Plasma Membrane Depolarization without Repolarization Is an Early Molecular Event in Anti-Fas-induced Apoptosis*

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The movement of intracellular monovalent cations has previously been shown to play a critical role in events leading to the characteristics associated with apoptosis. A loss of intracellular potassium and sodium occurs during apoptotic cell shrinkage establishing an intracellular environment favorable for nuclease activity and caspase activation. We have now investigated the potential movement of monovalent ions in Jurkat cells that occur prior to cell shrinkage following the induction of apoptosis. A rapid increase in intracellular sodium occurs early after apoptotic stimuli suggesting that the normal negative plasma membrane potential may change during cell death. We report here that diverse apoptotic stimuli caused a rapid cellular depolarization of Jurkat T-cells that occurs prior to and after cell shrinkage. In addition to the early increase in intracellular Na⁺, ⁸⁶⁸⁶Rh⁺ studies reveal a rapid inhibition of K⁺ uptake in response to anti-Fas. These effects on Na⁺ and K⁺ ions were accounted for by the inactivation of the Na⁺/K⁺-ATPase protein and its activity. Furthermore, ouabain, a cardiac glycoside inhibitor of the Na⁺/K⁺-ATPase, potentiated anti-Fas-induced apoptosis. Finally, activation of an anti-apoptotic signal, i.e. protein kinase C, prevented both cellular depolarization in response to anti-Fas and all downstream characteristics associated with apoptosis. Thus cellular depolarization is an important early event in anti-Fas-induced apoptosis, and the inability of cells to repolarize via inhibition of the Na⁺/K⁺-ATPase is a likely regulatory component of the death process.

Apoptosis is a fundamental physiological process where activation of specific biochemical and morphological events results in cellular suicide. Although programmed cell death is a normal physiologic process observed during development and cellular homeostasis, insufficient or excessive apoptosis can lead to various pathological conditions, such as Alzheimer’s and Parkinson’s disease, cancer, and AIDS. The loss of cell volume, chromatin condensation, and internucleosomal DNA fragmentation are all defining characteristics of this mode of cell death. Recently, a loss of intracellular monovalent ions has been shown to play a pivotal role in apoptosis (1–11). A major loss of both intracellular potassium and sodium occurs when apoptotic cells shrink and prior to the loss of membrane integrity (6, 7). Maintenance of the normal physiologic intracellular concentration of these monovalent ions was also shown to inhibit the activation of effector caspases (caspase-3-like enzymes) and the apoptotic nuclease activity during cell death, suggesting that the role ions play during apoptosis is more extensive than simply facilitating the loss of cell volume (7).

In most excitable cells, cellular depolarization occurs as a result of a movement of sodium ions, which can occur through a variety of mechanisms including opening of voltage-gated sodium channels (12), suppression of the Na⁺/K⁺-ATPase activity (13, 14), and activation of Na⁺/K⁺-dependent amino acid co-transport systems, which can act like sodium ionophores (15, 16). In contrast to excitable cells, lymphocytes have a relatively stable sodium concentration, and very little detail is known about the movement of sodium ions in these cells, although several studies have suggested that changes in sodium levels in lymphocytes may occur by similar mechanisms as in other cell types (17, 18). Nonetheless, the movement of ions, especially sodium, in lymphocytes would be likely to be reflected in a change in plasma membrane potential (PMP).1

The loss of the mitochondrial membrane potential has been shown to occur in a variety of apoptotic model systems (19–21). Mitochondrial depolarization is proposed to occur through the opening of permeability transition pores, located on the inner mitochondrial membrane, thus disrupting the member potential by permitting the redistribution of ions across the membrane (22–26). Recent evidence has shown that changes in the mitochondrial membrane potential, along with several other characteristics of apoptosis, appear to be restricted to the shrunken population of cells (27). These observations led us to investigate whether an early transit of monovalent ions, prior to the loss of cell volume, might promote the activation of apoptosis and lead to the downstream movement of ions and apoptotic events associated with cell death.

Flow cytometry allows multiple cell death characteristics to be analyzed at the single cell level and thus has been an invaluable tool in the study of apoptosis. We have used this technology to ascertain if an early movement of monovalent ions occurs during apoptosis. By using a fluorescent dye that measures changes in intracellular sodium, we show that an early increase in intracellular sodium occurs prior to the loss of cell volume. We hypothesized that this increase in intracellular sodium would be reflected in changes in the plasma membrane potential. By using flow cytometry and a dye that responds to acute changes in the plasma membrane potential (PMP), we show that cells depolarize very early during apoptosis, prior to

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1 The abbreviations used are: PMP, plasma membrane potential; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DiBAC_((3), bis-(1,3-dibutyl)rhodamine) triethylammonium salt; DiOC_((5), 3,3'-dihexyloxacarbocyanine iodide; SBFI-AM, sodium-binding benzofuran isothiophosphate acetylxylmethylster; PI, propidium iodide; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MMP, mitochondrial membrane potential.
a loss in cell volume, and in response to various apoptotic stimuli. We also observed that changes in PMP correlated with a population of cells with increased intracellular sodium. 

K<sup>+</sup> uptake studies using <sup>86</sup>Rb<sup>+</sup> suggested the rapid inactivation of the Na<sup>+</sup>/K<sup>+</sup>-ATPase, which was confirmed by using a functional enzyme activity assay and Western blot analysis. We also show that inhibition of the Na<sup>+</sup>/K<sup>+</sup>-ATPase by using ouabain enhances cellular depolarization and apoptosis, whereas treatment with an anti-apoptotic PKC activator prevents anti-Fas-induced cellular depolarization and cell death. These studies indicate that an early movement of monovalent ions, particularly sodium, results in plasma membrane depolarization that may orchestrate subsequent movement of ions during apoptosis.

**MATERIALS AND METHODS**

**Cell Culture and Reagents—**Jurkat cells, E6.1 (human lymphoma), were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 4 mM glutamine, 31 mg/liter penicillin, and 50 mg/liter streptomycin at 37 °C, 7% CO<sub>2</sub> atmosphere. Induction of apoptosis in Jurkat cells (5 x 10<sup>5</sup> cells per ml) was accomplished using either 10 or 50 ng/ml anti-human Fas IgM (Kamiya Biomedical), 2 μM A23187 (Calbiochem), or 10 μM thapsigargin (Sigma). The cells were incubated at 37 °C, 7% CO<sub>2</sub> atmosphere for the specified periods. The caspase-8 inhibitor benzoyloxycarbonyl-IETD-fluormethyl ketone was purchased from Calbiochem. Ouabain and the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma.

**Measurement of Acute Changes in Plasma Membrane Potential—**Acute changes in the plasma membrane potential were measured by flow cytometry using DiBAC<sub>4</sub>(3) (Molecular Probes). DiBAC<sub>4</sub>(3) was prepared in Me<sub>2</sub>SO according to the manufacturer's instructions. Graded potassium media were made by altering the KCl and NaCl concentrations in RPMI 1640 media containing glutamine and antibiotics. The normal KCl and NaCl concentrations in RPMI 1640 are 5.4 and 102.7 mM, respectively, totaling 108.1 mM for these salts. For graded potassium media, the KCl concentration was set at 5.4 (normal), 25, 50, 75, or 102.7 mM, whereas the NaCl concentration was adjusted such that the combined monovalent salt concentration equaled 108.1 mM. Heat-inactivated fetal calf serum, dialyzed against several changes of the KCl/NaCl-free RPMI 1640, was added to a final concentration of

**Phorbol 12-myristate 13-acetate (PMA; synthetic analog of diacylglycerol)** was purchased from Calbiochem.
37 °C, 7% CO₂ atmosphere. Immediately prior to flow cytometric examination, propidium iodide (PI, Sigma) was added to a final concentration of 10 μg/ml. Ten thousand cells were analyzed by sequential excitation of the cells containing SBFI-AM and PI at 340–350 and 488 nm, respectively, using a FACSVantage SE flow cytometer (Becton Dickinson). CellQuest software and CellQuest software. 

**Measurement of K⁺-Efflux and Uptake Using §6Rb⁺**—For the §6Rb⁺ efflux experiments, Jurkat cells (5 × 10⁶ cells per ml) loaded overnight with 12.5 μCi of §6Rb⁺ were washed twice in normal RPMI 1640 and then split into 2 samples at a final cell density of 1 × 10⁶ cells per ml. Anti-Fas antibody was added to one sample at a final concentration of 100 ng/ml at 37 °C, 7% CO₂ atmosphere. At 1-h intervals, 3 separate 1-ml aliquots of cells were harvested for each sample, and 800 μl of the supernatant was removed to be counted. The pellet was washed in RPMI 1640 and finally resuspended in RPMI 1640 containing 0.5% Triton X-100. Both the pellet and supernatant were counted in triplicate, and the average §6Rb⁺ in the pellet fraction from two independent experiments is shown ± S.E. For the §6Rb⁺ uptake experiments, 5 μCi of §6Rb⁺ was added to Jurkat cells (5 × 10⁶ cells per ml) in the presence or absence of 100 ng/ml of an anti-Fas antibody. All samples were incubated at 37 °C, 7% CO₂ atmosphere. At 1-h intervals, 3 separate 1-ml aliquots of cells were harvested for each sample. The pellets were washed twice in RPMI 1640 and then resuspended in RPMI 1640 containing 0.5% Triton X-100 and counted in triplicate, and the average §6Rb⁺ in the pellet fraction from two independent experiments is shown ± S.E. 

**DNA Analysis**—The DNA content for each sample was determined as described previously by flow cytometry (28). Briefly, 5 ml of cells were pelleted from the culture medium and fixed by the slow addition of cold 70% ethanol to a volume of ~1.5 ml. The volume of each sample was adjusted to 5 ml with cold 70% ethanol, and the cells were stored at 4 °C overnight. For flow analysis, the fixed cells were pelleted, washed once in 1× phosphate-buffered saline (PBS), and stained in 1 ml of 20 μg/ml PI, 1 mg/ml RNase in 1× PBS for 20 min. Seven thousand five hundred cells were examined by flow cytometry using a Becton Dickinson FACSort by gating on an area versus width dot plot to exclude cell debris and cell aggregates. The percentage of degraded DNA was determined by the number of cells with subdiploid DNA divided by the total number of cells examined under each experimental condition.

**Functional Expression Assay for Na⁺/K⁺-ATPase**—The functional expression of the Na⁺/K⁺-ATPase was assessed by the ouabain-sensitive uptake of Rb⁺. Cells cultured in RPMI 1640 were treated in the presence or absence of 100 ng/ml of an anti-Fas antibody for 3 h. Thirty minutes prior to the assay, 100 μM ouabain was added to the medium in the fraction transport. Cells were pelleted, washed with 1× PBS, and then resuspended in RPMI 1640 without K⁺ but containing 2.5 mM RbCl. After 10 min at 37 °C (time of transport), triplicates of 250-μl cell aliquots were immediately transferred to Eppendorf tubes on ice, centrifuged at 1000 × g for 2 min, and washed 3 times with cold 0.1 M MgCl₂. The pellet was resuspended in 250 μl of 0.1 M trichloroacetic acid. Cell extracts were analyzed for Rb⁺ by an atomic absorption flame photometer in an atomic absorption spectrophotometer AA100 (PerkinElmer Life Sciences). Transport activity is expressed as μmol of Rb⁺ uptake per million cells in 10 min.

**Western Blot Analysis**—Jurkat cells were treated in the presence or absence of 50 ng/ml of an anti-Fas antibody, harvested at the indicated times, and washed once in cold PBS. Protein extracts for each sample were prepared by resuspending the cells in a chilled lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl, and 0.5% Triton X-100) containing a mixture of protease inhibitors (1 μM pepstatin, 1 μM leupeptin, 1 μM aprotinin, 1 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride) and were homogenized with a Dounce homogenizer. After 15 min of centrifugation at 13,000 rpm in a microcentrifuge, the supernatant was collected and assayed for protein concentration by the method of Bradford using the Bio-Rad system. 20–50 μg of protein per sample equally diluted in Laemmli loading buffer and denatured for 5 min were examined by gel electrophoresis at 120 V for 2 h using 12% SDS-polyacrylamide gel electrophoresis gels (NOVEX, San Diego, CA). The gels were then electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) at 42 V for 1.5 h and stained with Ponceau S (Sigma) to verify the equal amount and quality of protein before the addition of blocking buffer. The membranes were blocked overnight at 4 °C in Tris-buffered saline (TBS) containing 0.05% Tween (Sigma) and 5% nonfat dried milk. Monoclonal anti-Na⁺/K⁺-recognizing human α and β isoforms (Affinity Bioreagents, Golden, CO) and monoclonal anti-caspase-3 (Calbiochem) were diluted 1:250 in TBS, 0.65% Tween, 0.5% milk and membranes were blotted with the correspondent antibody for 1 h at room temperature. Blots were washed 3 times with

**Determination of Changes in Plasma Membrane Potential during Apoptosis**—Jurkat cells treated with either 10 ng/ml of an anti-Fas antibody, 2 μM A23187, or 10 mM thapsigargin were incubated at 37 °C, 7% CO₂ atmosphere. Stock solutions of 20 mM DiBAC₄(3) and DiOC₃(3) (Molecular Probes) was prepared in Me₂SO. Thirty minutes prior to each time of examination, either DiBAC₄(3) or DiOC₃(3) was added to 1 ml of cells at a final concentration of 150 nM, and incubation was continued at 37 °C, 7% CO₂ atmosphere. Cells were examined as changes in their plasma membrane potential by flow cytometry using either a Becton Dickinson FACSort or FACSVantage SE as described above. Ten thousand cells were examined under each condition, and all flow cytometric analyses were accomplished using CellQuest software.

**Determination of Intracellular Sodium**—Jurkat cells treated with either 10 ng/ml of an anti-Fas antibody, 2 μM A23187, or 10 mM thapsigargin were incubated at 37 °C, 7% CO₂ atmosphere. One hour prior to each time of examination, SBFI-AM (Na⁺) was added to 1 ml of cells at a final concentration of 5 μM, and incubation was continued at 37 °C, 7% CO₂ atmosphere. Immediately prior to flow cytometric examination, propidium iodide (PI, Sigma) was added to a final concentration of 10 μg/ml. Ten thousand cells were analyzed by sequential excitation of the cells containing SBFI-AM and PI at 340–350 and 488 nm, respectively, using a FACSVantage SE flow cytometer (Becton Dickinson). CellQuest software and CellQuest software.
**RESULTS**

An Increase in Intracellular Sodium Occurs Early during Apoptosis—The maintenance of a homeostatic balance of intracellular and extracellular ions is crucial for cell survival. Alterations in this ionic balance can signal a cell to divide, differentiate, or even to undergo cell death. We have previously shown that a dramatic loss of intracellular ions, particularly sodium and potassium, is associated with the shrinkage of cells during apoptosis, thus altering the intracellular environment and permitting nuclease activity and effector caspase activation (6, 7). In this study, we were interested in determining if a change in monovalent cations could be detected in response to apoptotic stimulation, prior to cell shrinkage. Thus, we analyzed Jurkat cells treated with anti-Fas for changes in intracellular sodium using the sodium-binding fluorescent indicator SBFI-AM (Na\(^+\)) (6). Flow cytometric analysis of Jurkat cells treated with an anti-Fas antibody showed a time-dependent increase in the number of cells that had an increase in DiBAC\(_4(3)\) fluorescence, indicating plasma membrane depolarization, under each apoptotic condition. The histograms show a representative change in DiBAC\(_4(3)\) fluorescence for each experimental condition, and the percentage of cells with an increase in DiBAC\(_4(3)\) fluorescence reflects the average ± S.E. of 3 independent experiments.

Plasma Membrane Depolarization Occurs with a Variety of Apoptotic Stimuli—We initially used the plasma membrane-specific dye, DiBAC\(_4(3)\), to examine apoptotic cells for changes in their PMP at the single cell level by flow cytometry. DiBAC\(_4(3)\) is an anionic oxonal dye that responds with an increase in fluorescent intensity at 530 nm upon membrane depolarization. We determined the utility of this membrane potential dye in our model system, Jurkat T-cells, by analyzing cells for acute changes in their PMP. Jurkat cell were depolarized with increasing concentrations of extracellular KCl. In the presence of DiBAC\(_4(3)\), these KCl-treated Jurkat cells responded with a stepwise increase in DiBAC\(_4(3)\) fluorescence, indicating cellular depolarization (Fig. 2). To determine the specificity of this dye to measure changes specific to the PMP, we examined the response of DiBAC\(_4(3)\) to acute changes in the mitochondrial membrane potential (MMP) by using various concentrations of the protonophore CCCP to collapse the membrane potential of these organelles. We have previously shown that the concentrations of CCCP used in this study were effective in uncoupling the MMP when either JC-1, a mitochondrial membrane specific dye, or DiOC\(_6(3)\), a dye which responds to both changes in the mitochondrial and plasma membrane potential, were used to access changes in the MMP (27). In contrast to the results shown for acute plasma membrane depolarization, DiBAC\(_4(3)\) did not respond to changes in the mitochondrial membrane potential indicating a distinct ability of DiBAC\(_4(3)\) to measure strictly changes in the PMP (Fig. 2).

To determine whether changes in the PMP occur during apoptosis, Jurkat cells were treated with an anti-Fas antibody, the calcium ionophore A23187, or thapsigargin, all known
Plasma Membrane Depolarization during Apoptosis

apoptotic agents that differ in their mode of cell death activation (27). Under each apoptotic condition, a population of cells with an increase in DiBAC$_4$(3) fluorescence, indicating plasma membrane depolarization, was observed in a time-dependent manner (Fig. 3). Interestingly, the observed cellular depolarization was not a transient event, as might occur in electrically excitable cells, but rather was sustained, as the population of cells with increased DiBAC$_4$(3) fluorescence increased over time. This sustained cellular depolarization suggests that upon apoptotic stimulation, the ability of cells to repolarize is lost, thus maintaining a constant state of depolarization throughout the cell death process. In addition to the time-dependent nature of this cellular depolarization observed during cell death, we determined that this event was also sensitive to the concentration of apoptotic stimulus employed. Increasing concentrations of anti-Fas antibody added to Jurkat cells 3 h prior to flow cytometric examination in the presence of DiBAC$_4$(3) resulted in a concentration-dependent increase in the number of cells with increased DiBAC$_4$(3) fluorescence (Fig. 4A), thus indicating that cellular depolarization is intrinsically linked to the degree of apoptotic stimulation. The onset of plasma membrane depolarization is rapid, occurring between 1 and 2 h after stimulation with anti-Fas (Fig. 4B).

Plasma Membrane Depolarization Is Not Restricted to the Shrunken Population of Apoptotic Cells—Many characteristics of apoptosis such as changes in the mitochondrial membrane potential, the loss of intracellular ions, effector caspase activation, and DNA degradation have been shown to be restricted to the shrunken population of cells (6, 7, 27, 28). Thus, we determined if cellular depolarization was also restricted to the shrunken apoptotic cells or if it occurred prior to the loss of cell volume. Flow cytometry, which permits the simultaneous examination of multiple cellular characteristics at the single cell level, was used to determine the relationship between cellular depolarization and cell size by examining DiBAC$_4$(3) fluorescence and the forward light scattering property of the cell, respectively. When control Jurkat cells were examined on a forward-scatter versus side-scatter dot plot in the presence of DiBAC$_4$(3), a single major population of cells was observed (Fig. 5). Gating on this single population of cells, we examined these cells on a forward-scatter versus a DiBAC$_4$(3) fluorescence contour plot. Analysis of these control Jurkat cells showed only a single level of DiBAC$_4$(3) fluorescence. In contrast, gating on the minor population of cells with a decrease in forward-scattered light in the control sample, denoting cells with a decreased cell size, showed that the shrunken cells had an increase in DiBAC$_4$(3) fluorescence or a depolarized plasma membrane (Fig. 5). Treatment of Jurkat cells with an anti-Fas antibody resulted in an increase in the number of shrunken cells (Fig. 5). Similar to the control sample, which contained some spontaneously dying apoptotic cells, the shrunken, anti-Fas-induced apoptotic cells showed an increase in DiBAC$_4$(3) fluorescence, again suggesting that the cells fail to repolarize during the cell death process. However, gating on the normal or nonshrunken anti-Fas-treated cells resulted in 2 distinct similar sized cell populations on a forward-scatter versus DiBAC$_4$(3) fluorescence contour plot, one having a control level of DiBAC$_4$(3) fluorescence and a second having an increase in DiBAC$_4$(3) fluorescence (Fig. 5). These data suggest that cellular depolarization occurs in the normal population of cells, prior to the loss of cell volume, and is sustained after cell shrinkage.

Plasma Membrane Depolarization and Increased Intracellular Sodium Occur in the Same Population of Apoptotic Cells—In view of cellular depolarization and increased intracellular sodium being early occurrences during the apoptotic process, we subsequently wanted to determine whether these two events were related. Initial experiments using DiBAC$_4$(3) and SBFI-AM (Na$^+$) showed that these two fluorescent probes are incompatible when used simultaneously in flow cytometric experiments. Therefore, we examined the response of a different membrane potential dye, DiOC$_6$(3), in relation to changes in the PMP. DiOC$_6$(3) is a positively charged dye that has been shown to respond to changes in both the plasma and mitochondrial membrane potential by a loss of fluorescent emission upon membrane depolarization (27). We have previously shown that changes in the MMP resulting from the treatment of Jurkat cells with apoptotic agents used in this study are completely restricted to the shrunken population of cells (27). Therefore, by using flow cytometry to separate the normal and shrunken populations of cells, we can use this dye to examine only changes in the PMP by gating solely on the normal or nonshrunken cells. Preliminary experiments using DiOC$_6$(3) in Jurkat cells treated with an anti-Fas antibody, A23187, or thapsigargin showed a decrease of this positively charged dye...
in a time-dependent manner, indicating plasma membrane depolarization with this different dye (data not shown). Examination of only the normal or nonshrunk Jurkat cells using a variety of apoptotic stimuli resulted in an increase in SBFI-AM (Na⁺) fluorescence indicating a rise in intracellular sodium prior to the loss of cell volume (Fig. 6A). Additionally, examination of these normal or nonshrunk apoptotically treated cells in the presence of DiOC₆(3) showed a decrease in DiOC₆(3) fluorescence suggesting cellular depolarization prior to cell shrinkage (Fig. 6A). Simultaneous comparison of DiOC₆(3) fluorescence with SBFI-AM (Na⁺) fluorescence showed that cells that were depolarized had a rise in intracellular sodium (Fig. 6B), suggesting that apoptotic depolarization and increased intracellular sodium are linked. It is important to note that DiOC₆(3) and SBFI-AM are excited at 2 spatially separate wavelengths (488 and 350–360 nm, respectively), thus no spectral overlap exists when using these dyes in combination.

**Na⁺/K⁺-ATPase Inactivation Occurs during Anti-Fas-induced Apoptosis**—Mammalian cells normally have an extreme concentration gradient of Na⁺ and K⁺ across their plasma membrane which contributes significantly to the distinctive cellular negative resting membrane potential. Flow cytometric analysis of the normal or nonshrunk population of cells treated with various apoptotic agents failed to reveal any significant change in the concentration of intracellular potassium prior to the loss of cell volume (data not shown); however, previously we showed that a dramatic decrease in intracellular potassium occurs coincident with cell shrinkage during apoptosis (6, 7). We chose to concentrate on anti-Fas-induced apoptosis due to its importance in the normal physiological turnover of lymphoid cells and the well-defined receptor-mediated signal transduction pathway. Therefore, to determine whether changes in intracellular potassium occur early in the cell death process, we used ⁸⁶Rb⁺ as a tracer for K⁺ (30) in Jurkat cells treated with an anti-Fas antibody. In experiments examining ⁸⁶Rb⁺ uptake, anti-Fas-treated Jurkat cells showed a striking decrease in ⁸⁶Rb⁺ uptake, which could be detected as early as 2 h after apoptotic stimulation (Fig. 7). This finding suggests that a major disruption in the normal ionic balance across the plasma membrane occurs early in the cell death process. Since maintenance of a sodium/potassium gradient across the plasma membrane is primordial for cellular homeostasis and is closely regulated by the ubiquitous plasma membrane Na⁺/K⁺-ATPase, we examined the role of the Na⁺/K⁺-ATPase during anti-Fas-induced apoptosis.

To address this issue we examined anti-Fas-treated Jurkat cells in the presence or absence of the cardiac glycoside ouabain, a known inhibitor of the Na⁺/K⁺-ATPase, for changes in the PMP, cell size, and DNA content by flow cytometry. Treatment of Jurkat cells with ouabain resulted in a dramatic loss of PMP in the entire population of cells, suggesting that inhibition of the Na⁺/K⁺-ATPase induces cellular depolarization (Fig. 8). Interestingly, this complete cellular depolarization alone increased apoptosis, although modestly. However, the presence of ouabain in Jurkat cells treated with 10 ng/ml anti-Fas also resulted in a similar loss in PMP in the entire population of cells, but dramatically potentiated the extent of DNA degradation compared with either ouabain or anti-Fas alone, which was also reflected in the overall changes in cell size (Fig. 8). The occurrence of complete cellular depolarization in the presence of ouabain alone without a major enhancement of degraded DNA suggests that increased PMP alone is not by itself a sufficient activator of apoptosis but rather is necessary for cell death. To substantiate these inhibitor studies, we examined the functional expression of the Na⁺/K⁺-ATPase during apoptosis using a well-established ouabain-sensitive uptake of Rb⁺ assay (31–33). Total Rb⁺ uptake along with the Rb⁺ fraction that was ouabain-insensitive were measured in both control and anti-Fas-treated Jurkat cells (Fig. 9). The difference between these two fractions for each individual sample indicates the net ouabain-sensitive Na⁺/K⁺-ATPase activity. As shown in Fig. 9, anti-Fas treatment of Jurkat cells resulted in an overall net decrease in ouabain-sensitive Na⁺/K⁺-ATPase activity compared with control cells. Western blot analysis of both the α and β subunits of the Na⁺/K⁺-ATPase during anti-Fas-induced apoptosis revealed a rapid time-dependent decrease in both the α and β subunit steady state levels of the proteins (Fig. 10A). Interestingly, we also detected the specific, apparently proteolytic, cleavage of the β subunit (Fig. 10A). Congruent with our results on the uptake of ⁸⁶Rb⁺ during anti-Fas-induced cell death (Fig. 7), the decrease in α subunit protein and the cleavage of β subunit of the Na⁺/K⁺-ATPase.
could be detected as early as 1 h after apoptotic treatment, suggesting that the Na\(^+\)/K\(^+\)-ATPase is a specific target for inactivation during anti-Fas-induced apoptosis.

To determine whether a change in the Na\(^+\)/K\(^+\)-ATPase subunit expression was associated with the depolarized population of cells, we simultaneously sorted depolarized and nondepolarized anti-Fas-treated Jurkat cells by flow cytometry. Western blot analysis of the \(\alpha\) subunit of the Na\(^+\)/K\(^+\)-ATPase showed a decrease in the steady state level of this protein in the depolarized cells, compared with the nondepolarized cells (Fig. 10B), suggesting that the loss of Na\(^+\)/K\(^+\)-ATPase expression, and thus activity, is related to the depolarized apoptotic cells. For comparison we analyzed the activation of caspase-8 over the same time and under the same conditions as shown for the expression of the Na\(^+\)/K\(^+\)-ATPase. These data suggest that in anti-Fas-treated Jurkat cells cleavage of the \(\beta\) subunit and decreased levels of the \(\alpha\) subunit of the Na\(^+\)/K\(^+\)-ATPase occur prior to detectable levels of active caspase 8 (Fig. 10C). In Fas-induced cell death, both plasma membrane depolarization and increased intracellular sodium are prevented in the presence of either the pan-caspase inhibitor benzylxocarbonyl-Val-Ala-Asp (OMe) or the caspase-8 inhibitor IETD (data not shown). However, UV-induced caspase-8-deficient cells readily undergo a sustained plasma membrane depolarization, suggesting that more than one pathway likely exists to drive the degradation of the Na\(^+\)/K\(^+\)-ATPase. Together, these data suggest that the Na\(^+\)/K\(^+\)-ATPase plays a vital role in the early movement of monovalent ions during anti-Fas-induced apoptosis by contributing to an early change in PMP.

FIG. 6. An early rise in intracellular sodium occurs prior to the loss of cell volume and correlates with the depolarized population of apoptotic cells. A, the response of SBFI-AM (Na\(^+\)) to measure changes in intracellular sodium prior to the loss of cell volume under various apoptotic conditions. Jurkat cells were treated with various apoptotic stimuli and examined for changes in intracellular sodium as described in Figs. 1 and 3. Cells were initially examined on a forward-scatter versus a side-scatter dot plot. An analysis gate was drawn around the normal or nonshrunken control cells. Treatment of Jurkat cells with various apoptotic agents after 6 h were then examined in the presence of the sodium indicator dye on an SBFI-AM (Na\(^+\)) versus PI dot plot. Cells that had a normal and an increase in intracellular sodium were independently gated and subsequently examined on a SBFI-AM (Na\(^+\)) versus forward-scatter dot plot. Results of this analysis showed a population of cells with increased intracellular sodium (green) occurred prior to the loss of cell volume. DiOC\(_6\)(3) was used to examine changes in the PMP in the normal or nonshrunken cells under various apoptotic conditions. Jurkat cells treated with various apoptotic agents were examined by flow cytometry for changes in their plasma membrane potential after 6 h using the positively charged membrane potential dye, DiOC\(_6\)(3). Thirty minutes prior to flow cytometric examination, DiOC\(_6\)(3) was added to a final concentration of 150 ng/ml, and incubation was continued at 37 °C, 7% CO\(_2\) atmosphere. Gating on only the normal or nonshrunken cells resulted in a population of cells (green) with a decrease in DiOC\(_6\)(3) fluorescence, indicating plasma membrane depolarization prior to the loss of cell volume. B, relationship between increased intracellular sodium and cellular depolarization. Jurkat cells were treated with a variety of apoptotic agents for 6 h. Prior to flow cytometric examination, SBFI-AM (Na\(^+\)) and DiOC\(_6\)(3) were added as described above. Analysis of the normal or nonshrunken cells on a DiOC\(_6\)(3) fluorescence versus a SBFI-AM (Na\(^+\)) fluorescence dot plot showed that only the depolarized population of cells had an increase in intracellular sodium (green). The dot plots are representative of at least 2 independent experiments.

\(^2\) C. Vu, C. D. Bortner, and J. A. Cidlowski, unpublished observations.
We have recently shown that PKC stimulation is anti-apoptotic in Jurkat cells and affords a protective effect during anti-Fas-induced cell death at a site upstream of caspase-8 (34). Anti-Fas-induced cell shrinkage and loss of intracellular potassium were both blocked in the presence of the phorbol ester PMA, an activator of PKC. Additionally, all downstream characteristics of apoptosis were also inhibited, indicating a profound effect of PKC stimulation on preventing cell death (34). We now examined if PKC stimulation also prevented changes in the PMP as observed during anti-Fas-induced apoptosis. Jurkat cells treated with 50 ng/ml anti-Fas alone showed cellular depolarization, the loss of cell volume, and the expected increase in degraded DNA (Fig. 11). PMA alone resulted in a slight hyperpolarization of the PMP; however, the presence of this agent had no effect on cell size or the extent of degraded DNA (Fig. 11). When anti-Fas-treated Jurkat cells were examined in the presence of PMA for changes in their PMP, cell size, and DNA content, a striking inhibition of the apoptotic process was observed (Fig. 11). This inhibition included both early apoptotic events such as cellular depolarization, along with late apoptotic characteristics such as DNA degradation and cell viability (data not shown), suggesting that prevention of cellular depolarization can control the apoptotic process and perhaps promote tumor growth by its ability to inhibit apoptosis.

**DISCUSSION**

In this study, we show that an early increase in intracellular sodium induced by a variety of apoptotic stimuli is associated with a marked loss of potassium. This loss of potassium is associated with cellular depolarization and the loss of cell volume. The loss of potassium is associated with the activation of caspase-8 and the induction of apoptosis. The loss of potassium is associated with the activation of caspase-8 and the induction of apoptosis. The loss of potassium is associated with the activation of caspase-8 and the induction of apoptosis. The loss of potassium is associated with the activation of caspase-8 and the induction of apoptosis. The loss of potassium is associated with the activation of caspase-8 and the induction of apoptosis.

**Fig. 7. Anti-Fas stimulation regulates K⁺ uptake.** ⁸⁶Rb⁺ uptake experiments were accomplished by adding 5 μCi of ⁸⁶Rb⁺ to Jurkat cells (5 x 10⁶ cells per ml) in the presence or absence of 100 ng/ml of an anti-Fas antibody. All samples were incubated at 37 °C, 7% CO₂ atmosphere. At 1-h intervals, 3 separate 1-ml aliquots of cells were harvested for each sample. The pellets were washed twice in RPMI 1640 and then resuspended in RPMI 1640 containing 0.5% Triton X-100 and counted in triplicate, and the average ⁸⁶Rb⁺ in the pellet fraction from two independent experiments is shown ± S.E. In the presence of anti-Fas stimulation, a dramatic decrease in ⁸⁶Rb⁺ uptake was observed.

**Fig. 8. Ouabain potentiates anti-Fas-induced apoptosis in Jurkat cells.** Control and anti-Fas (10 ng/ml)-treated Jurkat cells were examined in the presence and absence of 100 μM ouabain after 6 h. Thirty minutes prior to flow cytometric analysis, DiBAC₄(3) was added to 1 ml of each sample, and incubation at 37 °C, 7% CO₂ atmosphere was continued. Analysis of degraded DNA was accomplished as described previously (28). Representative histograms and three-dimensional plots are shown under each experimental condition. The percentage of cellular depolarization and degraded DNA are the average ± S.E. of 3 independent experiments. The presence of ouabain in the anti-Fas treated Jurkat cells enhanced both early and late apoptotic characteristics.
with plasma membrane depolarization and occurs prior the loss of cell volume. Although changes in the PMP have been previously suggested during cell death (35–37), here we report the first demonstration that cellular depolarization directly correlates with an early increase in intracellular sodium upon apoptotic stimulation. Additionally, we show that cellular depolarization is not a transient event during apoptosis but is sustained throughout the early activation phase of the cell death process, asserting that cells that become depolarized are prevented from repolarizing back to a normal PMP. We have also observed plasma membrane depolarization and increased intracellular sodium in a variety of other lymphoid cells includ-

![Graph](image)

FIG. 9. The functional expression of the Na\(^+\)/K\(^+-\)ATPase in the presence or absence of anti-Fas antibody. The functional expression of the Na\(^+\)/K\(^+-\)ATPase was assessed by the ouabain-sensitive uptake of Rb\(^+\). Cells cultured in RPMI 1640 were treated in the presence or absence of 100 ng/ml of an anti-Fas antibody. Thirty minutes prior to the assay, 100 μM ouabain was added in the medium in the fraction of cells used for ouabain-insensitive transport. Cells were pelleted, washed with 1× PBS, and then resuspended in RPMI 1640 without K\(^+\) but containing 2.5 mM RbCl. After 10 min at 37 °C (time of transport), triplicates of 250-μl cell aliquots were immediately transferred to Eppendorf tubes on ice, centrifuged at 1000 × g for 2 min, and washed 3 times with cold 0.1 mM MgCl\(_2\). The pellet was resuspended in 250 μl of 0.1 M trichloroacetic acid. Cell extracts were analyzed for Rb\(^+\) by emission flame photometry in an atomic absorption spectrophotometer AA100 (PerkinElmer Life Sciences). Transport activity is expressed as micromoles of Rb\(^+\) uptake per million cells in 10 min. Data are the average of 3 independent experiments ± S.E.

FIG. 10. Western blot analysis shows a decrease in the α subunit and cleavage of the β subunit of the Na\(^+\)/K\(^+-\)ATPase during anti-Fas-induced apoptosis. A, Jurkat cells were treated in the presence or absence of 50 ng/ml of an anti-Fas antibody, harvested at the indicated times, and washed once in cold PBS. Protein extracts for each sample were prepared and examined on a 12% SDS-polyacrylamide gel electrophoresis gel. The gels were transferred to nitrocellulose membranes and examined for the expression of either the α or β subunit of the Na\(^+\)/K\(^+-\)ATPase. Ponceau S staining was employed to verify equal amounts and quality of protein between lanes prior the Western blotting. An early decrease in the α subunit of the Na\(^+\)/K\(^+-\)ATPase was observed along with the specific cleavage of the β subunit of the Na\(^+\)/K\(^+-\)ATPase occurred in the depolarized population of cells. B, kinetic analysis of caspase-8 activation during anti-Fas-induced apoptosis. Jurkat cells were treated with anti-Fas and prepared for Western blot analysis as described above. Activation of caspase-8 occurs by cleavage of the pro-form of enzyme (56–58 Kd) and was detected by the appearance of the cleaved active fragments (43–41 kDa). Cleaved caspase-8 products were detected within 2 h of anti-Fas treatment. Each Western analysis is representative of 2 independent experiments.
ing primary rat thymocytes and S49 Neo cells induced to undergo apoptosis with dexamethasone, A23187, thapsigargin, or UV irradiation. Additionally, we observed plasma membrane depolarization in anti-Fas-treated HeLa cells undergoing apoptosis. These findings suggest that cellular depolarization is a general component of the apoptotic process in both lymphoid and nonlymphoid cells.

We also show that a primary target associated with this early change in intracellular ions is the Na\(^+\)/K\(^+\)-ATPase, whose inhibition contributes to sustained apoptotic depolarization and thus failure of the cells to repolarize. We have made similar observations in dexamethasone-treated primary rat thymocytes. Furthermore, activation of PKC, which has previously been shown to inhibit anti-Fas-induced apoptosis, also prevented cellular depolarization, indicating a critical role of the PMP in regulating cell death.

The contribution of ion movement, especially potassium, to the mitogenic activation of lymphoid cells has been previously documented (38, 39). Potassium channel inhibitors such as 4-aminopyridine and tetraethylammonium at a one-to-one drug molecule to channel stoichiometry prevent mitogenesis induced by phytohemagglutinin. In addition, phytohemagglutinin-induced protein synthesis and interleukin-2 production are also inhibited by potassium channel blockers, emphasizing the physiological importance of ion gradients in regulating mitogenic activation. Our current study showing an early increase in intracellular sodium, cellular depolarization, along with inhibition of the Na\(^+\)/K\(^+\)-ATPase during apoptosis supports the notion of the importance of maintaining a normal, homeostatic balance of ions. However, the mechanism underlying PMA blockade of anti-Fas-mediated depolarization and cell death remains unclear. Recent studies from our laboratory suggest that PMA may effect both potassium efflux and uptake and that inhibition of PKC activity enhances the loss of intracellular potassium and apoptosis (34).

Several studies suggest a potential role for an early movement of monovalent ions in apoptosis. Several members of the anti- and pro-apoptotic Bcl-2 protein family are capable of modulating the cell membrane potential by hyperpolarizing the cells thus increasing their resistance to apoptosis, and these proteins have the potential to function as selective ion channels (40–43). Additionally, voltage-dependent n-type K\(^+\) channels, which are proposed to function to return the plasma membrane potential to control levels after cellular depolarization, are inhibited by ligation of the Fas receptor in Jurkat cells, ceramide (a lipid metabolite synthesized upon Fas receptor ligation), and by the generation of reactive oxygen species (44–47). Recently, the Drosophila genes reaper, grim, and hid, which induce apoptosis and contain N-terminal sequences similar to the
N-terminal inactivation domains of voltage-gated potassium channels, are capable of fast inactivation of Shaker-type potassium channels (48). These studies along with our present data suggest that an early movement of monovalent ions plays a strategic role in the activation of apoptosis.

Our current data also provides direct evidence that plasma membrane depolarization plays a critical role during apoptosis and is mediated by an early increase in intracellular sodium along with inactivation of the Na\(^+\)/K\(^+\)-ATPase. However, the rise in intracellular sodium and the inhibition of the Na\(^+\)/K\(^+\)-ATPase may contribute to different aspects of this early change in monovalent ions in relation to plasma membrane depolarization. Interestingly, upon apoptotic stimulation, cellular de-polarization may contribute to different aspects of this early change in monovalent ions in relation to plasma membrane depolarization. Interestingly, upon apoptotic stimulation, cellular de-polarization is not transient but is maintained through the initial stages of apoptosis. Since the addition of ouabain, which inhibits the Na\(^+\)/K\(^+\)-ATPase, does not by itself dramatically induce cell death in Jurkat cells, the early loss of this ion transport mechanism may play a more important role in sustaining cellular depolarization during apoptosis. Maintaining a sustained state of depolarization for a period after apoptotic stimulation could also occur through the inhibition of the voltage-activated potassium channels (46). Therefore, cellular depolarization may serve as a check point in the activation of apoptosis. Additionally, the rise in intracellular sodium which occurs in the presence of ouabain is not sufficient to fully activate the apoptotic process. Thus, inhibition of the Na\(^+\)/K\(^+\)-ATPase does not trigger apoptosis but enhances the early activation of the cell death signal (Fig. 8). This implies that the early rise in intracellular sodium must occur through a separate or independent sodium transport mechanism, which is intimately linked to the activation of the cell death process. Therefore, our demonstration that the Na\(^+\)/K\(^+\)-ATPase is inhibited during anti-Fas-induced apoptosis in Jurkat cells combined with the an early rise in intracellular sodium suggests a previously unexplored level of control during programmed cell death.

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