Synergistic movements of Ca$^{2+}$ and Bax in cells undergoing apoptosis

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ABSTRACT

Apoptosis is a physiological counterbalance to mitosis and plays important roles in tissue development and homeostasis. Cytosolic Ca\(^{2+}\) has been implicated as a proapoptotic second messenger involved in both triggering apoptosis and regulating cell death-specific enzymes. A critical early event in apoptosis is associated with the redistribution of Bax from cytosol to mitochondria and endoplasmic reticulum (ER) membranes, however the molecular mechanism of Bax translocation and its relationship to Ca\(^{2+}\) is largely unknown. Here we provide functional evidence for a synergistic interaction between the movements of intracellular Ca\(^{2+}\) and cytosolic Bax in the induction of apoptosis. Overexpression of Bax in cultured cells causes a loss of ER Ca\(^{2+}\) content. Depletion of ER Ca\(^{2+}\) through activation of ryanodine receptor enhances the participating of Bax into the mitochondrial membrane. Neither Bax translocation nor Bax-induced apoptosis is affected by buffering of cytosolic Ca\(^{2+}\) with BAPTA, suggesting that depletion of ER Ca\(^{2+}\) rather than elevation of cytosolic Ca\(^{2+}\) is the signal for cell apoptosis. This dynamic interplay of Ca\(^{2+}\) and Bax movements may serve as an amplifying factor in the initial signaling steps of apoptosis.
INTRODUCTION

Apoptosis or programmed cell death is a highly regulated process of selective cell deletion involved in development, normal cell turnover, cell-mediated immunity, pathological disorders and tumor regression (1,2). The Bcl-2 family members are active mediators of apoptosis, which either inhibit (e.g. Bcl-2, Bcl-xL, Mcl-1) or facilitate apoptotic cell death (e.g. Bax, Bad, Bak, Bid, Bik/Nbk, Bim) (3,4). The pro-apoptotic Bax proteins are largely cytosolic or loosely associate with intracellular membranes (5). Following an appropriate death signal, Bax targets and integrates into intracellular membranes, especially the mitochondrial outer membrane, which induces opening of the permeability transition pore (PTP), release of cytochrome c, and activation of downstream caspase pathways (6-8). Recent studies have shown that this process may involve Bax dimerization or oligomerization and conformational changes (9-11), but the precise mechanism by which Bax is activated to translocate from cytosol to intracellular membrane has not been elucidated.

Several studies have suggested that changes in intracellular Ca\(^{2+}\) homeostasis play an important role in the modulation of apoptosis. Many cell death stimuli, including growth factor withdrawal, hormonal stimulation, and drug treatment are known to alter the concentration of Ca\(^{2+}\) in the cytosol and the storage of Ca\(^{2+}\) in the intracellular organelles (12-14). Compounds that directly affect the intracellular Ca\(^{2+}\) homeostasis and capacitative Ca\(^{2+}\) entry, such as Ca\(^{2+}\) ionophores and thapsigargin (TG)*, have been shown to induce apoptosis in a variety of cells (15,16). Ca\(^{2+}\) release channels in the ER, i.e. inositol 1,4,5-trisphosphate receptor (IP\(_3\)R) and ryanodine receptor (RyR), appear to participate in the signal transduction pathway of apoptosis (17-19). Furthermore, recent evidence shows that Bcl-2 may suppress apoptosis via a mechanism that is linked to intracellular Ca\(^{2+}\) compartmentalization (20-22).

* Abbreviations: TG, thapsigargin; RyR, ryanodine receptor; IP\(_3\)R, inositol 1,4,5-trisphosphate receptor; CHO, Chinese hamster ovary cells; WT, wild type;
Our previous study demonstrated that depletion of ER Ca\(^{2+}\) by caffeine and ryanodine through activation of RyR/Ca\(^{2+}\) release channels induce apoptosis in Chinese hamster ovary (CHO) cells transfected with RyR; and Bcl-xL prevents cell death at a stage downstream of ER Ca\(^{2+}\) release and capacitative Ca\(^{2+}\) entry (23). However, the functional interplay between Ca\(^{2+}\) and pro-apoptotic Bcl-2 members in apoptosis, e.g. Bax, has not been studied. Here we report that over-expression of Bax affected the ER Ca\(^{2+}\) homeostasis, and perturbation of intracellular Ca\(^{2+}\) could feedback to the translocation process of Bax. Our data suggest a synergistic action between the movements of Bax and Ca\(^{2+}\) in the initiation of apoptosis.
MATERIAL AND METHODS

Cell Culture and Gene Transfection. Stable clones of Chinese hamster ovary (CHO) cells permanently expressing RyR and Bcl-xL proteins were maintained at 37 °C and 5% CO₂ in Ham’s F-12 medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (23). To eliminate possible bias due to antibiotic selection, the control cells (CHO-WT) and cells stably expressing RyR (CHO-RyR) were transfected with mock pCEP4 vector (Invitrogen), and subjected to double selection with G418 (0.5 mg/ml) and hygromycin (0.26 mg/ml), in the same way as cells co-expressing RyR and Bcl-xL (RyR-xL). The cDNA of mouse Bax gene was cloned into the pCMS-EGFP expression vector (Clontech) with transcription driven by the CMV promoter. The pCMS-EGFP plasmid also contains a separate SV40-driven green fluorescent protein (GFP) sequence, providing a convenient way of selecting cells transiently transfected with Bax and GFP genes. The GFP-Bax fusion construct was cloned into the pcDNA3 expression vector. Plasmids carrying Bax or GFP-Bax cDNAs were introduced into CHO cells using the LipofectAmine Plus reagents following manufacturer’s instructions (Life Technologies Inc.). The plasmid containing GFP alone was used as controls. Transient expressions of Bax and GFP-Bax proteins were assayed 10-36 hours after gene transfection, by confocal microscopy and Western blot.

Confocal Microscopy Imaging and Immunocytochemistry. For subcellular localization of transiently expressed GFP-Bax, CHO cells were grown on glass-bottomed ΔTC3 dishes (Bioptechs Inc., Butler, PA), and visualized with a Zeiss LSM-510 laser scanning confocal microscope using a Plan-Apochromat 63x oil or C-Apochromat 40x water immersion objective (24). Anti-cytochrome oxidase subunit IV (COX IV) monoclonal antibody (Molecular Probes) was used to label the mitochondria. 18 hours after transfection with GFP-Bax, cells were fixed with 4% paraformaldehyde for 15min and permeabilized with 0.2% Triton X-100 for 10 minutes. The cells were incubated for 1 hour with PBS with 2% normal goat serum, 2 hours with anti-COX IV mAbs (diluted 1:100 in PBS+5% normal goat serum), and developed with tetramethylrhodamine (TMRM)-conjugated goat anti-mouse IgG antibody.
(Molecular Probes, 1:1000 dilution). Relative green and red channels of LSM were used to observe the fluorescence of GFP-Bax and mitochondrial marker protein. The cell death process was monitored by Hoechst 33442 staining of the cell nucleus, as described (23).

**Intracellular Ca\(^{2+}\) Measurement.** CHO cells were grown in ΔTC3 dishes and loaded with 2 μM Fura-2 AM fluorescent Ca\(^{2+}\) indicator. The changes in intracellular Ca\(^{2+}\) were measured with a dual wavelength excitation (340 nm and 380 nm, emission at 510 nm) spectrofluorometer (Photon Technology International Inc.). Release of intracellular Ca\(^{2+}\) in individual cells was measured following exposure to caffeine, ryanodine, ATP, or thapsigargin in a Ca\(^{2+}\)-free balanced salt solution (BSS) (in mM): 140 NaCl, 2.8 KCl, 2 MgCl\(_2\), 0.5 EGTA, 10 HEPES, pH 7.2) by rapid solution exchange. A separate set of FITC filter was used to select CHO cells transiently transfected with the pcDNA3.1 (GFP-Bax) or pCMS-EGFP (Bax) plasmids, to test the effect of GFP-Bax and Bax on intracellular Ca\(^{2+}\) release.

**Quantitative assay of Bax-integration into intracellular membranes.** CHO cells transfected with GFP-Bax were visualized under confocal microscope. Cells were grouped according to the pattern of GFP-Bax distribution: healthy cells with diffuse pattern of green fluorescence indicating cytosolic distribution of GFP-Bax, and pre-apoptotic cells with punctate pattern of green fluorescence indicating integration of GFP-Bax into intracellular membranes. At different time points, cells with punctated and diffused patterns of GFP-Bax were counted from at least 20 fields, which were randomly selected. The percentage of transfected cells with Membrane-Bound Bax was defined as the number of cells with punctated pattern of GFP-Bax divided by the total number of green fluorescent cells (in both punctated and diffused patterns). The data are from 4-10 independent experiments.

**Apoptosis Assay.** The detailed procedure of quantitative assay of cell apoptosis upon treatment of cells with caffeine/ryanodine or after transient transfection with Bax has been described elsewhere (23, 25). Briefly, the differential uptake of fluorescent DNA binding dyes acridine orange (4 μg/ml) and ethidium bromide (8 μg/ml) was used to measure viable and nonviable cells in a given population. A viable cell will have a red cytoplasm with bright green nucleus due to intercalation of acridine orange into the...
DNA. Ethidium bromide is only taken up in nonviable cells after disruption of the plasma membrane, and this dye also intercalates into DNA, making it appear orange. Thus, a dying cell will have a bright nucleus (the ethidium overwhelms the acridine) and its cytoplasm will appear dark red.
RESULTS

Transient expression of Bax in CHO cells stably transfected with RyR and Bcl-xL - To study the effect of Bcl-2 related proteins on the Ca^{2+} signaling of apoptosis, we derived several clones of Chinese hamster ovary (CHO) cells stably transfected with RyR alone (CHO-RyR), or together with Bcl-xL (RyR-xL), an anti-apoptotic protein (Fig. 1a, lane 2 & 4) (23). The ryanodine receptors are located in the ER membrane and provide the conduction pore of Ca^{2+} release channels (26). Activation of these Ca^{2+} release channels by caffeine and ryanodine leads to sustained depletion of the intracellular Ca^{2+} stores (27, 28) (Fig. 1b), indicated by the lack of thapsigargin-induced Ca^{2+} release even after prolonged incubation with 2 mM Ca^{2+} in the bath solution.

The endogenous Bax proteins in CHO cells were not detectable with Western blot (data not shown). To study the interaction of Bax with Bcl-xL and Ca^{2+} in the apoptosis of CHO cells, we have used a transient expression system. Ten hours after transfection with either Bax or GFP-Bax gene into CHO-RyR cells, ample amount of Bax proteins could be detected, as revealed by the distinct band of 21 kDa protein recognized by the anti-Bax antibody (Fig. 1a, lane 3) or a band of 46 kDa protein recognized by the anti-GFP antibody (lane 5). Under identical transfection condition, the expression levels of Bax or GFP-Bax were similar in CHO-RyR and RyR-xL cells (not shown), suggesting that the presence of Bcl-xL does not affect the transient expression of Bax in CHO cells.

Apoptosis of CHO cells induced by overexpression of Bax -- In Figure 2, we show that overexpression of Bax in CHO-RyR and RyR-xL cells can induce apoptotic cell death, based on the following features of apoptosis: genomic DNA laddering, nuclear chromatin condensation, and mitochondrial membrane clustering. As shown in Fig. 2a, DNA laddering was clearly visible in CHO-RyR cells 24 h after transfection with either Bax or GFP-Bax (lane 4, 5), but not in control cells transfected with GFP only (lane 1). As described in our previous study (ref 23), depletion of ER Ca^{2+} stores via activation of RyR/Ca^{2+} release channel induced apoptosis in CHO-RyR cells but not in RyR-xL cells (Fig. 2a, lane 2 and 3), whereas genomic DNA isolated from RyR-xL cells overexpressing GFP-
Bax showed typical laddering pattern although the nucleus fragmentation process was delayed compared with that in CHO-RyR cells. This result indicates that Bxl-xL cannot prevent apoptosis completely when Bax is active.

More obvious condensation of the nucleus in individual transfected cells is revealed in Fig. 2b by using Hoechst 33342 dye staining. Clearly, most cells with punctate pattern of GFP-Bax distribution showed condensed nuclei with fragmented chromatin structures, a characteristic feature of cells undergoing apoptosis (Fig. 2b, indicated by arrows). And the translocation of Bax from cytosol to intracellular membranes appears to be essential for Bax-induced apoptosis, as only cells with diffuse pattern of GFP-Bax distribution remained healthy, morphologically intact, with normal nuclear structure, consistent with previous studies (5, 7).

Apoptosis is also accompanied with rearrangement of mitochondria from organized membrane network in cells with diffuse distribution of GFB-Bax, into clustered vesicular pattern in cells with punctate pattern of GFP-Bax. Fig. 2c shows the immunohistochemical structure of mitochondria labeled with monoclonal antibody against COX IV (a mitochondria marker protein). Similar phenomenon was also reported by Margineantu et al. (29). The punctated GFP-Bax was mostly in the peri-nuclear region, and co-localized with mitochondria (yellow spots in Fig 2c overlay), but not excluding ER or other intracellular membrane.

Depletion of ER Ca\(^2+\) enhances translocation of Bax from cytosol into intracellular membranes-

In the transient expression assays, we observed that GFP-Bax was predominantly distributed in the cytosol with diffuse pattern of green fluorescence, 4-10 hours after gene transfection. By 24-36 hours, most of transfected cells showed punctate distribution of GFP-Bax. Interestingly, close to 60% of the cells expressing RyR (CHO-RyR) exhibited punctate distribution of GFP-Bax as early as 6 hours after gene transfection, a number that is significantly higher than in control CHO cells (CHO-WT) (Fig. 3b, left panel). Thus, it seems that the presence of RyR in the ER membrane may serve as an intrinsic factor for the translocation of Bax from cytosol to intracellular membranes. We have exercised separate assays
with CHO cells permanently transfected with a non-conducting mutant of RyR, the E4032A-RyR1 mutant (ref 23), and found no difference between CHO-WT and CHO-E4032A cells in the initial distribution of GFP-Bax. Thus, it is likely the intrinsic Ca\(^{2+}\) release function of RyR may play a role in the movement of GFP-Bax. Consistent with the anti-apoptotic effect of Bcl-xL, the RyR-xL cells transfected with GFP-Bax maintained cytosolic distribution of GFP-Bax for as long as 24-36 hours, with delayed induction of apoptosis (see Fig. 3b right panel).

Application of caffeine and ryanodine to CHO-RyR and RyR-xL cells, which led to depletion of ER Ca\(^{2+}\) (see Fig. 1b), caused significant redistribution of GFP-Bax (Fig. 3a). The percentage of cells with punctate distribution of GFP-Bax, a measure of the membrane-bound Bax, was significantly increased, within 6 hours after depletion of ER Ca\(^{2+}\). This enhanced movement of Bax from cytosol to mitochondria and ER could be clearly seen with the RyR-xL cells, which appeared to maintain the cytosolic distribution of GFP-Bax under control conditions (Fig. 3b). 24 hours after transfection of GFP-Bax into RyR-xL cells, the addition of caffeine and ryanodine caused redistribution of GFP-Bax from mostly cytosolic (22±3%) to mostly membrane-bound (63%±9%). Thus, the combined activation of ER Ca\(^{2+}\) release and Bax overexpression overcame the anti-apoptotic effect of Bcl-xL, and led to eventual cell death.

*Overexpression of Bax causes depletion of intracellular Ca\(^{2+}\) stores*- The integration of Bax into intracellular membranes might have a reciprocal effect on the intracellular Ca\(^{2+}\) homeostasis, and contribute to the overall apoptotic cell death process. Therefore, we measured the ability of ATP, caffeine or thapsigargin to release Ca\(^{2+}\) from intracellular membranes in individual CHO-RyR cells after transient transfection with either GFP-Bax or Bax; in the latter case, the Bax cDNA was cloned into the pCMS-EGFP vector, allowing selection of transfected cells with GFP fluorescence. ATP is an agonist of the purinergic receptor on the plasma membrane, which activates IP\(_3\) receptors on the ER membrane via generation of IP\(_3\) (30). Thapsigargin is a specific inhibitor of the SERCA Ca\(^{2+}\) pump, permitting direct assessment of the ER Ca\(^{2+}\) pool (15,16, 31).
As shown in Fig. 4a, CHO-RyR cells transfected with GFP alone respond to rapid release of Ca$^{2+}$ from ER upon application of caffeine and ATP, indicating functional RyR and IP$_3$ receptor are present in the ER membrane and share a common pool of releasable Ca$^{2+}$. A striking difference was observed with CHO-RyR cells transfected with Bax, where the amount of ATP- and caffeine-induced Ca$^{2+}$ release was significantly smaller as a result of Bax overexpression (Fig. 4b). To further correlate the subcellular localization of Bax with its effect on ER Ca$^{2+}$ homeostasis, we selected the CHO-RyR cells based on their pattern of GFP-Bax distribution. CHO-RyR cells with GFP-Bax present in the cytosol contain an ER Ca$^{2+}$ pool that is significantly smaller than in control cells (compare Fig. 4c with 4a); those cells with GFP-Bax localized to the intracellular membranes appeared to have an empty ER Ca$^{2+}$ pool, since they lost response to caffeine, ATP and thapsigargin (Fig. 4d). Based on transmission images, these cells could have normal morphology with intact plasma membrane, even with punctate distribution of GFP-Bax, and thus are likely in the pre-apoptotic stages. Similar results, i.e. reduced or even empty ER Ca$^{2+}$ stores, were observed in CHO-wt and CHO-E4032A (mutant RyR protein lacking Ca$^{2+}$ channel activation) cells transfected with GFP-Bax by using ATP and thapsigargin stimulation (data not shown). Interestingly, RyR-xL cells with diffuse distribution pattern of GFP-Bax are capable of releasing intracellular Ca$^{2+}$ upon stimulation with caffeine, in a manner similar to the CHO-RyR cells (compare Fig. 4e with a); but the extent of ER Ca$^{2+}$ release is significantly reduced, in response to a second application of ATP.

The results with separate cells in multiple transfections are summarized in Fig. 5 and Table 1. Overexpression of Bax led to significant reduction in the ER Ca$^{2+}$ content, as ~70% of the CHO-RyR cells transfected with GFP-Bax contained an empty ER Ca$^{2+}$ pool, whereas only a small population of cells transfected with GFP alone (~18%) appeared to have lost their ER Ca$^{2+}$ content, likely due to the toxic effect of LipofectAmine reagent. Interestingly, the resting [Ca$^{2+}$] in CHO-RyR and RyR-xL cells did not appear to be affected by the transfection with either Bax or GFP-Bax genes (Table 1), suggesting
that the over expressed Bax, in addition to affecting the intracellular Ca\(^{2+}\) release process, may also interfere with the extracellular Ca\(^{2+}\) entry pathway (unpublished data by Pan et al.).

*Effects of BAPTA on Bax translocation and Bax-mediated apoptosis* - Depletion of intracellular Ca\(^{2+}\) results in transient elevation of cytosolic Ca\(^{2+}\), which may directly activate caspase-like enzymes or indirectly influence the release of cytochrome c from mitochondria and trigger the apoptotic cascade (32, 33). It is not clear whether the elevation of cytosolic Ca\(^{2+}\) per se, or the depletion of intracellular Ca\(^{2+}\) store serves as the primary trigger for apoptosis (20-22, 34, 35). To further test the effect of cytosolic Ca\(^{2+}\) on Bax translocation and Bax-mediated apoptosis, we pretreated the cells with BAPTA-AM, a membrane permeable Ca\(^{2+}\) chelator. As shown in Fig. 6a, in cells pretreated with BAPTA-AM, the caffeine/ryanodine-induced rapid cytosolic Ca\(^{2+}\) elevation was dramatically reduced (compare trace ii with trace i). Buffering of cytosolic Ca\(^{2+}\) by BAPTA had no effect on the movement of GFP-Bax in both CHO-RyR and RyR-xL cells, as neither the pattern of GFB-Bax distribution (Fig. 6b) nor the time-course of spontaneous GFP-Bax translocation changed with or without the addition of BAPTA-AM (Fig. 7a). In addition, Hoechst dye staining of the cell nucleus did not reveal visible difference between control and BAPTA treated cells (Fig. 6b). Notice that the un-transfected CHO-RyR and RyR-xL cells are not affected by the pretreatment with BAPTA-AM, in other words, buffering of cytosolic Ca does not appear to interfere with the apoptotic process of CHO cells. Furthermore, quantitative assays of cell apoptosis using differential uptake of fluorescent DNA binding dyes acridine orange and ethidium bromide give similar cell death indices irrespective of treatment with BAPTA, either 6 or 24 hours after transfection with GFP-Bax (Fig. 7b). Together, these data suggest that depletion of intracellular Ca\(^{2+}\) store rather than elevation of cytosolic Ca\(^{2+}\) serves as the signal in Bax-mediated apoptosis.
DISCUSSION

In this study, we demonstrate a functional interaction between the movements of intracellular Ca\(^{2+}\) and cytosolic Bax in the apoptotic pathway. We show that overexpression of Bax can affect the storage of Ca\(^{2+}\) in the intracellular membranes, and changes in intracellular Ca\(^{2+}\) storage can feedback to the translocation of Bax from cytosol to intracellular membranes. The spontaneous Bax redistribution between cytosol and membrane association appears to be accelerated by the release of Ca\(^{2+}\) from the ER, whereas it is not affected by the resting Ca\(^{2+}\) level as buffering of cytosolic Ca\(^{2+}\) by BAPTA does not influence the movement of Bax. These data add further insights into the cellular and molecular mechanism of Ca\(^{2+}\) signaling in apoptosis.

ER as a multifaceted organelle participates in protein synthesis and trafficking, cellular response to stress, and intracellular Ca\(^{2+}\) signaling. Accumulating evidence shows that ER plays important roles not only in cell proliferation, but also in apoptosis (35, 36). It has been shown that changes in ER luminal environment by overexpression of calreticulin affect cell sensitivity to apoptosis (37). Activation of caspase-12 by ER stress induces apoptosis in a mitochondria-independent pathway (32). In the initial stage of apoptosis, although majority of Bax distributes to mitochondrial outer membrane, a portion of Bax also participate into other intracellular membranes, such as ER (38). Presumably, that ER-bound Bax may affect apoptosis by interfering with the ionic homeostasis of the ER and introducing cellular stress to the intracellular organelles. The Bax-induced depletion of ER Ca\(^{2+}\) storage may represent one of the functional effects of Bax in apoptosis. Alternatively, the Bax-mediated reduction of ER Ca\(^{2+}\) may represent a secondary effect of cells entering the apoptotic cycle. In any event, the combined effects of Ca\(^{2+}\) and Bax movements add an amplifying factor in triggering the fast execution of apoptotic cell death.

Exactly how Bax affects the intracellular Ca\(^{2+}\) homeostasis is present unknown, and understanding its molecular and cellular mechanisms will require extensive studies. Several possibilities
can be proposed here. First, the membrane-bound Bax may oligomerize and create a non-selective ion pore across the ER membrane, and therefore cause leakage of Ca\(^{2+}\) from the intracellular stores (39). Second, Bax may interact directly or indirectly with the SERCA Ca\(^{2+}\) pump like Bcl-2 (40), the IP\(_3\)R or the RyR Ca\(^{2+}\) release channels, and thus affect the overall Ca\(^{2+}\) transport across the ER membrane.

Another intriguing possibility could be that Bax can affect the communication between ER and mitochondria in the movement of Ca\(^{2+}\). With the presence of Bax in both mitochondria and ER membranes, a toxic loading of Ca\(^{2+}\) into the mitochondria matrix may cause morphological changes in the mitochondria network and thus leading to apoptotic cell death. The anti-apoptotic effects of Bcl-2 have been implicated in changes of intracellular Ca\(^{2+}\) homeostasis. It is not surprising, therefore, that the balancing effect of Bax and Bcl-2 on ER Ca\(^{2+}\) homeostasis could play a role in determining the fate of the cells to either undergo proliferation or apoptosis (41, 42).

Calcium can function either as pro-mitotic or as pro-apoptotic messenger, depending on its localization, cytosolic concentration or oscillating pattern (23, 35, 36). One of the interesting results from this study is that Ca\(^{2+}\) movement across ER membrane accelerated Bax movement from cytosol to intracellular membrane, therefore accelerated apoptosis. The balance between mitosis and apoptosis is essential for tissue development and homeostasis. Abnormal cell growth or cell death has been implicated as the primary cause for cancer or degenerative diseases, respectively (1-3). Proper control of apoptosis requires rigorous signaling communication and ordered function of regulatory molecules, which provide rapid and effective response to diverse extracellular and intracellular apoptotic triggers. Ca\(^{2+}\) and Bcl-2 related proteins affect the morphology and function of ER and mitochondria, two of the key intracellular organelles participating in apoptosis. Our data establish the concept the dynamic interplay between the movements of Ca\(^{2+}\) and Bax may provide an important feedback mechanism for rapid execution of apoptosis.
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FIGURE LEGENDS

Figure 1. Expression of RyR, Bcl-xL, Bax, and GFP-Bax in CHO cells. 

a. Western blot of RyR, Bcl-xL, Bax or GFP-Bax expressed in CHO cells. CHO-RyR and RyR-xL are stable clones of CHO cells that were permanently transfected with RyR, or co-transfected with RyR and Bcl-xL, respectively. The Bax and GFP-Bax cDNAs were introduced into the cells using the LipofectAmine reagent. Cells were harvested 10 hours after transfection, and proteins (60µg/lane) from the cell lysate were separated on a 3-12% SDS-PAGE gradient gel, and probed with monoclonal antibodies against RyR (Mw, 560 kDa, upper portion), against Bcl-xL (Mw, 26 kDa) or Bax (Mw, 21 kDa) (lower portions). lane 1 – CHO-WT cells, lane 2 – CHO-RyR cells, lane 3 – Bax expressed in CHO-RyR cells, lane 4 – RyR-xL cells, lane 5 – GFP-Bax (Mw, 46 kDa) expressed in CHO-RyR cells. 
b. caffeine and ryanodine-induced Ca\(^{2+}\) release from CHO-RyR cells. Application of 10 µM ryanodine (rya) to the cell at resting state did not affect intracellular Ca\(^{2+}\) release, as ryanodine only binds to the open state of the Ca\(^{2+}\) release channel (26). Caffeine (caff, 10 mM) activated the RyR channel and caused rapid Ca\(^{2+}\) release from the ER. Subsequent binding of ryanodine to RyR induced permanent opening of the Ca\(^{2+}\) release channel (28), which emptied the ER Ca\(^{2+}\) content revealed by the lack of response to thapsigargin (TG).

Figure 2. Overexpression of Bax induced apoptosis in CHO cells. 
a. DNA laddering assay. Genomic DNA isolated from various CHO cells were separated on a 1.5% agarose gel. lane 1 - control CHO-RyR cells transfected with GFP only; lane 2 – CHO-RyR cells treated with caffeine and ryanodine; lane 3 – RyR-xL cells treated with caffeine and ryanodine; lane 4 – CHO-RyR cells 24 hours after transfection with GFP-Bax; lane 5 – CHO-RyR cells 24 hours after transfection with Bax; lane 6 – RyR-xL cells 36 hours after transfection with GFP-Bax. 
b. Representative confocal images of CHO cell with Hoechst 33342 staining and GFP fluorescence. Pictures were taken 12 hours after transfection with GFP-Bax. Hoechst dye staining (blue color) revealed characteristic nuclear condensation and chromatin fragmentation in cells undergoing apoptosis (indicated by arrows) with punctated GFP-Bax, but not in cells with diffuse pattern of GFP-Bax distribution. 
c. Changes in mitochondria morphology in cells
undergoing apoptosis. Immunostaining of COX IV (red fluorescence) revealed organized mitochondrial structure in RyR-xL cells with diffuse pattern of GFP-Bax (upper panel). Clustering of mitochondria became evident in cells with punctated distribution of GFP-Bax (lower panel). In overlay images, yellow color shows localization of GFP-Bax in mitochondria, and green color shows GFP-Bax traffics to other intracellular membranes.

Figure 3. Depletion of ER Ca\(^{2+}\) enhances translocation of Bax from cytosol to intracellular membranes in cells undergoing apoptosis. \(a\). Ca\(^{2+}\) release-mediated redistribution of GFP-Bax from cytosol to intracellular membranes. Representative images were taken from CHO-RyR (upper panels) and RyR-xL cells (lower panels) 12 hours after transfection with GFP-Bax (control). Most of the RyR-xL cells exhibited diffused pattern of GFP-Bax distribution, whereas a portion of the CHO-RyR cells exhibited punctated pattern of GFP-Bax distribution. Caffeine (10 mM) and ryanodine (10 µM) was added to the medium 6 hours after transfection of cells with GFP-Bax (+caff/rya). As a result, both CHO-RyR and RyR-xL cells exhibited more punctated distribution of GFP-Bax. \(b\). Time dependence of Bax translocation. The time-dependent changes in Bax redistribution under control condition (filled symbols), or after treatment with caffeine and ryanodine (arrow, open symbols) were followed in a period of 60 hours after transfection of cells with GFP-Bax into CHO-RyR (left panel) or RyR-xL cells (right panel). For comparison, CHO-WT cells were presented as filled diamonds. The Membrane-Bound Bax is defined as the percentage of cells with punctated pattern of GFP-Bax distribution in total green cells. 6 hour was the earliest time when measurable expression of GFP-Bax could be detected after transfection.

Figure 4. Bax-mediated changes in intracellular Ca\(^{2+}\) in cells undergoing apoptosis. The changes in cytosolic [Ca\(^{2+}\)] were monitored by Fura-2, a Ca\(^{2+}\) indicator, and represented as the ratio of fluorescence at 340/380 excitation wavelength. The release of intracellular Ca\(^{2+}\) was triggered by caffeine (caff, 10 mM), ATP (0.2 mM) or thapsigargin (TG, 1 µM) applied to a Ca\(^{2+}\)-free balanced salt solution. \(a\). CHO-RyR cells transfected with GFP alone (for control purpose) contain active RyR and IP\(_3\) receptor Ca\(^{2+}\) release channels, indicated by the rapid response to caffeine and ATP. TG induced slower
rise in cytosolic Ca\(^{2+}\) via inhibition of the SERCA Ca\(^{2+}\) pump. b. Transient transfection of pCMS-EGFP(Bax) into CHO-RyR cells led to significant reduction in the ER Ca\(^{2+}\) content, as the cell’s response to caffeine, ATP and TG were all smaller compared with the controls (a). c. CHO-RyR cells with cytosolic distribution of GFP-Bax also exhibited reduced ER Ca\(^{2+}\) content. d. CHO-RyR cells with intracellular membrane-bound GFP-Bax contained empty ER Ca\(^{2+}\) content, since the cell lost its response to caffeine, ATP and TG. The cell had intact plasma membrane structure, and was likely in the pre-apoptotic stage. e. RyR-xL cells with diffuse pattern of GFP-Bax distribution exhibited close to normal response to caffeine stimulation compared with the controls (a), but the second application of ATP resulted in significantly less intracellular Ca\(^{2+}\) release.

**Figure 5. Reduction of ER Ca\(^{2+}\) storage induced by Bax overexpression in CHO cells.** The bar chart represents data from multiple cells in multiple transfections. The percentage of cells with full ER Ca\(^{2+}\) content (ratio of 340/380 Fura-2 fluorescence > 1.3), reduced ER Ca\(^{2+}\) content (Fura-2 fluorescence range 0.3 to 1.2), or empty ER Ca\(^{2+}\) (Fura-2 fluorescence < 0.2) were calculated from a total of 33 cells (n = 11 transfections) with CHO-RyR cells transfected with GFP alone; a total of 168 cells (n=17) with CHO-RyR cells transfected with GFP-Bax, and a total of 126 cells (n=16) with RyR-xL cells transfected with GFP-Bax.

**Figure 6. BAPTA-AM reduced elevation of cytosolic Ca\(^{2+}\) and did not affect apoptosis in CHO cells.** Cells were incubated with 10 µM BAPTA-AM in extracellular medium at 37 °C for 90 min. a. Representative traces of caffeine/ryanodine-induced Ca\(^{2+}\) release were plotted in control (i, black line) and 2h after pretreatment with BAPTA-AM (ii, gray line) in CHO-RyR cells. b. Cells were incubated with BAPTA-AM 3 hours after transfection with GFP-Bax. The pretreatment of BAPTA did not alter the subcellular distribution of GFB-Bax. Blue color of Hoechst dye staining revealed similar characteristic nuclear condensation and chromatin fragmentation in cells undergoing apoptosis (indicated by arrows) irrespective of the treatment with BAPTA (compared with Fig.2 c).
Figure 7. Effects of BAPTA on Bax translocation and Bax-mediated apoptosis.  

a. The time course of GFP-Bax translocation from cytosol to intracellular membranes (defined as the membrane-bound Bax) was not affected by BAPTA treatment (open symbols), in either CHO-RyR (squares) or RyR-xL cells (circles).  
b. Quantitative assays of Bax-induced apoptosis. Plotted as the percentages of apoptosis in CHO-RyR cells, as a result of overexpression of Bax, GFP-Bax, or depletion of ER Ca\textsuperscript{2+} (with addition of caffeine and ryanodine). Pretreatment with BAPTA did not affect the degree of apoptosis either 6 hours or 24 hours after transfection with GFP-Bax.
Table 1. Bax-mediated changes in intracellular Ca\textsuperscript{2+} homeostasis.

| Cells      | Transfected DNA | $\Delta F_{340}/F_{380}$ after stimulation |
|------------|-----------------|------------------------------------------|
|            |                 | + caffeine                               | + ATP                     |
| CHO-RyR    | GFP             | 0.81± 0.03                               | 1.37± 0.30                |
|            | Bax             | 0.89± 0.08                               | 0.30± 0.10                |
|            | GFP-Bax         | 0.96± 0.08                               | 0.43± 0.22                |
| RyR-xL     | GFP             | 0.94± 0.19                               | 2.18± 0.39                |
|            | Bax             | 0.87± 0.04                               | 0.81± 0.26                |
|            | GFP-Bax         | 0.96± 0.06                               | 1.35± 0.46                |

Entry points are average values of $F_{340}/F_{380}$ (mean ± s.e.m., n = 5 - 90) in individual cells loaded with 2 μM Fura-2 AM. The resting [Ca\textsubscript{i}] was measured with 2 mM Ca\textsuperscript{2+} present in balanced salt solution (BSS). Caffeine- (10 mM) and ATP- (0.2 mM) induced Ca\textsuperscript{2+} release from ER were performed in Ca\textsuperscript{2+} free BSS (+0.5 mM EGTA).
Fig. 1/Pan et al. (JBC)
**Figure 3**

**Panel a**

Images showing the fluorescence distribution of CHO-RyR and RyR-xL under control and +caff/rya conditions.

**Panel b**

Graphs illustrating the membrane-bound Bax percentage over time for different conditions:
- CHO-RyR, control
- CHO-RyR, +caff/ryn
- CHO-WT, control
- RyR-xL, control
- RyR-xL, +caff/ryn

**X-axis:** Time (hr)
**Y-axis:** Membrane-Bound Bax (%)
Fig. 4/Pan et al. (JBC)
Fig. 5/Pan et al. (JBC)
a

Time (sec)

F$_{340nm}$/F$_{380nm}$

0 120 240 360 480

i

ii

b

CHO-RyR

Hoechst

Overlay

RyR-xL

Pan et al. (JBC)
Fig. 7/Pan et al. (JBC)
Synergistic movements of Ca2+ and Bax in cells undergoing apoptosis
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