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The subcellular compartmentalization of ions is perturbed during the process of apoptosis. In this work, we investigated the impact of K⁺ on the apoptotic process in thymocytes and T cell hybridoma cells. Irrespective of the death-inducing stimulus (glucocorticoids, topoisomerase inhibition, or Fas-crosslinking), a significant K⁺ outflow was observed during apoptosis, as determined on the single-cell level by means of the K⁺-sensitive fluochrome, benzofuran isothiopate. This loss of cytosolic K⁺ only occurs in cells that have completely disrupted their inner mitochondrial transmembrane potential. Inhibition of this mitochondrial transmembrane potential loss by Bcl-2 or by specific inhibitors acting on the mitochondrial permeability transition pore (bongkrekic acid, cyclosporin A) prevents K⁺ leakage. K⁺ drops at the same stage at which cells expose phosphatidylserine residues on the outer leaflet of the membrane and reduce the levels of nonoxidized glutathione, but before they hyperproduce reactive oxygen species, undergo massive Ca²⁺ influx, shrink, and lyse. In a cell-free system of apoptosis, isolated nuclei exposed to the supernatant of mitochondria that have undergone permeability transition only manifest chromatinolysis when the K⁺ concentration is lowered from physiologic to apoptotic levels. Accordingly, massive DNA fragmentation causing subdiploidy is confined to cells that have undergone K⁺ leakage. Together, these data point to the step-wise acquisition of membrane dysfunction in apoptosis and indicate an important role for the disruption of normal K⁺ homeostasis in apoptotic degradation. Derepression of endonucleases due to low K⁺ concentrations may be a decisive prerequisite for end-stage DNA fragmentation. The Journal of Immunology, 1998, 160: 5605–5615.

The apoptotic process can be subdivided into at least three different phases: the initiation phase, which is “private” in the sense that it depends upon the apoptosis-inducing stimulus; the common effector stage, during which the “decision to die” is made; and the degradation phase, during which the cell proceeds beyond the point-of-no-return and acquires the morphologic and biochemical hallmarks of apoptosis (1, 2). Among these phases, the effector stage has received much attention because it is logic and biochemical hallmarks of apoptosis (1, 2). Among these phases, the effector stage has received much attention because it is logic and biochemical hallmarks of apoptosis (1, 2). Among these phases, the effector stage has received much attention because it is logic and biochemical hallmarks of apoptosis (1, 2). Among these phases, the effector stage has received much attention because it is logic and biochemical hallmarks of apoptosis (1, 2).

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3 Abbreviations used in this paper: AIF, apoptosis-inducing factor; FSC, forward scatter; SSC, side scatter; BA, bongkrekic acid; DEX, dexamethasone; PS, phosphatidylserine; DCOC(3), 3,3’-dioctylacryloxyxymine iodide; ΔΨᵢₚᵢᵢ, AM, ace-toxymethyl ester; mitochondrial transmembrane potential; CsA, cyclosporin A; Eth, ethidium; GSH, glutathione; HE, hydroethidine; PI, propidium iodide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; PBFI, potassium-binding; benzofu ran isothiopate; PT, permeability transition; ROS, reactive oxygen species; Z-VAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp-7-amino-4-methyltrifluoromethyl coumarin; Z-VAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.
membrane permeability is lost. Commitment to cell death has already occurred at stage 1. DNA fragmentation is initiated during stage 2 (20–26).

We and others have investigated the subcellular distribution of Ca$^{2+}$ and protons during the apoptotic process (15, 20–26, 28–31). Moreover, it has recently been shown that K$^{+}$ extrudes from the cell during the apoptotic process (32, 33). Whereas Ca$^{2+}$ is much more abundant in extracellular fluid than in the cytosol, K$^{+}$ concentrates in the cytosol (physiologic concentration in mammalian cells is 100–140 mM) as compared with the extracellular medium (4–5 mM). The asymmetric distribution of K$^{+}$ is vital for the maintenance of plasma membrane integrity and function. Manipulations of cytosolic K$^{+}$ concentrations causing a decrease in K$^{+}$, such as extracellular K$^{+}$ depletion or the addition of K$^{+}$–specific ionophores, can cause apoptosis (34, 35). In contrast, an extra supply of external K$^{+}$ can prevent apoptosis, at least in neuronal cell lines (36–38).

Based on these premises, we decided to determine the role of K$^{+}$ in several models of apoptosis. As shown in this work, a loss of cytosolic K$^{+}$ occurs during programmed cell death and is located downstream of mitochondrial PT and caspase activation. Although this K$^{+}$ loss becomes detectable relatively late during apoptosis, it appears crucial for the activation of endonucleases as suggested by data obtained in intact cells and cell-free systems. Collectively, our data establish that the disruption of K$^{+}$ homeostasis constitutes an important step in the apoptotic degradation phase after commitment to death has occurred.

Materials and Methods

**Cells and culture conditions**

Thymocytes from female 4- to 8-wk-old BALB/c mice were cultured at 37°C with 5% CO$_2$ in RPMI 1640 supplemented with 10% FCS, L-glutamine, antibiotics, and β-mercaptoethanol (50 μM, Sigma, St. Louis, MO). Murine 2B4.11 T cell hybridoma cell lines that had been stably transfected with the SFFV.neo vector containing the human bcl-2 gene or the neomycin resistance gene only were a gift from J. Ashwell (National Institutes of Health, Bethesda, MD).

**Induction and inhibition of apoptosis**

The following cell death inducers were employed: the glucocorticoid receptor agonist dexamethasone (DEX) (at a final concentration 1 μM; Sigma), etoposide (10 μM), or an Ab specific for CD95/Fas (clone 154000D, 500 ng/ml; PharMingen, San Diego, CA). The following apoptosis inhibitors were tested: the antioxidant N-acetylcysteine (50 mM), the protein synthesis inhibitor cycloheximide (35 μM; Sigma), the IL-1β-converting enzyme inhibitors zVAD-fmk and L-NAME, and the caspase-3 inhibitor Nec-1s. The following apoptosis inhibitors were tested: the antioxidant N-acetylcysteine (50 mM), the protein synthesis inhibitor cycloheximide (35 μM; Sigma), the IL-1β-converting enzyme inhibitors zVAD-fmk and L-NAME, and the caspase-3 inhibitor Nec-1s.
enzyme-like caspase inhibitor acetyl-Tyr-Val-Ala-Asp-chloromethylketone (100 μM), the broad spectrum caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk) (at a final concentration of 50 μM) and bongkrekic acid (BA) (at 37°C), cells were kept on ice (60-min maximum) until analysis. For the experiments, cells were labeled in the presence of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) (at a final concentration of 100 μM; stock 500 μM in dimethylformamide) (40 – 42). The resulting PBFI fluorescence was elicited at 360 nM and measured at 485 nM.

Cytofluorometric determination of mitochondrial parameters, ROS, GSH levels, and cytosolic Ca²⁺ or K⁺

For the determination of the ΔΨm, 3,3’-dihexyloxacarbocyanine iodide (DiOC₃(3)) (at a final concentration of 40 nM; stock 40 μM in ethanol; excitation wave length of 488 nm, emission 525 nm; Sigma) was used (39). The generation of ROS was monitored with hydroethidine (HE) (at a final concentration of 5 μM; stock 10 mM in DMSO; excitation wave length of 488 nm, emission 620 nM; Molecular Probes); the content in nonoxidized GSH was determined using monochlorobimane (at a final concentration of 50 μM; stock 100 mM in ethanol; excitation wave length of 488 nm; emission 620 nM). Ca²⁺ levels were measured using Fluo-3 acetoxymethyl ester (AM) (at a final concentration of 250 nM; stock 1 mM in DMSO; excitation wave length of 488 nm, emission 525 nM; Sigma) in calcium-free HBSS (Eurobio, Paris, France). After incubation in the presence of the indicated fluorochrome (15–30 min, 37°C), cells were kept on ice (60-min maximum) until analysis. For the determination of intracellular K⁺ levels, cells were loaded during the 15 to 30 min with cell-permeant potassium-binding benzofuran isothiophosphate (PBFI)-AM (at a final concentration of 2.5 μM; stock 500 μM in dimethylformamide) (40 – 42). The resulting PBFI fluorescence was elicited at 360 nM and measured at 485 ± 20 nm.

Cytofluorometric analyses

The frequency of hypoploid cells was determined by ethanol fixation followed by staining with propidium iodine (PI) as previously described (43) using an EPICS Profile II Analyzer (Coulter, Hialeah, FL). All other stainings were analyzed using a FacsVantage cytofluorometer (Becton Dickinson). Data were analyzed in duplicate, and results were recorded for 10,000 cells while gating was performed either on cells exhibiting normal forward scatter (FSC) and side scatter (SSC) characteristics or on the whole cell population (normal cells plus shrunken cells) as indicated. In control experiments, cells were labeled in the presence of the proponent carbonyl cyanide m-chlorophenylhydrazone (CCCP) (at a final concentration of 100 μM; intermediate stock 10 mM in ethanol), the redox-cycling agent menadione (which causes the generation of superoxide anions; 1 mM; stock 1 M in water), the calcium ionophore A23187 (which allows for the influx of extracellular Ca²⁺; 500 nM; stock 500 μM in DMSO), or a combination of valinomycin (0.5 μM/gingerisin (2.5 μM) (which allows for the free distribution of K⁺) plus different concentrations of KCl/NaCl (with a final osmolarity of 300 mM) and 5 mM HEPES buffer (pH 7.4). Cell sorting was performed using an Elite II cytofluorometer (Coulter, Miami, FL).
37°C, 30 min; Enzyme Systems, Livermore, CA) cleaving activity of mitochondrial supernatants was assessed. Z-VAD.fmk, an inhibitor of AIF (45, 46), was used for control purposes at a final concentration of 100 μM.

Results and Discussion
Cells undergoing apoptosis exhibit a reduction in cytosolic potassium levels

Upon loading with the K⁺-sensitive dye PBFI (40–42), thymocytes that undergo apoptosis in response to DEX exhibit a lower fluorescence as compared with untreated cells (Fig. 1). Control experiments revealed that the difference in PBFI-dependent fluorescence between viable (PI-excluding) DEX-treated cells and viable control thymocytes disappeared in the presence of a combination of the potassium ionophore valinomycin and the K⁺/H⁺ exchanger nigericin (Fig. 1A), which together allow for the free distribution of K⁺ between the cell and the extracellular environment (41). Thus, the difference in PBFI fluorescence is due to a difference in K⁺ concentration rather than in dye uptake. Calibration of K⁺-permeant cells with variable doses of extracellular K⁺ (Fig. 1, A and B) revealed that viable, PI-excluding cells, which retain PBFI, exhibit a low (0.6–0.8 logs) although consistent (n = 5) K⁺-dependent variation of fluorescence. Although this relatively low signal/background ratio would render this dye inappropriate for the quantitation of apoptosis in single parameter studies, it is possible to assess the loss of PBFI fluorescence in combination with

![Graph](http://www.jimmunol.org/)
additional fluorochromes (see below, Figs. 2-4). In the presence of K\textsuperscript{+} ionophores and external K\textsuperscript{+}, all cells, including the fraction of DEX-treated thymocytes with an originally low PBFI-dependent fluorescence (PBFI\textsubscript{low} cells), acquire a PBFI\textsubscript{high} phenotype. The reduction of K\textsuperscript{+}-dependent PBFI fluorescence is detectable in a fraction of normal-sized cells before major volume loss occurs, at least according to the criterium of the FSC (Fig. 1C), and is also detectable before cells become permeant to PI (Fig. 1A). A gas spectroscopic method for K\textsuperscript{+} determination confirmed that K\textsuperscript{+} loss occurred during apoptosis (see below, Fig. 5). In conclusion, DEX-induced thymocyte apoptosis is accompanied by both a loss of intracellular K\textsuperscript{+} that occurs before cell shrinkage and a complete loss of plasma membrane barrier function.

Kinetics of K\textsuperscript{+} changes and temporal relationship with other features of the apoptotic process

In most cell types, including thymocytes, the first cytofluorometrically detectable sign of apoptosis is a loss of \(\Delta\Psi\textsubscript{m}\), and the subsequent hyperproduction of ROS on the uncoupled respiratory chain (21, 22, 26, 28, 47). To determine the temporal relationship between these signs of apoptosis and K\textsuperscript{+} loss, we performed a multiparameter analysis taking advantage of the \(\Delta\Psi\textsubscript{m}\)-sensitive dye DiOC\textsubscript{6}(3) (green fluorescence), the ROS-dependent conversion of hydroethidine into ethidium (Eth) (red fluorescence), and PBFI as a K\textsuperscript{+}-sensitive marker (blue fluorescence). As shown in Figure 2, phenotypically normal (DiOC\textsubscript{6}(3) high HE\textsubscript{high} Eth\textsubscript{low} cells) are homogeneously PBFI\textsubscript{high}, whereas DiOC\textsubscript{6}(3)\textsubscript{low} HE\textsubscript{high} Eth\textsubscript{high} cells are PBFI\textsubscript{low}. In contrast, cells under- going apoptosis manifest the \(\Delta\Psi\textsubscript{m}\) disruption before K\textsuperscript{+} leakage. Of note, DiOC\textsubscript{6}(3)\textsubscript{low} but not DiOC\textsubscript{6}(3)\textsubscript{intermediate} cells emit low K\textsuperscript{+}-dependent fluorescence (Fig. 3A). K\textsuperscript{+} loss exhibits a strong

**FIGURE 4.** Functional association between apoptotic \(\Delta\Psi\textsubscript{m}\) disruption, K\textsuperscript{+} loss, and nuclear apoptosis. Thymocytes were incubated in the presence of the indicated apoptosis inducers and inhibitors during a 4-h culture period as described in Materials and Methods and stained with PBFI plus DiOC\textsubscript{6}(3). The percentage of cells in each gate is shown. Numbers in dark circles indicate the percentage of subdiploid cells. The results are representative of two to three different determinations.
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Incubation of thymocytes with DEX (1 μM or 10 μM etoposide) (Fig. 4). Hence these agents have no effect on Caspase-induced apoptosis in any of its manifestations at the levels of mitochondria, nuclei, and K⁺. In contrast, acetyl-Tyr-Val-Ala-Asp-chloromethylketone (an inhibitor of caspase-1, formerly called IL-1β-converting enzyme) prevents caspase-1-dependent Caspase-induced apoptosis and K⁺ outflow but has no effect on DEX- and etoposide-induced apoptosis (Fig. 4). These data support the conclusion that apoptotic ΔΨₘ loss, nuclear apoptosis, and K⁺ outflow are functionally associated with each other.

Functional relationship between K⁺ outflow and mitochondrial features of apoptosis

As shown in Figures 2 through 5, K⁺ loss is always secondary to ΔΨₘ loss. No K⁺ loss is observed in cells whose ΔΨₘ remains stable. Cytofluorometric separation of DiOC₆(3)/PBFI-stained cells and flame spectroscopic determination of K⁺ revealed that DEX-treated DiOC₆(3)³⁰⁰⁰⁰PBFI³⁰⁰⁰⁰ cells contained 10.9 ± 3.6 nmol K⁺/10⁶ cells (mean ± SEM, n = 10). This value is comparable with that of nontreated control cells. In contrast, DiOC₆(3)³⁰⁰⁰⁰PBFI<sub>low</sub> cells contain only 3.1 ± 1.1 nmol K⁺/10⁶ cells (n = 10; p < 0.001, paired Student's t test) (Fig. 5, A and B). Since this determination has been performed on cells with a normal volume (i.e., cells with normal FSC and SSC parameters), it appears that the K⁺ concentration drops to less than one-third of the control value.

To further investigate the relationship between mitochondrial PT and K⁺ loss, cells were treated with two inhibitors of the mitochondrial megachannel, BA and CsA. BA is a ligand of the adenine nucleotide translocator that prevents ΔΨₘ loss in most models of apoptosis (12, 24, 27, 48) but that fails to protect mitochondria against Fas-elicted caspase-1 (46). This agent stabilizes the ΔΨₘ of DEX- or etoposide-treated cells and simultaneously prevents the loss of K⁺-dependent PBFI fluorescence (Fig. 4, lower panels), suggesting that K⁺ loss is secondary to mitochondrial PT.

To confirm this hypothesis, cells were stained with DiOC₆(3) and PBFI and then cytofluorometrically separated into DiOC₆(3)³⁰⁰⁰⁰PBFI³⁰⁰⁰⁰, DiOC₆(3)³⁰⁰⁰⁰PBFI<sub>inter</sub>, DiOC₆(3)³⁰⁰⁰⁰PBFI³⁰⁰⁰⁰, and DiOC₆(3)³⁰⁰⁰⁰PBFI<sub>low</sub>.
cells (Fig. 5A). Upon culture at 37°C, a fraction of DiOC6(3)high cells became DiOC6(3)intermediate and a significant percentage of DiOC6 (3)intermediate cells became DiOC6(3)low and concomitantly reduced PBFI fluorescence (Fig. 5C), thus corroborating the precursor product relationship (DiOC6(3)high PBFIhigh $\rightarrow$ DiOC6(3)intermediate PBFIhigh $\rightarrow$ DiOC6(3)low PBFIlow) that was suggested by the kinetic analyses (Fig. 3). CsA, a transient inhibitor of mitochondrial PT which acts on matrix cyclophilin D (16, 49), was employed as a probe to assess the role of mitochondrial dysfunction in this system. CsA (1–10 μM, Fig. 5 and data not shown) prevents the initial transition (DiOC6(3)high PBFIhigh $\rightarrow$ DiOC6(3)intermediate PBFIhigh) but not the second one (DiOC6(3)intermediate PBFIhigh $\rightarrow$ DiOC6(3)low PBFIlow), suggesting that the CsA-suppressible PT is only responsible for the first phase of $\Delta \Psi_{m}$ disruption (Fig. 5C). The transfection-enforced overexpression of Bcl-2, an endogenous inhibitor of PT (12, 45, 50), also prevents both $\Delta \Psi_{m}$ dissipation and the extrusion of $K_{\text{+}}$ ions (Fig. 6).

Taken together, these data indicate that mitochondrial PT is a prerequisite for $K_{\text{+}}$ loss. However, PT itself is not the mechanism that accounts for $K_{\text{+}}$ release.

Functional relationship between $K_{\text{+}}$ loss and caspase activation

Since caspases play a major role in apoptosis, we evaluated the effect of caspase inhibition on cytofluorometrically detectable apoptotic changes. Z-VAD.fmk, a broad spectrum inhibitor of caspases and other proteases including AIF (45, 46), completely prevents Fas-induced apoptosis at the DiOC6(3)high stage, thus confirming the involvement of caspase cascades early during Fas-induced apoptosis (46, 51, 52). In contrast, Z-VAD.fmk arrests DEX- and etoposide-treated cells at a later stage of the process, namely the DiOC6(3)intermediate stage (Fig. 4). Thus, in support of previous results (30, 48, 53), caspase inhibitors fail to prevent mitochondrial PT. Instead, they arrest the apoptotic process in a postmitochondrial phase of the apoptotic process. Cells that have been exposed to DEX plus Z-VAD.fmk (or etoposide plus Z-VAD.fmk) tend to accumulate at the DiOC6(3)intermediate stage (Figs. 4 and 7).

At this Z-VAD.fmk-arrested stage, cells still maintain high concentrations of nonoxidized intracellular GSH (as detected by monochlorobimane), lack PS exposure, and remain PBFIhigh (Fig. 7).

This observation indicates that the quasisimultaneous acquisition of these features of apoptosis (complete $\Delta \Psi_{m}$ disruption, GSH depletion/oxidation, PS exposure, and $K_{\text{+}}$ loss) requires the action of Z-VAD.fmk-inhibitable proteases. Moreover, these data imply that caspase activation occurs before the $K_{\text{+}}$ loss. Accordingly, physiologic concentrations of $K_{\text{+}}$ (100–140 mM) fail to inhibit the acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin-cleaving protease activity that accumulates in the cytosol of DEX-treated thymocytes. Similarly, in the presence of 140 mM $K_{\text{+}}$, recombinant human caspases-3, -6, and -7 retain 60 to 80% of their enzymatic activity (100% value at 140 mM NaCl) (data not shown) (32). Thus, caspases can act upstream of $K_{\text{+}}$ loss in cells with a normal $K_{\text{+}}$.

$K_{\text{+}}$ loss is a prerequisite for endonuclease activation

In several models of apoptosis induction and inhibition, the $K_{\text{+}}$ loss exhibits a strong correlation with nuclear DNA loss (Fig. 4). This correlation has been confirmed by cytofluorometric purification of DiOC6(3)high PBFIhigh, DiOC6(3)intermediate PBFIhigh, and DiOC6(3)low PBFIlow cells (as Fig. 5A) and by the subsequent determination of the frequency of hypoploid cells. Only DiOC6(3)low PBFIlow cells exhibit advanced DNA fragmentation (Fig. 8), suggesting the possibility that $K_{\text{+}}$ may act as an endogenous regulator of apoptotic degradation. Since manipulations of potassium have pleiotropic and toxic effects on intact cells (Fig. 1) (54), we tested the effect of variable $K_{\text{+}}$ concentrations on a cell-free system of apoptosis in which isolated HeLa nuclei are exposed to supernatants from mitochondria that have been treated with atracyloside, an inducer of PT leading to the release of AIF (12) and cytochrome c (55). Physiologic concentrations of KCl corresponding to the normal cytosolic $K_{\text{+}}$ concentration (100–140 mM) inhibit chromatin condensation and
Concluding remarks

The data reported in this work are compatible with a step-wise sequence of events of apoptosis (3–5, 12–14). One of the first events of the common phase of apoptosis is the disruption of mitochondrial membrane integrity, with the dissipation of the ΔΨm and/or the release of proteins that are normally sequestered in the intermembrane space (cytochrome c, AIF) in a sequence that remains to be determined and that may be cell type- and/or inducer-specific (13–15, 20–26, 29–31, 46). Thus, the sequence of molecular and cellular events leading to apoptosis, as described here, applies to thymocytes and lymphoid cells but may be different in other cell types.

The dissipation of the inner membrane proton gradient (stage 1) is mediated by PT, a process that is inhibited by CsA, BA, and Bcl-2 (Figs. 4–6) (3, 15, 16, 21, 24, 45, 49). After initial ΔΨm disruption, cells are irreversibly committed to death and consequently have entered the effector phase of apoptosis (20, 30, 53). However, early after ΔΨm disruption, cells still maintain a normal morphology (20) and lack major changes in K+/Ca2+ homeostasis (Figs. 1–3), redox potentials (Figs. 2, 3, and 7), or plasma membrane structure (Figs. 1, 3, and 7) (stage 1, Fig. 10).

The activation of Z-VAD.fmk-inhibitable proteases, which are triggered as a consequence of mitochondrial changes (5, 12–14, 24, 26, 31, 45, 46), is rate-limiting for the manifestation of a subsequent block of alterations (stage 2, Fig. 10), namely PE exposure on the cell surface, loss of nonoxidized GSH, and K+ leakage (Figs. 4 and 7). The functional relationship among these events is unknown. As a possibility, the rearrangement of those plasma membrane lipids that are linked to PE exposure might favor the extrusion of GSH and K+ (56). Alternatively, the inhibition of Na/K adenosine triphosphatase and/or the opening of K+ channels may be connected to redox regulation and/or plasma membrane function (37, 57). Thus, perturbation of redox metabolism by protonophores or menadione favors rapid K+ efflux (Fig. 3). However, the exact molecular mechanism of K+ loss that occurs during apoptosis remains elusive. The cytosolic accumulation of those apoptogenic activities (AIF, cytochrome, etc.) that are normally confined to mitochondria (12, 45, 46, 58) stimulates nuclear endonucleases that can only be activated in the context of a low K+ concentration (Fig. 9).

The progressive exhaustion of antioxidant defense subsequently causes the manifest (HE-detectable) hyperproduction of ROS, which entails massive Ca2+ influx into the cytosol (stage 3, Fig. 10) shortly before cells shrink, form apoptotic bodies, and lyse (Figs. 1–3) (25).

As a caveat, it would be erroneous to assume that the pleiotropic molecules that are involved in the common pathway of apoptosis such as K+, Ca2+, ROS, or caspases only participate in the degradation phase of apoptosis. Indeed, it has been established that elevations in cytosolic Ca2+ and ROS can serve as facultative transmitters in particular (private) proapoptotic signal transduction pathways (reviewed in Ref. 3). Similarly, caspases can be activated at the premitochondrial stage of apoptosis, for instance after Fas crosslinking (46, 51, 52), although this is not a general feature of apoptosis. By analogy, it is possible that the K+ efflux can participate in special cases of apoptosis initiation. Indeed, a reversible K+ loss has been observed early after IL-3 withdrawal in a murine hematopoietic cell line (59) and an outward K+ current may be decisive for neuronal apoptosis that is induced by staurosporine or serum deprivation (38). However, at least in the models that we have studied in this work, K+ release occurs at a relatively late stage of apoptosis, after commitment to cell death.

After submission of this work, several studies reporting the efflux of K+ from cells undergoing apoptosis have been published (32, 33, 60). These studies clearly establish the role of...
the K\(^+\) (and Na\(^+\)) efflux as a mechanism leading to cytosolic H\(_2\)O loss and consequent volume loss (32, 33, 60). Thus, as shown here (Fig. 1), all cells with a shrunken phenotype exhibit low K\(^+\)-dependent PBFI fluorescence, and only a minor fraction of cells that still have a normal FSC demonstrate a PBFI low phenotype (33). Moreover, in agreement with our data, a rise in the concentration of extracellular K\(^+\) preventing the drop in cytosolic K\(^+\) can prevent nuclear DNA fragmentation (32, 33).

However, in contrast to our observations, Cidlowski and co-workers (32, 33) suggest that K\(^+\) is an endogenous inhibitor of caspase activation and has a primary role in the regulation of cell death. This interpretation is based on the observations that 1) the cytochrome c/deoxy ATP-driven activation of caspase-3 is inhibited by K\(^+\) (32), and that 2) exogenous K\(^+\) can prevent endonuclease activation both in cells and in cell-free systems of apoptosis. As shown here, the action of a Z-VAD.afc-specific enzyme that is thought to be responsible for caspase-3 activation (46) is not inhibited by K\(^+\) (Fig. 9). This may imply that during early apoptosis, when K\(^+\) is still high, caspase activation is triggered by factors other than cytochrome c that are present in mitochondrial supernatants (46). Clearly, in our cell-free system of nuclear apoptosis, in which isolated nuclei are mixed with mitochondrial supernatant in the absence of cytosol (46, 61), cytochrome c and deoxy ATP are inactive, because they require the addition of cytosolic proteins for caspase-3 and endonuclease activation (62). In intact cells, the inhibition of caspase activation by Z-VAD.fmk prevents the K\(^+\) efflux, suggesting that caspase activation occurs upstream of this efflux (Fig. 7). Since the inhibition of caspases by Z-VAD.fmk fails to prevent the initial \(\Delta W_m\) dissipation (Fig. 7) and the subsequent cytolysis (53), it appears that the commitment point which seals the fate of the cell is located upstream of caspase activation and consequently upstream of K\(^+\) efflux. Therefore, although K\(^+\) outflow is critical for the acquisition of several hallmarks of apoptotic morphology (cell shrinkage and DNA fragmentation), it is probably not important for the life/death decision, which is made before K\(^+\) drops and before caspases and endonucleases are activated (53).

In conclusion, we show in this paper that a loss of cytosolic K\(^+\) occurs as a common event in the apoptotic degradation phase of thymocytes, after mitochondrial PT and caspase activation but before cell shrinkage and before the plasma membrane becomes permeable. K\(^+\)
leakage is closely associated with the loss of plasma membrane asymmetry and facilitates endonuclease-mediated chromatin degradation. These findings emphasize the functional impact of ion gradient dissipation on the apoptotic process.

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