

Apoptosis and Autophagy: Decoding Calcium Signals that Mediate Life or Death

Michael W. Harr^{1,4} and Clark W. Distelhorst^{1,2,3}

¹Division of Hematology and Oncology, Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

²Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106

³Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio 44106

⁴Molecular Pharmacology and Chemistry Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Correspondence: cwd@case.edu

Calcium is a versatile and dynamic 2nd messenger that is essential for the survival of all higher organisms. In cells that undergo activation or excitation, calcium is released from the endoplasmic/sarcoplasmic reticulum to activate calcium-dependent kinases and phosphatases, thereby regulating numerous cellular processes; for example, apoptosis and autophagy. In the case of apoptosis, endogenous ligands or pharmacological agents induce prolonged cytosolic calcium elevation, which in turn leads to cell death. In contrast, there is now evidence that calcium regulates autophagy by several mechanisms, and these may be important for maintaining cell survival. Here we summarize what is known about how calcium regulates these life and death decisions. We pay particular attention to pathways that have been described in lymphocytes and cardiomyocytes, as these systems provide optimal models for understanding calcium signaling in the context of normal cell physiology.

Apoptosis is a process of programmed cell death or suicide that occurs when cells have undergone irreversible stress or damage. It is required to maintain normal cell homeostasis or to eliminate a population of cells that may be harmful to the organism or unnecessary during organ development (Green 2003). For example, it is the primary mechanism by which potentially autoreactive T cells are eliminated from the immune system. There are two conventional apoptosis pathways: the extrinsic pathway, which is typically initiated by death receptors (e.g., Fas) on the plasma membrane

and the intrinsic (mitochondrial) pathway, which involves permeabilization of the outer mitochondrial membrane followed by the release of cytochrome c. In this review, we primarily focus our attention on the intrinsic pathway due to the importance of intracellular calcium in the regulation of this process.

In brief, cytochrome c release stimulates apoptosis via its interaction with the protein Apaf-1, which in turn activates the initiator caspase-9 and the executioner caspase-3 (Green 2005). Caspases comprise a family of cysteine proteases that are essential for the classically



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observed cellular and biochemical characteristics of apoptosis, which include (but are not limited to) membrane blebbing, chromatin condensation, and DNA fragmentation. Another class of cysteine proteases, calpains, require calcium for their activation and are important mediators of apoptosis following ER stress. As discussed later in this review, calpains are reported to directly activate caspases, thus promoting apoptotic cell death independent of mitochondrial cytochrome *c* release. The following sections provide a more detailed explanation of the varied ways in which calcium signals induce cell death and are themselves regulated.

APOPTOSIS REGULATION BY ANTIGEN RECEPTORS: A MODEL FOR PROGRAMMED CELL DEATH

Much that is known about calcium signaling came from immunological studies using activated lymphocytes (Berridge 1997). Immature T cells are an ideal model for investigating apoptosis because they are programmed to die during development. This is evident by the fact that 95% of double positive (i.e., CD4/CD8) thymocytes undergo apoptosis as a consequence of negative selection (Starr et al. 2003). Apoptosis of thymocytes occurs when self antigen presented on thymic epithelial cells binds to T-cell receptors with strong avidity (Hogquist 2001). As depicted in Figure 1, ligation of the T-cell receptor activates a signaling pathway that results in autophosphorylation of Src family kinases Lck and Fyn, which are recruited to the plasma membrane to phosphorylate the zeta chain of the T-cell receptor (Latour and Veillette 2001; Mustelin and Tasken 2003; Palacios and Weiss 2004). Activation of these kinases facilitates the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C γ , thereby generating diacylglycerol and inositol 1,4,5-trisphosphate (IP₃) (Lewis 2001). IP₃ mediates ER calcium release through the opening of IP₃ receptors (IP₃Rs), which in turn stimulates calcineurin-mediated activation of the nuclear factor for the activation of T cells (NFAT) (Gallo et al. 2006; Winslow and Crabtree 2005; Winslow et al. 2003). Cytosolic

calcium release is also mediated by ryanodine receptors, which are calcium channels expressed in lymphocytes, cardiomyocytes, and neurons. It has been suggested that ryanodine receptors facilitate calcium flux in response to nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic adenosine dinucleotide phosphate ribose, both of which are produced during T-cell activation (Berg et al. 2000; Dammermann et al. 2009; Guse et al. 1999). However, unlike ADP ribose, the notion that NAADP is a direct activator of ryanodine receptors is not yet certain and may be context- or cell-type specific (Galione and Petersen 2005). Nevertheless, the generation of these 2nd messengers, along with IP₃, may be required for robust calcium elevation in response to antigen receptor stimulation.

In general, lymphocyte activation encodes distinct patterns of calcium signaling, which ultimately regulate cell proliferation, survival, and apoptosis (Berridge 1997; Lewis 2001) (Fig. 1). It has been proposed that strong agonist stimulation of T-cell receptors generates calcium transients that trigger apoptosis, whereas weak stimulation produces calcium oscillations that are needed for cell survival (Randriamampita and Trautmann 2003). The patterns of calcium elevation following strong and weak agonist stimulation are vastly different. For example, calcium transients can be detected in cells 1–2 minutes following activation of the T-cell receptor. They are generally synchronized and characterized by a broad peak that is high in amplitude. On the other hand, calcium oscillations consist of asynchronous and repetitious spikes that persist for as long as one hour following activation. Using fluorescence-activated cell sorting, we have shown that T cells with a high level of cytosolic calcium more readily undergo apoptosis compared to those that have lower levels (Zhong et al. 2006). Further, in those cells that undergo calcium oscillations in response to weak agonist stimulation, NFAT is rapidly de-phosphorylated, and this is associated with increased levels of IL-2 mRNA (Harr et al. 2009; Zhong et al. 2006). These observations are consistent with the strength of signal theory, which states that T cells undergo positive or negative selection

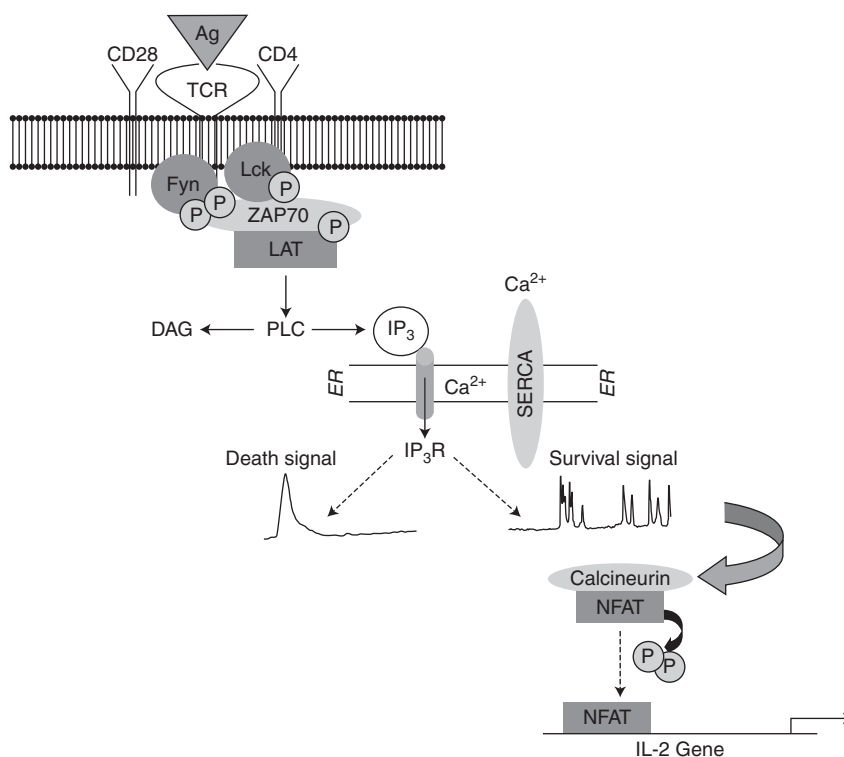


Figure 1. Calcium signaling mediated by T-cell activation. In a CD4 positive T cell, activation is induced by antigen binding to the T-cell receptor (TCR) and a co-stimulatory signal mediated by ligation of CD28. Src family kinases Fyn and Lck are activated by autophosphorylation and recruited to the plasma membrane to associate with CD3 (TCR) and CD4, respectively. This in turn leads to the phosphorylation of ZAP-70 and the adaptor protein LAT in order to activate phospholipase C and generate 2nd messengers IP₃ and diacylglycerol (DAG). IP₃ binds to the IP₃R, resulting in ER calcium release. Calcium is released into the cytosol by way of a single calcium transient (left), which is associated with cell death by apoptosis. On the other hand, calcium oscillations activate calcineurin, which dephosphorylates NFAT, thereby sending it to the nucleus to activate transcription of IL-2. The Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) is responsible for maintaining the appropriate concentration of luminal calcium by actively transporting calcium across the ER membrane.

according to the avidity of T-cell receptor activation (Hogquist 2001; Mariathasan et al. 1998). This provides one example in which the amplitude and frequency of calcium signals encodes information that regulates apoptosis or cell survival.

APOPTOSIS REGULATION BY HORMONES AND OTHER SIGNALING MOLECULES

Corticosteroids

Knowledge of calcium-dependent apoptosis evolved from studies examining the effects of glucocorticoids on immature T cells. Glucocorticoids, such as cortisol, are physiological

immunomodulatory hormones that regulate immune cell development. Pharmacologically, synthetic glucocorticoids, such as prednisone and dexamethasone, are widely used to treat autoimmune disease and cancer (e.g., leukemia and lymphoma) because of their ability to suppress the immune system and selectively kill immature lymphocytes, respectively. Glucocorticoid hormones are secreted from thymic epithelial cells to antagonize self-antigen recognition in immature thymocytes (Ashwell et al. 2000). Thus, glucocorticoids negatively regulate T-cell activation by attenuating T-cell receptor signaling (Baus et al. 1996; Lowenberg et al. 2005; Van Laethem

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et al. 2001). Conversely, T-cell receptor signaling can also inhibit glucocorticoid-induced apoptosis, a concept known as mutual antagonism (Ashwell et al. 2000; Jamieson and Yamamoto 2000; Tolosa and Ashwell 1999).

While studying these inhibitory effects on T-cell activation, our laboratory discovered that short-term treatment with glucocorticoids modulates T-cell receptor-mediated calcium elevation by converting calcium transients to oscillatory signals (Harr et al. 2009). However, prolonged glucocorticoid treatment with pharmacological concentrations of prednisone or dexamethasone results in thymocyte apoptosis by a mechanism that is dependent, in part, on *de novo* transcription (Herold et al. 2006). Importantly, glucocorticoid-induced apoptosis in thymocytes is consistently associated with a sustained rise in cytosolic calcium (Bian et al. 1997; Cohen and Duke 1984; Kaiser and Edelman 1977; Lam et al. 1993; McConkey et al. 1989; Orrenius et al. 1991). This increase in cytosolic calcium is associated with the classically observed biochemical characteristics of apoptosis, including DNA fragmentation and endonuclease activity. While there is evidence that cytosolic calcium elevation contributes to the induction of apoptosis, this mechanism has not been firmly established. Marks and colleagues found that anti-sense mediated knock-down of IP₃R1 protected cells from apoptosis induced by dexamethasone (Jayaraman and Marks 1997). However, we observed that dexamethasone-mediated up-regulation of IP₃Rs did not contribute to cytosolic calcium elevation or apoptosis following glucocorticoid treatment (Davis et al. 2008). An alternative theory is that dexamethasone down-regulates the sarcoplasmic endoplasmic reticulum ATPase (SERCA) that pumps calcium into the ER (Chai et al. 2009), thereby decreasing ER luminal calcium and inducing apoptosis.

Angiotensins

Angiotensins are a second class of hormone that induce calcium-dependent apoptosis, specifically Angiotensin II (Cigola et al. 1997; Kajstura et al. 1997; Palomeque et al. 2009; Yamada et al.

1996). Angiotensins are oligomeric peptides released in response to steroid hormones, such as glucocorticoids and estrogen. They are powerful vasoconstrictors, and consequently, angiotensin receptors are targets for antihypertensive medications (Gradman 2009). In a cardiomyocyte, muscle contraction is stimulated by the opening of an L-type calcium channel that enables calcium release via ryanodine receptors (Fig. 2). Angiotensins bind to their receptors (AT1 and AT2) resulting in the generation of IP₃ followed by transient calcium elevations (Mattiuzzi 1997). While ryanodine receptors are more abundant than IP₃Rs in cardiomyocytes, both calcium and IP₃ are required for IP₃R channel opening. While still not universally accepted, it is likely that calcium release via neighboring ryanodine receptors facilitates IP₃R-opening, enabling both channels to function cooperatively in response to angiotensin ligands (Kockskamper et al. 2008).

Much like glucocorticoids, treatment with higher concentrations of angiotensin II results in apoptosis that can be blocked by receptor antagonists (Andreka et al. 2004). As depicted in Figure 2, stimulation of cardiomyocytes with angiotensin II causes an acute release of cytosolic calcium, and several reports suggest that calcium elevation contributes to apoptosis (Kajstura et al. 1997). For instance, verapamil, an L-type calcium channel blocker, inhibits angiotensin-induced apoptosis (Goldenberg et al. 2001). Further, ectopic expression of angiotensin receptors (AT1 and AT2) results in apoptosis by a calcium-dependent mechanism (Aranguiz-Urroz et al. 2009).

Testosterone

Testosterone is a steroid hormone that has rapid effects on cardiomyocytes. In one study, it was shown that testosterone increased cytoplasmic calcium concentrations in 1–7 minutes by an IP₃R-dependent fashion (Vicencio et al. 2006). A second study reported that testosterone signals activate the extracellular signal-regulated kinase (ERK), and this activation could be inhibited by 2-aminoethyldiphenyl borate and the phospholipase C inhibitor U-73122, suggesting

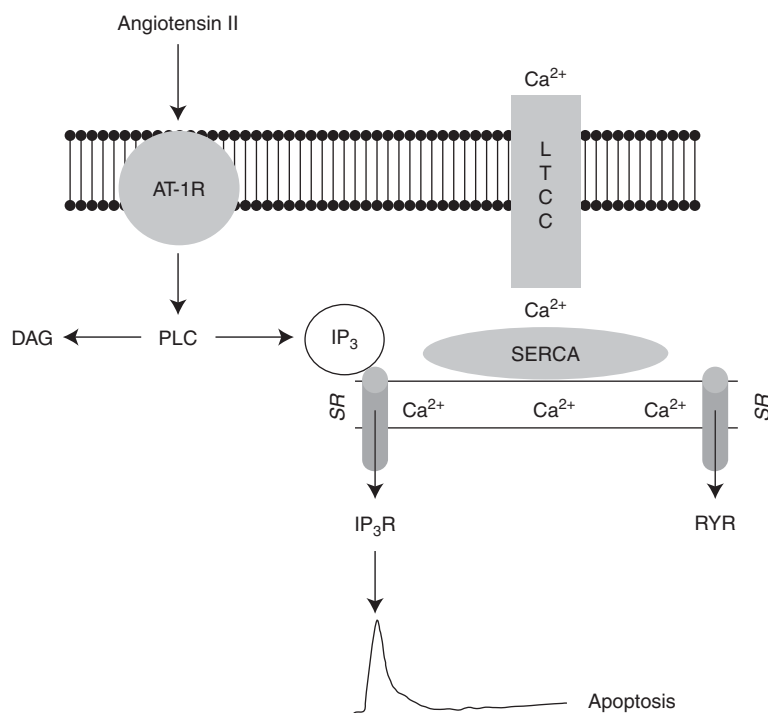


Figure 2. Calcium signaling mediated by angiotensin II hormone in a cardiomyocyte. In a cardiomyocyte, calcium signaling is mediated by the influx of calcium through L-type calcium channels (LTCC). The Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) is responsible for maintaining the appropriate concentration of luminal calcium by actively transporting calcium ion across the SR membrane, while ryanodine receptors and IP₃Rs promote its release into the cytosol. Angiotensin, a peptide hormone, binds to the AT-1 receptor (AT-1R), a G-protein coupled receptor that activates phospholipase C (PLC) following GTP hydrolysis, thereby generating IP₃ and diacylglycerol (DAG). It should be noted that ryanodine receptors are 50- to 100-fold more abundant than IP₃Rs in cardiomyocytes. Therefore, calcium release via ryanodine receptors have shown to be much more robust compared to calcium responses that are mediated by IP₃Rs (Kockskamper et al. 2008). Nevertheless, there is unequivocal evidence for the contribution of IP₃Rs during SR-calcium release, which is likely due to synergy between the two calcium channels in mediating calcium-induced calcium release.

that testosterone generates IP₃ in cardiomyocytes (Altamirano et al. 2009). Similar rapid non-genomic effects of testosterone have been observed in T cells, where calcium influx was observed within seconds (Benten et al. 1997). However, it remains to be determined whether these calcium signals would eventually lead to apoptosis. Interestingly, in neuronal cells, low concentrations of testosterone result in calcium oscillations, whereas higher concentrations induce apoptosis, also by a mechanism that is IP₃R dependent (Estrada et al. 2006). Thus, steroid hormones can have direct apoptotic effects on multiple cells types, as is the

case for glucocorticoids and androgens, and perhaps indirectly via glucocorticoid regulation of angiotensin.

Nitric Oxide

Nitric oxide is an endogenous signaling molecule that regulates muscle contraction, oxygen consumption, and mitochondrial metabolism in the heart (Massion et al. 2003). It is produced by a family of calcium-dependent enzymes called nitric oxide synthases (NOS). NOS enzymes have been found to localize to the sarcoplasmic reticulum and can inhibit L-type channel



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and ryanodine-receptor activity (Barouch et al. 2002; Sears et al. 2003; Xu et al. 1999). Further, there is evidence that NOS enzymes can inhibit calcium channel activity by S-nitrosylation of thiol residues (Davidson and Duchen 2006; Razavi et al. 2005), which in turn decreases mitochondrial calcium uptake, thereby preventing cytochrome c release and mitochondrial metabolism (Brooks et al. 2000; Dedkova and Blatter 2005; Khan and Hare 2003). In addition, nitric oxide can also attenuate apoptosis by nitrosylation of caspases on cysteine residues (Dimmeler et al. 1997). The ability of nitric oxide to prevent apoptosis in a calcium-dependent manner may be important for understanding certain pathophysiologicals such as ischemia or reperfusion injury.

APOPTOSIS REGULATION BY PHARMACOLOGICAL AGENTS

Although calcium-mediated apoptosis can occur by physiological signals, there are multiple cytotoxic agents that function to disrupt calcium homeostasis leading to apoptotic cell death. Among these are thapsigargin, staurosporine, and cisplatin.

Thapsigargin

Thapsigargin decreases the ER calcium pool by inhibiting SERCA pumps, which results in ER stress and apoptosis (Lam et al. 1993). Apoptosis induced by thapsigargin occurs by a mechanism that is dependent on the activation of caspase-12, a mammalian protease that localizes to the ER and is important for mediating apoptosis in response to ER stress (Szegezdi et al. 2003). This is exemplified by experiments performed in mice in which caspase-12 had been deleted (Nakagawa et al. 2000). In vitro studies have demonstrated that a calpain activates caspase-12 leading to the subsequent activation of caspase-9 (Morishima et al. 2002; Nakagawa and Yuan 2000; Rao et al. 2002). These data suggest the possibility that apoptosis induced by thapsigargin can occur independently of cytochrome c release and is

thus directly induced by calcium via calpain activation.

Staurosporine

Staurosporine is a natural apoptosis-inducing alkaloid originally isolated from *Streptomyces staurosporeus*. It directly provokes calcium leak from the ER by activating caspase-3 mediated cleavage of IP₃R1 (Hirota et al. 1999). Additionally, it was shown that cleavage of IP₃R1 contributed, in part, to the induction of apoptosis by accelerating calcium leak (Assefa et al. 2004; Verbert et al. 2008). In these experiments, transfection of a mutant IP₃R resistant to caspase-mediated cleavage partially inhibited apoptosis induction by staurosporine in B cells lacking wild type IP₃Rs. A recent study by Mikoshiba and colleagues further identified IP₃Rs as being important mediators of apoptosis induction by staurosporine. They determined that G protein-coupled receptor kinase interacting proteins (GITs) bind to IP₃Rs to inhibit their function and suppress apoptosis in the presence of staurosporine (Zhang et al. 2009b). Finally, staurosporine also promotes the activation of a mitochondrial calpain that positively regulates apoptosis (Norberg et al. 2008), thus illustrating similarities with ER stress-driven pathways.

Cisplatin

A third example is cisplatin, a platinum-based chemotherapeutic agent used to treat several types of cancer. Cisplatin also causes an IP₃-R-dependent increase in cytosolic calcium and subsequent activation of a calpain prior to the induction of apoptosis (Mandic et al. 2003; Schrodler et al. 2009; Spletstoeser et al. 2007). Further, cisplatin treatment results in ER stress as suggested by increased expression of Grp78 and activation of caspase-12 (Mandic et al. 2003). Interestingly, IP₃R1 contributes to cisplatin sensitivity in bladder cancer, as knocking down its expression in cell lines mediates resistance to apoptosis (Tsunoda et al. 2005). These results collectively indicate that cisplatin induces apoptosis, in part, by disrupting calcium homeostasis in a variety of cell types.

APOPTOSIS REGULATION BY ANTI-APOPTOTIC Bcl-2 FAMILY PROTEINS

In each of the previous examples in which apoptosis is regulated by calcium, cell death can be readily inhibited by anti-apoptotic proteins such as B cell leukemia/lymphoma-2 (Bcl-2) and other Bcl-2 family members. As its name implies, Bcl-2 was first identified because it was overexpressed in B cell follicular lymphoma (Tsujiimoto et al. 1985). Membership in the Bcl-2 family is defined by the presence of Bcl-2 homology domains (BH domains) (Chipuk et al. 2010). Bcl-2 has four BH domains. BH1, BH2, and BH3 are located within the C-terminal half, where they participate in forming a hydrophobic groove that binds and thereby inhibits proapoptotic family members. The BH4 domain is located near the N-terminus and connected to the C-terminal half of Bcl-2 by an unstructured loop, facilitating intra- and intermolecular interactions. It is now known that Bcl-2 is overexpressed in a number of cancers because of its ability to inhibit cell death and promote survival of malignant cells (Cory and Adams 2002). In fact, Bcl-2 localizes not only to the outer mitochondrial membrane but also to the ER, where it regulates IP₃-mediated calcium release. The observation that Bcl-2 regulates calcium release from the ER was initially made more than 15 years ago (Baffy et al. 1993; Lam et al. 1994) and the overall importance of Bcl-2 on the ER is exemplified in studies in which ER-targeted Bcl-2 inhibited apoptosis in response to agents that depolarize the mitochondrial membrane (Annis et al. 2001).

The Bcl-2-IP₃ Receptor Interaction

Our laboratory was the first to show that Bcl-2 directly interacts with IP₃Rs to inhibit IP₃-dependent calcium flux (Fig. 3) (Chen et al. 2004). This interaction, as well as an interaction of the Bcl-2 homologue Bcl-xL with the IP₃R, has subsequently been detected by a number of laboratories (Rong and Distelhorst 2007). Bcl-2 directly inhibits IP₃R channel opening in vitro in lipid bilayer experiments and also inhibits IP₃-induced calcium release in T cells (Chen et al. 2004; Zhong et al. 2006). We have

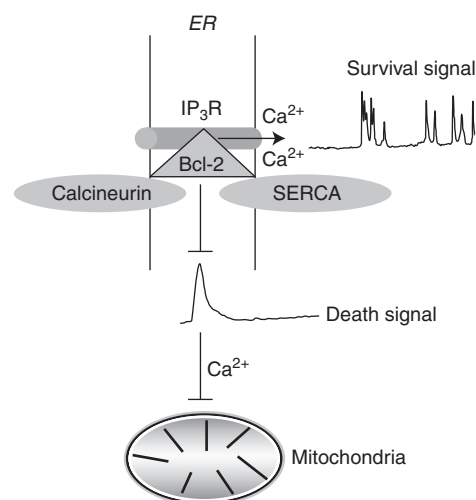


Figure 3. The Bcl-2-IP₃R interaction inhibits ER-calcium release. Bcl-2 localizes to the ER where it binds IP₃Rs to inhibit calcium transients. In T cells, calcium transients are activated in response to strong T-cell receptor ligation, which results in apoptosis that can be inhibited by Bcl-2. In contrast, calcium oscillations that are associated with cell survival are promoted by Bcl-2 and Bcl-xL. In addition, Bcl-2 regulates the level of ER luminal calcium by increasing membrane permeability or by interacting with the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA). Bcl-2 also interacts with calcineurin, thereby forming a complex with both calcineurin and IP₃Rs on the ER membrane.

now further elucidated the mechanism of the Bcl-2-IP₃R interaction by demonstrating that the BH4 domain of Bcl-2 associates with the regulatory and coupling domain of IP₃R1, specifically an 80 amino acid sequence within domain 3 (Rong et al. 2008). Further analysis indicated that the BH4 domain was both necessary and sufficient to inhibit IP₃-mediated calcium signals and subsequently apoptosis in T cells (Rong et al. 2009). This is particularly interesting given that the BH4 domain of Bcl-2 and Bcl-xL seem to be involved in preventing apoptosis. Although our work has emphasized the interaction of Bcl-2 with the regulatory and coupling domain of the IP₃R, there is also evidence that Bcl-2 and/or Bcl-xL may interact with the C-terminus of the IP₃R, and through this interaction, enhance IP₃-mediated calcium

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oscillations (White et al. 2005). In light of these discoveries, there is now increased interest in using small molecules to inhibit Bcl-2 and enhance proapoptotic calcium transients. One example of this was the use of a Bcl-2 inhibitor HA14-1 that induced cytochrome c release and apoptosis by a calcium-dependent mechanism in myeloid leukemia (An et al. 2004).

Bcl-2 and IP₃R Phosphorylation

Other proteins within the Bcl-2-IP₃R complex may regulate ER calcium release by altering phosphorylation of IP₃Rs. As shown in Figure 3, Bcl-2 binds the phosphatase calcineurin (Erin et al. 2003; Shibasaki et al. 1997), and calcineurin also interacts with IP₃Rs (Cameron et al. 1995). Because IP₃R channel activity is positively regulated by phosphorylation (DeSouza et al. 2002), it is reasonable to speculate that Bcl-2 may facilitate the dephosphorylation of IP₃R by interacting with calcineurin. Although such a mechanism has not been definitively established, we have observed that IP₃R phosphorylation is decreased in Bcl-2 overexpressing T cells (Chen et al. 2004). Furthermore, Bcl-2 has been reported to regulate IP₃R phosphorylation in Bax/Bak double knockout cells (Oakes et al. 2005). Moreover, Xu et al. have implicated protein phosphatase 1 in the regulation of IP₃R phosphorylation by Bcl-2 (Xu et al. 2007). Thus, much remains to be determined regarding the specific kinases and phosphatases that regulate IP₃R channel opening, how these are regulated by Bcl-2-IP₃R interaction, and how they contribute overall to the regulation of calcium signals by Bcl-2 and its relatives.

Bcl-2 and Mitochondrial Cross Talk

Another important function of Bcl-2 and Bcl-xL is to inhibit calcium-mediated cross talk between ER and mitochondria (Kruman and Mattson 1999; Pinton et al. 2008). Because both organelles are in close proximity, calcium is rapidly taken up by mitochondria via the calcium uniporter on the outer mitochondrial membrane (Hajnóczky et al. 2006; Hanson et al. 2004; Rizzuto et al. 2003; Szalai et al.

1999; Szlufcik et al. 2006). Bcl-2 and Bcl-xL inhibit calcium redistribution to the mitochondria, thereby limiting calcium uptake (Hanson et al. 2008; Pinton et al. 2008) (Fig. 3). In the context of apoptosis, it was shown that Bcl-2 inhibited mitochondrial calcium uptake following IL-3 and serum withdrawal in hematopoietic cells and fibroblasts, respectively (Baffy et al. 1993; Magnelli et al. 1994). Interestingly, Bcl-2 inhibited apoptosis in cardiomyocytes exposed to ceramide and staurosporine, which caused depolarization of the mitochondrial membrane and cytochrome c release (Pacher and Hajnóczky 2001). A more recent study suggests that Bcl-2 inhibits calcium release through L-type channels, thereby preventing mitochondrial calcium uptake (Diaz-Prieto et al. 2008). Finally, Mcl-1, an anti-apoptotic protein overexpressed in myeloid and lymphoid leukemia, also blocks calcium redistribution following exposure to staurosporine (Minagawa et al. 2005).

Bcl-2 Regulation of Luminal Calcium

In contrast to experiments reporting that Bcl-2 inhibits IP₃R opening, there is also substantial evidence that Bcl-2 and Bcl-xL directly regulate the concentration of luminal calcium (Fig. 3). Initial studies showed that Bcl-2 increased membrane permeability, thereby resulting in calcium leak and decreased signaling in response to ATP (Foyouzi-Youssefi et al. 2000; Pinton et al. 2000). Another study documented that knocking down Bcl-2 prevented the loss of ER luminal calcium (Oakes et al. 2005). While these studies also cite the significance of the Bcl-2-IP₃R interaction in regulating the calcium pool, others have found that Bcl-2 depletes luminal calcium by interacting with SERCA (Dremina et al. 2004; Dremina et al. 2006; Vanden Abeele et al. 2002). In spite of these differences in experimental findings, it cannot be refuted that Bcl-2 localizes to the ER to inhibit calcium signaling. Thus, mechanistic differences may be attributed to cell type in which the relative expression and localization of Bcl-2 family members are considerably distinct. Accordingly, the effect of luminal calcium is dependent upon the predominant IP₃R isoform expressed, yet not

necessary for Bcl-2 or Bcl-xL mediated effects on calcium signaling (Li et al. 2007).

Bcl-2 Regulation of Prosurvival Calcium Signals

Although Bcl-2 inhibits calcium transients that are associated with apoptosis, anti-apoptotic Bcl-2 family members can also enhance calcium oscillations that promote survival (Fig. 3). In T cells, Bcl-2 enhances calcium oscillations induced by weak T cell receptor stimulation and thus increases NFAT activation (Zhong et al. 2006). Similarly, Bcl-xL enhances pro-survival oscillations following weak ligand stimulation (Distelhorst and Zhong, unpubl). In DT40 B lymphocytes, Bcl-2, Bcl-xL, and Mcl-1 enhance calcium oscillations by sensitizing cells to lower concentrations of IP₃ (Eckenrode et al. 2010; Li et al. 2007; White et al. 2005). This in turn leads to accelerated mitochondrial metabolism and cell survival. Additional studies in other cell types support this hypothesis by demonstrating that overexpression of Bcl-2 enhances calcium oscillations in epithelial cells following stimulation with ATP and also in neuronal cells to facilitate survival (Jiao et al. 2005; Palmer et al. 2004).

APOPTOSIS REGULATION BY PROAPOPTOTIC Bcl-2 FAMILY PROTEINS

In contrast to Bcl-2, proapoptotic Bcl-2 family proteins are missing a classic BH4 domain, although recent findings suggest the presence of consensus BH4 sequence in proapoptotic family members (Chipuk et al. 2010). Multidomain BH proteins (i.e., BH1-3) include Bax and Bak, both of which are essential for apoptosis driven by the mitochondrial pathway. Multiple studies using Bax/Bak knockout models have demonstrated that the loss of these proteins confers resistance to numerous apoptotic stimuli (Wei et al. 2001).

Calcium Regulation by Bax and Bak

Like Bcl-2 and Bcl-xL, Bax and Bak also localize to the ER where they regulate calcium homeostasis. In Bax/Bak knockout cells, ER luminal

calcium is decreased compared to wild type cells, which compromises ER calcium release as well as mitochondrial uptake (Oakes et al. 2005; Scorrano et al. 2003). Consistent with these observations, the Bax Inhibitor-1 protein facilitates ER calcium leak, depleting the available calcium pool (Chae et al. 2004; Kim et al. 2008). Although the mechanism by which Bax and Bak decrease luminal calcium has not been determined, it is generally inferred that Bax/Bak ordinarily prevents Bcl-2-mediated ER calcium leak, and thus their deficiency promotes depletion of ER luminal calcium by Bcl-2. Moreover, Bax alone is required for calcium elevation in response to cytotoxic agents, such as staurosporine (Nutt et al. 2002a). For example, reconstitution of Bax in a prostate cancer cell line augmented cytosolic calcium elevation and restored mitochondrial uptake. The same group also reported that Bax/Bak overexpression induced calcium elevation followed by cytochrome c release and apoptosis (Nutt et al. 2002b). Interestingly, when Bak is targeted to the ER, it facilitates cytosolic calcium elevation and activates caspase-12; yet this does not occur when Bak is specifically targeted to mitochondria (Zong et al. 2003). In addition, ER-targeted Bak requires calcium in conjunction with ER stress for apoptosis to occur (Klee et al. 2009). This suggests that the localization of Bax and/or Bak may determine which effector pathway is induced. It is possible that Bax/Bak localization to the ER is favored when cells undergo apoptosis induced by ER stress.

Calcium Regulation by BH3-only Proteins

Other proapoptotic Bcl-2 family members have only the BH3 domain and therefore are designated as “BH3-only” proteins. These include Bim, Bad, Bik, BNIP3, PUMA, and NOXA (Cory and Adams 2002). Like their multidomain counterparts, these proteins also localize to both ER and mitochondria. At the mitochondria, BH3-only proteins facilitate Bax/Bak oligomerization by two potential mechanisms. In brief, one model suggests they function to directly activate Bax and Bak, whereas another suggests they do so indirectly by sequestering

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anti-apoptotic Bcl-2 family members (Cheng et al. 2001; Willis et al. 2007). Although the mechanism remains controversial, it is now certain that BH3-only proteins are necessary for Bax/Bak activation and the induction of apoptosis.

While the role of these proteins at the mitochondria has been extensively studied, there are sufficient data to conclude that they also regulate apoptosis at the ER. For example, Bim translocates to the ER following ER stress and may be required for the activation of caspase-12 (Morishima et al. 2004). Thus, ER localization of Bim as well as Bak may be necessary for apoptosis in response to ER stress-inducing agents. Interestingly, dexamethasone induces Bim transcript and protein levels in T cells (Lu et al. 2006; Wang et al. 2003), a process that is associated with the elevation of cytosolic calcium and required for a robust apoptotic response. Engagement of T-cell receptors also stimulates de novo transcription of Bim by a calcium-dependent mechanism (Cante-Barrett et al. 2006). It has yet to be determined if this process contributes to apoptosis induced by T-cell receptor activation. Intriguingly, T cells deficient in Bim have impaired calcium release following their stimulation, and this is associated with increased binding of Bcl-2 to IP₃Rs (Ludwinski et al. 2009). Thus, another role of Bim may be to enhance calcium elevation by sequestering Bcl-2 away from IP₃Rs at the ER membrane.

Bik is a BH3-only protein that promotes ER calcium depletion in a Bax/Bak dependent manner (Mathai et al. 2005). Similarly, BNIP3 causes a leak of ER luminal calcium when selectively targeted to the ER (Zhang et al. 2009a). NOXA and PUMA are both p53 target genes that are up-regulated in response to genotoxic stress. A study by Shore and colleagues has shown that NOXA may function cooperatively with Bik to promote the activation of Bax and Bak (Germain et al. 2005). A recent study indicates that the mitochondrial targeting region of NOXA functions to increase mitochondrial permeability and release calcium. Interestingly, a peptide corresponding to this region was able to induce calcium-dependent cell death by necrosis (Seo et al. 2009). This demonstrates

that BH3-only proteins are not only important for regulating calcium flux and homeostasis, but may also function to regulate other mechanisms of cell death. Collectively, the Bcl-2 family makes up a network of proteins and each contributes to the regulation of normal calcium homeostasis. It is clear that alterations in expression or localization of these proteins can have profound effects on cell viability by inducing apoptosis.

AUTOPHAGY

Autophagy is a process of self-eating whereby cellular organelles and proteins are phagocytosed in order to produce energy during metabolic stress (Levine and Klionsky 2004). It is an evolutionarily conserved physiological process that is thought to promote cell survival. Some cellular contexts in which autophagy may be induced include nutrient deprivation, hypoxia, ER stress, abnormal cell growth, and microbial infection (Mizushima et al. 2008). On the other hand, autophagy has also been shown to promote cell death under certain conditions and stimuli. In fact, autophagy is often referred to as type II programmed cell death (distinct from type I programmed cell death) because it does not require caspase activation or DNA fragmentation, which are classical characteristics of apoptosis (Levine and Yuan 2005). However, it is likely that both processes occur simultaneously, and thus, it is important to understand the signaling pathways that govern autophagy, especially when considering that many of the same mechanisms regulate apoptosis.

During autophagy, double membrane vesicles, or autophagosomes, fuse with the lysosome, leading to the degradation of cellular proteins (Mizushima 2007). An example of an autophagosome is illustrated by the electron micrograph in Figure 4; shown are autophagosomes from a WEHI 7.2 T cell stably expressing Bcl-2 and cultured in normal growth media containing dexamethasone. Glucocorticoids promote autophagy (Laane et al. 2009; Swerdlow et al. 2008), and this process is most evident when apoptosis is inhibited by Bcl-2 (Swerdlow et al. 2008).

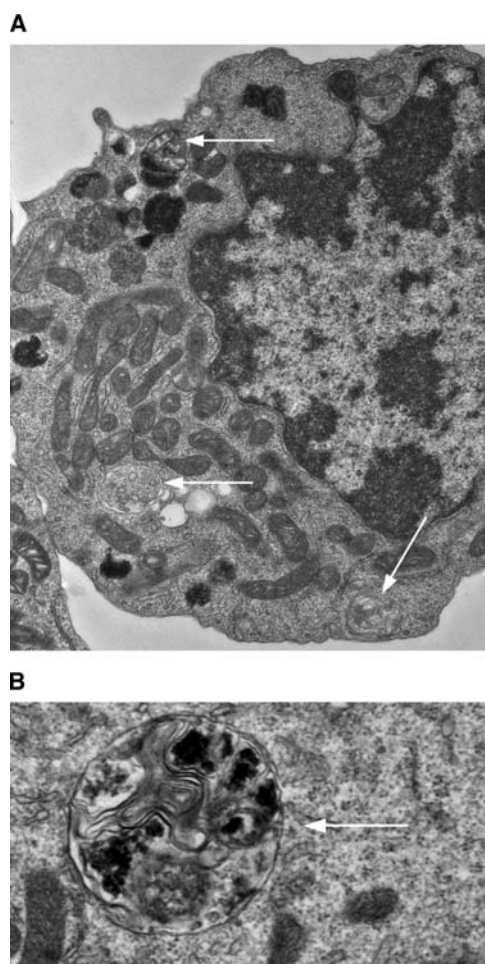


Figure 4. Electron micrograph of a malignant T cell undergoing autophagy. T cells that ectopically express Bcl-2 readily undergo autophagy in the presence of glucocorticoid hormones. Here, WEHI7.2 T cells stably expressing Bcl-2 (to inhibit apoptosis) were treated with 10^{-6} M dexamethasone for 72 hours and visualized by electron microscopy. (A) An electron micrograph of a single cell or (B) a region from within a cell. Examples of autophagosomes are shown next to the arrows.

Methods for the detection of autophagy are very well described by Klionsky and colleagues (Klionsky et al. 2008). Briefly, the most common method of analysis is the assessment of microtubule-associated protein-1 light chain-3 (LC3) by immunoblotting. LC3 is considered a marker for autophagy when it is proteolytically

processed and conjugated to phosphatidylethanolamine (LC3II). GFP-tagged LC3 is commonly transfected into cells to detect LC3 aggregates or punctate GFP-LC3II, which is indicative of autophagosome formation. A second marker of autophagy is p62 degradation. The signaling adaptor p62, which is implicated in the activation of the transcription factor NF κ B, is rapidly degraded by autolysosomes. Thus, p62 expression is inversely correlated with the conversion of LC3I to LC3II. Finally, as previously stated and shown in Figure 4, autophagosome formation can be readily visualized by electron microscopy, although this method is less amenable than others to quantitative interpretation.

Autophagy Induction by ER Stress Pathways

The link between calcium and autophagy was initially discovered by several groups reporting that autophagy could be provoked by ER stress (Bernales et al. 2006; Ogata et al. 2006; Yorimitsu et al. 2006). For example, both thapsigargin and tunicamycin stimulate autophagy (Ogata et al. 2006). ER stress affects Autophagy-Related Genes (*atg*), which are evolutionarily conserved and indispensable for autophagy in many cell systems. In yeast, the transcription factor Hac-1 (an ortholog of the ER-stress mammalian XBP-1) transactivates *atg8* during the unfolded protein response (Bernales et al. 2006). Other studies have shown that mutations in ER stress-related proteins such as PERK or EIF2 α can inhibit autophagy (Kouroku et al. 2007). Further, knockout of several *atg* genes prevents autophagy-mediated survival in the presence of tunicamycin (Ogata et al. 2006). While these data indisputably demonstrate that ER stress induces autophagy, a direct role for calcium had not been implicated at this point in time.

Autophagy Regulation by Calcium Signaling

Direct evidence that calcium signaling stimulates autophagy was first reported by Jaattala and colleagues (Hoyer-Hansen et al. 2007). They demonstrated that ER calcium mobilization induces autophagy when stimulated by

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agents such as vitamin D, ionomycin, and thapsigargin. Moreover, GFP-LC3 aggregates were inhibited with BAPTA-AM, suggesting that autophagosome formation was calcium dependent. They provided further evidence that autophagy occurred by the calcium-dependent activation of AMP activated protein kinase, which required upstream activation of the calcium/calmodulin kinase kinase β . AMPK is activated during nutrient deprivation to inhibit activity of the target of rapamycin (mTOR), a negative regulator of autophagy (Hoyer-Hansen and Jaattela 2007). Further evidence supporting a direct role for calcium in the induction of autophagy was the finding that calcium phosphate precipitates could induce autophagy when transfected into HEK293 cells (Gao et al. 2008). Importantly, autophagy mediated by calcium phosphate was also Beclin-dependent. Beclin is a newly discovered BH3-only protein that mediates autophagy by forming a complex between the class III PI3 kinase Vps34 and p150, which facilitates assembly of the autophagosome (Sinha and Levine 2008).

Autophagy in the Context of T-cell Activation

As previously described, significant contributions in the calcium field have been made by investigating signal transduction pathways in activated lymphocytes. Accordingly, autophagy may also be important for regulating lymphocyte activation. For example, T cells from *atg5* knockout mice do not proliferate following ligation of the T-cell receptor, nor do they survive in the periphery (Pua et al. 2007), suggesting that autophagy is required for T-cell activation. Interestingly, it was shown that T-cell activation increases autophagy by NF κ B-dependent transcription of Beclin-1 (Copetti et al. 2009a; Copetti et al. 2009b). In this study, the authors provide evidence that NF κ B directly binds to the Beclin-1 promoter following activation of Jurkat T cells. Although a direct link has not been observed, it is possible that calcium-dependent activation of calcineurin stimulates this process, thus implicating a role for calcium in Beclin-1 transcription and autophagy.

In addition to producing IP₃, T-cell activation also generates reactive oxygen species (Devadas et al. 2002; Hildeman et al. 2003). Thiol groups are found on IP₃Rs and ryanodine receptors, and oxidation of both calcium channels favors their opening (Bootman et al. 1992; Bultynck et al. 2004; Joseph et al. 2006; Sun et al. 2001; Xia et al. 2000). Further, cyclic ADP ribose and NAADP govern redox reactions and are also endogenous ligands for ryanodine receptors and natural 2nd messengers produced by T-cell activation (Fliegert et al. 2007; Guse 2009). These studies have implicated a role for reactive oxygen species in regulating calcium signals. Not surprisingly, reactive oxygen species also contribute to the induction of autophagy. For example, hydrogen peroxide directly facilitates formation of the autophagosome by oxidizing Atg4 (Scherz-Shouval et al. 2007). Another study demonstrated that neurons undergo autophagy when mitochondrial fission is induced by nitric oxide (Barsoum et al. 2006). This observation is attractive in light of the fact that nitric oxide protects cardiomyocytes from apoptosis. Together, these data suggest the possibility that oxidative metabolites function as signaling molecules by activating calcium and triggering autophagy in lymphocytes, although more definitive data is necessary to support this important conclusion.

Autophagy Regulation by IP₃Rs

There is now substantial evidence that autophagy is directly regulated by IP₃ as well as by IP₃Rs. For example, inhibition of inositol monophosphate by agents such as lithium and L-690,330 induced autophagy (Sarkar et al. 2005; Sarkar and Rubinsztein 2006). Kroemer and colleagues first showed that IP₃Rs act as inhibitors of autophagy by demonstrating that knocking down IP₃Rs or inhibiting their channel activity was sufficient to induce conversion of LC3I to LC3II. (Criollo et al. 2007). Moreover, Beclin-1 is in complex with IP₃Rs along with Bcl-2 and perturbation of the Beclin-IP₃-R-Bcl-2 interaction with Xestospongion B or RNA interference is sufficient for autophagy to occur (Vicencio et al. 2009). Additionally,

phosphorylation of Beclin promotes its dissociation from Bcl-xL in order to induce autophagy (Zalckvar et al. 2009). Interestingly, inhibition of IP₃Rs or siRNA knockdown of Beclin-1 did not affect ER luminal calcium as measured in aequorin-expressing cells (Criollo et al. 2007; Vicencio et al. 2009). However, because Xestospingin significantly inhibited calcium responses following stimulation with histamine, it is possible that a lack of calcium release promotes autophagy without altering steady state levels under these conditions. Nevertheless, these findings indicate a role for calcium channels and signaling in the induction of autophagy.

CONCLUDING REMARKS

Calcium is a dynamic signal transducing ion that is absolutely required for life. Slight alterations in the frequency and/or amplitude of a calcium signal can lead to apoptosis or autophagy by numerous mechanisms. In addition, abnormal signaling not only alters calcium homeostasis in cells, but may contribute to several pathogenic states such as cancer, heart failure, diabetes, and Alzheimer's disease (Berridge 2003; Berridge 2010; Huang et al. 2010; Luciani et al. 2009; Roderick and Cook 2008). It is therefore essential to understand the pathways by which calcium regulates life and death decisions, as they will not only provide insight into normal cell physiology, but may also facilitate the development of novel targets and treatments for chronic diseases.

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