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Bcl-2 interaction with the inositol 1,4,5-trisphosphate receptor: role in Ca²⁺ signaling and disease

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Summary

The Bcl-2 protein, best known for its ability to inhibit apoptosis, interacts with the inositol 1,4,5-trisphosphate receptor (IP₃R) Ca²⁺ channel to regulate IP₃-mediated Ca²⁺ release from the endoplasmic reticulum. This review summarizes the current state of knowledge regarding the interaction of Bcl-2, and also its homologue Bcl-xl, with the IP₃R and how these interactions regulate Ca²⁺ signaling. The dual role of these interactions in promoting prosurvival Ca²⁺ signals, while at the same time inhibiting proapoptotic Ca²⁺ signals, is discussed. Moreover, this review will elucidate the recently recognized importance of the Bcl-2-IP₃R interaction in human disease.

Introduction

The *bcl-2* (B cell lymphoma 2) gene was discovered through analysis of a chromosomal translocation, t(14;18), commonly associated with follicular lymphoma [1-3]. By placing *bcl-2* under control of immunoglobulin gene enhancer elements, this translocation produces abnormally high-level expression of Bcl-2, the protein product encoded by the *bcl-2* gene. In a closely related malignancy, chronic lymphocytic leukemia (CLL), Bcl-2 is elevated not by a chromosomal translocation but by loss of micro RNAs required to regulate Bcl-2 expression levels [4,5]. Bcl-2 elevation contributes to the pathogenesis and therapeutic resistance of follicular lymphoma and CLL by inhibiting apoptosis. Therefore, agents that decrease Bcl-2 expression or inhibit its activity are currently under investigation for the treatment of cancer.

Conversely, a *bcl-2* gene polymorphism associated with decreased Bcl-2 expression levels has been recently implicated in the pathogenesis of bipolar disorder [6,7]. This is fascinating, since abnormal Ca²⁺ signaling is a hallmark of this disorder [8] and lithium, used for many years to treat patients with this illness, works at least in part by increasing Bcl-2 levels [8,9].

The involvement of both increased and decreased levels of Bcl-2 in the pathogenesis of various disease states indicates that Bcl-2 plays a critical role in cellular homeostasis,

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perhaps even beyond its well-known role in regulating apoptosis. The recent discovery that Bcl-2 interacts with the inositol 1,4,5-trisphosphate receptor (IP₃R) and both positively and negatively regulates IP₃-mediated Ca²⁺ signals has opened a new window into Bcl-2's fundamental mechanism of action, as well as its role in various disease processes. These topics are the subject of this review. Ultimately, an in depth understanding of Bcl-2's role in regulating Ca²⁺ signals may provide novel ways to target Bcl-2 therapeutically for diseases associated with abnormal Bcl-2 expression.

Bcl-2 and its family members

Bcl-2 is the founding member of a large protein family known for its role in cell death regulation. Although this review primarily focuses on Bcl-2-IP₃R interaction, related contributions of other family members to Ca²⁺ homeostasis and signaling will also be discussed. Membership in the Bcl-2 protein family is defined by the presence of domains of sequence homology, or Bcl-2-homology domains (BH domains) [10]. Each Bcl-2 family member is grouped into one of three subfamilies, based its BH domain content. Members of the Bcl-2 subfamily, including Bcl-2 and Bcl-xl, have four BH domains (BH1, BH2, BH3, BH4). These proteins typically inhibit apoptosis. Members of the Bax subfamily, including Bax and Bak, have three BH domains (BH1, BH2, BH3). These proteins have a pro-apoptotic function, based primarily on their ability to oligomerize and form cytochrome C-releasing pores in the outer mitochondrial membrane. Members of the third subfamily, also proapoptotic in function, have only a BH3 domain. Members of this BH3-only subfamily function as sentinels of cellular dysfunction, activating Bax subfamily members to trigger apoptosis.

Bcl-2 and its anti-apoptotic relatives inhibit apoptosis primarily by binding, and thereby inhibiting, the activity of pro-apoptotic Bcl-2 family members [10] (Figure 1). The tertiary structures of Bcl-2 and its relatives have been characterized in detail, demonstrating that the BH1-3 domains of Bcl-2 form a hydrophobic surface groove responsible for interacting with the BH3 domain of pro-apoptotic proteins, including Bax and Bak, and BH3-only proteins. The role of the BH4 domain in apoptosis inhibition is less clear, although the presence of a typical BH4 domain is the major structural feature that distinguishes anti-apoptotic Bcl-2 family members from proapoptotic family members. Moreover, deleting the BH4 domain converts Bcl-2 into a pro-apoptotic protein. As discussed below, the BH4 domain appears to play a critical role in mediating interactions between Bcl-2 and proteins outside the Bcl-2 protein family, including the IP₃R.

Ca²⁺ and cell death

Elevation of cytosolic Ca²⁺ concentration is evoked by numerous stimuli (hormones, growth factors, neurotransmitters, etc) and has many functions [11], but one principal effect is to stimulate energy production by activation of mitochondrial respiration [12-14]. Three key citric acid cycle enzymes are regulated by the Ca²⁺ concentration within the mitochondrial matrix. The sequestration of Ca²⁺ by mitochondria and the subsequent increase in respiration is a key mechanism for coupling cellular activity with energy demand [13]. Under conditions that promote autophagy, transfer of Ca²⁺ to mitochondria can protect cells from death by enhancing ATP production [15-17]. Conversely, excessive mitochondrial Ca²⁺ uptake, or Ca²⁺ sequestration in the presence of factors such as ceramide, arachidonic acid or reactive oxygen species, leads to the activation of the intrinsic apoptotic pathway via release of a number pro-apoptotic factors including cytochrome C, apoptosis-inducing factor (AIF) and second mitochondrial activator of apoptosis (Smac/Diablo) [18]. Exactly how Ca²⁺ loading leads to release of these factors is not always clear. A commonly evoked mechanism is the activation of a protein complex that effectively permeabilizes the inner

mitochondrial membrane. This mitochondrial permeability transition correlates with loss of mitochondrial membrane potential, mitochondrial swelling leading to rupture of the outer mitochondrial membrane and release of cytochrome C (Figure 1).

Mitochondria can rapidly sequester substantial amounts of Ca^{2+} , and by doing so can influence the profile of cytosolic Ca^{2+} changes [19]. Mitochondria sequester Ca^{2+} signals arising from both Ca^{2+} release and Ca^{2+} entry channels, and then slowly return Ca^{2+} back to the cytoplasm when the cellular Ca^{2+} signal declines [14, 20, 21]. The buffering role of mitochondria is critical to cell function, and it has been suggested that loss of mitochondrial Ca^{2+} uptake could predispose cells to the pathological consequences of Ca^{2+} overload. For example, the debilitating progression of amyotrophic lateral sclerosis is believed to result from mitochondrial dysfunction leading to the selective loss of neurons in the voluntary motor system [22]. Cells generally use transient elevations of cytosolic Ca^{2+} , rather than sustained Ca^{2+} responses [19]. The rationale for employing transient Ca^{2+} elevations is to provide high-fidelity digital signals, whilst avoiding the deleterious effects of sustained Ca^{2+} rises. Prolonged Ca^{2+} elevation can activate proteases and mitochondrial Ca^{2+} overloading that precipitates permeability transition, culminating in caspase activation and cell death.

The complexity and versatility of Ca^{2+} regulation by Bcl-2 family members

A number of laboratories have actively pursued the role of Bcl-2 and its family members in regulating Ca^{2+} homeostasis and signaling. Thus, many puzzle pieces have been generated that, as yet, do not all fit together into a complete picture. At least the emerging evidence indicates that the regulation of Ca^{2+} by the Bcl-2 family is both complex and versatile. The complexity derives from the different roles that various Bcl-2 family members appear to play. The versatility shows in the ability of Bcl-2, and perhaps also Bcl-x1, to both inhibit and enhance Ca^{2+} signals. Although at first this may seem paradoxical, that a single protein might both inhibit and enhance IP_3R activity, it is not at all surprising in view of the versatility of Ca^{2+} itself, which can mediate a variety of processes, including opposing processes such as cell survival and cell death [11, 19, 23]. Furthermore, there are other examples of proteins with dual stimulatory and inhibitory effects on Ca^{2+} signalling. Calmodulin, for example, can both facilitate and inhibit voltage-activated Ca^{2+} channels [24]. With respect to IP_3Rs , 'calcium- and integrin-binding protein 1' [25] and 'Ca²⁺-binding protein 1' [26, 27] have been demonstrated to have both activatory and inhibitory actions. Therefore, precedents exist for allosteric regulators both enhancing and diminishing Ca^{2+} signalling.

A key aspect of the versatility of Ca^{2+} as a messenger arises through the ability of cells to generate Ca^{2+} signals with different amplitudes and temporal patterns [28-30]. As this review will summarize, Bcl-2 impacts on the magnitude and temporal characteristics of cellular Ca^{2+} signals to promote cell survival. Essentially, Bcl-2 inhibits high-amplitude, sustained Ca^{2+} elevations that lead to cell death, while promoting lower amplitude, oscillatory Ca^{2+} elevations that favor cell survival.

There have been numerous reports that Bcl-2 inhibits Ca^{2+} release from the ER, thereby preserving mitochondrial integrity and inhibiting apoptosis following growth factor withdrawal [31, 32] or treatment with thapsigargin [33], hydrogen peroxide [34], ceramide [35], and tumor necrosis factor [36]. Exactly how ER Ca^{2+} release is reduced is a contentious topic, with substantial evidence for at least three different, non-exclusive, mechanisms (Figure 3). One prominent mechanism is thought to involve a constitutive Bcl-2-mediated leakage of Ca^{2+} from the ER, which in turn reduces luminal Ca^{2+} concentration [45-50,92], thereby depleting the amount of Ca^{2+} that can be liberated during a triggered response. For example, Bassik et al demonstrated that stable expression of Bcl-2,

in a Bcl-2 null background, reduced luminal Ca^{2+} and consequently decreased mitochondrial Ca^{2+} uptake and inhibited apoptosis [92]). Furthermore, phosphorylation of Bcl-2 in its unstructured loop region reversed the effect of Bcl-2 on ER Ca^{2+} concentration, suggesting that ER Ca^{2+} levels could be titrated by dynamic regulation of Bcl-2 phosphorylation. Related studies reported that knocking out Bax and Bak, proapoptotic members of the Bcl-2 protein family, also produces a significant reduction in ER Ca^{2+} concentration [60, 74, 81]. In this regard, the studies of Scorrano et al [81] indicated that Bax and Bak associate with the ER, as well as mitochondria, causing a leak of Ca^{2+} from the ER and thus reducing ER Ca^{2+} content. Their findings pointed to ER Ca^{2+} release as a critical step in apoptosis induction by several agents including hydrogen peroxide, C2-ceramide and arachidonic acid, but not to the BH3-only protein tBid. Strikingly, the defect in cell death caused by Bax/Bak knock out could be reversed by expression of SERCA Ca^{2+} pumps that restore ER Ca^{2+} . The molecules involved in the putative constitutive Ca^{2+} leak induced by Bcl-2 have not been definitively demonstrated. However, work by Oakes et al [60] indicated that Bax/Bak double knockout is associated with an increase in IP_3R phosphorylation, attributed to Bcl-2- IP_3R interaction. It was suggested that IP_3R phosphorylation causes constitutive channel activity that lowers ER Ca^{2+} content, and reduces the Ca^{2+} available for subsequent release. Overall, these findings implicate the ratio of pro- and anti-apoptotic Bcl-2 family members in regulating the phosphorylation status of the IP_3R and thereby regulating the level of ER Ca^{2+} . Thus, in the absence of Bax and Bak, unopposed Bcl-2 leads to IP_3R hyperphosphorylation, enhanced ER Ca^{2+} leak and decreased steady-state ER Ca^{2+} stores.

Although the preceding work provides compelling evidence that Bcl-2 decreases ER Ca^{2+} , a number of reports concluding that Bcl-2 does not reduce ER Ca^{2+} need also to be taken into account. These include findings suggesting that Bcl-2 actually preserves ER luminal Ca^{2+} concentration when cells are stimulated with hormones or death-inducing agonists [31, 33, 34, 37-44]. Moreover, it has been reported that high-level expression of Bcl-2 disrupts ER architecture and in that way falsely appears to decrease luminal Ca^{2+} [53], consistent with reports that over-expression of Bcl-2 can induce cell death [51, 52] (Figure 2). Thus, there has been some controversy among investigators as to why Bcl-2 appears to decrease ER Ca^{2+} concentration under some but not all circumstances. Whilst there is a substantial literature supporting an effect of Bcl-2 on ER luminal Ca^{2+} content, published by various groups, including some collaborators, the authors of this article have not obtained evidence for reduced ER Ca^{2+} content within their own labs. However, in the presence of Bcl-2 we clearly see a reduction of Ca^{2+} release from the ER in response to acute stimulation of IP_3Rs . We therefore favor a model in which Bcl-2 directly acts on IP_3Rs .

Bcl-2 distinguishes between different modes of Ca^{2+} signalling

The observation that Bcl-2, an anti-apoptotic protein, inhibits ER Ca^{2+} release is consistent with abundant evidence that Ca^{2+} elevation triggers apoptosis [18, 54, 55]. On the other hand, this observation seems incompatible with the many known physiological actions of Ca^{2+} signaling, many of which promote cell survival [19, 56, 57]. This paradox was specifically addressed by comparing the effects of Bcl-2 on Ca^{2+} elevation induced by strong versus weak T cell receptor activation [58]. Bcl-2 inhibited high amplitude transient Ca^{2+} elevation induced by strong T cell receptor activation, but enhanced the development of Ca^{2+} oscillations in response to weak T cell receptor activation (Figure 3). Importantly, the former pattern of Ca^{2+} elevation promotes cell death, whereas the latter pattern of Ca^{2+} elevation supports cell survival. Thus, in keeping with its role as an anti-apoptotic protein, Bcl-2 discriminates between lethal Ca^{2+} elevation and pro-survival Ca^{2+} elevation, inhibiting the former while supporting the latter.

The concept that Bcl-2 family members enhance IP₃-mediated Ca²⁺ oscillations is supported by substantial evidence. A report by Palmer et al [49] indicated that Bcl-2 over-expressed in an epithelial cell line inhibited the large release of ER Ca²⁺ into the cytoplasm by ATP, but enhanced ATP-induced Ca²⁺ oscillations. Also, Bcl-2 and another anti-apoptotic Bcl-2 family member, Mcl-1, were recently reported to increase the sensitivity of IP₃Rs to low levels of IP₃ and thereby increase the probability of IP₃-dependent Ca²⁺ oscillations, providing protection against apoptosis [59].

Overall, therefore, Bcl-2 supports cell survival both by inhibiting high amplitude elevations of Ca²⁺ that trigger apoptosis, and enhancing Ca²⁺ oscillations that mediate a variety of pro-survival functions. Bcl-2's relatives, Bcl-xl and Mcl-1, also support cell survival by enhancing Ca²⁺ oscillations, although much less is known about their ability to inhibit pro-apoptotic Ca²⁺ elevation (see discussion of this topic later in this review). Thus, these concepts need to be examined at a molecular level using tools that facilitate investigation of endogenously expressed anti-apoptotic proteins. An opportunity to do so was provided by the recent discovery of an interaction between anti-apoptotic Bcl-2 family members and IP₃Rs.

Bcl-2-IP₃R Interaction

The discovery that Bcl-2 interacts with the IP₃R was originally based on two types of evidence: coimmunoprecipitation of Bcl-2 with IP₃Rs, and IP₃Rs with Bcl-2; and, co-migration of Bcl-2 and IP₃Rs in complexes detected by blue native gel electrophoresis [43]. Co-immunoprecipitation of these proteins by additional laboratories indicated that the interaction was not unique to a particular experimental system [44, 60, 61]. Also, Bcl-2 and IP₃Rs were shown to co-immunoprecipitate from cells that naturally express Bcl-2 at levels considerably lower than in cells where Bcl-2 had been exogenously expressed [43, 62]. This indicates that Bcl-2-IP₃R interaction is not dependent upon Bcl-2 over-expression. Finally, Bcl-2-IP₃R interaction was detected by FRET both in fixed cells and in living cells [62], providing substantial evidence that Bcl-2 does interact with IP₃Rs within cells, not just in cell extracts where one might imagine the interaction to be an artifact of disrupting cellular integrity.

Identifying regions of interaction between Bcl-2 and the IP₃R provided an opportunity to gain insight into nature of the physical interaction and to develop tools to manipulate the interaction for experimental purposes [62]. The initial experimental approach was to pull-down Bcl-2 from extracts of cells where it is naturally expressed, using GST-tagged IP₃R fragments representing recognized functional domains of the IP₃R isoform 1. These experiments localized Bcl-2 binding to an eighty amino acid sequence within the IP₃R regulatory and coupling domain. This domain is strategically located between the IP₃-binding domain near the N-terminus of the IP₃R and the trans-membrane regions that form the Ca²⁺ channel, located near the C-terminus. This domain couples IP₃ binding to channel opening, and is subject to interaction with a variety of previously recognized regulatory proteins and post-translational modifications [63, 64]. Thus, an interaction of Bcl-2 with this region is consistent with previous evidence that Bcl-2 regulates IP₃-dependent IP₃R channel opening [43].

To formally address the role of Bcl-2 interaction with the regulatory and coupling domain in regulating Ca²⁺, we developed a novel tool with which to disrupt Bcl-2-IP₃R interaction within cells [62]. This tool is a synthetic twenty amino acid peptide corresponding in sequence to the Bcl-2-binding site in the regulatory and coupling domain of the IP₃R. This sequence is the same in IP₃R isoforms 1 and 2, and very similar in isoform 3. This peptide, referred to as IP₃R-derived peptide (IDP), functions as a competitive inhibitor of Bcl-2-IP₃R

interaction, measured both by co-immunoprecipitation and by GST-IP₃R pull-down. When introduced into cells by pre-incubation with Chariot peptide uptake reagent or by fusion with the cell penetrating peptide of HIV TAT, IDP reverses Bcl-2-mediated inhibition of Ca²⁺ elevation induced either by T cell receptor activation or by a cell permeable IP₃ ester (Figure 4). Also, IDP reverses the Bcl-2-mediated inhibition of IP₃-dependent channel opening in artificial lipid bilayers and IP₃-evoked ⁴⁵Ca²⁺ release from the ER of permeabilized cells [62]. Thus, there is convincing evidence that Bcl-2's interaction with the regulatory and coupling domain of the IP₃R is responsible, at least in part, for its inhibition of IP₃-mediated Ca²⁺ elevation. Conversely, the enhancement of IP₃-mediated Ca²⁺ oscillations by the anti-apoptotic proteins Bcl-xL, Mcl-1 and Bcl-2 is attributed to interaction with a carboxyl terminal region of the IP₃R [59, 65, 66] (further discussed below).

The BH4 domain of Bcl-2 mediates interaction with the IP₃R regulatory and coupling domain [67]. As discussed above, the BH4 domain is the major structural feature that distinguishes anti-apoptotic family members from pro-apoptotic family members. Moreover, the BH4 domain is tethered to the remainder of the Bcl-2 protein by a long unstructured loop, a structural feature thought to facilitate interaction of the BH4 domain with other proteins [10]. The evidence that the BH4 domain is necessary for Bcl-2-IP₃R receptor interaction is based on experiments in which a BH4 deletion mutant of Bcl-2 failed to interact with IP₃Rs, based on coimmunoprecipitation assays [67]. In addition, Bcl-2 with a point mutation within the BH4 domain also failed to co-immunoprecipitate with IP₃Rs. Furthermore, a synthetic peptide corresponding in sequence to the Bcl-2 BH4 domain was documented to interact with a GST-IP₃R fragment corresponding to the regulatory and coupling domain of IP₃R isoform 1 both in pull-down experiments [67] and by surface plasmon resonance [68]. These findings indicate that the BH4 domain of Bcl-2 is both necessary and sufficient for interaction with the IP₃R. This concept is further substantiated by functional studies indicating that a synthetic peptide corresponding to the BH4 domain inhibits IP₃-dependent IP₃R channel opening in vitro in artificial lipid bilayers. Moreover, this BH4 peptide inhibits IP₃-evoked ⁴⁵Ca²⁺ release from the ER of permeabilized cells and, when introduced into cells with Chariot reagent or fusion with the cell penetrating peptide of HIV TAT, inhibits Ca²⁺ elevation induced by T cell receptor stimulation or a cell permeable IP₃ ester [67] (Figure 4).

How the interaction between Bcl-2 and the IP₃R is regulated is presently uncertain. In studies using cell lines the interaction has been detected without any initiating stimulus. But in primary neuronal cells it has been reported that the interaction of Bcl-2 with IP₃Rs is induced by stress [69-71]. Conversely, pro-apoptotic Bcl-2 family members that are generally induced or activated by stress are reported to disrupt the interaction of Bcl-2 with IP₃Rs. This concept is based on evidence that Bcl-2-IP₃R co-immunoprecipitation is increased in cells lacking Bax and Bak, compared to cells expressing Bax and Bak [60]. Further supporting this concept, Bcl-2-IP₃R interaction appears increased in cells lacking the pro-apoptotic BH3-only family member Bim [72]. Moreover, although there are several reports indicating that Bax and Bak induce Ca²⁺ release from the ER [41, 42, 73], we are unaware of evidence indicating that pro-apoptotic Bcl-2 family members interact with the IP₃R. Notwithstanding these reports, potential crosstalk between anti-apoptotic and pro-apoptotic family members at the IP₃R level has not yet been fully established and deserves further investigation.

An additional question is whether Bcl-2 acts alone in binding to the IP₃R, or whether Bcl-2 may be a component of a larger complex. In neuronal cells, Bcl-2 associates with the IP₃R in complex with the protein phosphatase calcineurin, delaying stress-induced neuronal cell death [69-71]. The Bcl-2 BH4 domain also binds the protein phosphatase calcineurin and blocks calcineurin-induced Ca²⁺-dependent cell death [75-77]. Also, calcineurin was

reported to bind indirectly to IP₃Rs, resulting in IP₃R dephosphorylation and inhibition of ER Ca²⁺ release [78, 79]. Thus, a reasonable hypothesis, yet unproven, is that a Bcl-2-calcineurin complex decreases IP₃R phosphorylation, thereby inhibiting IP₃-induced Ca²⁺ release from the ER. Alternatively, the interaction of calcineurin with Bcl-2 may be more important in dephosphorylating Bcl-2, increasing the anti-apoptotic action of Bcl-2 [80]. Yet another theory, put forth by Xu et al [61], is that Bcl-2 regulates a macromolecular complex of IP₃R1 with protein phosphatase 1 (PP1) and protein kinase A (PKA). They present evidence that Bcl-2 interacts with PP1 and competes with the IP₃R for binding PP1, thus modulating IP₃R phosphorylation and hence regulating IP₃-evoked Ca²⁺ release from the ER. Oakes et al [60] provide another explanation, that Bcl-2-IP₃R interaction leads to IP₃R1 hyperphosphorylation, enhanced ER Ca²⁺ leak and decreased steady-state ER Ca²⁺ stores. They also propose that this effect of Bcl-2 is counter-balanced by the pro-apoptotic protein Bax, perhaps explaining previous reports that Bax (and Bak) deficiency reduces luminal Ca²⁺ concentration and thereby inhibits IP₃-evoked Ca²⁺ release from the ER [60, 81].

Bcl-2-IP₃R interaction and disease

One testament to the importance of a biologic process is that its dysfunction produces or contributes to a disease state. Recent findings indicate that this is true for Bcl-2's interaction with the IP₃R and inhibition of ER Ca²⁺ release. Bcl-2 is expressed in many, but not all tissues [82]. Based on immunocytochemistry, Bcl-2 is normally present in the hematopoietic system including the lymphoid system, epithelium (e.g., glandular epithelium of the breast and thyroid; epithelium of the small and large bowel, as well as the basal layer of the epidermis), and neurons of the central nervous system [82]. Altered Bcl-2 levels, and thus altered Bcl-2-IP₃R interaction, contribute to disease states involving two systems in which Bcl-2 is normally expressed, the neuronal system and the lymphoid system. Bipolar disorder is associated with decreased levels of Bcl-2, whereas follicular lymphoma and chronic lymphocytic leukemia (CLL) are associated with elevated levels of Bcl-2 (Figure 2).

Bipolar disorder is a major medical problem characterized by recurrent changes in mood. This disorder is associated with alterations in intracellular Ca²⁺ homeostasis and signaling, including elevation of basal cytoplasmic Ca²⁺ levels and increased Ca²⁺ elevation following agonist stimulation or thapsigargin (reviewed in [8]). Mood stabilizers used to treat bipolar disorder, such as lithium, attenuate these Ca²⁺ abnormalities, among their other known actions (reviewed in [8, 9]). Moreover, these agents elevate Bcl-2 levels in the brain [83-87]. A *bcl-2* gene polymorphism responsible for decreasing Bcl-2 expression levels increases both basal cytosolic Ca²⁺ and IP₃R-mediated Ca²⁺ release in lymphocytes from individuals with bipolar disorder [6]. These abnormalities in Ca²⁺ homeostasis were reversed by chronic lithium treatment and mimicked Bcl-2 inhibition. Collectively, these findings are consistent with the concept that Bcl-2, by interacting with the IP₃R, plays an important role in modulating IP₃-evoked Ca²⁺ elevation. Moreover, these findings suggest that a reduction in Bcl-2 levels may lead to abnormally increased ER Ca²⁺ release, contributing to the pathogenesis of bipolar disorder, and that the therapeutic action of mood stabilizers, such as lithium, may derive from their ability to increase Bcl-2 levels, and hence Bcl-2-IP₃R interaction.

CLL, the most common leukemia in the Western world, is invariably associated with an elevation of Bcl-2 levels, thus presenting a scenario opposite to that of bipolar disorder. Recently, an IP₃R-derived peptide (IDP, discussed above) that disrupts Bcl-2-IP₃R interaction was found to induce high amplitude Ca²⁺ elevation and apoptosis in primary CLL cells isolated from CLL patients [68]. These findings suggest that Bcl-2-mediated repression of IP₃-mediated Ca²⁺ elevation may contribute to the pathophysiology of CLL. Remodeling of Ca²⁺ homeostasis and signaling through a variety of mechanisms supports

the survival and proliferation of cancer cells [88]. The interaction of Bcl-2 with IP₃Rs may contribute to this remodeling process by repressing Ca²⁺ signals driven by tonic BCR signaling, a hallmark of CLL pathogenesis (reviewed in [89]). Since BCR signaling increases IP₃ synthesis by activating phospholipase C, exposure of CLL cells to the IP₃R-derived peptide alone would be sufficient to expose these Ca²⁺ signals by displacing Bcl-2 from the IP₃R, thus triggering apoptosis. We hypothesize that tonic BCR-mediated Ca²⁺ signals might eliminate CLL cells if it were not for their repression by elevated levels of Bcl-2 interacting with and inhibiting IP₃Rs.

Both of these examples illustrate the importance of the Bcl-2-IP₃R interaction and how abnormalities in Bcl-2 expression contribute to disease processes through this interaction. Moreover, these findings raise the prospect of targeting Bcl-2-IP₃R interaction for therapeutic purposes. Agents that mimic the effects of this interaction on Ca²⁺ homeostasis and signaling would be predicted to have therapeutic value in bipolar disorder, whereas agents that inhibit Bcl-2-IP₃R interaction would likely be effective in the treatment of CLL.

Interaction of Bcl-xl with the IP₃R and effects on Ca²⁺

Bcl-xl has significant sequence homology with Bcl-2 and also functions as an anti-apoptotic protein. Both Bcl-xl and Bcl-2 block BH3-only and Bax/Bak-mediated outer mitochondrial membrane permeabilization and apoptosis [90, 91]. But these proteins differ in terms of lineage specificity [91] and also in terms of their interaction with intracellular membranes, and hence their intracellular location. Although a portion of Bcl-xl has been detected on the ER [92], Bcl-xl predominantly associates with mitochondria, in contrast to Bcl-2, which is readily detected on both ER and mitochondria. This difference in intracellular localization is governed by differences in the COOH-terminal transmembrane (TM) domain anchor of each protein. The TM domain of Bclxl is flanked at both ends by at least two basic amino acids, targeting Bcl-xl specifically to the outer mitochondrial membrane [93]. On the other hand, the transmembrane domain of Bcl-2 lacks this signaling property; hence, Bcl-2 is less selective in its interaction with intracellular membranes and can be found on the ER, the nuclear envelope, and the outer mitochondrial membrane [93]. It has therefore been proposed that Bcl-xl specifically acts on mitochondria, whereas Bcl-2 can also control ER events associated with apoptosis [93]. Also, although both are in possession of all four BH domains, the BH4 domain sequences are not identical. Moreover, the BH4 domain of Bcl-2 is tethered to the Bcl-2 protein structure by a long, unstructured loop, whereas this loop region is much shorter in Bcl-xl. One might speculate that this loop region may be critically important in mediating an interaction of the BH4 domain with other proteins, including the IP₃R.

Despite these differences, there is considerable evidence that Bcl-xl interacts with all three IP₃R isoforms, based on findings of co-immunoprecipitation and GST-Bcl-xl pull-down experiments [65]. In contrast to evidence described above indicating that Bcl-2 binds to the regulatory and coupling domain, Bcl-xl was reported to bind to a COOH-terminal region of the IP₃R that includes a portion of the transmembrane channel sequence [65]. This interaction increases the sensitivity of IP₃Rs to IP₃, increasing IP₃R channel activity and thereby enhancing both spontaneous Ca²⁺ oscillations and Ca²⁺ oscillations induced by low-level agonist stimulation [65, 66]. In turn, Bcl-xl-supported Ca²⁺ oscillations promote mitochondrial metabolism, thus favoring cell survival [65]. In nuclear patch-clamp studies, purified recombinant pro-apoptotic proteins Bax and Bid displaced GST-Bcl-xl from IP₃Rs and abolished the functional effects of Bcl-xl on Ca²⁺ [65], thus providing strong evidence that Bcl-xl-IP₃R interaction is indeed responsible for the enhancement of Ca²⁺ oscillations.

Reminiscent of the reported effects of Bcl-2 on ER luminal Ca^{2+} , summarized above, a Bcl-xl-induced reduction in luminal Ca^{2+} has been reported by several labs [65, 66, 92], although not by all [94]. It was suggested that Bcl-xl-IP₃R interaction induces a diminution of luminal Ca^{2+} through its enhancement of IP₃-mediated Ca^{2+} oscillations [65]. But a subsequent report by the same investigators found that Bcl-xl expression was associated with a reduction in luminal Ca^{2+} only in cells expressing type 3 IP₃Rs, not in cells expressing types 1 or 2 IP₃Rs [66]. Yet, Bcl-xl enhanced spontaneous Ca^{2+} oscillations in DT40 cells expressing each IP₃R subtype, suggesting that reduction of luminal Ca^{2+} was not due to enhancement of spontaneous Ca^{2+} oscillations or necessary for the enhancement of Ca^{2+} oscillations. Similar to the situation with Bcl-2, it is widely accepted that Bcl-xl alters cellular Ca^{2+} signalling, but does not consistently affect ER luminal Ca^{2+} .

Although the ability of Bcl-xl to enhance Ca^{2+} oscillations has been emphasized by the recent studies just summarized, there is also evidence that Bcl-xl, like Bcl-2, can prevent apoptosis by inhibiting Ca^{2+} elevation. Pacher et al [95] reported that the apoptotic agents ceramide and staurosporine induce Ca^{2+} spikes generated by IP₃-mediated release of Ca^{2+} from the sarcoplasmic reticulum of cardiac myotubes. In turn, these Ca^{2+} spikes generate mitochondrial Ca^{2+} waves that induce permeability transition pore (PTP) opening, leading to cytochrome *c* release, caspase activation and apoptosis. They observed that Bcl-xl inhibits these mitochondrial Ca^{2+} waves, although it was not determined if this Bcl-xl action was based at the mitochondrial membrane or sarcoplasmic reticulum membrane. In another study, Bcl-xl over-expression inhibited Ca^{2+} elevation following T cell receptor activation, attributed to a Bcl-xl-imposed reduction in IP₃R levels [96]. We also have detected an inhibitory effect of Bcl-xl over-expression on Ca^{2+} elevation following T cell receptor activation, although the magnitude of Bcl-xl effect was more variable than observed in our earlier studies with Bcl-2 in the same experimental system [43, 58] and a reduction in IP₃R levels was not detected (CWD, unpublished observations). Finally, in GST-pull-down experiments used previously to identify the site of Bcl-2 interaction on the IP₃R, an interaction of Bcl-xl with the regulatory and coupling domain was also detected, although the strength of this interaction did not appear to be as strong as that of Bcl-2 (CWD, unpublished observations).

Summary

Ca^{2+} signalling is intimately linked with cell survival. In using Ca^{2+} as a messenger cells tread a tightrope between appropriate activity and catastrophic demise. Bcl-2 and its homologues play a critical part in tuning cellular Ca^{2+} signals to provoke survival, or to guide cells into physiological forms of cell death. Alteration of Bcl-2 expression or function can critically adjust the balance between malignant cell survival and untimely cell loss. Bcl-2 family members interact with cellular Ca^{2+} signalling systems at many levels, leading to a complex web of potential interactions. For Bcl-2, it is becoming clear that a key interaction occurs with IP₃R and their ability to mobilize Ca^{2+} from the ER. There is still a lack of consensus concerning how Bcl-2 reduces IP₃R activity, with data supporting a number of different concepts. However, a growing body of evidence supports a model whereby the binding of Bcl-2 via its BH4 domain to a region within the regulatory domain of IP₃R leads to reduced channel activity and attenuated Ca^{2+} signals. Consequently, less Ca^{2+} is transferred to mitochondria so that the intrinsic apoptotic pathway is not triggered. At the same time, Bcl-2 has the dual function of promoting Ca^{2+} release with lower levels of cellular stimulation. Plausibly, an enhanced release of Ca^{2+} prevents cell death by activating pro-survival activities such as mitochondrial respiration. The molecular mechanism of this enhanced IP₃R activity is unknown. However, it is evident that Bcl-2, and perhaps also Bclxl and Mcl-1, can discriminate between high and low levels of cellular stimulation, or different cellular contexts, to promote Ca^{2+} signals that support survival. The

characterization of the Bcl-2/IP₃R binding opens the possibility of using targeted inhibitors/activators to treat conditions such as CLL and bipolar disorder where this endogenous interaction is aberrant.

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Figure 1. The relative expression/activity of pro- and anti-apoptotic Bcl-2 family members determines cell survival

Box i depicts how anti-apoptotic Bcl-2 family members promote cell survival by inhibiting proapoptotic family members, and also by reducing ER-to-mitochondria Ca^{2+} flux. Box ii illustrates the consequences of a change in the expression/activity of anti-apoptotic family members on cell survival. In this situation, the pro-apoptotic family members lead to the activation/insertion of Bax/Bak into mitochondrial membranes, subsequently causing mitochondrial permeability changes, loss of cytochrome C and activating apoptosis. In addition, the loss of anti-apoptotic Bcl-2 family members leads to increased ER-to-mitochondrial Ca^{2+} flux, which also precipitates mitochondrial permeability transition and cell death.

Figure 2. Variation in Bcl-2 expression has dramatic effects on cell viability

This figure illustrates the different cellular outcomes associated with variations in Bcl-2 expression. Only 'moderate expression' correlates with normal cell survival. It is important to note that not all cells express Bcl-2, and in those situations other anti-apoptotic Bcl-2 family members are present. Whether they possess the same concentration-dependent consequences is not clear.

Figure 3. Bcl-2 converts sustained high-amplitude Ca^{2+} signals into Ca^{2+} oscillations

This diagram depicts the action of Bcl-2 on agonist-evoked Ca^{2+} signals. Various mechanisms have been proposed to account for Bcl-2's ability to reduce cellular Ca^{2+} signals. Any, or all, of the proposed mechanisms could plausibly convert sustained Ca^{2+} responses (that arise from chronic IP_3R activity) into Ca^{2+} oscillations (arising from periodic bursts of IP_3R activity).

Figure 4. The BH4 domain of Bcl-2 is necessary and sufficient for inhibition of IP₃Rs

This figure depicts the interaction of Bcl-2 and the BH4 domain of Bcl-2 with an IP₃ receptor, and the consequently reduced Ca²⁺ response that would be observed at a whole-cell level. Antagonism of the Bcl-2/IP₃R interaction using a peptide that corresponds to the BH4 domain-binding domain on the IP₃R enhances Ca²⁺ release.