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David J. McConkey
The University of Texas M.D. Anderson Cancer Center, David_McConkey@isqm.mda.uth.tmc.edu

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THE ROLE OF CALCIUM IN THE REGULATION OF APOPTOSIS

David J. McConkey

Department of Cell Biology - 173, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030

Telephone number: (713) 792-8591 / FAX number (713) 792-8747
E.mail: David_McConkey@isqm.mda.uth.tmc.edu

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Abstract

The recognition that apoptosis is regulated by an evolutionarily conserved set of polypeptides from the nematode *Caenorhabditis elegans* to humans suggests that a conserved set of biochemical mechanism(s) may also be involved in the response. Early evidence suggested that the endogenous endonuclease implicated in apoptosis in most model systems is Ca^{2+}-dependent, and subsequent work from a number of independent laboratories suggests that alterations in cytosolic Ca^{2+} homeostasis are one of the conserved biochemical pathways regulating the response. Molecular targets for Ca^{2+} are now being identified and include signal transduction intermediates, endonuclease(s) and proteases, and the enzymes involved in the maintenance of phospholipid asymmetry in the plasma membrane. Furthermore, interesting preliminary work suggests that BCL-2 suppresses apoptosis via a mechanism that is linked to intracellular Ca^{2+} compartmentalization, and it appears that Ca^{2+} alterations in some examples of apoptosis occur as the result of changes within the mitochondria. This review will summarize what is known about the role of Ca^{2+} in the regulation of apoptosis and discuss how Ca^{2+} might interact with some of the other biochemical signals implicated in cell death.

Key Words: Apoptosis, calcium, endonuclease, oxidative stress, BCL-2.

Introduction

Apoptosis (programmed cell death) is a highly regulated process of selective cell deletion involved in development, normal cell turnover, hormone-induced tissue atrophy, cell-mediated immunity, tumor regression, and a growing number of pathological disorders, typified by acquired immune deficiency syndrome (AIDS) and Alzheimer's diseases (Thompson, 1995; Wyllie *et al.*, 1980). The response is characterized by stereotyped morphological alterations, including plasma and nuclear membrane blebbing, organelle relocalization and compaction, chromatin condensation, and the formation of membrane-enclosed structures termed "apoptotic bodies" that are extruded into the extracellular millieu (Savill *et al.*, 1993; Wyllie *et al.*, 1980). Uptake of apoptotic debris is carefully controlled, as apoptotic cells and bodies are specifically recognized and cleared by neighboring epithelial cells and professional phagocytic cells (macrophages) before their contents can be released into the extracellular millieu, thereby allowing for cell death to occur in the absence of inflammation (Savill *et al.*, 1993).

Apoptosis has historically been characterized biochemically by endogenous endonuclease activation, resulting first in the production of domain-sized large (50-300 kilobase) DNA fragments (Brown *et al.*, 1993; Filipski *et al.*, 1990; Oberhammer *et al.*, 1993) and subsequently into oligonucleosomal cleavage products commonly referred to as "DNA ladders" (Wyllie, 1980). It appears that DNA ladders are derived from the larger DNA fragments but that these events may be mediated by different enzymatic activities that can be distinguished by their divalent cation requirements (Sun and Cohen, 1994). In addition to endonuclease activation, more recent work has demonstrated that a family of cysteine proteases homologous to the *Caenorhabditis elegans* (*C. elegans*) cell death gene *ced-3* and human interleukin-1 converting enzyme (ICE) are also critically involved in the response, as inhibitors of these enzymes block both endonuclease activation and cell death (Fernandes-Alnemri *et al.*, 1994, 1995; Kumar *et al.*, 1994; Martin

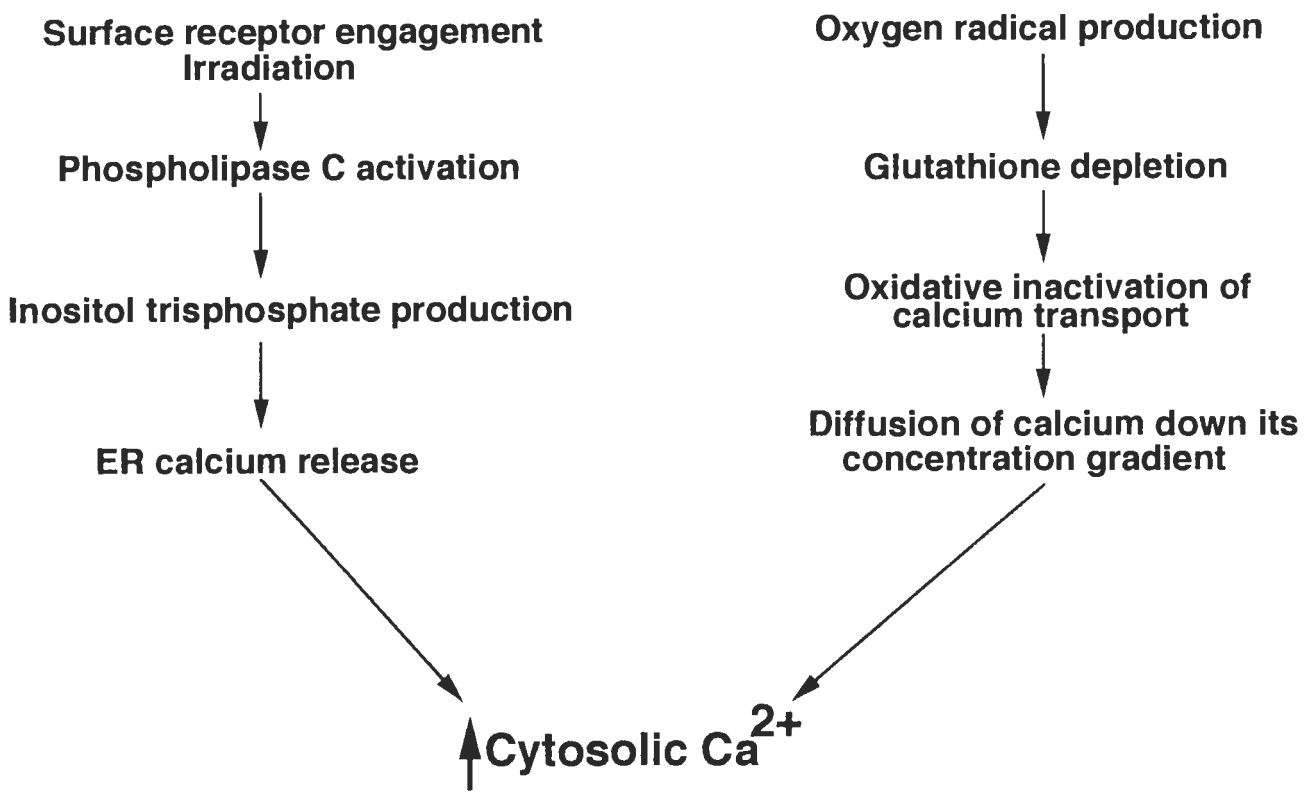

Figure 1. Schematic representation of phospholipase C-dependent and damage-mediated pathways involved in promoting Ca^{2+} elevations in apoptotic cells.

and Green, 1995; Nicholson *et al.*, 1995; Tewari *et al.*, 1995; Wang *et al.*, 1994; Yuan *et al.*, 1993). The important substrates for these proteases remain largely unidentified but include poly (ADP-ribose) polymerase (PARP), the lamins, and a viral inhibitor of their activity (baculovirus p35) (Bump *et al.*, 1995; Kaufmann *et al.*, 1993; Lazebnik *et al.*, 1993; Tewari *et al.*, 1995; Xue and Horvitz, 1995). Although many of the important biochemical alterations associated with apoptosis occur within the nucleus, recent work indicates that apoptosis can also be initiated in enucleated cells (cytoplasts) (Jacobson *et al.*, 1994; Schulze-Osthoff *et al.*, 1994), and may lead investigators to suspect that the process is initiated in the cytoplasm.

At the molecular level, apoptosis is regulated by many familiar oncogenes (*bcl-2*, *myc*, *ras*, *abl*, *fos*) and tumor suppressor genes (*p53*, *Rb*) (Thompson, 1995). However, the family of polypeptides homologous to the *bcl-2* appear to collectively be the most important. Regulation of apoptosis susceptibility is their only established function to date. One class of *bcl-2* homologs (including *bcl-2*, *bcl-xL*, *mcl-1*, and several viral proteins) suppress apoptotic cell death, while another group (*bax*, *bcl-xs* and *bak*) promotes apoptosis sensitivity (Oltvai and Korsmeyer, 1994). Thus, overexpression of BCL-2 or BCL-XL blocks apoptosis induced by very diverse stimuli, including growth factor withdrawal, tumor necrosis factor, engagement of the Fas antigen, ionizing radiation, oncogenes such as myc, and chemical chemotherapeutic agents (Thompson, 1995). Moreover, the apoptosis-regulatory functions of BCL-2 and its homologs are evolutionarily conserved, as the *C. elegans* cell death suppressor *ced-9* is a structural and functional homolog of human *bcl-2* (Hengartner *et al.*, 1992; Hengartner and Horvitz, 1994). Together, these observations have suggested to many investigators that BCL-2 regulates a central biochemical signal in the pathway to cell death, the identity of which is still unknown. However, several strong candidates are emerging. Work from our laboratory (McConkey *et al.*, 1989a, 1989b, 1994) and others (Shi *et al.*, 1989; Martikainen and Isaacs, 1990; Story *et al.*, 1992) over the past five years has demonstrated that alterations (Baffy *et al.*, 1993; Lam *et al.*, 1994) in intracellular Ca^{2+} homeostasis are commonly involved in promoting apoptosis, and more recent work suggests that one aspect of BCL-2 function involves preventing these alterations (Baffy *et al.*, 1993; Lam *et al.*, 1994). Here, we will discuss the role of

Ca^{2+} in regulating apoptosis and compare it with other general mediators of apoptotic cell death.

Calcium Alterations in Apoptosis

Early studies by Kaiser and Edelman (1977) demonstrated that glucocorticoid-stimulated apoptosis is associated with enhanced Ca^{2+} influx, work that provided the first evidence that increases in intracellular Ca^{2+} might be involved in triggering apoptosis. We have since confirmed that glucocorticoids induce elevations in the cytosolic Ca^{2+} concentration in thymocytes via enhanced Ca^{2+} influx across the plasma membrane (McConkey *et al.*, 1989b). Recent results from Synder's laboratory have implicated the type 3 receptor for inositol trisphosphate (IP_3R) in Ca^{2+} influx, DNA fragmentation, and cell death, suggesting that these physiologically relevant Ca^{2+} channels play a central role in the response (Khan *et al.*, 1996). However, intracellular Ca^{2+} storage sites also appear to be affected, as the Ca^{2+} pool located in the endoplasmic reticulum is depleted in a lymphoid cell line in response to glucocorticoid treatment (Lam *et al.*, 1993), and a similar phenomenon has been documented in an interleukin 3 (IL-3)-dependent myeloid cell line undergoing apoptosis following IL-3 withdrawal (Baffy *et al.*, 1993). Circumstantial evidence suggests that the mitochondrial Ca^{2+} pool may also be affected (Richter, 1993), as mitochondrial membrane potential drops very early during apoptosis (Petit *et al.*, 1995; Zamzani *et al.*, 1995a, 1995b), and it is well known that the maintenance of mitochondrial Ca^{2+} homeostasis is dependent upon mitochondrial membrane potential (Richter, 1993). Apoptosis in other systems also appears to involve elevations in the cytosolic Ca^{2+} concentration. For example, rapid, sustained Ca^{2+} increases precede the cytolysis of the cellular targets of cytotoxic T lymphocytes (Allbritton *et al.*, 1988) and natural killer (NK) cells (McConkey *et al.*, 1990). In developing T lymphocytes, high affinity engagement of the T cell receptor induces apoptosis (McConkey *et al.*, 1989a; Murphy *et al.*, 1990; Shi *et al.*, 1989; Smith *et al.*, 1989) that involves a sustained Ca^{2+} elevation (McConkey *et al.*, 1989a; Nakagama *et al.*, 1992).

Both second messenger- and damage-mediated mechanisms can be involved in promoting Ca^{2+} increases in apoptotic cells (Fig. 1). In an example of the former, T cell receptor engagement on thymocytes leads to a sustained increase in the cytosolic Ca^{2+} concentration that involves protein tyrosine kinase activation, phosphorylation of the γ-isoform of phospholipase C, phosphoinositide hydrolysis leading to the production of inositol trisphosphate (IP3), and mobilization of Ca^{2+} from the endoplasmic reticulum and extracellular millieu that promote cell death (McConkey *et al.*, 1989a, 1994). Similarly, surface antigen receptor engagement on B cells leads to Ca^{2+} increases that promote cell death (Norvell *et al.*, 1995; Parry *et al.*, 1994; Tsubata *et al.*, 1993; Yao and Scott, 1993). Thus, in these examples of apoptosis, Ca^{2+} increases occur via a controlled, physiological mechanism that is also utilized in alternative responses such as cellular activation leading to proliferation.

Work from our laboratory (Fernandez *et al.*, 1995) and others (Richter, 1993) has revealed another mechanism that is involved in promoting sustained cytosolic Ca^{2+} increases in apoptotic cells. It is well known that cytosolic Ca^{2+} concentration is maintained at roughly 100 nM in resting cells, whereas the concentrations in the extracellular millieu, and the ER, are much higher (in the millimolar range). Early work on the biochemical mechanisms underlying the cytotoxicity of agents that generate reactive oxygen species in cells (oxidative stress) indicated that the Ca^{2+} transport systems localized to the ER, mitochondria, and plasma membrane can be damaged by oxygen radicals (Orrenius *et al.*, 1989). This leads to diffusion of Ca^{2+} down its concentration gradient, a disruption of intracellular Ca^{2+} homeostasis, and sustained Ca^{2+} increases. Oxidative stress is now known to be commonly involved in apoptosis (Buttke and Sandstrom, 1994; Dypbukt *et al.*, 1994; Fang *et al.*, 1995; Hockenbery *et al.*, 1993; Lennon *et al.*, 1990), and it is therefore possible that oxidative disruption of intracellular Ca^{2+} homeostasis is involved in these systems. Supporting this idea, Fernandez *et al.* (1995) have recently shown that the glucocorticoid-induced Ca^{2+} increase observed in thymocytes is blocked by antioxidants, and Kroemer's laboratory has presented evidence that oxidative stress leads to disruption of mitochondrial Ca^{2+} stores (Zamzani *et al.*, 1995b).

Direct evidence that Ca^{2+} increases can mediate apoptotic endonuclease activation and cell death has been obtained from experiments with intracellular Ca^{2+} buffering agents and extracellular Ca^{2+} chelators. Our group (Aw *et al.*, 1990; Bellomo *et al.*, 1992; McConkey *et al.*, 1989a, 1989b, 1990, 1994; Zhivotovsky *et al.*, 1993) and others (Robertson *et al.*, 1993; Story *et al.*, 1992) have shown that these agents can inhibit both DNA fragmentation and death in apoptotic cells. The Ca^{2+}-dependent regulatory cofactor calmodulin may link these Ca^{2+} alterations to the effector machinery, as we and others have shown that calmodulin antagonists can interfere with apoptosis in some of these systems (Dowd *et al.*, 1991; McConkey *et al.*, 1989b) and increases in calmodulin expression are linked to apoptosis in glucocorticoid-treated thymoma cells (Dowd *et al.*, 1991) and in prostatic epithelial cells following withdrawal of androgen (Furuya and Isaacs, 1993). Independent evidence for the involvement of Ca^{2+} influx in the trigger-

ing of apoptosis has come from studies with specific Ca^{2+} channel blockers, which abrogate apoptosis in the regressing prostate following testosterone withdrawal (Martikainen and Isaacs, 1990) and in pancreatic β-cells treated with serum from patients with type I diabetes (Juntti-Berggren *et al.*, 1993).

Other support for the involvement of Ca^{2+} in apoptosis comes from the observation that agents which directly mobilize Ca^{2+} can trigger apoptosis in diverse cell types. Early work by Kaiser and Edelman (1978) demonstrated the cytolytic effects of glucocorticoids on lymphoid cells can be mimicked by treating the cells with Ca^{2+} ionophores. Subsequently, Wyllie *et al.* (1984) demonstrated that Ca^{2+} ionophores cause endonuclease activation as well as many of the morphological changes that are typical of apoptosis in thymocytes. Calcium ionophores also trigger apoptosis in prostate tumor cells (Martikainen and Isaacs, 1990) and in non-metastatic melanoma lines (McConkey, unpublished). Independent evidence for the general relevance of this mechanism has come from studies with the endoplasmic reticular Ca^{2+}-ATPase inhibitor thapsigargin, the product of the plant, *Thapsa garganica*, which can also trigger all of the morphological and biochemical events of apoptosis in thymocytes (Jiang *et al.*, 1994) and some other cell types (Choi *et al.*, 1995; Kaneko and Tsukamoto, 1994; Levick *et al.*, 1995).

A final argument for a central role for Ca^{2+} in regulating apoptosis comes from recent and ongoing work on the biochemical mechanisms of apoptosis suppression by the BCL-2 oncoprotein (Baffy *et al.*, 1993; Lam *et al.*, 1994; Zornig *et al.*, 1995; Marin *et al.*, 1996). The possible relationship between Ca^{2+} and BCL-2 was first suggested by work by Baffy *et al.* (1993), who showed that BCL-2 can block the depletion of the endoplasmic reticular Ca^{2+} pool in transfectants of an IL-3-dependent cell line (32D). Interestingly, Baffy *et al.* (1993) also demonstrated that constitutive levels of Ca^{2+} in mitochondria (measured following treatment with an uncoupler that promotes rapid and selective depletion of this intracellular Ca^{2+} store) were significantly lower in BCL-2-expressing cells compared to vector control transfectants, consistent with the notion that BCL-2 may also regulate Ca^{2+} compartmentalization in mitochondria. More recently, Lam *et al.* (1994) have shown that overexpression of BCL-2 interferes with thapsigargin-induced Ca^{2+} mobilization from the ER in the WEHI7.2 T lymphoma cell line, an effect that is associated with preservation cell viability. Other work has shown that BCL-2 prevents the Ca^{2+} increase induced by hydrogen peroxide in B cells from BCL-2 trangenic mice (Zornig *et al.*, 1995). Finally, a screen for polypeptides capable of physically interacting with BCL-2 has identified a protein (Nip-2) containing a putative Ca^{2+}-binding domain that colocalizes with BCL-2 in the ER and perinuclear region (Boyd *et al.*, 1994). Precisely how BCL-2 regulates intracellular Ca^{2+} is still unclear, although given its co-localization with Ca^{2+} transport sites in mitochondria, the ER, and the nuclear envelope (deJong *et al.*, 1994), a direct effect of BCL-2 on Ca^{2+} channel(s) is possible. Alternatively, given the tight interrelationship between Ca^{2+} and oxidative stress, BCL-2 could be influencing Ca^{2+} homeostasis via effects on cellular redox status (Hockenbery *et al.*, 1993; Kane *et al.*, 1993). It is also possible that BCL-2 regulates the localization and/or enzymatic activities of other polypeptides implicated in the control of apoptosis, especially nuclear proteins such as p53, Myc, AP-1 and the catalytic component of protein kinase A (Marti *et al.*, 1994).

Calcium Coupling to the Effector Pathway

An important aspect of ongoing research involves defining the biochemical consequences of Ca^{2+} mobilization in apoptotic cells, and at present there are two models to explain how these alterations might trigger apoptosis. In one, depletion of intracellular stores and possibly influx of Ca^{2+} across the plasma membrane promote a sustained Ca^{2+} increase that acts as a signal for apoptosis, perhaps in part by activating key catabolic enzymes that make up the effector machinery (Fig. 2). In the second, it is not the Ca^{2+} increase but the emptying of intracellular Ca^{2+} stores that triggers apoptosis, perhaps by disrupting intracellular architecture and allowing key elements of the effector machinery to gain access to their substrates. These models are certainly not mutually exclusive. Evidence for both models will be presented below, but it should be emphasized at the outset that definitive proof for either one is lacking at present.

Possible Targets for Ca^{2+} Elevations

Signal transduction intermediates

Activation of Ca^{2+}-dependent protein kinases and/or phosphatases leading to alterations in gene transcription represents one possible way that Ca^{2+} might regulate apoptosis. The most convincing support for this hypothesis has come from experiments with the immunosuppressant cyclosporin A, a compound that binds a family of cytosolic receptors termed cyclophilins and in so doing, forms a composite molecular surface that binds to and inhibits the Ca^{2+}/calmodulin-dependent protein serine/threonine phosphatase, calcineurin (Liu *et al.*, 1991). Studies by several independent laboratories have shown that cyclosporin A can block Ca^{2+}-dependent apoptosis in lymphoid model systems (Amendola *et al.*, 1994; Bonnefoy-Berard *et al.*, 1994; Makrigiannis *et al.*,

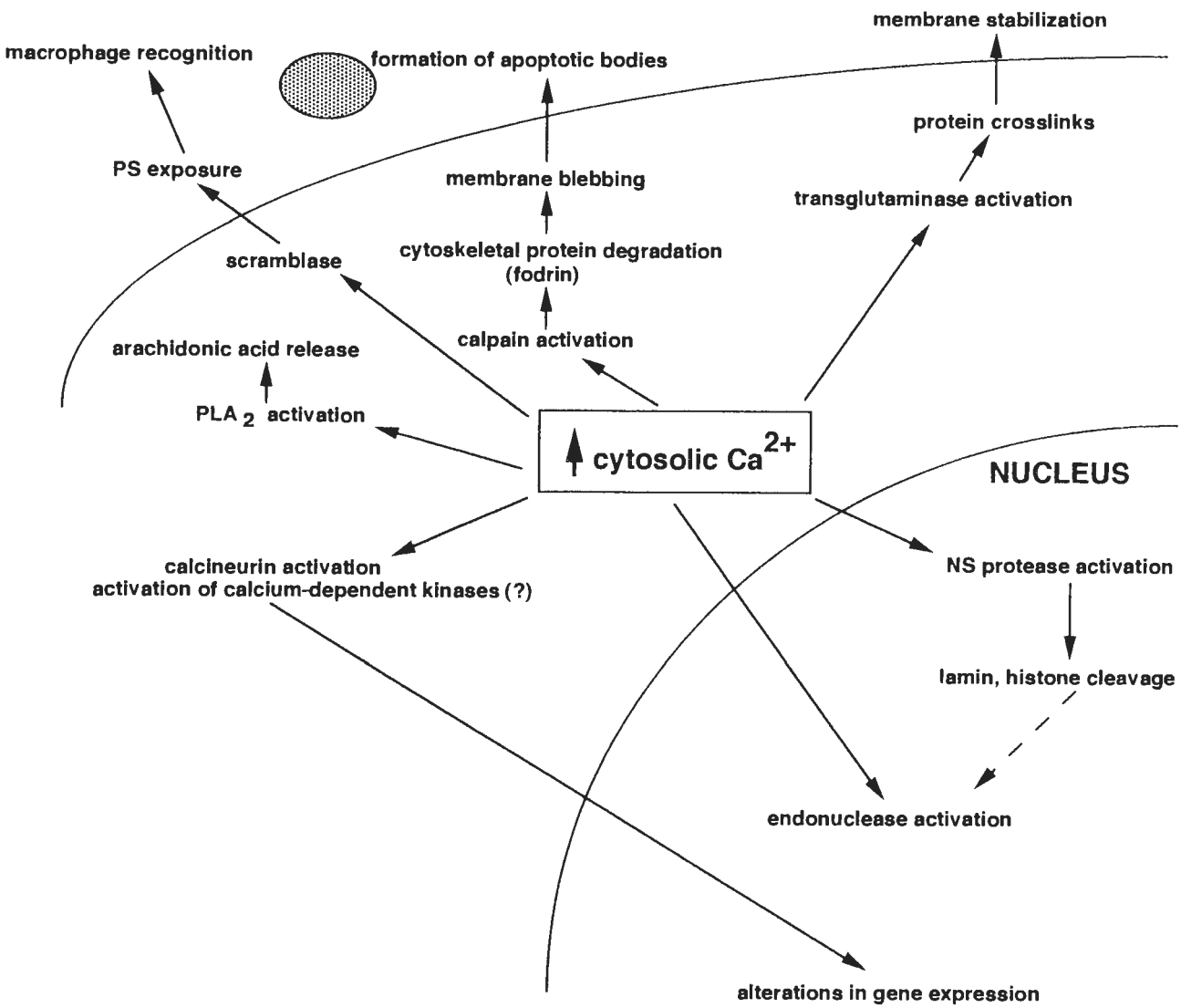

Figure 2. Targets for Ca^{2+} in the regulation of apoptosis. The figure schematically presents the processes involved in apoptosis for which a role for Ca^{2+} is strongly supported by recent investigation. PS: phosphatidylserine; PLA_2: phospholipase A_2; NS: nuclear scaffold.

1994; Shi *et al.*, 1989), indicating that calcineurin activation may be required for these responses. Recent work (Jiang, Chow, Orrenius, manuscript submitted) confirms that cyclosporin A and FK506 block Ca^{2+} stimulated apoptosis in T cell hybridomas but they are without effect on Ca^{2+}-dependent apoptosis in immature rodent thymocytes *in vitro*. Induction of the orphan steroid receptor Nur77 and the Fas ligand represent at least two of the confirmed molecular targets of calcineurin in mature T cells and T cell hybridomas (Anel *et al.*, 1994; Yazdanbakhsh *et al.*, 1995). The involvement of calcineurin in Ca^{2+} stimulated apoptosis could also potentially explain, in part, the sensitivity of various apoptotic pathways to inhibition by calmodulin antagonists.

Phospholipase A_2 (PLA_2) is another signaling intermediate that has been suggested to be a target for Ca^{2+} in apoptosis. Stimulation of T cell clones with retroviral or bacterial superantigens is associated with the generation of reactive oxygen intermediates and PLA_2 activation (Wever *et al.*, 1995). Similarly, several groups have shown that tumor necrosis factor-induced apoptosis is associated with PLA_2 activation (Hayakawa *et al.*, 1993; Hollenbach *et al.*, 1992; Mutch *et al.*, 1992; Voelkel-Johnson *et al.*, 1995), and that the sensitivity of a large panel of melanoma cell lines to TNF plus inhibitors of macromolecular synthesis is associated with the level of cytosolic PLA_2 (Voelkel-Johnson *et al.*, 1995). Arachidonic acid release has been documented in several

other models of apoptosis (Agarwal *et al.*, 1993), most notably radiation-induced apoptosis in thymocytes (Korystov *et al.*, 1993; Shaposhnikova *et al.*, 1994), and inhibitors of PLA_2 block both arachidonic acid release and cell death (Korystov *et al.*, 1993). Radiation and TNF-induced apoptosis may actually be linked by PLA_2 activation, as irradiation triggers TNFα production via a PLA_2-dependent mechanism (Hallahan *et al.*, 1994). Direct effects of X-rays on PLA_2 activation in isolated membrane preparations have also been reported (Cohen and DeLeo, 1993). It has also been suggested that BCL-2 and BCL-X may inhibit TNF- and Fas-induced apoptosis in breast carcinoma cells via a mechanism that involves inhibition of PLA_2 activation (Jaattela *et al.*, 1995). Therefore, Ca^{2+} may act in these and other systems to promote PLA_2 activation, which may in turn result in oxidative stress and DNA damage via release and metabolism of arachidonic acid.

Ca^{2+}-activated proteases

There is some evidence that Ca^{2+}-sensitive protease(s) might represent direct targets for Ca^{2+} elevations in apoptosis. Recent work has shown that the Ca^{2+}-dependent neutral protease, calpain, is rapidly activated in T lymphocytes following treatment with glucocorticoids or exposure to γ-irradiation, and that calpain antagonists can block DNA fragmentation associated with the response (Squier *et al.*, 1994). Similarly, Henkart's group has demonstrated that some (but not all) pathways of apoptosis in mature T lymphocytes can be inhibited by calpain antagonists (Sarin *et al.*, 1993; 1995). The cytoskeletal protein, fodrin, is at least one substrate for calpain that is cleaved in T cells following treatment with glucocorticoids or engagement of the Fas antigen (Martin *et al.*, 1995). Similarly, we have recently obtained evidence that the cytoskeletal protein, vimentin, is also cleaved in apoptotic cells by a calpain-sensitive mechanism (J. Kiefer *et al.*, unpublished observation). Precisely how fodrin and vimentin cleavage participate in the apoptosis effector mechanism is unclear, although they may be involved in cellular shrinkage, membrane blebbing, or other structural alterations associated with apoptosis.

Other Ca^{2+}-activated proteases may also participate in the process. Previous work has demonstrated that incubation of isolated nuclei in the presence of Ca^{2+} promotes the rapid degradation of a family of nuclear matrix proteins, the lamins (Clawson *et al.*, 1992; Tokes and Clawson, 1989). The protease responsible for lamin cleavage in this system is directly associated with the nuclear matrix and is activated by Ca^{2+}. In parallel, independent work from several laboratories has shown that lamins are also degraded in cells undergoing apoptosis (Kaufmann, 1989; Lazebnik *et al.*, 1993; Neamati *et al.*, 1995; Oberhammer *et al.*, 1994), and in thymocytes lamin cleavage occurs via a Ca^{2+}-dependent mechanism (Neamati *et al.*, 1995), inspiring investigations into the possible involvement of the lamin protease in the response. Thus, we have found that a specific peptide inhibitor of the putative lamin protease {also known as "nuclear scaffold (NS) protease"} blocks cellular shrinkage and DNA fragmentation in thymocytes exposed to antibodies to the T cell receptor, thapsigargin and glucocorticoids, but not in cells treated with etoposide, a cancer chemotherapeutic agent that acts via induction of DNA damage (Zhivotovsky *et al.*, 1995; McConkey, 1996). We have also found that inhibitors of this protease selectively block both lamin cleavage and DNA fragmentation in isolated nuclei incubated with Ca^{2+}, while inhibitors of the ICE family of proteases do not (McConkey, 1996). Importantly, however, the NS protease is not the only protease that can cleave the lamins, as indicated by recent work by Lazebnik *et al.* (1995), who have shown that an inhibitor of the ICE/ced-3 family of cysteine proteases blocks lamin cleavage in another isolated nuclei system, whereas treatment with inhibitors of the NS protease does not. Moreover, in this system, lamin cleavage, chromatin condensation, and DNA fragmentation do not require exogenous Ca^{2+}. Whether Ca^{2+} is involved in generating the apoptosis-promoting activity found within the extracts these authors use to promote nuclear lamin cleavage is not clear at present. In addition, whether BCL-2 is capable of blocking the nuclear alterations induced by these extracts is also not known, and it is therefore possible that the activity present in them is an irreversibly activated component of the effector machinery (i.e., an ICE protease) that is no longer subject to Ca^{2+} regulation.

Ca^{2+}-activated endonuclease(s)

As introduced above, endonuclease activation resulting in the formation of oligonucleosome-length DNA fragments (DNA ladders) remains the most characteristic biochemical feature of apoptotic cell death. Early work by Hewish and Burgoyne (1973) and later by Vanderbilt *et al.* (1982) demonstrated that a Ca^{2+}/Mg^{2+}-dependent enzyme activity, capable of generating characteristic apoptotic chromatin cleavage patterns, is constitutively present within nuclei of a variety of different cell types. Subsequent work by Cohen and Duke (1984), and Wyllie *et al.* (1984), demonstrated the involvement of this activity in the DNA fragmentation observed in thymocytes undergoing apoptosis, and it is now thought that it mediates DNA fragmentation in a variety of other model systems as well. The search for, and purification of potential Ca^{2+}-dependent apoptotic nucleases has subsequently been undertaken by several laboratories. Thus, Gaido and Cidlowski (1991) have described a low-

molecular weight nuclease (NUC18) with Ca^{2+} and Mg^{2+} dependence activity in apoptotic lymphoid cells in response to several kinds of apoptotic stimuli. Interestingly, the purified NUC18 shares amino acid sequence homology with cyclophilin, and human recombinant cyclophilin A has biochemical and pharmacological properties identical to native NUC18 (Montague *et al.*, 1994). NUC18 is also present in untreated thymocytes in precursor form or as part of a higher molecular weight complex (> 100 kDa), suggesting that the enzyme is maintained in an inactive complex from which the nuclease dissociates in response to apoptotic signals. Although the precise mechanism of liberation of active enzyme from its precusors is unknown, an attractive possibility is that it may involve proteolysis.

The Ca^{2+}-dependent endonuclease DNase I is another excellent candidate apoptotic nuclease (Peitsch *et al.*, 1993). Addition of the enzyme to isolated nuclei and other reconstitution systems promotes the formation of DNA strand breaks that possess the same 5'-PO_4 and 3'-OH end groups found in DNA fragments isolated from apoptotic cells. Although the enzyme is localized within the rough endoplasmic reticulum, the Golgi complex, and small (secretory) vesicles in viable cells, it is also found within the perinuclear space of apoptotic cells, and it is possible that structural alterations in the ER and/or nuclear envelope associated with apoptosis may promote the entry of DNase I into the nucleus (see below). A similar mechanism may promote entry of an ER-localized fraction of the NS lamin protease into the nucleus. Several other proteins with Ca^{2+}/Mg^{2+} endonuclease activity have been isolated (Ishida *et al.*, 1974; Nikonova *et al.*, 1993; Ribeiro and Carson, 1993; Wyllie *et al.*, 1992), but to date proof that any one of these activities is directly involved in oligonucleosomal DNA fragmentation in apoptosis is lacking.

Transglutaminase activation

Transglutaminases are a group of Ca^{2+}-dependent enzymes that catalyze the post-translational coupling of amines (including polyamines) into proteins and the crosslinking of proteins via gamma glutamyl lysine bridges when the amine is a peptide-bound lysine residue. Tissue transglutaminase has been implicated in a number of physiological processes, including crosslinking of integral plasma membrane proteins with the cytoskeleton. Recent work indicates that tissue transglutaminase is also involved in induction of apoptosis (Fesus *et al.*, 1987, 1989). Expression of transglutaminase mRNA and protein levels increase markedly in dying cells. The enzyme appears to be activated by elevations of the cytosolic Ca^{2+} concentration, which are involved in apoptosis in many different systems. Isolation of apoptotic bodies from a number of different tissues has shown that they are resistant to dissolution by detergents and chaotrophic agents; this may in part be explained by the fact that surface polypeptides in these structures are crosslinked via gamma glutamyl lysine isopeptide bonds (Taresa *et al.*, 1992). The resistance of these structures to proteolysis may allow them to accumulate, and they can be detected in the media of cell cultures containing high rates of apoptotic cell death (Fesus *et al.*, 1991). Isodipeptide can also be detected in normal plasma, and its concentration increases following induction of apoptosis in various organs, including the thymus and liver.

The role of transglutaminase promoting cell death and/or phagocytosis is still poorly understood. One possibility is that protein crosslinking stabilizes apoptotic cells and bodies, preventing leakage of intracellular contents into the extracellular millieu (which can trigger inflammation). Alternatively, transglutaminase modification may target proteins for subsequent degradation. Intriguingly, overexpression of the enzyme has been reported to trigger apoptotic cell death (Melino *et al.*, 1994), suggesting that transglutaminase may be a component of the death effector pathway. Further efforts are required to identify the substrates for transglutaminase in apoptotic cells and to determine the consequences of their modification.

Exposure of phosphatidylserine and macrophage recognition

Recent work indicates that the movement of phosphatidylserine (PS) from the inner to the outer surface of the plasma membrane, a process that functions in the removal of apoptotic cells and bodies by both professional phagocytic cells (macrophages) and neighboring cells in tissues (Fadok *et al.*, 1992a, 1992b), is another component of apoptosis that is regulated by alterations in cytosolic Ca^{2+}. Phospholipids are known to be distributed asymmetrically across the plasma membrane, with phosphatidylcholine and sphingomyelin localized primarily to the extracellular surface and PS and phosphatidylethanolamine restricted almost exclusively to the intracellular surface under normal conditions (Verkleij *et al.*, 1973; Zwaal *et al.*, 1975). Most of the work on plasma membrane lipid asymmetry has been conducted with red blood cells, where it is known that PS localization is regulated by energy-dependent processes involving specific lipid transporters ("flipases" and "flopases") that move PS to the outside or inside surface, respectively. Transport in both directions is ATP-dependent and sensitive to sulfhydryl modifying agents. Interestingly, inhibition of lipid movement with reagents that abrogate the activities of both the flipase and flopase does not result in loss of membrane asymmetry (Connor and Schroit, 1990), suggesting that these enzymes may primarily function to restore lipid asymmetry following its disrup-

tion. However, transport can also be inhibited by increasing the cytosolic Ca^{2+} concentration, which does result in rapid non-specific redistribution of all phospholipids (Bevers *et al.*, 1990). The mechanism of Ca^{2+}-mediated PS exposure is not yet clear, but in red blood cells, it closely parallels formation of cytoskeleton-free lipid microvesicles (Sims *et al.*, 1989) and other phenomena, such as, calpain activation (Fox *et al.*, 1991) and the formation of phosphatidylinositol(4,5)-bisphosphate-Ca^{2+} complexes (Sulpice *et al.*, 1994). However, neither direct Ca^{2+} effects, calpain-mediated protein degradation (Basse *et al.*, 1993; Orrenius *et al.*, manuscript submitted), Ins(4,5)P_2 accumulation (Bevers *et al.*, 1995), nor inhibition of the flipase can singularly accommodate the membrane rearrangements that occur. More recent work suggests that Ca^{2+}-mediated redistribution is mediated by a Ca^{2+} and sulfhydryl-sensitive, energy-dependent lipid scramblase (Williamson *et al.*, 1995). Therefore, elevations in the cytosolic Ca^{2+} concentration probably promote PS exposure and allow for macrophage recognition primarily by inactivating the PS translocase and by activating the scramblase (Verhoven *et al.*, 1995). Interestingly, PS exposure on aged red blood cells is associated with increased cytosolic Ca^{2+} levels and increased cell density, suggesting that these events may be mechanistically related in both red cells and apoptotic cells. In addition, given its regulation by Ca^{2+} and calpain-dependent mechanisms, it is tempting to speculate that red blood cell microvesiculation may be functionally related to the formation of apoptotic bodies by nucleated cells undergoing apoptosis.

Plasma membrane lipid asymmetry is critically involved in several other important physiological functions. For example, surface-exposed PS serves as the point of assembly for the coagulation factors Va and Xa into the prothrombinase complex (Rosing *et al.*, 1985). In addition, PS exposure enhances membrane fusion events and appears to be involved in the initiation of microvesiculation in red blood cells (Schewe *et al.*, 1992). Finally, surface PS is also detectable on certain tumor cells (Utsugi *et al.*, 1991). Although the mechanisms underlying the latter have not been defined, it is possible that PS exposure is involved in the prominent macrophage infiltration observed in most solid tumors and that surface PS may represent a potential target for anti-tumor therapies.

Possible Consequences of Intracellular Ca^{2+} Pool Depletion

In some cellular systems, extracellular or intracellular Ca^{2+} chelators can actually promote DNA fragmentation (Treves *et al.*, 1994), even though other triggers of apoptosis in these systems (i.e., glucocorticoids, growth factor withdrawal) have been shown to deplete the ER Ca^{2+} store. These observations have led Baffy *et al.* (1993) and more recently Lam *et al.* (1993) to propose that depletion of the ER Ca^{2+} store may itself serve as a signal for apoptosis. How could this occur? At least two of the catabolic enzymes proposed to be involved in the effector mechanism of apoptosis (DNase I and an extranuclear pool of the NS protease) are localized to the ER, and it is therefore possible that loss of Ca^{2+} leads to release of these factors into the perinuclear region or into the nuclear matrix itself. In addition, it is known that ER Ca^{2+} pool depletion results in the release of a small biomolecule that participates in a retrograde signal for plasma membrane Ca^{2+} influx, and it is possible that it or another molecule released in a similar fashion can also promote cell death.

Depletion of mitochondrial Ca^{2+} stores may also participate in the signal for apoptosis. Mitochondrial Ca^{2+} uptake is driven by mitochondrial membrane potential ($\Delta\Psi$) (Richter, 1993). In de-energized mitochondria, Ca^{2+} can be released by a reversal of the uptake pathway. Under conditions of oxidative stress, mitochondrial Ca^{2+} cycling can reach critical levels, leading to increased energy expenditure and a dramatic fall in $\Delta\Psi$. Recent work has shown that a fall in mitochondrial $\Delta\Psi$ is an early event in apoptosis (Petit *et al.*, 1995; Zamzani *et al.*, 1995a, 1995b), and ruthenium red, an inhibitor of the mitochondrial Ca^{2+} uptake pathway, blocks apoptosis in L929 fibroblasts (Hennet *et al.*, 1993) and inhibits the progression of apoptosis in glucocorticoid-treated splenocytes (Zamzani *et al.*, 1995a), suggesting that mitochondrial Ca^{2+} release is involved. Again, further efforts are required to determine the relationship between this event and the activation of the proteases and nucleases of the effector pathway.

Alternative Signals for Apoptosis

Oxidative stress

Several lines of evidence indicate that reactive oxygen species are involved in promoting apoptosis in diverse model systems. Treatment of cells with low to moderate doses of exogenous oxidants (i.e., hydrogen peroxide, tert-butyl peroxide, menadione) can trigger apoptosis (Buttke and Sandstrom, 1994; Hennet *et al.*, 1993; Hockenbery *et al.*, 1993; Kane *et al.*, 1993; Lennon *et al.*, 1990, 1991; McConkey *et al.*, 1988). Moreover, apoptosis induced by agents that are not direct oxidants (TNF, glucocorticoids, thapsigargin, chemotherapeutic agents) is associated with oxygen radical production and depletion of intracellular antioxidants (i.e., reduced glutathione) (Fernandez *et al.*, 1995; Hennet *et al.*, 1993; Hockenbery *et al.*, 1993; Kane *et al.*, 1993; Mayer and Noble, 1994; Richter, 1993; Slater *et al.*,

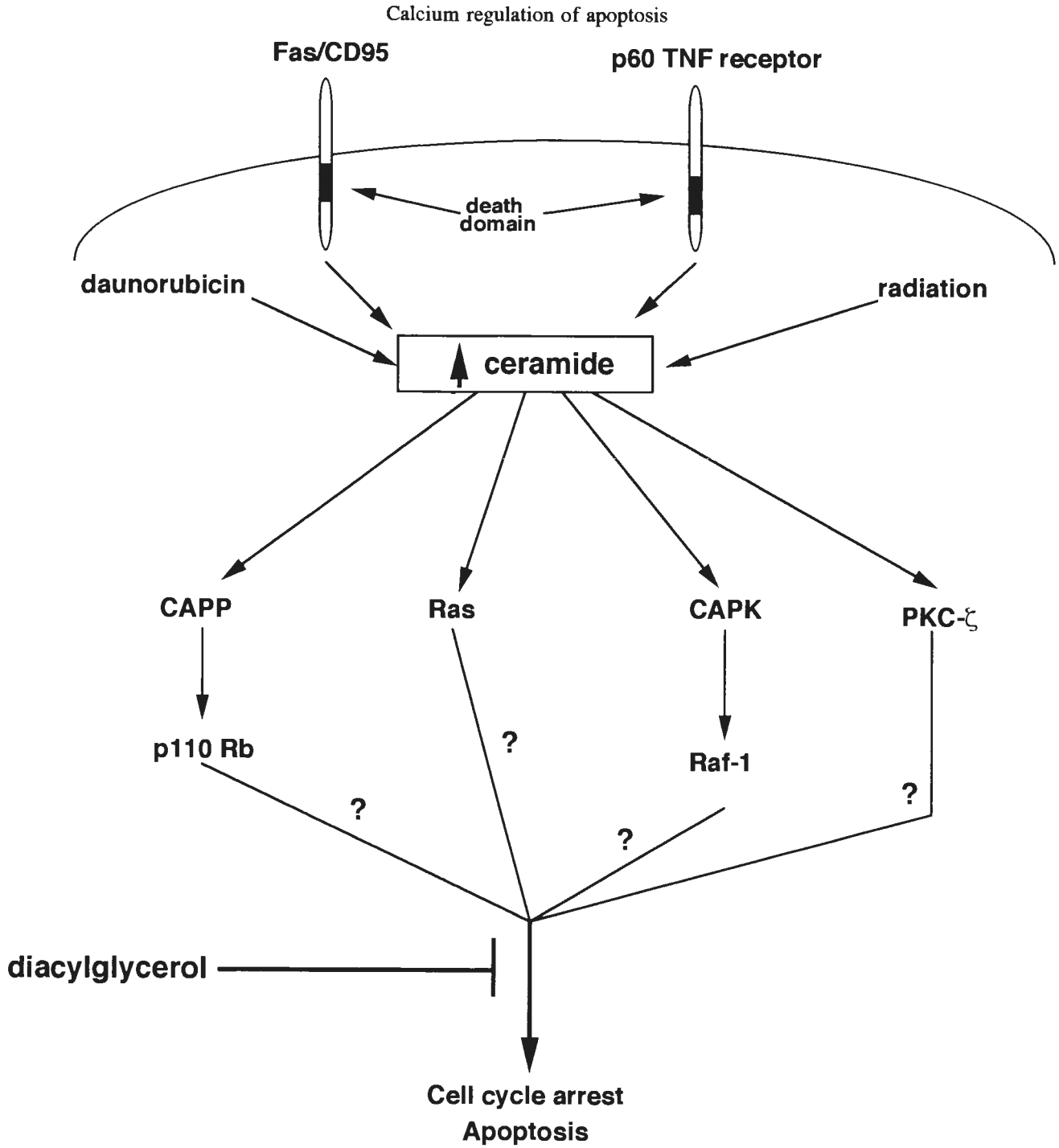

Figure 3. Candidate downstream mediators of ceramide-induced apoptosis. Production of ceramide via sphingomyelinase activation or ceramide synthase stimulation (daunorubicin) can lead to elevations in ceramide levels that can trigger cell death. Signaling intermediates known to be directly regulated by ceramide include ceramide-activated protein phosphatase (CAPP) and ceramide-activated protein kinase (CAPK). More recent work suggests that the former can promote the dephosphorylation and activation of the p110 retinoblastoma protein, resulting in cell cycle arrest (Dbaibo *et al.*, 1995), whereas the latter has been shown to interact with and regulate the protein serine/threonine kinase Raf-1 (Yao *et al.*, 1995). We (Trent *et al.*, 1996) and others (Gulbins *et al.*, 1995) have obtained evidence that activation of Ras is also involved, and preliminary work suggests that the ζ isoform of protein kinase C (PKCζ) represents another target for ceramide (Lozano *et al.*, 1994). Importantly, diacylglycerol and phorbol esters can block ceramide-induced apoptosis, presumably by activating one or more PKC isozymes. How cell cycle arrest and apoptosis are differentially regulated by ceramide is not known at present.

1995; Wolfe *et al.*, 1994; Zamzani *et al.*, 1995a). Although the identities of the oxygen radicals involved in each system are still under active investigation, roles for superoxide (Hennet *et al.*, 1993; Zamzani *et al.*, 1995b), lipid peroxides (Hockenbery *et al.*, 1993), nitric oxide (Xie *et al.*, 1995), and hydroxyl radicals (Wolfe *et al.*, 1994) have been proposed. In these systems, exogenous antioxidants such as N-acetyl cysteine and free radical scavengers block DNA fragmentation and cell death (Fernandez *et al.*, 1995; Mayer and Noble, 1994; Slater *et al.*, 1995; Wolfe *et al.*, 1994). Finally, several laboratories have now presented strong evidence that BCL-2 (Hockenberry *et al.*, 1993; Kane *et al.*, 1993) and BCL-XL (Fang *et al.*, 1995) possess antioxidant properties that may be involved in their abilities to inhibit cell death. Thus, oxidative stress is another good candidate for a central cell death signal, one which may affect intracellular Ca^{2+} homeostasis. However, it should be noted that BCL-2 and BCL-XL can still inhibit apoptosis under conditions of low oxygen (Jacobson and Raff, 1995; Shimizu *et al.*, 1995), which has been raised as an argument against a universal role for oxidative stress in the response.

Intracellular acidification

Eastman's group (Barry and Eastman, 1993) have proposed another alternative to Ca^{2+} that may serve as a general signal for cell death. In their efforts to characterize the biochemical mechanisms underlying chemotherapy-induced apoptosis, they determined that isolated nuclei from their cellular models possessed an endonuclease activity that was stimulated by acidic pH (Dnase II) (Barry and Eastman, 1993). Interestingly, Mary Collins and her co-workers (personal communication) have recently identified an acidic nuclease in IL-3-dependent hematopoietic cells that can also be activated by Ca^{2+} under the appropriate conditions, suggesting that intracellular acidification and alterations in intracellular Ca^{2+} homeostasis may represent independent ways of arriving at the same endpoint (endonuclease activation) in apoptotic cells. Other efforts have shown that a drop in cytoplasmic pH precedes the morphological and biochemical features of apoptosis in certain models (Barry and Eastman, 1992; Li and Eastman, 1995), observations that have since been confirmed by other investigators in other systems (Gottlieb *et al.*, 1995). Moreover, the protective effects of certain survival factors and agents that activate protein kinase C have been linked to activation of the Na^+/H^+ antiporter and intracellular alkylinization (Gottlieb *et al.*, 1995; Rajotte *et al.*, 1992). Finally, the drop in pH is prevented by overexpression of BCL-2 (A. Eastman, personal communication), suggesting that acidification may represent another cellular target for this family of apoptosis suppressors. It will be interesting to determine whether intracellular acidification is related to oxidative stress and intracellular Ca^{2+} alterations in these systems.

Ceramide production

The generation of bioactive signal transduction regulators via the hydrolysis of plasma membrane phospholipids is emerging as an important general means of regulating apoptotic cell death. Of all of the second messengers shown to be involved, most recent attention has been focussed on ceramide as a possible ubiquitous trigger of apoptosis (Obeid *et al.*, 1993; Pushkareva *et al.*, 1995) (Fig. 3). Two major mechanisms have been identified that appear to contribute to the formation of ceramide under different circumstances. The most common pathway involves activation of the enzyme sphingomyelinase, which catalyzes the hydrolysis of sphingomyelin to form ceramide and diacylglycerol, a response that is involved in apoptosis induced by tumor necrosis factor, engagement of the Fas antigen, and ionizing radiation (Cifone *et al.*, 1994; Gulbins *et al.*, 1995; Haimovitz-Friedman *et al.*, 1994; Jarvis *et al.*, 1994b; Obeid *et al.*, 1993; Pushkareva *et al.*, 1995; Tepper *et al.*, 1995). Ceramide can also be formed *de novo* via activation of ceramide synthase, and it has been shown that the cytotoxic effects of the chemotherapeutic agent daunorubicin on leukemic cell lines are mediated by this pathway (Bose *et al.*, 1995). Notably, exogenous hydrolysis-resistant ceramide analogs or sphingomyelinase can mimic the effects of TNF and the other apoptosis-inducing agents to trigger endonuclease activation and cell death, indicating that ceramide production is sufficient to induce apoptosis (Bose *et al.*, 1995; Cifone *et al.*, 1994; Gulbins *et al.*, 1995; Haimovitz-Friedman *et al.*, 1994; Jarvis *et al.*, 1994a, 1994b; Obeid *et al.*, 1993; Tepper *et al.*, 1995). In addition, the possibility that ceramide is an evolutionarily conserved trigger of apoptosis is suggested by the fact that overexpression of the Drosophila cell death protein, Reaper, leads to upregulation of cellular ceramide levels (Pronk *et al.*, 1996). Interestingly, phorbol esters and diacylglycerol (DAG) antagonize the death-promoting effects of ceramide in diverse models (Jarvis *et al.*, 1994a; Obeid *et al.*, 1993), suggesting that a balance between ceramide and DAG may determine the outcome of death signals. The downstream targets for ceramide remain unclear, but candidates include a ceramide-dependent protein phosphatase (CAPP) (Wolff *et al.*, 1994), a ceramide-activated protein kinase (CAPK) (Kolesnick and Golde, 1994), the ζ isoform of protein kinase C (Lozano *et al.*, 1994), and the H-ras protooncogene (Gulbins *et al.*, 1995; Trent *et al.*, 1996). Whether ceramide acts upstream, downstream, or independently of the other candidate biochemical mediators of apoptosis remains to be determined.

Conclusions and Future Directions

The independent efforts of many laboratories over the past several years have established that alterations in intracellular Ca^{2+} homeostasis are commonly involved in initiating apoptosis. Ongoing work suggests that activation of Ca^{2+} stimulated signalling networks and catabolic enzymes represents one way these signals are translated into responses. In addition, preliminary evidence indicates that the depletion of intracellular Ca^{2+} pools can itself serve as a signal for cell death, perhaps by promoting relocalization of some of the key catabolic enzymes involved and by enhancing oxidative stress in mitochondria. In some systems, other signals have been defended as key regulators of the response, and further efforts are therefore required to determine if and how all of these signals are interrelated.

An important component of the defense of Ca^{2+} as a central cell death regulator is the idea that BCL-2 and its homologs specifically regulate intracellular Ca^{2+} compartmentalization, an idea that has strong preliminary support but requires a good deal of additional investigation. In particular, further efforts are required to determine how BCL-2 exerts its effects on Ca^{2+} and whether these effects are required for its cell death-suppressing function. Similarly, it is maintained at present that any central cell death signal would directly or indirectly activate one or more members of the ICE family of cysteine proteases, and at present there is no evidence available that any of the candidate central cell death signals are capable of this. Elucidation of these relationships over the next few years should provide new targets for therapeutic intervention that may aid in the treatment of the expanding number of diseases, including cancer, AIDS, and neurodegenerative diseases, in which apoptosis is thought to play a central role in their pathologies.

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Discussion with Reviewers

K.M. Kim: Abnormal increases in Ca^{2+} play a key role in regulation of apoptosis by activation of enzymes that leads to DNA fragmentation. How does the increase in Ca^{2+} come about in the first place? What initially triggers apoptosis?

Author: It appears that Ca^{2+} evaluations in apoptotic cells occur via one of two general mechanisms. The first involves receptor-coupled activation of phospholipase(s) C leading to polyphosphoinositide hydrolysis and production of inositol triphosphate, which promotes release of Ca^{2+} from the endoplasmic reticulum and Ca^{2+} influx across the plasma membrane. This mechanism is analogous to the one involved in growth factor-induced cell proliferation and can therefore be regarded as "physiological." The second involves damage, resulting in redistribution of Ca^{2+} down the concentration gradients maintained between the cytosol and intra- and extracellular Ca^{2+} down the concentration gradients

maintained between the cytosol and intra- and extracellular Ca^{2+} pools. Oxidative stress represents at least one common trigger for the damage-dependent Ca^{2+} increase pathway, and depletion of cellular ATP represents an attractive (hypothetical) second. The initial trigger for apoptosis is unknown, although Zamzani *et al.* (1995b) have advanced the hypothesis that alterations in mitochondrial membrane potential and/or function are involved which could initiate the irreversible disruption of intracellular Ca^{2+} homeostasis.

K.M. Kim: Activation of ICE/CED-3 is said to be involved in the earliest stage of apoptosis. What is the mechanism?
Author: The biochemical mechanisms involved in the activation of the ICE family are not known but are under active investigation. Importantly, the ICE family proteases are not directly Ca^{2+} dependent.

K.M. Kim: Abrogation of apoptosis by BCL-2 is said to involve inhibition of Ca^{2+} transports by membranes. Would the author elaborate on the phenomenon?
Author: Changes in intracellular Ca^{2+} compartmentalization occur in many examples of apoptosis. In a number of cellular systems the protective effects of BCL-2 have been linked to preventing these Ca^{2+} compartmentalization changes, but the mechanism(s) involved have not been elucidated to date. It is possible that BCL-2 serves as a channel regulator or that its effects on Ca^{2+} are secondary to its primary action on mitochondrial membrane potential and/or oxidative stress.

A.R. Eastman: This review focuses on the possible activation of calcium-dependent endonucleases n apoptosis. However, this conclusion is questionable when one considers the potential role of calcium in activation of proteases, calmodulin and tissue transglutaminase. Given these multiple functions, can one really suggest that calcium is required for any one particular step? I agree that evidence clearly supports the presence of a calcium endonuclease in many nuclei, but is there really evidence for its involvement in apoptosis? Most of the evidence is based upon experimentally increasing or decreasing calcium, both these manipulations will equally modify proteases, calmodulin and tissue trans-glutaminase. The author ignores protein kinase C, yet, this is a calcium-dependent kinase that frequently has been shown to protect cells from apoptosis. This is yet another step in apoptosis that can be modified by changes in calcium.

In a similar vein, the author suggests that calcium is required for lamin degradation, but is lamin degradation required for apoptosis?

Author: Dr. Eastman is absolutely correct in emphasizing the pleiotropic effects of Ca^{2+} in apoptotic cells (and indeed in those protected from apoptosis), and I agree that Ca^{2+} is most likely not acting at one step in the apoptotic pathway but at many. Ongoing investigation aimed at directly identifying the molecular targets for Ca^{2+} in apoptotic cells should help to clarify this issue.

Our hypothesis is that lamin degradation promotes DNA fragmentation. Since Lazebnik has shown that DNA fragmentation can occur under certain circumstances where lamin degradation is blocked (Lazebnik *et al.*, 1995), we would not even state that lamin degradation is absolutely required for endonuclease activation. However, our preliminary work indicates that inhibitors of the lamin protease dramatically delay apoptotic cell death in many of our models; in fact, they appear more potent than are inhibitors of the ICE family. Therefore, we would suggest that activation of the lamin protease may be required for cell death, although its "death substrates" may be quite numerous and diverse.

A.R. Eastman: The author describes an apparent paradox that increasing calcium sometimes induces apoptosis but at other times inhibits it. What are the levels of calcium in each case? Upon withdrawal of IL3, I believe that there is a drop in intracellular calcium, and that A23187 permits recovery, not really an increase in calcium. In contrast, in other cases A23187 causes a dramatic influx of calcium. Could the answer be that cells undergo apoptosis at either low or high calcium, and that in the IL-3 situation, A23187 helps restore normal calcium homeostasis?
Author: It is definitely possible that is the magnitude of the change in Ca^{2+} concentration, rather than the absolute level achieved in the cytoplasm, that determines whether cell undergoes apoptosis or not. How this $\Delta[Ca^{2+}]_i$ would engage the enzymatic machinery for apoptosis is not clear, but disruption of subcellular structure (i.e., endoplasmic reticular permeability changes) could be postulated.

A.R. Eastman: Is there evidence for a calcium-dependent and independent mechanism of apoptosis in a single cell line, or does the calcium dependence vary with cell line?
Author: The only clear example I know of involves work with an IL-3-dependent hematopoietic cell line, where Mary Collins and her co-workers (personal communication) have found evidence for the co-expression of both Ca^{2+}/Mg^{2+} and acidic endonucleases in isolated nuclei.