A Selective Requirement for Elevated Calcium in DNA Degradation, but Not Early Events in Anti-Fas-induced Apoptosis*

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Jurkat cells undergo apoptosis in response to anti-Fas antibody through a caspase-dependent death cascade in which calcium signaling has been implicated. We have now evaluated the role of calcium during this death cascade at the single cell level in real time utilizing flow cytometric analysis and confocal microscopy. Fluo-3 and propidium iodide were employed to evaluate calcium fluxes and to discriminate between viable and non-viable cells, respectively. Anti-Fas treatment of Jurkat cells resulted in a sustained increase in intracellular calcium commencing between 1 and 2 h after treatment and persisting until subsequent loss of cell membrane integrity. The significance of this rise in calcium was evaluated by buffering intracellular calcium with BAPTA and/or removing calcium from the extracellular medium and monitoring the effects of these manipulations on calcium signaling and components of the apoptotic process. Complete inhibition of the anti-Fas-induced rise in intracellular calcium required both chelation of [Ca$^{2+}$]$_i$ and removal of extracellular calcium. Interestingly, this condition did not abrogate several events in Fas-induced apoptosis including cell shrinkage, mitochondrial depolarization, annexin binding, caspase activation, and nuclear poly(A)DP-ribose polymerase cleavage. Furthermore, calcium-free conditions in the absence of anti-Fas antibody weakly induced these apoptotic components. In marked contrast, calcium depletion did not induce DNA degradation in control cells, and inhibited apoptotic DNA degradation in response to anti-Fas. These data support the concept that the rise in intracellular calcium is not a necessary component for the early signal transduction pathways in anti-Fas-induced apoptosis in Jurkat cells, but rather is necessary for the final degradation of chromatin via nuclelease activation.

Apoptosis, also known as programmed cell death, is a physiological mode of cellular suicide that plays a vital and important role during embryogenesis, development, and normal tissue homeostasis. It is characterized by distinct morphological and biochemical characteristics, including cell shrinkage, protein degradation, alterations in plasma membrane lipid asymmetry, depolarization of mitochondria, and chromatin condensation and fragmentation. Diverse internal and external stimuli can initiate apoptosis through complex signal transduction pathways that eventually lead to a central cascade of effectors that carry out the final stages of cell death although the precise signaling pathways that regulate apoptosis are not yet fully understood. One of the better defined pathways for the activation of apoptosis is the Fas-dependent killing of T-lymphocytes, where the framework for the death cascade has now been conceptualized and involves the formation of a death inducing complex which recruits death effectors and activates downstream catalytic pathways (1).

Recent studies from several laboratories have indicated that the Fas-induced death pathway may effectively activate lymphocyte apoptosis via mechanisms dependent on ionic changes in the cell, such as those involving cell volume regulation (2) or calcium mobilization (3–7). Indeed calcium, a major factor in many cellular signaling pathways, has long been implicated in apoptosis since many of the nucleases activated during this form of cell death appear to require calcium (8–10). However, the precise role of calcium in the cell death process remains controversial with some studies suggesting that it is essential (3, 5–7) whereas others suggest that it is not (11, 12). These differences may reflect differences in the model systems under investigation or perhaps the end points used to evaluate apoptosis.

Early studies of glucocorticoid-induced cell death by Kaiser and Edelman (13) suggested an increase in intracellular calcium as a key component. The characteristic chromatin condensation and DNA degradation of glucocorticoid-induced death could be mimicked by agents which elevated intracellular calcium (14, 15). Other investigators have shown that the Ca$^{2+}$-ATPase inhibitor, thapsigargin, which causes a rise in intracellular calcium by inhibiting the uptake of calcium into the endoplasmic reticulum also induces apoptosis (16). Buffering of intracellular calcium with BAPTA, or removal of extracellular calcium with EGTA, has been reported to protect cells against apoptosis, suggesting that a rise in intracellular calcium is an important cell death signal (17). Perhaps the best evidence for the involvement of calcium in Fas-mediated apoptosis in T-lymphocytes comes from the work of Jayaraman and Marks (18). They showed that T-lymphocytes with a mutated inositol 1,4,5-trisphosphate receptor, which are incapable of mobilizing intracellular calcium, were resistant to Fas-mediated apoptosis when DNA degradation was used as the sole criterion for apoptosis.

Conversely several studies argue against an increase in intracellular calcium as a necessary component of apoptosis. In MIN6 cells thapsigargin-induced apoptosis is not blocked by

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The abbreviations used are: BAPTA, 1,2-bis(O-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; MMP, mitochondrial membrane potential; PI, propidium iodide; FITC, fluorescein isothiocyanate; PARP, poly(ADP-ribose) polymerase; [Ca$^{2+}$]$_i$, intracellular [Ca$^{2+}$].
buffering intracellular calcium with BAPTA (11). Additionally, previous work from our laboratory has also shown that glucocorticoid-induced apoptosis of S49<sup>neo</sup> cells resulted only in a modest increase in intracellular calcium which was not required for cell death (12). Furthermore, maintenance of intracellular calcium stores via expression of Bcl-2 protected cells against apoptosis (19, 20). Thus where and how calcium is involved in lymphocyte apoptosis remains unclear.

Most studies on the role of calcium in apoptosis used mixed populations of apoptotic and non-apoptotic cells and relied on DNA degradation, a late event in the death cascade, as an index of apoptosis. In the present study, we have addressed the roles that calcium mobilization may play in multiple events in the apoptotic cascade by using the well defined anti-Fas antibody-treated Jurkat cells as a model system. Apoptosis was examined at the single cell level by confocal microscopy as well as by flow cytometry, which permits simultaneous analysis of multiple parameters. We show that activation of the Fas pathway results in release of intracellular stores of calcium and influx of calcium from the extracellular space, however, this elevation appears to be unnecessary for most components of the apoptotic pathway other than DNA degradation.

**MATERIALS AND METHODS**

**Cell Culture—**Jurkat cells (human lymphoma) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 4 mM glutamine, 75 units/ml streptomycin, and 100 units/ml penicillin at 37 °C, 7% CO<sub>2</sub> atmosphere. Jurkat cells were treated with 100 ng/ml anti-human Fas IgM (Kamiya Biomedical, Seattle, WA) for the time periods indicated. Calcium-free media were prepared from powdered RPMI (Hyclone) without added calcium nitrate and was supplemented with normal calcium with BAPTA loading protocol was optimal for buffering a thapsigargin-induced calcium rise, without significantly affecting the viability of cells tested previously with a Becton Dickinson FACSort equipped with a Becton Dickinson FACSort equipped with CellQuest software (21). For each sample 10,000 cells were examined by flow cytometry as described previously with a Becton Dickinson FACSort equipped with CellQuest software (21). For each sample 10,000 cells were examined by flow cytometry as described previously with a Becton Dickinson FACSort equipped with CellQuest software (21). For each sample 10,000 cells were examined by flow cytometry as described previously with a Becton Dickinson FACSort equipped with CellQuest software (21). For each sample 10,000 cells were examined by flow cytometry as described previously with a Becton Dickinson FACSort equipped with CellQuest software (21). For each sample 10,000 cells were examined by flow cytometry as described previously with a Becton Dickinson FACSort equipped with CellQuest software (21). For each sample 10,000 cells were examined by flow cytometry as described previously with a Becton Dickinson FACSort equipped with CellQuest software (21). For each sample 10,000 cells were examined by flow cytometry as described previously with a Becton Dickinson FACSort equipped with CellQuest software (21). For each sample 10,000 cells were examined by flow cytometry as described previously with a Becton Dickinson FACSort equipped with CellQuest software (21). For each sample 10,000 cells were examined by flow cytometry as described previously with a Becton Dickinson FACSort equipped with CellQuest software (21).
RESULTS

Anti-Fas Antibody Promotes Calcium Mobilization in Jurkat Cells—We initially examined Jurkat cells treated in the presence or absence of anti-Fas for changes in their intracellular calcium concentration. The data shown in these and subsequent studies include only those cells negative for PI staining indicative of cells with an intact plasma membrane. When control Jurkat cells were examined on a forward scatter versus Fluo-3 (calcium) fluorescence contour plot, only a single population of cells was observed, which remained constant over a
period of 6 h (Fig. 1, left-hand column). Interestingly, treatment of cells with anti-Fas resulted in three distinct populations of cells, which became apparent between 1 and 2 h after treatment (Fig. 1, right-hand column). One population of cells had a normal cell size and intracellular level of calcium, similar to the control sample; a second small population (16% at 4 h) had a marked reduction in cell size, but a normal level of intracellular calcium and a third major population (26% at 4 h) had an increased intracellular calcium level and were of a slightly smaller size, as judged by forward-scatter measurements. The number of cells with elevated \([\text{Ca}^{2+}]\), increased over the course of the experiment and at 6 h two major populations predominated; one with normal cell size and normal \([\text{Ca}^{2+}]\) (33%) and one with slightly reduced cell size and elevated \([\text{Ca}^{2+}]\) (36%) while the numbers of cells with reduced cell size and normal \([\text{Ca}^{2+}]\) remained constant (16%). Prolonged incubation with anti-Fas antibody (24 h) resulted in complete conversion of the normal size/small \([\text{Ca}^{2+}]\) population to this major population of cells with elevated \([\text{Ca}^{2+}]\). This increase in the population of cells with elevated \([\text{Ca}^{2+}]\), during anti-Fas treatment suggests that calcium elevation may have some important downstream consequences on effector components of apoptosis.

Cells undergoing apoptosis shrink and caspase-3 activity and DNA degradation only occur in this cellular condensation phase of the death process (21, 23). We therefore examined \([\text{Ca}^{2+}]\) in the shrunken and normal sized cells at the 6 h time point by selecting these populations on a forward-scatter versus side-scatter dot plot (Fig. 2, center) and then displaying the Fluo-3 fluorescence parameter for cells from that specific gated population (Fig. 2, right and left). In the control sample the majority of cells had a normal cell size and normal levels of \([\text{Ca}^{2+}]\), (Fig. 2, top right). Additionally, in the control sample, a small percentage (4.8%) of the population had a decrease in cell size and a slightly lower \([\text{Ca}^{2+}]\), than normal sized control cells (Fig. 2, top left). Examination of membrane integrity showed that these cells were all propidium iodide negative, indicative of an intact cell membrane (data not shown). In contrast, anti-Fas-treated cells which had a normal cell size showed two distinct populations; one population of cells had control levels of \([\text{Ca}^{2+}]\), and a second population of cells had a higher \([\text{Ca}^{2+}]\) (Fig. 2, bottom right). Interestingly, the shrunken apoptotic cells in the anti-Fas sample showed a population of cells with increased \([\text{Ca}^{2+}]\), (Fig. 2, bottom left) in contrast to the control shrunken apoptotic cells, which had a lower intracellular calcium. These data suggest that although intracellular calcium is elevated during anti-Fas-induced apoptosis it may not be necessary to induce cell shrinkage during apoptosis, since control cells which spontaneously die in the absence of anti-Fas do not have elevated \([\text{Ca}^{2+}]\), (Fig. 2).

**Direct Visualization of Intracellular Calcium Concentration and Nuclear Morphology**—Since flow cytometry does not provide information on the subcellular distribution of calcium we attempted to determine if the rise in intracellular calcium seen in Fas-induced apoptosis is evenly distributed between the cytoplasm and the nucleus using confocal microscopy. Highly elevated calcium or low calcium (based on Fluo-3 fluorescence) cells were obtained by sorting on the FACS Vantage flow cytometer (Fig. 3A). These distinct populations were then visualized on a Zeiss LSM410 confocal microscope, which has the capability of exciting both the Fluo-3 and the Hoechst (nuclear) dyes (Fig. 3B). The nuclear morphology of the untreated Jurkat cells is uniformly large, uncondensed, and dimly fluorescent. In comparison the anti-Fas-treated cells have condensed and highly fluorescent nuclei, regardless of the intensity of the Fluo-3 fluorescence (Fig. 3B, first column). Untreated cells, which have a low (or normal) intracellular calcium concentration, show the Fluo-3 fluorescence both in the cytoplasm and the nucleus (Fig. 3B, second column). In marked contrast, cells undergoing Fas-induced apoptosis show very little Fluo-3 fluorescence in their condensed nuclei, which could reflect lower intranuclear calcium or dye exclusion from the highly com-

**Fig. 2.** Rise in intracellular calcium occurs in both normal and shrunken cells. Center, Jurkat cells were treated without (top) or with (bottom) anti-Fas antibody for 6 h and loaded with 1 \(\mu\text{M Fluo-3/AM plus 10 ng/ml PI for 30 min.} \) The cells were analyzed on a forward-scatter versus side-scatter plot and gates drawn around cells of normal size (R1) and smaller size (R2). Right, contour plots of forward-scatter versus intracellular calcium of cells from the normal size gate show the increase in calcium over time in the anti-Fas-treated cells. Left, contour plots of forward-scatter versus intracellular calcium of cells from the shrunken population also show increased calcium over time in the anti-Fas-treated cells. The number of cells in the R1 and R2 gates for this experiment are displayed as percentages of the total population.
intracellular calcium buffering reduced by BAPTA (Fig. 4). The micrographs of Fluo-3 fluorescence are shown at the same contrast and brightness settings, however, the attenuation of the laser excitation was varied between 3, 10, and 30, highlighting the difference of fluorescence intensity between the low and highly fluorescent populations.

Consequences of Inhibition of the Rise in Intracellular Calcium Concentration on Effectors of Apoptosis—To define further the roles of an increase in [Ca$^{2+}$]i during anti-Fas-induced apoptosis we examined specific components of the apoptotic effector pathway while buffering the anti-Fas-induced rise in calcium. We initially analyzed treated and non-treated Jurkat cells for changes in cell viability. Control cells in normal medium with or without BAPTA-AM showed no significant loss of cell viability over 6 h (Table I) whereas anti-Fas treated Jurkat cells resulted in a significant loss of viability over the same period of time in normal medium. The loss of cell viability observed during anti-Fas antibody treatment was not abrogated in the presence of BAPTA-AM (Table I). In calcium-free media control cells also maintained high viability, while in the presence of BAPTA-AM alone a small but significant loss of cell viability was observed (Table I). This procedure of loading of cells with BAPTA while in the absence of extracellular [Ca$^{2+}$], would be expected to induce depletion of [Ca$^{2+}$], from intracellular calcium containing organelles, primarily the endoplasmic reticulum. The decreased viability under this condition is consistent with the evidence suggesting that depletion of calcium from endoplasmic reticulum stores can serve as an initiating stimulus for apoptosis (12). Anti-Fas-treated Jurkat cells also showed a significant reduction in viability with or without BAPTA loading in the presence or absence of extracellular
calcium (Table I). Thus the elevation in intracellular calcium does not appear to be necessary for anti-Fas-induced cell death. Since the loss of cell volume is a fundamental component of apoptosis (23, 24) we next examined the relationship between calcium mobilization and cell size. Control Jurkat cells in normal medium showed the main population of cells having high forward light scatter properties, indicating normal cell size (Fig. 5A). Similar results were observed for the BAPTA-AM-treated cells in normal medium (Fig. 5B). Cells undergoing apoptosis induced by anti-Fas antibody (Fig. 5C) showed a population of cells with decreased cell volume indicated by a decrease in forward scattered light and loading the cells with BAPTA (Fig. 5D) did not prevent the loss of cell volume resulting from anti-Fas treatment. Control cells in calcium-free medium existed as a main population of cells, which displayed a normal cell size, similar to that observed in the presence of extracellular calcium (Fig. 5E). Cells treated with anti-Fas in the absence of extracellular Ca\(^{2+}\) also showed an increase in the number of cells with a reduced cell size which was greater than that observed in the presence of calcium (Fig. 5G). Also, consistent with the data for cell viability in the presence of BAPTA-AM alone under calcium-free conditions a significant population of cells showed a loss of cell volume (Fig. 5F). Similarly, BAPTA-AM plus anti-Fas treatment did not prevent apoptotic cell shrinkage (Fig. 5H). These results suggest that: 1) the elevation of [Ca\(^{2+}\)]\(_i\) during anti-Fas-induced cell death is not necessary for cell shrinkage and 2) that depletion of intracellular calcium by BAPTA-loading in calcium-free medium is sufficient to cause cell shrinkage.

Because the elevation in [Ca\(^{2+}\)]\(_i\) seen during anti-Fas-induced cell death was not directly involved with loss of viability or cell volume, we wished to evaluate this elevation relationship with other events in the death cascade. We next examined another relatively early apoptotic event, an increase in phosphatidylserine exposure on the cell surface. These molecules are reoriented to the outside of the plasma membrane during the early stages of apoptosis and can be detected by binding to fluorescein isothiocyanate (FITC)-tagged annexin V. When used in combination with propidium iodide as a vital dye, one can distinguish between early apoptotic cells which have increased phosphatidylserine exposure (annexin positive) but maintain their membrane integrity (PI negative) and cells which lose membrane integrity (PI positive). Control cells in normal medium after 6 h showed 95% of the cell population having little phosphatidylserine exposure (annexin negative) (Fig. 6A). Whereas cells loaded with BAPTA-AM in normal medium showed a slight increase in the number of annexin-positive cells (Fig. 6B), anti-Fas treatment caused a greater increase in the number of annexin-positive cells (Fig. 6C). BAPTA loading of cells before anti-Fas treatment did not block the increase in the number of annexin-positive cells (Fig. 6D). In calcium-free medium there is a small increase in the number of annexin-positive cells in the control sample (Fig. 6E). Either BAPTA loading alone (i.e. calcium depletion) or anti-Fas treat-
ment caused a greater increase in the number of annexin-positive cells (Fig. 6, F and G). It should be noted that the combination of BAPTA loading and anti-Fas treatment caused an increase in the number of annexin-positive (Fig. 6H) and PI-positive cells (see legend of Fig. 6). This accelerated activation of apoptosis decreased the total number of cells shown in the histograms because only the PI-negative cells are shown. Suppressing the anti-Fas-induced intracellular rise in calcium with BAPTA and depletion of extracellular calcium did not inhibit the increase in the number of annexin-positive cells.

The importance of changes in the mitochondrial membrane potential during apoptosis has emerged from numerous recent investigations (24, 25). Mitochondria in cells undergoing apoptosis under a variety of conditions have been shown to lose membrane potential and to release pro-apoptotic factors. Additionally, under certain conditions, mitochondria can sequester calcium; thus we examined the correlation between calcium mobilization and mitochondrial membrane depolarization during anti-Fas-induced apoptosis. We used JC-1, a dye that is highly selective for detecting changes in the mitochondrial membrane and which was shown to be unresponsive to changes in plasma membrane potential (22, 24). Under normal conditions the dye is in an aggregate state and depolarization of the mitochondrial membrane causes disassociation of the dye into monomers with a resulting change in fluorescent emission. Control cells in normal media showed high aggregate fluorescence with only a slight loss of MMP in the presence of BAPTA-AM (Fig. 7A and B, respectively). Anti-Fas-treated cells showed a significant decrease in the number of cells with high aggregate fluorescence (Fig. 7C) with a concurrent increase in the number of cells with high monomer fluorescence (data not shown) indicating a substantial loss of MMP during apoptosis. The combination of BAPTA loading and anti-Fas treatment resulted in a greater number of cells with depolarized mitochondria (Fig. 7D). Control cells, cultured in calcium-free media had a small but significant loss of MMP, which was enhanced with BAPTA loading (Fig. 7E and F). Treatment with anti-Fas increased the number of cells that displayed a loss of MMP compare with cells in Ca²⁺-containing medium (Fig. 7G). Anti-Fas treatment of BAPTA-loaded cells under calcium-free conditions showed the greatest increase in the number of cells with depolarized mitochondria (Fig. 7H). Together these data support our previous observation, suggesting that the elevation in intracellular calcium is not necessary for...
anti-Fas-induced mitochondrial membrane depolarization.

A critical component of many apoptotic pathways is the initiation of the caspase cascade. Caspase-3 is considered one of the effector caspases and it cleaves many non-caspase substrates, such as poly(ADP-ribose) polymerase, and is downstream from caspase-8 (FLICE) in the Fas pathway. To evaluate the effect of calcium mobilization on caspase-3-like activity in control and anti-Fas-treated Jurkat cells, we used a cell permeable caspase-3 substrate, phi-phi-lux. Phi-phi-lux is a 33-amino acid peptide with rhodamines conjugated on both termini. The inherent fluorescence is unquenched upon cleavage of an internal DEVD or caspase-3-like consensus sequence. Control cells cultured in normal medium or those loaded with BAPTA for 6 h had low caspase-3-like activity (Fig. 8, A and B). An increase in the number of cells showing increased caspase-3-like activity was seen in anti-Fas-treated cells (Fig. 8C). Anti-Fas treatment plus BAPTA loading resulted in a marked increase in the number of cells with increased caspase-3-like activity (Fig. 8D). Removing extracellular calcium did not affect caspase-3-like activity as evidenced by control cells cultured in calcium-free media for 6 h (Fig. 8E). However, in the absence of extracellular calcium, BAPTA-AM treatment or anti-Fas treatment alone resulted in substantial increases in the number of cells with increased caspase-3-like activity (Fig. 8, F and G, respectively). These effects were additive following simultaneous treatment with both BAPTA-AM and anti-Fas under calcium-free conditions (Fig. 8H). These results strongly suggest that the anti-Fas induced elevation of [Ca\(^{2+}\)]\(_i\) is also not necessary for the activation of caspase-3 whereas depletion of Ca\(^{2+}\) can also serve as a sufficient signal for this response.

**Effects of Calcium Mobilization on Nuclear Aspects of Apoptosis**—Our results thus far have focused mainly on events that occur in the cytoplasm or at the cell membrane. Since there is evidence that calcium may be differentially distributed between the cytoplasm and the nucleus under apoptotic conditions (26) and since we were unable to directly visualize that compartmentalization with confocal microscopy, we examined whether inhibiting the rise in [Ca\(^{2+}\)]\(_i\), caused by anti-Fas treatment affected nuclear aspects of apoptosis. Since caspase-3 activity was not suppressed under calcium-free conditions, we initially examined PARP, a constitutive nuclear protein involved in DNA repair and a known caspase-3 substrate, to determine if this was cleaved under calcium-free conditions. Western blots of SDS-polyacrylamide electrophoresis gels using an anti-PARP antibody showed intact protein in all cells at the start of the experiments (Fig. 9A). After 6 h, control cells still showed intact PARP, however, anti-Fas treatment of cells resulted in degradation of PARP (Fig. 9A). This degradation...
was not blocked by inhibiting the rise in $[\text{Ca}^{2+}]_i$ in both normal and calcium-free media (Fig. 9A). Interestingly, PARP cleavage also occurred in BAPTA-AM-treated cells in normal media and to a greater extent under calcium-free conditions after 6 h. This increase in PARP cleavage is consistent with the earlier result that showed increased caspase-3-like activity in cells treated with BAPTA-AM alone under both normal and calcium-free conditions. Furthermore, elevated calcium appears to be unnecessary for the transit of caspases from the cytoplasm to the nucleus.

As a final indicator of apoptosis, we examined the DNA integrity of cells treated with anti-Fas under normal and calcium-free conditions. Control cells in the presence of extracellular calcium showed a normal cell cycle histogram with a very small amount of subdiploid or degraded DNA (Fig. 9B). Interestingly, control cells in calcium-free media showed a cell cycle block at the G2/M phase indicating that extracellular calcium is needed for normal cell cycle progression. Treatment with anti-Fas antibody resulted in an increase in subdiploid DNA under both normal and calcium-free conditions (Fig. 9B). However, this DNA degradation was largely inhibited by BAPTA loading of cells. The combination of BAPTA loading and removal of extracellular $\text{Ca}^{2+}$ did not induce DNA degradation but rather this combination protected the cells from the effects of anti-Fas treatment (Fig. 9B) even when a higher dose (200 ng/ml) of anti-Fas was used to activate death (data not shown). Thus DNA degradation, unlike the other apoptotic events evaluated in this study, appeared to be dependent on the presence of intracellular free calcium. These data are consistent with the role of calcium in the activation of the nucleases responsible for the degradation of chromatin.

**DISCUSSION**

In this study we have used flow cytometry and confocal microscopy to define the relationship between calcium mobilization and the apoptotic effectors in the induction of programmed cell death. We show that engagement of the Fas signaling pathway in Jurkat cells results in a sustained increase in intracellular calcium concentration that occurs prior to cell shrinkage, suggesting that alterations in calcium homeostasis may be involved in the activation of various apoptotic characteristics. In light of this finding we inhibited the anti-Fas induced rise in $[\text{Ca}^{2+}]_i$, by removing extracellular calcium and/or chelating intracellular free calcium. Individually, each
of these treatments diminished the elevation of [Ca\(^{2+}\)], in anti-Fas-treated cells. However, simultaneous removal of both intra- and extracellular components resulted in essentially a complete blockade of the anti-Fas-induced calcium signal, suggesting that anti-Fas acts by mobilizing Ca\(^{2+}\) from both intracellular and extracellular sites. Despite inhibiting the elevation of [Ca\(^{2+}\)], the apoptotic process still proceeded arguing that a rise in [Ca\(^{2+}\)], is not necessary for the signaling of most of the apoptotic components we examined.

Numerous studies have suggested a role for calcium in apoptosis, stemming from initial observations of changes in calcium homeostasis during the cell death process. However, varying results from these studies did not define the role of calcium in relation to other apoptotic events. Many studies suggest that initiation of cell death in various apoptotic systems involves a rise in intracellular calcium concentration which is the stimulus for downstream events (5, 13, 14, 16, 17, 27). Additionally, recent studies suggest that depletion of calcium from intracellular pools such as the endoplasmic reticulum is the primary stimulus with the resulting rise in 

[Ca\(^{2+}\)] being a secondary event and thus not necessary to carry out the apoptotic process (11, 12, 19, 20, 28).

In contrast, several studies show that increasing [Ca\(^{2+}\)], can protect cells that undergo apoptosis by interleukin-3 withdrawal (29–31). Neuronal cells can also be protected from apoptosis by increasing intracellular calcium (32). Our results suggest that control of calcium homeostasis does indeed play a role in the apoptotic process, although the elevation in [Ca\(^{2+}\)], is not required for many of the components of apoptosis. In addition, buffering intracellular free calcium in conjunction with removal of extracellular calcium activates the cell death pathway in Jurkat cells in the absence of additional apoptotic stimuli. Interestingly, buffering intracellular Ca\(^{2+}\), and/or removal of extracellular Ca\(^{2+}\), each had small effects on cell viability. None of the procedures significantly affected basal [Ca\(^{2+}\)], in the cells, and so it is likely that the ability of BAPTA loading to deplete stored Ca\(^{2+}\) is responsible for the observed loss of viability, cell shrinkage, changes in the MMP, and increased caspase-3 activity in BAPTA-AM treated cells under calcium-free conditions.

Previous work has also suggested that depletion of Ca\(^{2+}\) from intracellular stores can serve as a trigger for apoptosis. Bian et al. (12) found that removal of extracellular Ca\(^{2+}\) augmented, rather than inhibited apoptosis due to thapsigargin. Lam et al. (20) also reported a significant role for endoplasmic reticulum calcium stores in signaling apoptosis. The conclusion from these studies is that depletion of intracellular stores can provide a potent stimulus for apoptosis, independent of the activation of Ca\(^{2+}\) entry. Depletion of intracellular calcium stores is known to generate a signal that activates plasma membrane calcium channels (33). It is conceivable that the same or a similar signaling mechanism could be involved in triggering apoptosis, although the available data do not address this possibility. Alternatively, it is possible that the inhibition of protein synthesis or loss of chaperone function resulting from severe depletion of intracellular stores is responsible since inhibition of protein synthesis by cycloheximide can initiate apoptosis (14, 34).

Since some studies have suggested that calcium may be differentially distributed between the cytoplasm and the nucleus during apoptosis (26) we examined the Fluo-3-loaded cells using confocal microscopy to try and distinguish calcium distribution. After initially separating the high and low [Ca\(^{2+}\)], populations we attempted to visualize calcium compartmentalization in these cells. Using Hoechst nuclear dye in conjunction with Fluo-3, we were able to see the nuclei of anti-Fas-treated cells become highly condensed. However, this chromatin condensation excluded the Fluo-3 dye from the nuclei so we were unable to discriminate cytoplasmic and nuclear compartmentalization of calcium. Nevertheless cell shrinkage and nuclear condensation were seen in both the high and low calcium populations of anti-Fas-treated cells, suggesting that the rise in calcium is not directly affecting these apoptotic events.

A component of the apoptotic process that we determined was sensitive to the anti-Fas-induced rise in [Ca\(^{2+}\)], was the activation of the nucleases involved in degradation of the chromatin. It has been previously reported that some of the nucleases responsible for apoptotic DNA degradation are Ca\(^{2+}\)/Mg\(^{2+}\)-dependent (35) and our finding that inhibiting a rise in [Ca\(^{2+}\)], also inhibits DNA degradation is in agreement with previously published results. Our study may also explain some of the conflicting results in earlier studies of calcium’s role in apoptosis. For example, a critical role for calcium in Fas-induced apoptosis has been suggested by studies which show that a functional inositol 1,4,5-trisphosphate receptor is necessary for apoptosis (18). These investigators, however, used only DNA degradation as a measure of apoptosis; the sole component of the death cascade that requires an elevation of intracellular calcium. Obviously one needs to consider all of the components within the death cascade to assign a specific role for calcium. Our results show that activation of the Fas pathway of apoptosis in lymphocytes is associated with an elevation in intracellular calcium but this rise is only necessary for the late catabolism of DNA.

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