Depletion of Intracellular Ca$^{2+}$ by Caffeine and Ryanodine Induces Apoptosis of Chinese Hamster Ovary Cells Transfected with Ryanodine Receptor*

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Recent studies have suggested a central role for Ca$^{2+}$ in the signaling pathway of apoptosis and certain anti-apoptotic effects of Bcl-2 family of proteins have been attributed to changes in intracellular Ca$^{2+}$ homeostasis. Here we report that depletion of Ca$^{2+}$ from endoplasmic reticulum (ER) leads to apoptosis in Chinese hamster ovary cells. Stable expression of ryanodine receptor (RyR) in these cells enables rapid and reversible changes of both cytosolic Ca$^{2+}$ and ER Ca$^{2+}$ content via activation of the RyR/Ca$^{2+}$ release channel by caffeine and ryanodine. Sustained depletion of the ER Ca$^{2+}$ store leads to apoptosis in Chinese hamster ovary cells, whereas co-expression of Bcl-xL and RyR in these cells prevents apoptotic cell death but not necrotic cell death. The anti-apoptotic effect of Bcl-xL does not correlate with changes in either the Ca$^{2+}$ release process from the ER or the capacitative Ca$^{2+}$ entry through the plasma membrane. The data suggest that Bcl-xL likely prevents apoptosis of cells at a stage downstream of ER Ca$^{2+}$ release and capacitative Ca$^{2+}$ entry.

Apoptosis, an evolutionarily conserved programmed cell death process, plays a central role in both development and homeostasis of tissues. Malfunctions in this process contribute to many diseases, such as Alzheimer’s disease, cancer, and AIDS (1). Characteristic features of apoptosis include DNA fragmentation, condensation of nuclear chromatin, cell membrane blebbing, and activation of a number of specific biochemical pathways (2). Although the sequence of events from the initial apoptotic trigger leading up to DNA fragmentation and eventual cell death is not completely understood, several genes that play essential roles in the regulation of apoptosis have been identified. Among them are the ones encoding a family of Bcl-2-related proteins that either inhibit (e.g. Bcl-2 and Bcl-xL) or facilitate apoptosis (e.g. Bax and Bad) (3, 4). Several studies have suggested a central role for Ca$^{2+}$ in the initiation of apoptosis. Many apoptotic stimuli, including growth factor withdrawal (5) and activation of surface antigen receptors (6), are known to alter the concentration of Ca$^{2+}$ in the cytosol and the storage of Ca$^{2+}$ in the intracellular organelles (7). Compounds that directly affect the intracellular Ca$^{2+}$ homeostasis, such as Ca$^{2+}$ ionophores and thapsigargin (TG), have been shown to induce apoptosis in a variety of cells (8–11). However, the elevated cytosolic Ca$^{2+}$ does not necessarily correlate with apoptosis (12, 13). 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. The anti-apoptotic effect of Bcl-2 has been implicated with changes in intracellular Ca$^{2+}$ (5, 14), but the cellular mechanism underlying the correlation between Bcl-2 and Ca$^{2+}$ remains largely unknown.

Ca$^{2+}$ release from the ER can occur through inositol 1,4,5-trisphosphate receptor (IP$_3$R) and/or ryanodine receptor (RyR), both of which function as Ca$^{2+}$ release channels in the ER membrane. The level of IP$_3$R has been shown to increase in lymphocytes induced to undergo apoptosis, and lymphocytes deficient in IP$_3$R are resistant to glucocorticoid-induced apoptosis (15, 16). Furthermore, dantrolene, an agent that blocks Ca$^{2+}$ release from the ER could prevent apoptosis in cultured PC-12 cells (17). Transforming growth factor-$eta_1$, one of the potent inducers of apoptosis, has been shown to up-regulate the expression of a form of RyR in pulmonary epithelial cells (18, 19). Thus, Ca$^{2+}$ release channels in the ER appear to play important roles in the signal transduction pathway of apoptosis.

In the present study, we examined the possibility that depletion of the ER Ca$^{2+}$ stores by activation of RyR/ER Ca$^{2+}$ release channel can directly induce apoptosis in cultured Chinese hamster ovary (CHO) cells. We have stably transfected CHO cells with the RyR and Bcl-xL genes, and measured the changes in cytosolic Ca$^{2+}$ as well as ER Ca$^{2+}$ content through activation of the Ca$^{2+}$ release channel with caffeine and ryanodine. The changes in morphology and chromatin structure of cells undergoing apoptosis or necrosis were characterized with confocal microscopic imaging and DNA ladder assays. Our data show that depletion of ER Ca$^{2+}$ store can serve as a trigger for apoptosis in CHO cells, and the anti-apoptotic effect of Bcl-xL likely occurs at a stage downstream of ER Ca$^{2+}$ release.

**EXPERIMENTAL PROCEDURES**

Plasmids—The full-length cDNA (~15.3 kilobases) of rabbit skeletal muscle RyR (RyR1) was cloned into the expression vector pRRE11 with transcription occurring under the control of SV40 promoter (20). The E403A mutant of RyR1 was generated using the Altered Sites II mutagenesis kit purchased from Promega (Madison, WI). A 4.25-kilobase cDNA fragment (nucleotide 10982–12230) encoding the carboxyl-terminal portion of RyR was subcloned into the pAlter vector, and a

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§ The abbreviations used are: TG, thapsigargin; ER, endoplasmic reticulum; RyR, ryanodine receptor; IP$_3$R, inositol 1,4,5-trisphosphate receptor; CHO, Chinese hamster ovary; BSS, balanced salt solution; CCE, capacitative Ca$^{2+}$ entry; BAPTA-AM, (acetoxymethyl-1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; AMP-PNP, adenosine 5’-(β,γ-iminotriphosphate).
designed oligonucleotide was used to change the glutamate residue at amino acid position 4032 (Glu1032) into alanine (Ala). The fragment containing the E4032A mutation was subcloned back into its original position in pRRS11, to generate the E4032A-RyR1 mutant. The mutagenesis was confirmed by restriction enzyme digestion and sequencing. The plasmid DNA encoding the human Bcl-xL or RyR1 cDNAs was transfected into the eukaryotic expression vector (Invitrogen, Carlsbad, CA). The pRRS11 vector contains the neomycin (G418) resistance gene and the pCEP4 vector contains the hygromycin resistance gene, which allow selection of cells co-transfected with the RyR and Bel-xL cDNAs, using G418 and hygromycin, separately.

Cell Culture and Gene Transfection—CHO cells were grown 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. The expression plasmids were introduced into the cells (60–70% confluent) using LipoDeCTAMINE reagent (Life Technologies Inc., Gaithersburg, MD) followed by the manufacturer’s instructions. 48 h after transfection, the cells were selected with G418 (0.5 mg/ml, for pRRS11 based plasmids), or both (for co-expression). Resistant colonies were isolated and characterized for RyR1 or Bel-xL expressing using Western blot analysis.

Western Blot Assay—Control and transfected CHO cells were harvested with ice-cold phosphate-buffered saline, and lysed with modified RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) in the presence of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM pepstatin, 1 mM benzamidine, 10 μM leupeptin, 1 μg/ml aprotinin). The whole cell lysate was mixed with a 2× sample buffer (200 mM Tris-Cl, pH 6.7, 5% SDS, 6% β-mercaptoethanol, 15% glycerol, 0.01% bromphenol blue) and separated on a 3–12% linear gradient SDS-polyacrylamide gel after heating the samples at 60 °C for 15 min. The proteins were transferred to a polyvinylidene difluoride membrane using a mini trans-blot electrophoretic transfer cell (Bio-Rad, Hercules, CA). The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline, 0.1% Tween-20 (TBS-T) before incubation with horseradish peroxidase-linked secondary antibody and the signal detected on Kodak films using chemiluminescent kit (Pierce, Rockford, IL).

Confocal Microscopic Images—The ~70% confluent CHO cells were treated with 10 μM caffeine and 10 μM ryanodine for 18 h. Hoechst 33342 (10 μM, Sigma) was added to the culture medium for 15 min at 37 °C in a dark chamber. The cell death process was monitored with a Zeiss LSM510 scanning confocal microscope using a 63× oil immersion objective, with the excitation wavelength set at 351 nm and the emission filter set at 417–482 nm (23). Intracellular Ca2+ Measurement in Single Cell—CHO cells were grown in 3T3 dishes (Bioprotech, Inc., Butler, PA) and loaded with 2 μM Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) for 30 min at 37 °C in a balanced salt solution (BSS) (140 mM NaCl, 2.8 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, pH 7.2). The cells were then left for a further 30-min period in the bathing solution at room temperature to allow ester hydrolysis to go to completion. Using a dual-wavelength spectrofluorometer, with excitation wavelengths at 340 and 380 nm and emission at 510 nm, fluorescence measurements were performed at 37 °C in a temperature-regulated chamber, mounted on the stage of an inverted fluorescence microscope (Olympus IX-70). Single-cell fluorescence spectra were continuously monitored at a sampling frequency of 50 Hz and collected with a PTI spectrofluorometer (Photon Technology International, Monmouth Junction, NJ) (24). The release of intracellular Ca2+ in individual cells was measured following exposure to caffeine, ryanodine, ATP, or thapsigargin in a Ca2+-free BSS solution (plus 0.5 mM EGTA) by rapid solution exchange. Intracellular Ca2+ was measured using confocal microscopy (Zeiss LSM510). Cells were grown on 100-mm tissue culture dishes at exponential growth phase and allowed to reach 70% confluence. Caffeine (10 μM), ryanodine (10 μM), or EGTA (0.5 mM) were added into the medium directly. 18 h later, both floating and attached cells were collected and washed with ice-cold PBS. The cells were pelleted and lysed in 500 μl of lysis buffer (10 mM EDTA, 150 mM NaCl, 0.1% Triton X-100, 0.1% sodium citrate). After the cell suspension was dispersed by 50 μl of 10% N-lauroyl sarcosine, 50 μl of proteinase K (1 mg/ml) were added separately. DNA was extracted and analyzed for fragmentation following the procedures of McGahan et al. (25).

Quantification of Cell Viability and Apoptosis—Differential quantifications of fluorescent DNA binding dyes acridine orange (4 μg/ml) and ethidium bromide (8 μg/ml) were used to determine viable and nonviable cells in a given population (25). 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 by nonviable cell after disruption of plasma membrane, and this dye also intercalates into DNA, making it appear orange. Thus a dying cell will have a bright nucleus (ethidium overwhelms acridine) and its cytoplasm will appear dark red. Early apoptotic cells have intact plasma membranes, but expression of annexins, but the initiation of DNA fragmentation will result in patch labeling of the nuclei by acridine orange. As the cells progress through the apoptotic pathway and membrane blebbing starts to occur, ethidium bromide enters the cell resulting in orange staining of the nucleus. Cells entering the late apoptotic stage will have bright orange areas of condensed chromatin that will distinguish them from necrotic ones, which have a uniform orange color. For each measurement, a minimum of 200 total cells were counted and the percentage of total apoptotic cells was obtained by adding early and late apoptotic cells. The percentage of necrotic cells was recorded by counting cells with bright orange chromatin with organized structure. Different extracellular agonists, i.e., 10 μM caffeine, 10 μM ryanodine, 0.5 mM EGTA, 0.5 mM ATP or AMP-PNP (Sigma), were added to the medium directly. To suppress the elevation of cytosolic Ca2+, cells were pretreated with 10 μM BAPTA-AM (Molecular Probes) added to the culture medium at 37 °C for 90 min.

RESULTS

Introduction—Stable Expression of RyR and Bel-xL in CHO Cells—The cDNAs encoding the wild type or E4032A-RyR1 were introduced into CHO cells using the LipofectAMINE reagent. Individual clones of CHO cells stably expressing RyR were obtained after selection with G418. As shown in the Western blot (Fig. 1A), the parental CHO cells do not contain detectable amounts of endogenous RyR protein (lane 2, CHO-WT), whereas an ample amount RyR proteins can be detected in the cells transfected with wt-RyR1 (lane 2, CHO-RyR) and E4032A-RyR1 (lane 3, CHO-E4032A), respectively. Stable clones of CHO cells co-expressing Bel-xL and wt-RyR1 were generated by transfection of Bel-xL cDNA into CHO-RyR cells, as revealed by the distinct band of 30-kDa protein recognized by the anti-Bcl-xL antibody, and ~560-kDa protein corresponding to the wt-RyR1 (lane 4, RyR-xL). The level of Bel-xL protein in parental CHO cells was not detectable with the anti-Bcl-xL antibody. As controls, we have also transfected the mock pCEP4 vector in the CHO-wt and CHO-RyR, and upon selection with hygromycin, the following clones were generated, CHO-C4 and RyR-C4, respectively.

The levels of protein expression in these cells were maintained in over 30 passages, suggesting that the CHO cells can take up well these exogenous RyR1 or Bel-xL proteins. Morphologically, CHO cells expressing wt-RyR1 appear to be slightly larger than the parental cells, but otherwise they exhibit normal growth and proliferation.

Depletion of ER Ca2+ Stores and Activation of RyR—Our previous studies have shown that the expressed RyR1 proteins are localized to the ER membrane of CHO cells, and they maintain functional Ca2+ release channel activity in response to stimulation with caffeine (Ref. 20, and see Fig. 1B). The early apoptotic markers have been identified as the Ca2+-dependent activation of the RyR1/Ca2+ release channel, as mutation of the corresponding residue in RyR3 (E3872A), a brain isoform of RyR1, produces a mutant Ca2+ release channel with reduced Ca2+ activation profile (26). As shown in Fig. 1B, addition of 10 mM caffeine to CHO-E4032A cells did not cause release of Ca2+ from the intracellular stores. These cells maintain an intact ER Ca2+ pool, since addition of thapsigargin caused release of Ca2+ from the ER (see Fig. 1C). Moreover, selection with hygromycin did not affect the intracellular Ca2+ movement, as reflected by the similar response to caffeine in CHO-RyR and
Fig. 1. Caffeine- and ryanodine-mediated movement of intracellular Ca\(^{2+}\) in CHO cells transfected with ryanodine receptor. A, Western blot of RyR and Bcl-xL stably expressed in CHO cells. Proteins from the whole cell lysate were separated on a 3–12% linear SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The top portion of the membrane was blotted with the 34C antibody (against RyR1) and the bottom portion of the membrane was blotted with the H-62 antibody (against Bcl-xL). Lane 1, parental CHO cells; lane 2, CHO-RyR; lane 3, CHO-E4032A; lane 4, RyR-xL. B, caffeine-induced release of intracellular Ca\(^{2+}\). a, CHO-E4032A; b, CHO-RyR; c, RyR-C4; d, RyR-xL. 10 mM caffeine (in Ca\(^{2+}\)-free BSS) stimulated Ca\(^{2+}\) release from ER in CHO-RyR, RyR-C4, or RyR-xL, but not in CHO-E4032A. The traces are representative of 5–6 cells in 3–4 independent experiments. C, depletion of ER Ca\(^{2+}\) stores by caffeine and ryanodine. e, CHO-E4032A; f, CHO-RyR; g, RyR-xL. The cells were pretreated with 10 \(\mu\)M ryanodine for 3 min, and 10 mM caffeine in Ca\(^{2+}\)-free BSS solution was applied to the cells. Following the caffeine-induced release of Ca\(^{2+}\) from the ER membrane, the cells were re-loaded with 2 mM Ca\(^{2+}\)-BSS for 3–6 min. The bath solution was then changed to a Ca\(^{2+}\)-free solution (0.5 mM EGTA) followed by quick addition of 10 \(\mu\)M TG. Inhibition of the Ca\(^{2+}\)-ATPase by TG resulted in gradual increase of cytosolic Ca\(^{2+}\) in CHO-E4032A, but not in CHO-RyR1 or RyR-xL. Each trace represents 4–12 cells in four independent experiments.

RyR-C4 cells (Fig. 1B). The inability of E4032A-RyR to release Ca\(^{2+}\) in response to caffeine and ryanodine serves as important controls for our subsequent experiments (see below).

Interestingly, the RyR-xL cells respond to caffeine with intracellular Ca\(^{2+}\) release that is not very different from the CHO-RyR cells (Fig. 1B, lower trace). On average, the peak of caffeine-induced Ca\(^{2+}\) release had a ratio of \(F_{340}/F_{380}\) = 4.5 ± 0.6 (\(n = 15\)) and 4.3 ± 0.4 (\(n = 6\)) for CHO-RyR and RyR-xL cells, respectively. In addition, the resting cytosolic [Ca\(^{2+}\)] did not show significant difference between CHO-RyR (\(F_{340}/F_{380} = 1.0 ± 0.4\) (\(n = 32\)) and RyR-xL cells (\(F_{340}/F_{380} = 1.2 ± 0.3\) (\(n = 11\)). The data suggest that Bcl-xL does not interfere with the caffeine- and ryanodine-induced Ca\(^{2+}\) release from the ER membrane.

Ryanodine, as a specific ligand for RyR, induces a global conformational change in the RyR protein, and locks the Ca\(^{2+}\) release channel in a permanent open state at micromolar concentrations (27). It binds only to the open state of the Ca\(^{2+}\) release channel, such that no significant release of intracellular Ca\(^{2+}\) was observed when 10 \(\mu\)M ryanodine alone was applied to cells at the resting state prior to stimulation with caffeine (Fig. 1C). But the combined application of ryanodine and caffeine resulted in fast elevation of cytosolic Ca\(^{2+}\) in CHO-RyR and RyR-xL cells but not in CHO-E4032A cells, as shown in Fig. 1C. After ryanodine/caffeine-induced Ca\(^{2+}\) release, although extracellular solution was changed back to BSS-Ca\(^{2+}\) to allow cells to reload with Ca\(^{2+}\), there was no detectable amount of thapsigargin-induced Ca\(^{2+}\) release from the ER in CHO-RyR and RyR-xL cells. Thus, with sustained opening of the Ca\(^{2+}\) release channel by ryanodine, the Ca\(^{2+}\) content in the ER had been completely depleted. Notice that the capacitative Ca\(^{2+}\) entry (CCE) becomes significantly larger in cells pretreated with ryanodine. The result is consistent with the suggestion that sustained depletion of ER Ca\(^{2+}\) is required for full activation of the CCE pathway (28, 29).

Apoptosis of CHO Cells Induced by Activation of RyR/Ca\(^{2+}\) Channels—Perturbation of ER Ca\(^{2+}\) homeostasis with caffeine and ryanodine has significant impact on the propagation and proliferation of CHO cells. Typically, within 16–24 h after treatment with caffeine and ryanodine, less than 10% of the CHO-RyR cells remained viable. To monitor the cell death process, confocal microscopic imaging of individual CHO cells stained with Hoechst 33342 was used. The Hoechst dye is a fluorescent indicator that binds specifically to the nuclear chromatin, which is traditionally used to monitor the apoptotic status of cells. As shown in Fig. 2A, 18 h after treatment with 10 mM caffeine and 10 \(\mu\)M ryanodine, the majority of the adhering CHO-RyR cells had a condensed nucleus with fragmented chromatin structure, which is the hallmark of cells undergoing apoptosis. In contrast, most of the CHO-E4032A cells remained healthy with normal nuclear structure following identical treatment with caffeine and ryanodine. Interestingly, the RyR-xL cells had a unique pattern of Hoechst dye staining that is different from those of CHO-RyR and CHO-E4032A cells, with the same treatment with caffeine and ryanodine. Although the nuclei of RyR-xL cells exhibited extended shape, they remained intact with no apparent chromatin fragmentation.

One of the common features of cells undergoing apoptosis is the fragmentation of genomic DNA into ladder patterns of oligonucleosome-sized fragments. Fig. 2B is a representative agarose gel of extracted genomic DNA from CHO cells following 18 h treatment with 10 mM caffeine, 10 \(\mu\)M ryanodine, or 0.5 mM EGTA. In CHO-RyR cells, DNA laddering was clearly visible after treatment with caffeine, especially with caffeine plus ryanodine (Fig. 2B, lane 3, 4). However, the CHO-E4032A cells showed no detectable DNA fragmentation following treatment with caffeine and ryanodine (lane 1). Prolonged incubation of cells with 0.5 mM EGTA (with nominal Ca\(^{2+}\) present in the culture medium) caused DNA laddering in both CHO-RyR and CHO-E4032A cells (lanes 2, 5, and 6). In contrast, no DNA fragmentation was observed in RyR-xL, whether treated with caffeine, caffeine plus ryanodine, or combination of caffeine, ryanodine, and EGTA (lane 8, 9, and 10).

Based on the morphological changes and the DNA laddering patterns of cells treated with caffeine, ryanodine, and EGTA, the data indicate that depletion of ER Ca\(^{2+}\) stores via activation of the RyR/Ca\(^{2+}\) release channel can induce apoptosis in CHO cells. Furthermore, Bcl-xL as an anti-apoptotic regulator
can prevent the ER-Ca\(^{2+}\) depletion induced apoptosis in CHO cells.

**Time-dependent Effect of Caffeine, Ryanodine, and EGTA on CHO Cell Viability**—To further characterize the time-dependent changes in cell viability following perturbation of ER Ca\(^{2+}\) homeostasis, we did the following studies. Apoptotic and necrotic cells were recorded by using DNA binding dyes acridine orange and ethidium bromide following the protocol of McGa- hon et al. (25). As shown in Fig. 3, combined application of caffeine and ryanodine, by virtue of their ability to deplete ER Ca\(^{2+}\) stores, induced substantial loss of viability in CHO-RyR cells (Fig. 3B). By 12 h, about 50% of the CHO-RyR cells had entered the apoptotic process, and by 24 h, less than 3% of cells remained viable. In contrast, 82% of the CHO-E4032A cells remained viable by 24 h and the majority of cell death was by necrosis, likely due to the pleiotropic effects of caffeine (Fig. 3A).

As shown in Fig. 1, depletion of ER Ca\(^{2+}\) stores induced a large and sustained increase in cytosolic Ca\(^{2+}\) due to the CCE across the plasma membrane (Fig. 1C). To determine if the sustained high concentration of Ca\(^{2+}\) or the entry of Ca\(^{2+}\) from the extracellular space is important in the induction of apoptosis, we studied the ability of caffeine and ryanodine to induce apoptosis in a Ca\(^{2+}\)-free medium (with 0.5 mM EGTA). This Ca\(^{2+}\)-free medium significantly augmented apoptosis instead of increasing the viability of CHO-RyR cells (compare left versus right panels of Fig. 3B). By 9 h, only 16% cells remained viable and most of the dying cells had entered the apoptosis stages. Moreover, pronounced apoptosis could be observed in CHO-E4032A cells, 12 h after the addition of EGTA (Fig. 3A, right), which is also evident in the DNA laddering patterns (Fig. 2B, lane 2). The results are consistent with the observation that prolonged incubation of cells with EGTA lead to eventual depletion of the intracellular Ca\(^{2+}\) stores (30). Together, our data suggest that it is the depletion of the ER Ca\(^{2+}\) pool, rather than the elevation of cytosolic Ca\(^{2+}\), that is responsible for the apoptotic cell death.

Consistent with the anti-apoptotic effect of Bcl-xL, the RyR-xL cells had less than 5% apoptotic death even after 24 h treatment with caffeine and ryanodine. Although Bcl-xL had a dramatic effect on cell viability, it is interesting to note that a significant portion of the RyR-xL cells died via necrosis, rather than apoptosis, following depletion of the ER Ca\(^{2+}\) stores. This is clearly seen when 0.5 mM EGTA was added to the extracellular medium (Fig. 3C, right). Thus, co-expression of Bcl-xL and wt-RyR1 in CHO cells prevented the apoptotic cell death pathway, but not the necrotic cell death process.

**Effect of Extracellular ATP on Intracellular Ca\(^{2+}\) Release and Cell Viability**—CHO cells contain purinergic receptors on the surface membrane, and binding of extracellular ATP to this receptor leads to generation of IP\(_3\), in the cytosol which, in turn, activates the IP\(_3\)-R channel in the ER membrane. Studies from other investigators have shown that application of ATP could induce apoptosis in cultured cells (31, 32). To compare the effect of ATP with those of caffeine and ryanodine on the
apoptosis of CHO cells, the following assays were used. First, the ability of extracellular ATP to release Ca$^{2+}$ from intracellular stores was compared in CHO-C4 and RyR-C4 cells. As shown in Fig. 4A, addition of 0.5 mM ATP to the extracellular medium caused fast release of Ca$^{2+}$ from ER with a magnitude that is comparable between CHO-C4 and RyR-C4 cells. After ATP-induced Ca$^{2+}$ release, addition of 10 mM caffeine caused further Ca$^{2+}$ release from intracellular stores; and pre-treatment of the RyR-C4 cells with caffeine and ryanodine also abolished the ATP-induced calcium release (Fig. 4B). The results indicate that the ATP-induced Ca$^{2+}$ release share the same pool with the caffeine- and ryanodine-induced Ca$^{2+}$ release, and overexpression of RyR in the ER membrane does not affect the function of the IP$_3$ receptor.

Next, we compared the effect of extracellular ATP on the viability of CHO-E4032A, CHO-RyR, and RyR-xL cells. As shown in Fig. 4C, addition of 0.5 mM ATP produced progressive cell death in all three types of cell lines. But the onset of ATP-induced cell death is significantly slower than that generated by caffeine and ryanodine. 24 h after the addition of ATP, only about 12% of CHO-RyR cells had entered the apoptotic process (Fig. 4C, middle), which is in contrast to the near complete apoptosis observed after exposure to caffeine and ryanodine (Fig. 3B). Similar ATP-induced apoptosis processes were also observed in CHO-E4032A (Fig. 4C, top). The slow ATP-induced apoptosis process is unlikely due to the hydrolysis of extracellular ATP by the ecto-ATPase, since AMP-PNP, a poorly hydrolyzable analog of ATP, produced similar effect (not shown). Thus, overexpression of RyR in CHO cells does not affect the intrinsic ability of cells to undergo apoptosis triggered by extracellular ATP. The difference between the ATP- and caffeine-induced cell death could reflect functional differences between RyR and IP$_3$R. The IP$_3$ receptor is known to enter a desensitized state with sustained stimulation with IP$_3$, and the IP$_3$R channel has a narrower window of intracellular Ca$^{2+}$ dependence compared with the RyR channel, i.e., the Ca$^{2+}$-dependent activation and inactivation phases of the IP$_3$R channel exhibit significant overlap (33, 34). Thus, transient activation of IP$_3$R is unlikely to cause sustained depletion of the ER Ca$^{2+}$ store, which may be sufficient to maintain certain viability of the cells.

**Effect of Bcl-xL on Capacitative Ca$^{2+}$ Entry and Cytosolic Ca$^{2+}$**—Studies from other investigators have suggested that part of the anti-apoptotic effect of Bcl-2 family proteins could be correlated with the altered CCE pathway across the plasma membrane (5, 14). This does not seem to be the case with CHO cells, based on the following two observations. First, the amount of Ca$^{2+}$ stored in ER membrane did not change with or without the presence of Bcl-xL, as reflected by the magnitude of caffeine-induced Ca$^{2+}$ release in CHO-RyR and RyR-xL cells (see Fig. 1B). Second, the amount of CCE following ER Ca$^{2+}$ depletion remained essentially the same, whether or not Bcl-xL is present in the cells. As shown in Fig. 5A, there is no significant difference in either the basal level of cytosolic Ca$^{2+}$ ([Ca$^{2+}$]$_{i}$)$_{\text{in}}$ or the peak of Ca$^{2+}$ entry ([Ca$^{2+}$]$_{i}$)$_{\text{max}}$ following the addition of ATP (2 mM) to the extracellular solution. However, significant differences between the CHO-RyR and RyR-xL cells was observed in the cytosolic [Ca$^{2+}$]$_{i}$ following sustained treatment with caffeine and ryanodine. 2–8 h after the depletion of ER Ca$^{2+}$, the cytosolic [Ca$^{2+}$]$_{i}$ in RyR-xL cells appear to be significantly higher than that in CHO-RyR cells (Fig. 5B).

**Effect of BAPTA-AM on ER Ca$^{2+}$ Release and Cell Viability**—One of the observations in Fig. 3 was that chelation of extracellular Ca$^{2+}$ with EGTA appeared to accelerate the cell death process in CHO cells. A potential caveat of using EGTA is that it might introduce an oxidative stress to the mitochondria, such as glutathione efflux from mitochondria, which may affect the apoptosis process (35). And to some extent, prolonged exposure to low Ca$^{2+}$ may cause cells to lose adhesion, which may also lead to apoptosis. As an alternative way of buffering cytosolic Ca$^{2+}$, CHO cells were treated with a membrane-permeable Ce$^{3+}$ chelator, BAPTA-AM (10 μM). As shown in Fig. 6A, in cells pretreated with BAPTA-AM, the ATP-induced cytosolic Ca$^{2+}$ elevation, as well as the caffeine-induced cytosolic Ca$^{2+}$ elevation, were dramatically reduced (trace b comparing with trace a). And BAPTA appeared to be stably remained in the cytosol since its effects on ATP-induced or caffeine-induced cytosolic Ca$^{2+}$ elevation in CHO-RyR cells after another 2 h were similar with the effects produced immediately after BAPTA-AM treatment (trace c comparing with b). The effects of BAPTA pretreatment on cell viability at 24 h after addition of 10 mM caffeine and 10 μM ryanodine are summarized in Fig. 6B. In 4 paired experiments with control (–dimethyl sulfoxide) or 10 μM BAPTA-AM-treated cells, there were no significant differences in the percentage of cells undergoing apoptosis, studied in all three types of cell lines, CHO-E4032A, CHO-RyR, and RyR-xL. But, the percentage of cells undergoing necrosis was dramatically reduced in RyR-xL cells. This is likely due to the buffering capacity of the cytosolic Ca$^{2+}$ by BAPTA (see Fig. 5C). With the measurement of cytosolic Ca$^{2+}$ in individual RyR-xL cells, we found that pretreatment with BAPTA lead to
significant reduction of the sustained cytosolic \(\text{Ca}^{2+}\), 2 h after stimulation by caffeine and ryanodine: the ratio of \(F_{340}\)/\(F_{380}\) decreased from 1.7 ± 0.3 (+dimethyl sulfoxide) to 1.2 ± 0.1 (+BAPTA-AM).

**DISCUSSION**

Stable expression of RyR enables rapid and reversible changes of \(\text{Ca}^{2+}\) release across the ER membrane, thus providing a convenient means for studying the role of \(\text{Ca}^{2+}\) signaling in apoptosis. Our data demonstrate that depletion of ER \(\text{Ca}^{2+}\) stores is capable of inducing apoptosis in CHO cells, which is in agreement with previous studies showing that inhibition of the ER \(\text{Ca}^{2+}\)-ATPase by thapsigargin leads to apoptotic cell death (8–11). Moreover, the anti-apoptotic effect of Bcl-xL in our system appears to be independent of changes in ER \(\text{Ca}^{2+}\) release and capacitative \(\text{Ca}^{2+}\) entry. This suggests that Bcl-xL prevents apoptosis at a step that is downstream of ER \(\text{Ca}^{2+}\) homeostasis.

The presence of RyR in the ER membrane of CHO-RyR cells has several advantages over the endogenous IP\(_3\)-R present in the parental CHO cells. First, it allows control of \(\text{Ca}^{2+}\) release in a reversible manner with caffeine, as well as control of the ER \(\text{Ca}^{2+}\) content with ryanodine. With the combined applica-

**FIG. 5.** Capacitative \(\text{Ca}^{2+}\) entry and sustained cytosolic \(\text{Ca}^{2+}\) in CHO cells following depletion of the ER \(\text{Ca}^{2+}\) stores. \(A\), capacitative \(\text{Ca}^{2+}\) entry was defined as the changes of fluorescence ratio 340 nm/380 nm in 0.5 mM EGTA-BSS ([Ca\(_{\text{next}}\)]\text{min}) and in 2 mM Ca\(_{\text{2+}}\)-BSS ([Ca\(_{\text{2+}}\)]\text{max}). CHO-RyR cells and RyR-xL were treated with 10 mM caffeine and 10 mM ryanodine in 0.5 mM EGTA-BSS, then bath solutions were changed to 2 mM Ca\(_{\text{2+}}\)-BSS. Neither [Ca\(_{\text{2+}}\)]\text{min} nor the peak Ca\(_{\text{2+}}\) entry [Ca\(_{\text{2+}}\)]\text{max} shows significant differences between the two cell types. \(B\), at 2 and 8 h after addition of caffeine and ryanodine in culture medium, fluorescence ratio 340/380 in CHO-RyR and RyR-xL cells were measured to represent the concentration of cytosolic \(\text{Ca}^{2+}\) ([Ca\(_{\text{2+}}\)]\text{cyt}) at both time points in RyR-xL cells (hatched bar) was significantly higher than that in CHO-RyR cells (blank bar).

**FIG. 6.** BAPTA-AM reduces elevation of cytosolic \(\text{Ca}^{2+}\) and inhibits necrosis in CHO cells after caffeine and ryanodine treatment. Cells were incubated with 10 \(\mu\)M BAPTA-AM (dissolved in dimethyl sulfoxide) in extracellular medium at 37 °C. Control cells were pretreated with equal volumes of dimethyl sulfoxide without BAPTA-AM. After 90 min, the cells were changed back to standard medium. \(A\), representative traces of ATP-induced \(\text{Ca}^{2+}\) release and caffeine-induced \(\text{Ca}^{2+}\) release were plotted in control (a), immediately (b, black lines), or 2 h (c, gray lines) after pretreatment with BAPTA-AM in CHO-RyR cells. \(B\), the effect on BAPTA on apoptosis (top) and necrosis (bottom) was measured 24 h after addition of 10 caffeine (10 mM) and ryanodine (10 \(\mu\)M). The number of necrotic cells in RyR-xL has significant difference (*).
Ryanodine results in activation of the capacitative Ca\textsuperscript{2+} channels. Mitochondria's Ca\textsuperscript{2+} signals enhance mitochondria's Ca\textsuperscript{2+} influx. However, it remains controversial whether Bcl-2-related pro-apoptotic proteins enhance mitochondrial Ca\textsuperscript{2+} loading capacity or prevent intracellular Ca\textsuperscript{2+} stores from overloading (46–48). Further studies to investigate the relationship between ER Ca\textsuperscript{2+} release and mitochondrial Ca\textsuperscript{2+} uptake, and to understand the effect of Bcl-xL on the communication between ER and mitochondria, should lead to new insights into the cellular and molecular mechanism of Ca\textsuperscript{2+} signaling in the initial phase of apoptosis.

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REFERENCES

Depletion of Intracellular Ca$^{2+}$ by Caffeine and Ryanodine Induces Apoptosis of Chinese Hamster Ovary Cells Transfected with Ryanodine Receptor
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