclosed fragments, termed apoptotic bodies, which are rapidly recognized and engulfed by neighboring cells or macrophages. Considerable biochemical changes occur within the apoptotic cell to facilitate neat packaging and removal of the apoptotic bodies by phagocytosis.

The execution stage of apoptosis involves the proper function of several enzyme systems activated through elaborate signaling pathways. The proteolytic activity of caspases provides a biochemical basis for the apoptotic phenotype [5]. Caspases constitute a family of proteases, which are synthesized as pro-enzymes with very low intrinsic activity and, therefore, require activation, either by proteolytic maturation or by interaction with an allosteric activator. Based on the size of the pro-domain, caspases can be divided into long and short pro-domain containing enzymes. Long pro-domain caspases, *i.e.* caspase-2, -8, -9, and -10, belong to the group of initiator caspases, while short pro-domain caspases, *i.e.* caspase-3, -6, and -7, belong to the group of effector enzymes. The effects of caspases in apoptosis are accomplished by the cleavage of numerous proteins located in the cytoskeleton, cytoplasm and nucleus. Structural components, such as nuclear lamins and cytoskeletal proteins, are cleaved by caspases, and this cleavage precedes nuclear condensation and plasma membrane blebbing. Furthermore, caspases cleave negative regulators of apoptosis and either inactivate them or produce fragments that promote cell death.

The efficient recognition of apoptotic cells by phagocytes requires rearrangement of the infrastructure and molecular composition of the plasma membrane in the dying cell. For example, alteration of the distribution of carbohydrates on the cell surface promotes the preferential binding of macrophages to apoptotic cells. Loss of phospholipid asymmetry in the plasma membrane with the externalization of phosphatidylserine (PS) further facilitates the recognition of dying cells by macrophages. The development of fluorescently labeled Annexin-V, which binds specifically to phosphatidylserine residues on the cell surface, enables detection of apoptotic cells with externalized PS both *in vitro* and *in vivo*. However, it should be noted that under *in vitro* culture conditions, where phagocytic cells are normally absent, apoptotic cells and their fragments lyse in a process similar to necrosis. This is termed "secondary necrosis" or "post-apoptotic necrosis".

3. Apoptosis signaling in mammalian cells

Several major signaling pathways lead to apoptosis in mammalian cells (Fig. 2). In the extrinsic, receptor-mediated pathway the ligation of surface receptors (e.g. CD95, TNFR1) is followed by the formation of the death-inducible signaling complex (DISC), resulting in the activation of pro-caspase-8. In type I cells, caspase-8 activates pro-caspase-3, which cleaves target proteins, leading to apoptosis. In type II cells, caspase-8 cleaves the Bcl-2 protein, Bid, which, in turn, triggers the translocation and insertion of the pro-apoptotic Bcl-2 protein, Bax into the outer mitochondrial membrane (OMM). This leads to the permeabilization of the OMM and the release of multiple proteins from the mitochondrial intermembrane space, including cytochrome *c* that forms a cytosolic apoptosome complex with apoptosis activating factor-1 (Apaf-1) and pro-caspase-9 in the presence of dATP. This results in the activation of pro-caspase-9, which triggers the caspase cascade by activation of pro-caspase-3.

In the intrinsic pathway, death signals act directly or indirectly on the mitochondria to cause the release of pro-apoptotic proteins from their intermembrane space. This cell death pathway is controlled by Bcl-2 family proteins (regulation of cytochrome *c* release), inhibitor of apoptosis proteins (IAPs) (inhibition of cas-

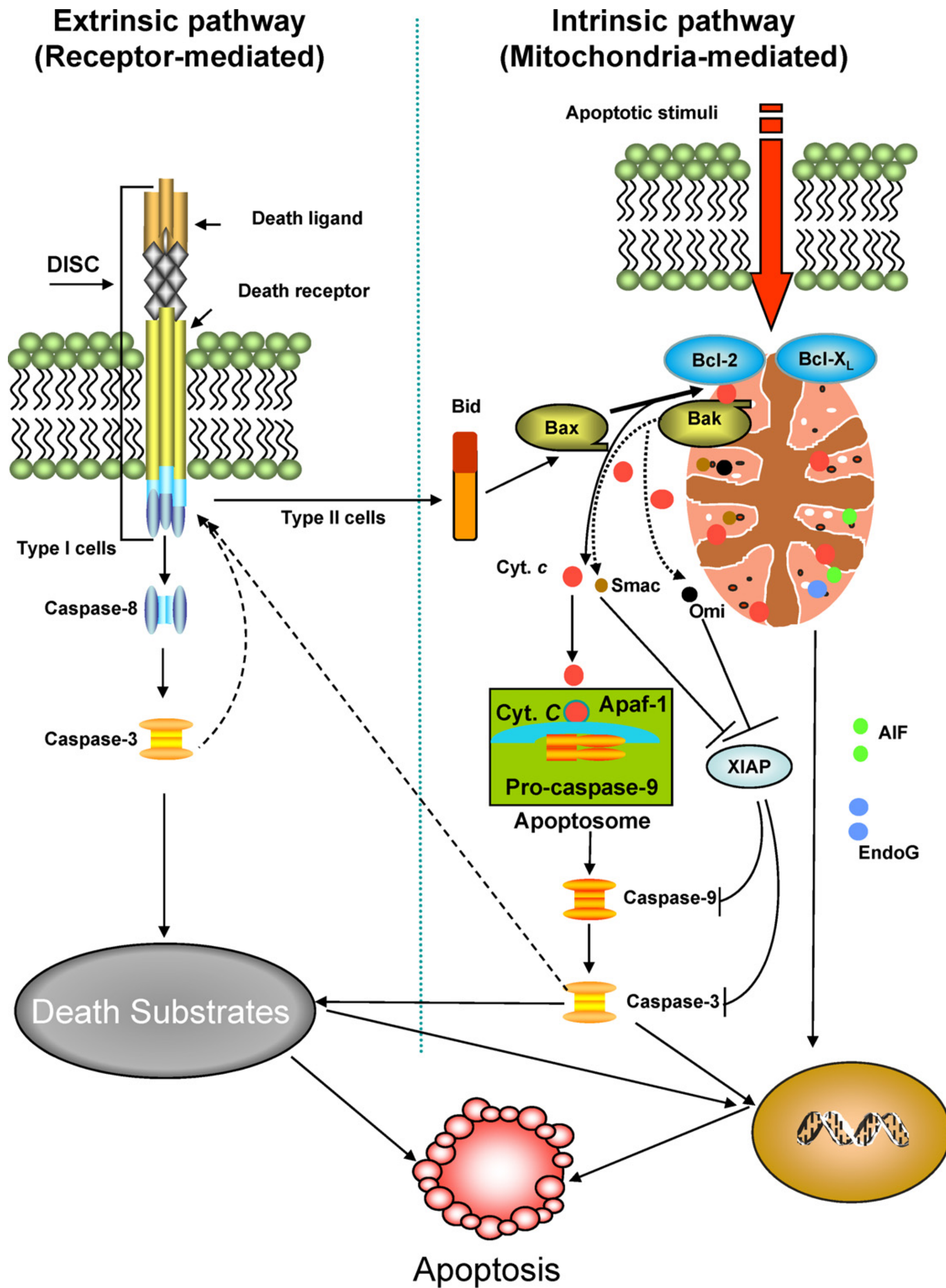

Fig. 2. Schematic illustration of apoptotic cell death pathways. The extrinsic pathway is triggered by ligands binding to receptors on the cell surface (TNFR1, CD95, TRAIL), resulting in their oligomerization, formation of DISC and caspase-8 activation. In Type I cells caspase-8 activates caspase-3, which results in the cleavage of a host of target proteins and apoptosis. In Type II cells, caspase-8 cleaves Bid, resulting in the engagement of the mitochondrial pathway. The latter involves the release of pro-apoptotic proteins (cytochrome *c*, Smac, Omi, AIF and EndoG) from the mitochondrial intermembrane space into the cytosol via Bax/Bak-mediated pores in the outer membrane. Here, cytochrome *c*, Smac and Omi participate in the activation of the caspase cascade, while AIF and EndoG are further translocated to the nucleus, where they participate in chromatin condensation and large-scale DNA fragmentation. XIAP is a cytosolic inhibitor of apoptosis protein, whose activity is blocked by Smac and Omi.

pases), second mitochondrial activator of caspases (Smac), and Omi (negative regulation of IAPs). The intrinsic pathway may also operate via caspase-independent mechanisms, which involve the release from mitochondria and translocation into the nucleus of at least two proteins, apoptosis inducing factor (AIF) and endonuclease G (EndoG). Nuclear effects of AIF include chromatin condensation and formation of high-molecular-weight DNA fragments. The role of EndoG in cell death is still unclear. When DNA damage is the trigger of the apoptotic response, the initially activated caspase is pro-caspase-2. Its activation also leads to the release of cytochrome *c* and apoptosome formation, although the precise mechanisms for this are unclear.

4. Ca^{2+} and the phagocytosis of apoptotic cells

Apoptotic cell death is terminated by the phagocytosis of the dying cell by macrophages or neighboring cells. PS exposure on the apoptotic cell surface appears to be the most important "eat me" signal, although its binding to macrophages is usually not direct but mediated by a number of bridging molecules [6]. Phospholipids are distributed asymmetrically across the plasma membrane, with the bulk of phosphatidylethanolamine, and all of the PS, being present in the inner leaflet of the plasma membrane in healthy cells. In apoptotic cells this situation is changed, such that part of the PS pool is translocated to the cell surface, whereas the distribution of the other major phospholipids is not affected. PS exposure stimulates phagocytic activity via several bridging molecules and receptors [7,8].

In the plasma membrane, the distribution of PS between the two leaflets is regulated by a Ca^{2+}-dependent scramblase that catalyzes the non-specific randomization of phospholipids across the bilayer and an ATP-dependent aminophospholipidtranslocase that mediates movement of aminophospholipids from the outer to the inner leaflet. Although their relative contribution to the regulation of phospholipid asymmetry in the plasma membrane is not known in detail, it seems that the aminophospholipidtranslocase is critically dependent on the intracellular ATP level for activity, but is inhibited by Ca^{2+}. Conversely, phospholipid scrambling is stimulated by Ca^{2+}, and recent findings suggest that transmembrane protein 16F is the Ca^{2+}-responsive component in the plasma membrane and of critical importance for PS exposure by apoptotic cells [9].

5. The endoplasmic reticulum, Ca^{2+} and apoptosis

Ca^{2+} storage and signaling, as well as folding, modifying and sorting of newly synthesized proteins, are among the main functions of the ER in mammalian cells. Disturbances of any of these functions can lead to so-called ER stress. Tunicamycin and a variety of other drugs can induce apoptosis by this mechanism. In rodent cells prolonged ER stress stimulates the activation of procaspase-12 [10]. This enzyme is localized in the ER membrane and cleaved and activated by calpain during ER stress, or in response to the mobilization of intracellular Ca^{2+} stores. Pro-caspase-12 may also be activated by its recruitment into a complex with Ire1α and adaptor protein TRAF-2 (TNF receptor-associated factor-2), which is formed as part of the UPR (unfolded protein response) pathway [11]. Once activated, caspase-12 acts on effector caspases to trigger apoptosis. These findings indicate that ER stress caused by Ca^{2+} depletion, or alterations in the Ca^{2+} transport systems, can be linked directly to caspase activation.

Recently, a pharmacological target for the modulation of Ca^{2+} signaling by the ER was identified [12]. Extranuclear PML (the promyelocytic leukemia tumor suppressor) was shown to be specifically enriched in the ER and in the mitochondria-associated membranes. Its signaling domain is involved in the ER-to-mitochondria Ca^{2+} transport and in the induction of apoptosis. Importantly, PML was found within a large-molecular-weight complex together with the IP3 receptor (IP3R), protein kinase Akt, and protein phosphatase 2a (PP2a), acting as an Akt- and PP2a-dependent modulator of IP3R phosphorylation and IP3R-mediated Ca^{2+} release. It is interesting that this effect appears to be specific for Ca^{2+}-dependent apoptotic stimuli, because alteration of PML did not influence etoposide-induced apoptotic signaling, which is largely independent of Ca^{2+}.

It is well-known that constitutive Ca^{2+} release from the ER is regulated by the IP3R, and that some of this Ca^{2+} is taken up by the mitochondria and is required for optimal respiration and ATP production. It was further shown that cleavage of the IP3R by either calpain or caspase-3 promotes Ca^{2+} accumulation by the mitochondria and apoptosis [13]. Moreover, another link between the ER and the mitochondria has been suggested by the demonstration that mitochondrially released cytochrome *c* can bind not only to Apaf-1, but also to the IP3R and thereby block the inhibition of its function by a rising cytosolic Ca^{2+} concentration [14]. This results in sustained, oscillatory Ca^{2+} increases, leading to augmented cytochrome *c* release and amplification of the apoptotic signal. Interestingly, during apoptosis IP3R-mediated Ca^{2+} signaling might also be modulated by its direct interaction with Bcl-2 [15]. A Bcl-2-interacting region in the regulatory and coupling domain of the IP3R has been identified. Specific peptides can efficiently displace Bcl-2 from this complex and reverse Bcl-2-mediated inhibition of IP3R channel activity, suggesting Bcl-2-IP3R interaction as a potential therapeutic target in diseases associated with the inhibition of cell death by Bcl-2.

Several ER membrane proteins have been reported to interact with members of the Bcl-2 family and influence the apoptotic process. Bax inhibitor 1 protein (BI-1), a mammalian apoptosis suppressor, which is also present in plants and yeast, specifically localizes to the ER membrane [16]. The cytoprotective function of BI-1 was originally discovered in cDNA library screens for human proteins capable of suppressing death of yeast induced by ectopic expression of mammalian Bax protein, Although the exact mechanism of action is unclear, it has been shown that this protein alters Ca^{2+} handling by the ER in a manner resembling the effects of overexpressing Bcl-2, or knocking out Bax and Bak [17] (for more details see the paper by A. Methner in this issue). In addition, BI-1 inhibits IRE1α, an essential mediator of the UPR, thereby facilitating cross-talk between apoptosis and the ER stress pathways. Another protein, BAP31 (Bcl-2-associated protein-31), a 28-kDa integral membrane protein of the ER contains a cytosolic domain, which interacts preferentially with pro-caspase-8, Bcl-X_L and Bcl-2 [18]. In addition, Spike, also a member of the BH3-only family, was isolated and found to be present exclusively in the ER, where it inhibits the formation of a complex between BAP31 and Bcl-X_L [19]. Active caspase-8 can cleave BAP31, and the amino-terminal fragment remains integrated into the ER and involved in the induction of apoptosis. Overexpression of this fragment caused an early release of Ca^{2+} from the ER, a concomitant uptake of Ca^{2+} by the mitochondria, and mitochondrial recruitment of a dynamin-related protein that mediates scission of the OMM, which has been shown to sensitize mitochondria to caspase-8-induced cytochrome *c* release. Moreover, this fragment is also involved in the regulation of mitochondrial fission via interaction with the mitochondrial fission protein, Fis 1 [20]. It seems that the recruitment of procaspase-8 to the Fis 1–Bap31 platform is an early event during apoptosis induction in certain experimental models. The association of pro-caspase-8 with the Fis1-Bap31 complex is dependent on the variant of death effector domain (vDED) in Bap31 and is required for the activation of pro-caspase-8. Hence, the Fis 1–Bap31 complex might serve as an additional platform for activation of pro-caspase-8, and thereby as a bridge between two critical organelles involved in apoptosis signaling.

6. Ca^{2+} as a cell death signal

It has long been known that Ca^{2+} signals govern a host of vital cell functions and hence are necessary for cell survival. However, it is also well established that cellular Ca^{2+} overload, or perturbation of intracellular Ca^{2+} compartmentalization, may cause cytotoxicity and result in either apoptotic, necrotic or autophagic cell death. Thus, cell death can be brought about by a loss of Ca^{2+} homeostatic control, but can also be triggered by more subtle changes in Ca^{2+} distribution within intracellular compartments.

Historically, the role of the Ca^{2+} ion as a death trigger dates back to Fleckenstein's observation that excess Ca^{2+} entry into cardiomyocytes might be the mechanism that underlies cardiac pathology after ischemia [21]. Subsequent studies emphasized the general importance of this observation, as both receptor overstimulation [22] and cytotoxic agents [23] were found to cause lethal Ca^{2+} influx into cells. An early link between Ca^{2+} and apoptosis was the finding that Ca^{2+} induced a typically apoptotic, ladder-like DNA fragmentation pattern in isolated thymocyte nuclei through the activation of a Ca^{2+}- and Mg^{2+}-dependent endonuclease [24]. More recently, it became clear that also non-disruptive changes in Ca^{2+} signaling could have adverse effects, including the induction of apoptosis. For example, as mentioned above, compromising Ca^{2+} sequestration by the ER can be sufficient to trigger apoptosis as part of a stress response. In addition, Ca^{2+}-dependent processes can be responsible for the processing of AIF, resulting in its mobilization from the mitochondria and release into the cytosol [25], or recruited to warrant the final elimination of dead or dying cells by promoting either their phagocytosis or their lysis.

6.1. Mitochondrial role in Ca^{2+} signaling

The mitochondria have long been known to actively participate in intracellular Ca^{2+} compartmentalization [26]. They take up Ca^{2+} electrophoretically from the cytosol through a uniport transporter and can release it again via several different routes. The affinity for Ca^{2+} of the uniporter is low, and the size of the mitochondrial Ca^{2+} pool is small under physiological conditions. However, much larger amounts of Ca^{2+} can accumulate in the mitochondria under pathological conditions, when intracellular Ca^{2+} concentrations rise [27].

Hence, for many years mitochondrial Ca^{2+} uptake was regarded primarily as a safety device in situations of temporary intracellular Ca^{2+} overload. However, this view has changed thanks to the development of novel indicators, which can sense Ca^{2+} fluctuations in specific intracellular compartments [28]. Employing this technology, it has become apparent that mitochondrial Ca^{2+} fluxes are integrated parts of intracellular Ca^{2+} signaling. The low affinity of the mitochondrial Ca^{2+} import system is overcome by the proximity of the mitochondria to the ER and by the formation of Ca^{2+} 'hotspots' at the mouth of ER release channels, where the local Ca^{2+} concentration may reach very high levels [29]. Subsequent uptake of Ca^{2+} by the mitochondria stimulates the Ca^{2+}-sensitive matrix dehydrogenases, which are key sources of NADH for the respiratory chain and thereby for mitochondrial ATP production. The importance of this mechanism for normal cellular energy metabolism has recently been demonstrated convincingly [30].

6.2. Ca^{2+}-mediated mitochondrial permeabilization

As mentioned above, the recruitment of the mitochondrial pathway of apoptosis signaling results in the permeabilization of the OMM and the release of a host of mitochondrial proteins, some of which trigger distinct apoptotic events in other subcellular compartments. OMM permeabilization may be achieved by several different mechanisms, including pore formation by pro-apoptotic Bcl-2 family proteins (notably Bax and Bak) and membrane rupture as a result of mitochondrial swelling (for review, see [31]). Except for the activation of Bad by calcineurin-catalyzed dephosphorylation, there is no established role for Ca^{2+} in the activation of the Bcl-2 family proteins during apoptosis (for more details, see the paper by C. Distelhorst and M. Bootman in this issue). Instead, Ca^{2+} can trigger another mechanism of OMM permeabilization that involves the opening of a permeability transition pore (PTP) made of a large proteinaceous complex whose exact composition is still unclear. The pore has been localized to the contact sites between the inner and outer mitochondrial membranes and behaves as a voltage-operated channel. Pore opening is activated by a high level of Ca^{2+} in the mitochondrial matrix and is further stimulated by oxidative stress, pyridine nucleotide and thiol oxidation, alkalinization, and low transmembrane potential. Persistent pore opening allows Ca^{2+} and low-molecular-weight matrix components ($M_r < 1500$) to escape from the mitochondria, while influx of water and solutes from the cytosol results in mitochondrial swelling and membrane rupture.

7. Cell death by mitochondrial permeabilization

Although PTP opening has mostly been associated with necrotic cell death, a number of agents have been found to trigger apoptosis via Ca^{2+}-mediated mitochondrial permeability transition. Such treatments include Ca^{2+}ionophores and thapsigargin, neurotoxins, chemotherapeutics, and pro-oxidants (e.g. arachidonic acid and peroxynitrite). In some instances, cell death could be prevented by inhibitors of mitochondrial Ca^{2+} uptake or PTP formation, such as ruthenium red and cyclosporin A (for more details, see the paper by A. Rasola and P. Bernardi in this issue). PTP formation usually results in the release of cytochrome *c*, AIF and other pro-apoptotic proteins from the mitochondria. However, this is not always the case, and it has been speculated that only a fraction of the mitochondrial population might undergo permeability transition and release pro-apoptotic factors. Or, resealing of PTP may occur, allowing the mitochondria to recover in spite of the partial loss of cytochrome *c* and other proteins from their intermembrane space [32]. Of particular interest is the observation that apoptotic stimuli, notably ceramide, can induce a switch in mitochondrial Ca^{2+} signaling at the beginning of the apoptotic process by facilitating Ca^{2+}-induced opening of the PTP [33]. This is in accordance with the recent observation that resistance of leukemic cells to 2-chlorodeoxyadenosine (CDA) was associated with an increased ability of their mitochondria to sequester Ca^{2+} without concomitant PTP induction [34]. The CDA-resistant cells were selectively cross-resistant to thapsigargin, but not to staurosporine or to CD95-mediated apoptosis.

Opening of pores and subsequent uncoupling of mitochondria would lead to active hydrolysis of cytosolic ATP (uncoupling-stimulated ATPase activity). As a result, the ATP content would drop, causing a perturbation of cytosolic Ca^{2+} homeostasis and activation of various catabolic enzymes (proteases, phospholipases, etc.). Hence, this model of OMM permeabilization may be most relevant under conditions associated with localized mitochondrial Ca^{2+} overload [35]. However, transient pore opening might also occur whereby a small fraction of mitochondria would have open pores at a given time [36]. In this case, mitochondrial protein release would occur without observable large-amplitude swelling, or drop in membrane potential, of the entire organelle population. This process can be observed also under normal physiological conditions, especially in mitochondria located in close proximity to calcium "hot spots", microdomains, in which the local concentration of ionized calcium far exceeds the average concentration measured throughout the cytosol [37]. This local Ca^{2+} concentration might be high enough to induce Ca^{2+} overload and subsequent pore opening. Therefore, under the influence of apoptotic stimuli the frequency of such spontaneous pore opening and closure might increase, contributing to translocation of intermembrane space proteins into the cytosol.

Recent observations have questioned the importance of PTP for the release of cytochrome *c* from the mitochondria under apoptotic conditions. Thus, overexpression of cyclophilin-D, a component of the pore complex, had opposite effects on apoptosis and necrosis; whereas NO-induced necrosis was promoted, NO- and staurosporine-induced apoptosis was inhibited. These find-

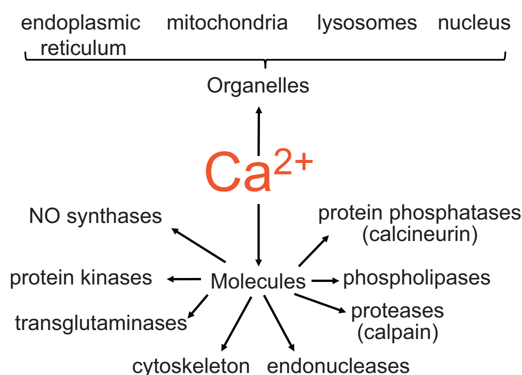

Fig. 3. Some intracellular targets in Ca^{2+}-mediated cytotoxicity. Presence of calcium in various intracellular compartments leads to activation of different molecules, involved in regulation of cell death. Modulation/disruption of Ca^{2+} signaling might result in the activation of multiple cytotoxic mechanisms, including mitochondrial permeability transition and disruption of cytoskeletal organization. Ca^{2+} activation of NO synthases, calcineurin, transglutaminase, calpain, phospholipases or endonucleases has been shown to be involved in various forms of apoptotic cell death.

ings suggest that PTP leads to cell necrosis, but argue against its involvement in apoptosis [38]. Similarly, cyclophilin-D-deficient cells died normally in response to various apoptotic stimuli, but were resistant to necrotic cell death induced by ROS and Ca^{2+} overload. In addition, cyclophilin-D-deficient mice showed resistance to ischemia/reperfusion-induced cardiac injury. These results suggest that the cyclophilin-D-dependent PTP regulates some forms of necrotic, but not apoptotic cell death [39].

8. Ca^{2+}-activated effector mechanisms

There are multiple targets for Ca^{2+} in cell death signaling, including protein kinases and phosphatses, NO synthases, endonucleases, transglutaminases, phospholipases, and proteases (Fig. 3). Each one of these targets has been reported to be involved in cell death signaling in one or more experimental systems. Of particular interest in this context is the role of the calpains.

Although the vast majority of studies have focused on caspases, there is convincing evidence that additional proteases, including members of the calpain family of Ca^{2+}-activated cysteine proteases, participate in apoptosis. Inhibitor studies suggest that the involvement of calpain in apoptosis seems to be more prominent in certain cell types, e.g. thymocytes, monocytes, cardiomyocytes, and neurons [40]. Further, calpain has been reported to cleave the endogenous calcineurin inhibitor cain/cabin 1, resulting in the activation of calcineurin and the promotion of cell death [41]. Moreover, accumulating data indicate that cross-talk between calpain and caspases is important in the regulation of the apoptotic process, and that a host of cellular proteins can be cleaved by both calpain and caspases during apoptosis.

8.1. Ca^{2+}-triggered AIF processing

Recent studies have revealed a novel function of Ca^{2+}/calpain in apoptosis signaling, notably in the processing of mitochondrial AIF [25]. AIF was the first protein reported to mediate caspase-independent, apoptotic cell death [42]. This 62 kDa flavoprotein is anchored to the mitochondrial inner membrane, in the neighborhood of complex I, by part of its peptide chain and needs to be cleaved before a 57 kDa pro-apoptotic fragment can be released into the cytosol for further translocation into the nucleus, where it promotes large-scale DNA fragmentation and chromatin condensation by a not yet clearly defined mechanism. Responsible for AIF cleavage is a calpain localized to the intermembrane space of the mitochondria, which is activated by a sustained elevation of the intracellular Ca^{2+} level. AIF proteolysis is further stimulated by the oxidative modification of AIF by mitochondrially produced ROS, leading to carbonylation of the protein and increased susceptibility to calpain cleavage [43]. In lung cancer cells of neuroendocrine origin and in cortical neurons treated with protein kinase C inhibitors, *i.e.* staurosporine or PKC 412, the activating Ca^{2+} signal originates from the import of extracellular Ca^{2+} via a hyperpolarization-activated cyclic nucleotide-gated (HCN2) ion channel in the plasma membrane [44]. Down-regulation of this channel blocks Ca^{2+} influx as well as AIF processing and apoptosis in both tumor cells and neurons.

8.2. Ca^{2}-mediated nuclear changes

Nuclear Ca^{2+} signaling has been controversial for many years; however, recently it has been shown that nicotinic acid adenine dinucleotide phosphate (NAADP) can release Ca^{2+} from the nuclear envelope, which has an ER-type Ca^{2+} store [45]. In fact, NAADP might act in a manner similar to IP3, being able to reduce the Ca^{2+} concentration within the nuclear envelope; this is associated with a transient rise in the nucleoplasmic Ca^{2+} concentration. It has further been shown that Ca^{2+} overload, as well as calpain activation, might result in a perturbation of macromolecular partitioning across the nuclear membrane with an increased leakiness of the nuclear barrier that affects nucleocytoplasmic transport [46]. Intranuclear Ca^{2+} fluctuations can also affect chromatin organization, induce gene expression, and influence several enzyme activities, such as proteases, endonucleases and protein kinases.

Ca^{2+} may affect chromatin organization by different mechanisms. Earlier work in our group has shown that nuclear Ca^{2+} sequestration is associated with chromatin unfolding [47]. Chromatin unfolding initiated by ion changes in the nuclear matrix may facilitate histone Hl redistribution or topoisomerase II activation. This could produce changes in the supercoiling of a particular chromatin domain. The resulting torsional stress could distort the chromatin structure and impose DNase sensitivity on a portion of that domain. The chromatin high-order structure may be influenced, and the interaction of histone proteins with the nucleosomes may change further. Activation of specific gene(s) within the unfolded region involves the activation and binding of a specific transcription factor protein, which would presumably not occur without the initial chromatin unfolding. Importantly, it was indicated that apoptosis can be delayed by agents that increase chromatin compaction (*i.e.* polyamines) [48].

Several genes involved in cell death signaling are directly or indirectly regulated by Ca^{2+}. Examples include *calmodulin*, *c-fos*, growth-arrest genes, such as *gadd 153*, and several others. The role of Ca^{2+} and calmodulin in the regulation of the cell cycle is also well established. Expression of a constitutive form of Ca^{2+}/calmodulin-stimulated protein kinase II can cause cell cycle arrest in G2; cell cycle alterations might in turn result in apoptosis. Further, Ca^{2+} may directly regulate 'death gene products' as suggested by the observation that two regions of the Ced-4 protein in *Caenorhabditis elegans* show similarity to Ca^{2+}-binding domains [49]. Finally, in many cells apoptosis is preceded by the expression of early response genes (*i.e. c-fos*, *c-jun*), which are regulated by Ca^{2+}. It is noteworthy that all these genes are components of signal transduction systems. Thus, their products might be required to promote downstream events leading to apoptosis . Indeed, long-term exposure of human macrophages to TNF resulted in sustained activation of *c-jun* and NFkB, signaling events that culminated in the induction and activation of Ca^{2+}-dependent transcription factor (NFAT), priming macrophages to terminal differentiation and cell death [50]. Recently, it was shown that Ca^{2+} was also required for nuclear accumulation of clusterin, a heterodimeric glycoprotein that is produced almost ubiquitously in

mammalian tissues. This accumulation led to inhibition of cell proliferation and caspase-dependent death of human prostate cells [51].

Studies of endonucleases represented an early approach to the biochemistry of apoptosis, and a number of both Ca^{2+}-dependent and Ca^{2+}-independent enzymes have since been reported to be involved in apoptotic DNA degradation [52]. The discovery of the caspase-3-activated endonuclease and its inhibitor, DNA fragmentation factor, DFF40/DFF45 (also known as caspase-activated DNase and its inhibitor; CAD/ICAD), and its involvement in nuclear apoptosis, led to a decrease in the search for new nucleases, as it seemed that the identity of the apoptotic endonuclease activity had finally been resolved [53,54]. However, the fact remains that the incubation of isolated cell nuclei with Ca^{2+} and ATP, *i.e.* in the absence of caspase-mediated DNA cleavage activity, results in a chromatin fragmentation pattern that is indistinguishable from that found in apoptotic cells. So, a possible contribution of other nucleases to overall DNA degradation during apoptosis cannot yet be conclusively excluded.

Of considerable interest is the finding that DNase II is critically involved in the phagocytic degradation of engulfed apoptotic nuclear material [55]. Interestingly, apoptotic bodies can be phagocytosed by macrophages after exposure of PS on their surface. They contain only a very low level of ATP, but a high concentration of Ca^{2+}. The Ca^{2+} accumulation is the result of inactivation of the plasma membrane Ca^{2+}-ATPase, which normally pumps Ca^{2+} out of the cells in an ATP-dependent manner. Accordingly, as a result of the perturbed Ca^{2+}/ATP balance in the apoptotic bodies, the aminophospholipidtranslocase is inactivated, while the scramblase is activated, resulting in the exposure of phosphatidylserine on their surface [56].

9. Caspases and intracellular ion homeostasis

As discussed above, Ca^{2+} signals can trigger apoptosis and lead to the activation of caspases, which may in turn cleave key components of the Ca^{2+} regulatory system and cause perturbation of Ca^{2+} homeostasis. For example, it has been shown that caspase-3 can cleave the type 1 IP_3 receptor in apoptotic cells, and that such cleavage resulted in a decrease in channel activity as the receptor was degraded [57]. In addition, caspase-3 can also cleave the plasma membrane Ca^{2+}-ATPase [58]. This enzyme is of critical importance for the long-term control of cellular Ca^{2+} homeostasis, and cleavage-associated loss of function during apoptosis results in Ca^{2+} overload and secondary cell lysis or necrosis. Moreover, the Na^+/Ca^{2+}-exchanger is also cleaved by caspase-3 during apoptosis, which further decreases the ability of the apoptotic cell to extrude excess Ca^{2+}.

The Na^+, K^+-ATPase is another ion pump in the plasma membrane that can act as a caspase substrate during apoptosis. Previous studies have shown that volume shrinkage in apoptotic cells is accompanied by a net efflux of ions due to the inactivation of the Na^+, K^+-ATPase [59], and that apoptosis is accelerated via Bcl-2-sensitive activation of outward K^+ currents. The β-subunit of the Na^+, K^+-ATPase, which regulates the K^+ affinity of the enzyme at the extracellular site, seems to be the caspase target. Apart from a role in cell shrinkage during apoptosis, inactivation of the Na^+, K^+-ATPase may promote the apoptotic process by lowering the intracellular K^+ concentration to levels that are compatible with optimal caspase activity [60].

10. Role of AIF in cell death execution

As discussed above, AIF is N-terminally anchored to the inner mitochondrial membrane and has to be liberated from its membrane anchor prior to being released into the cytosol and translocated to the nucleus, where it participates in large-scale DNA fragmentation and chromatin condensation. The available evidence suggests that AIF plays a critical role in cell death only in certain cell types, such as neurons and some tumor cells [61,62]. Furthermore, the type of lethal stimulus also determines whether AIF will be important in the cell death process to follow. Preferential AIF triggers should either perturb intracellular Ca^{2+} homeostasis, or cause early lysosomal permeabilization, in order to make the AIF-mediated pathway an essential mechanism of subsequent apoptosis. However, both these events are frequent components of cell death signaling, particularly in ischemia–reperfusion injury and after treatment with cytotoxic drugs.

Several studies suggest a critical role for AIF in neuronal cell death. Hence, microinjection of neutralizing AIF antibodies, or siRNA downregulation of AIF have been found to suppress glutamate-, hypoxia- and NMDA-induced neuronal death in cultures. Further, AIF knockdown in PC12 cells reduced the toxic effects evoked by MPP+ (1-methyl-4-phenylpyridinium) [63]. There are also several *in vivo* observations demonstrating the importance of the AIF-mediated pathway in neuronal cell death. For instance, as compared to wild-type mice, AIF-deficient Harlequin (Hq) mice were protected against NMDA- and kainic acid-induced neuronal damage in the hippocampus [64]. Cell death was also found to be suppressed in Hq mice subjected to hypoxia/ischemia. Neuroprotection was also observed in different mouse models, when AIF processing was prevented by oral administration of HIV protease inhibitors. Finally, it was reported that inhibition of the nuclear translocation of AIF caused neuroprotection in a rat model of retinal degeneration [65].

11. Role of Ca^{2+} in other cell death modalities

Regardless of the main execution pattern, death programs have at least two fundamental features in common: (1) they are evolutionarily conserved, which may be explained by a dual role of many components as both life and death signals, and (2) they involve the activation of one or more families of proteases and/or other degradative enzymes. In many instances, developmental cell death and death under pathological conditions share similar morphological features as well as signaling and execution systems. This is true not only for apoptosis, but also for other cell death modalities. For example, autophagic cell death, which is characterized by the presence of cytoplasmic vacuoles, occurs frequently in both neuronal development and neurodegenerative disease [66]. The existence of conserved biochemical pathways for cell death signaling and execution has reduced the previous focus on the morphological characteristics of cell death. Despite efforts to define individual forms of cell death based on their appearance, it appears that cell disassembly always involves nuclear fragmentation/dissolution, organelle disruption (sooner or later) and, eventually, membrane lysis and phagocytosis. In apoptosis, like in necrosis, the mitochondria can be partially or totally damaged and, again, in both apoptosis and necrosis the cytoskeletal structure is compromised and molecules are exposed on the cell surface to promote recognition and scavenging of the dying cell. Therefore, a particular death program is not necessarily linked to morphological appearance. It seems more likely that several death-executing routines may be activated concomitantly within injured cells, and that one or the other becomes predominant, depending on the stimulus and the metabolic state of the tissue. Thus, under pathological conditions several protease families may cooperate to disassemble cells, targeting different organelles or subcellular structures. While the predominance of one or the other death executing mechanism may be dictated by factors as different as energy requirement, signaling

molecules or the intensity of a given insult, in many instances the differentiation program within a given tissue dictates the way to die. This is particularly true of neurons, where the spatial selectivity of death signals and promiscuity of execution systems can result in the complex and relatively slow demise that occurs in neurodegenerative disease. The promiscuity of death subroutines is also evident in brain ischemia, where both caspase-dependent and caspase-independent death pathways are activated. The latter include Ca^{2+} activation of the calpain family of proteases. The predominance of one or the other death pathway can be explained in this case by the availability of ATP for the activation of the caspase-dependent routines [4].

11.1. Ca^{2+}-induced necrosis

Although it is now evident that chemical toxicity might be associated with multiple modes of cell death, toxic cell death was initially thought to be of the necrotic type and often related to a perturbation of intracellular Ca^{2+} homeostasis. Killing of hepatocytes by exposure to carbon tetrachloride, acetaminophen or bromobenzene may serve as classical examples thereof. Acetaminophen- and bromobenzene-induced cell death was studied intensely in the 1970s and found to be preceded by cytochrome P-450-mediated metabolic activation, glutathione depletion, and disruption of Ca^{2+} homeostasis; it was usually monitored by cellular leakage of lactate dehydrogenase or uptake of trypan blue, traditional assays of the increased plasma membrane permeability associated with necrotic cell death.

Following the recognition of apoptosis as a distinct mode of cell death, in 1987 Andrew Wyllie put forward two hypotheses related to its role in toxic cell death, namely (1) that apoptosis might be induced by injurious agents of lesser amplitude than those causing necrosis in the same cells, and (2) that this might occur more readily in cell populations primed for apoptosis [67]. An early example thereof was our finding that increasing concentrations of the redox-cycling quinone, 2,3-dimethoxy-1,4-naphthoquinone could stimulate proliferation, induce apoptosis, or cause necrosis in a pancreatic cell line dependent on the dose administered [48]. More recently, studies with arsenic trioxide revealed that low doses of this agent triggered Bax/Bak-dependent apoptosis, whereas higher doses caused mitochondrial permeability transition and necrotic cell death. The latter observation illustrates the frequent finding *in vivo* that distinct cell death modalities may co-exist within the same lesion, and that interference with certain signaling pathways, or critical cell functions, might result in cell killing by a different mechanism. Post-apoptotic necrosis due to intracellular Ca^{2+} overload following caspase cleavage and inactivation of vital Ca^{2+}-extruding proteins in the plasma membrane is one example thereof.

11.2. Role of Ca^{2+} in autophagic cell death

Autophagic cell death is morphologically defined (by transmission electron microscopy) as a type of cell death that occurs in the absence of chromatin condensation, but is accompanied by massive autophagic vacuolization of the cytoplasm [68]. Autophagy was initially described as a survival mechanism, and some reports indicate that cells presenting features of 'autophagic cell death' can still recover upon withdrawal of the death-inducing stimulus. Nevertheless, in some instances autophagy may be responsible for the destruction of cells as a result of a protracted atrophy of the cytoplasm beyond a not yet clearly defined point-of-no-return.

Several years ago it was reported that, like apoptosis, autophagy could be triggered by ER stress, suggesting a potential link between Ca^{2+} and autophagy (for review, see [69]). Indeed, stimulated Atg7-deficient cells display impaired influx, but not efflux, of calcium, and ER Ca^{2+} storage is increased in Atg7-deficient cells. Stimulation of Ca^{2+} mobilization by thapsigargin, ionomycin, or vitamin D was shown to induce autophagy [70]. Moreover, aggregation of GFP-LC3 was inhibited by BAPTA-AM, suggesting that autophagosome formation is Ca^{2+}-dependent. Autophagy was shown to occur by the Ca^{2+}-dependent activation of AMP-activated protein kinase (AMPK), which required upstream activation of the Ca^{2+}/calmodulin kinase β.

Another Ca^{2+}/calmodulin-regulated kinase, DAPK (death-associated protein kinase), also was shown to be an essential regulator of autophagy [71]. This enzyme interacts with LC3-binding protein, MAP1B, which is involved in autophagosome trafficking along microtubules. It interacts with and phosphorylates Beclin-1 (originally identified as a Bcl-2 interacting protein), disrupting the inhibitory Beclin-1-Bcl-2/X_L interaction. On the other hand, interaction of MAP1B with TSC2, a negative regulator of mTOR, leads to DAPK phosphorylation and inactivation [72].

A direct role for Ca^{2+} in the induction of autophagy was also supported by the observation that calcium phosphate precipitates could induce autophagy upon transfection in various cell lines. In this case, autophagy was Beclin-dependent. Recently, Kroemer and colleagues have shown that the IP3R regulates autophagy through its interaction with Beclin-1, establishing a functional and physical link between the ER and mitochondria not only in apoptosis, but also in autophagy [73]. Another link between Ca^{2+} and autophagy was observed when cells were treated with the autophagy inducer, fluspirilene, an inhibitor of Ca^{2+} fluxes. Finally, lowering of intracellular Ca^{2+} prevents calpain-mediated cleavage of Atg5, which, in turn, increases the levels of full-length Atg5 and of the Atg5-Atg12 conjugate [74].

Depending on the type of lethal agent, the cell death process can be initiated in different intracellular compartments, and cross-talk between these compartments appears essential for all cell death modalities. This inter-organelle cross-talk involves several molecular "switches" within the signaling network. Thus, p53 can be activated in response to DNA damage, or because of changes in the redox balance in the mitochondria, and Bcl-2 family proteins might act at the level of the mitochondria, ER or nucleus. Nuclear p53 promotes the transcription of pro-apoptotic and cell cycle-arresting genes, but also can act as an autophagy-inducing transcription factor. In contrast, cytoplasmic p53 might trigger apoptosis and/or inhibit autophagy, although the precise molecular mechanisms of this dual function are not known [75]. Further, it was recently shown that DAPK modulates upstream signaling events that regulate autophagy induction by activation of p53 in a p19ARF-dependent manner, leading to upregulation of both proteins. p53, in turn, activates AMPK, which suppresses mTOR signaling through TSC2m and upregulates the lysosomal protein, DRAM.

Another example of cross-talk between apoptosis and autophagy was described recently. As mentioned above, the pro-autophagic function of Beclin 1 can be inhibited by both Bcl-2 and Bcl-X_L [76]. Notably, although Beclin-1 possesses a BH3-only domain, and all BH3-only proteins of the Bcl-2 family are well-known inducers of apoptosis, Beclin 1 fails to trigger apoptosis. In fact, by stimulation of autophagy it offers protection against various pro-apoptotic agents. However, upon growth factor withdrawal, when autophagy precedes apoptosis, caspase-mediated cleavage of Beclin 1 inactivates autophagy and stimulates apoptosis by promoting the release of pro-apoptotic factors from the mitochondria. In this model, a caspase-generated fragment of Beclin 1 seems to trigger an amplifying loop enhancing apoptosis [77].

Finally, depending on the nature and severity of the stimulus, and on the cell type, the hierarchy of interorganelle cross-talk might result in different cell death modalities. Moreover, in some cases suppression of the function of a particular intracellular compartment might switch one mode of cell death to another. For example,

inhibition of mitochondrial energy metabolism (lowering of ATP) can change the mode of cell death from apoptosis to necrosis. Similarly, inhibition of caspase activity might change apoptosis to necrosis, or to autophagic cell death, whereas activation of calpain-mediated cleavage of autophagy-regulated protein, Atg-5, switches the mode of cell death from autophagy to apoptosis [78].

11.3. Calcium and anoikis

Anoikis is defined as a form of programmed cell death, which is induced when anchorage-dependent cells detach from the surrounding extracellular matrix. It is involved in a wide diversity of tissue-homeostatic, developmental and oncogenic processes. The signaling events culminating in anoikis are still unclear; however, similarly to other cell death modalities, Ca^{2+} and ROS have been implicated in the regulation of anoikis [79]. Hence, ROS-mediated changes in intracellular Ca^{2+} homeostasis led to activation of RhoA/Rho kinase signaling, altering vascular pathophysiology, and resulted in loss of cell–cell interaction [80].

In certain tumor cells treated with anticancer drugs, the level of calpain was reported to significantly influence their detachment from the matrix [81]. Notably, in another study Ca^{2+}-activated channels were implicated in the regulation of anoikis [82]. In breast cancer cells down-regulation of the Ca^{2+}-activated chloride channel was shown to result in resistance to detachment-induced cell death. Transfection of cells with plasmids encoding this channel led to significant reduction of colony formation and cell death via anoikis.

In an effort to identify mechanism(s) that can inhibit anoikis, β-catenin (a major oncoprotein) was recently found to down-regulate DAPK-2, leading to anoikis resistance and promotion of anchorage-independent growth [83]. DAPK activity also can be regulated by protein phosphatase-2 (PP2A), although this pathway seems to be most relevant for ceramide-induced anoikis [84]. Hence, it seems that similar proteins, with a requirement for Ca^{2+}, might be involved in the regulation of multiple cell death modalities; however, what regulates this switch is still unclear

12. Concluding remarks

Recent studies have further emphasized the crucial role of the Ca^{2+} ion in the regulation of cell death. Hence, technical progress during the last several years has helped us understand how ER–mitochondrial Ca^{2+} fluxes might influence cell death signaling and further revealed the critical role of the mitochondrion in Ca^{2+}-regulated cell death programs. It has also become clear that Ca^{2+} is an important player not only in apoptosis signaling, but also in the regulation of many other cell death modalities, including necrosis, autophagy, and anoikis. Future research will have to delineate these Ca^{2+}-mediated pathways in further detail and explore the role of Ca^{2+} in other, less well-defined cell death programs. The potential importance of these signaling pathways in disease pathogenesis should also be further substantiated. Although we have already entered the era of developing new pharmaceuticals that target Ca^{2+}-mediated processes, the clinical advantage as well as the potential adverse effects of such therapy must be critically evaluated.

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