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Plasma Membrane Potential in Thymocyte Apoptosis

Bruno Dallaporta,* Philippe Marchetti,*+ Manuel A. de Pablo,* Carine Maise,* Huynh-Thien Duc,‡ Didier Métivier,* Naoufal Zamzami,* Maurice Geuskens,§ and Guido Kroemer*##

Apoptosis is accompanied by major changes in ion compartmentalization and transmembrane potentials. Thymocyte apoptosis is characterized by an early dissipation of the mitochondrial transmembrane potential, with transient mitochondrial swelling and a subsequent loss of plasma membrane potential (Δψp) related to the loss of cytosolic K⁺, cellular shrinkage, and DNA fragmentation. Thus, a gross perturbation of Δψp occurs at the postmitochondrial stage of apoptosis. Unexpectedly, we found that blockade of plasma membrane K⁺ channels by tetratetraethylammonium (TPA), which leads to a Δψp collapse, can prevent the thymocyte apoptosis induced by exposure to the glucocorticoid receptor agonist dexamethasone, the topoisomerase inhibitor etoposide, γ-irradiation, or ceramide. The TPA-mediated protective effect extends to all features of apoptosis, including dissipation of the mitochondrial transmembrane potential, loss of cytosolic K⁺, phosphatidylserine exposure on the cell surface, chromatin condensation, as well as caspase and endonuclease activation. In strict contrast, TPA is an ineffective inhibitor when cell death is induced by the potassium ionophore valinomycin, the specific mitochondrial benzodiazepine ligand PK11195, or by primary caspase activation by Fas/CD95 cross-linking. These results underline the importance of K⁺ channels for the regulation of some but not all pathways leading to thymocyte apoptosis. The Journal of Immunology, 1999, 162: 6534–6542.

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poptosis may be defined as a cell death process in which the activation of catabolic processes and enzymes occurs before cytolysis, thereby facilitating the recognition, uptake, and digestion of the apoptotic cell by neighboring cells. For a long time, it has been assumed that the activation of endonucleases and specific proteases (caspases) would not only constitute a distinctive feature of apoptosis but also determine the decisive step (the “decision to die” or “commitment point”) that distinguishes living from dying cells. It is now clear that, in most models of apoptosis, inhibition of nucleases and caspases does not prevent cytolysis (1–5), indicating that the decision to die is made before catabolic enzymes are activated and that the activation of such enzymes can be a byproduct of the cell death process rather than a regulatory event (reviewed in Refs. 6 and 7). Accordingly, current attempts to understand the decisive step of the apoptotic process are focusing on the mechanisms leading to caspase and endonuclease activation rather than on the action of these hydrolyases.

We and others have shown that defective ion compartmentalization and membrane potentials may have a major impact on apoptotic regulation (7–10). Lipophilic cations accumulate in the mitochondria matrix, driven by the electrochemical gradient following the Nernst equation, according to which every 61.5-mV increase in membrane potential (usually 120–170 mV) corresponds to a 10-fold increase in cation concentration in mitochondria. Therefore, the concentration of such cations is 2–3 logs higher in the mitochondrial matrix than in the cytosol (CS) (11). During the process of apoptosis, cells reduce the mitochondrial retention of such lipophilic cations, including the fluorescent dye 3,3’-dihexyloxacarbocyanine iodide (DiOC₆(3)) (7, 8). Thus, the inner mitochondrial transmembrane potential (Δψm), which is mainly a proton gradient, dissipates relatively early during apoptosis (12, 13) via a process that involves the Bcl-2-inhibited and Bax-facilitated opening of the permeability transition (PT) pore (14, 15). Opening of the PT pore (which allows for the free diffusion of solutes of ≤1500 Da on the inner mitochondrial membrane) causes an increase in mitochondrial matrix volume (16). Because the surface of the outer mitochondrial membrane is smaller than that of the inner membrane, the PT pore opening may cause local mechanical disruption of the outer mitochondrial membrane (17, 18), with consequent release of soluble intermembrane proteins (19, 20). Several of the mitochondrial intermembrane proteins released upon PT pore opening (e.g., cytochrome c, apoptosis-inducing factor, and procaspases-2, -3, and -9) are endowed with the capacity to activate caspases or nucleases (21–25). As a result, mitochondrial membrane permeabilization has been viewed as a phenomenon that marks the “point-of-no-return” of the apoptotic process (6, 7, 12, 26, 27).

A further apoptosis-relevant alteration in ion compartmentalization affects plasma membrane K⁺ homeostasis. Under normal circumstances, the K⁺ concentration is much higher in the CS (100–
140 mM) than in the extracellular (EC) fluid (≤5 mM). A continuous, low K⁺ efflux, via so-called K⁺ leakage channels, accounts for the maintenance of the charge differences on the plasma membrane, the plasma membrane potential ($\Delta\Psi_p$), and thus is vital for ion and volume homeostasis. A major decrease of the intracellular K⁺ levels has been observed in several cell types (S49, Jurkat, HL60, and thymocytes) undergoing apoptosis in response to a variety of different inducers (glucocorticoids, A23187, anisomycin, thapsigargin, staurosporine, anti-Fas/CD95, UV irradiation, and etoposide) (10, 28, 29). Complete loss of the K⁺ gradient may be expected to have severe metabolic consequences and to be accompanied by a loss of $\Delta\Psi_p$ because K⁺ efflux (which normally follows the concentration gradient) would be interrupted. Moreover, a loss of intracellular K⁺ may be linked to cellular shrinkage (10, 28), one of the hallmarks of apoptosis. Importantly enough, K⁺ efflux is mandatory for endonuclease activation, given that physiological intracellular K⁺ concentrations inhibit nuclear endonucleases in both cells and cell-free systems of apoptosis (10, 29, 30). This has lead to the proposal that the derepression of endonucleases due to low intracellular K⁺ concentrations may be a decisive prerequisite for end-stage DNA fragmentation (29).

The mechanisms accounting for K⁺ efflux during the apoptotic process have been elusive thus far. On theoretical grounds, this K⁺ efflux could be due to the activation of K⁺ channels and/or due to the inhibition of active K⁺ transport systems such as K⁺/Na⁺ ATPase. Inhibition of K⁺/Na⁺ ATPase may be expected to result in an increase in cellular volume, which is not found in apoptosis and rather is a feature of necrosis (7). Therefore, we reasoned that an increased activity of K⁺ channels rather than an inhibition of active K⁺ transport might account for the loss of cellular K⁺, a hypothesis that we have tested in this work using K⁺ channel inhibitors. Because K⁺ efflux causes a local charge asymmetry on the plasma membrane and is the major determining factor for $\Delta\Psi_p$ (60–70 mV in most cell types, negative inside), we wondered whether the apoptosis-associated K⁺ loss would culminate in a loss of $\Delta\Psi_p$. Therefore, we determined the retention of DiOC₃(3) in the CS of cells undergoing apoptosis (which, based on the Nerst equation, should reach a cytosolic concentration $\sim$1 log higher than that of the EC medium at a $\Delta\Psi_p$ of 60 mV) and comparatively monitored the $\Delta\Psi_p$ and the $\Delta\Psi_m$ in cells undergoing apoptosis.

Like other cells, thymocytes possess voltage-dependent K⁺ channels that participate in sustaining the resting $\Delta\Psi_p$, in regulating cell volume, and in enabling cellular activation processes (31). Here, we addressed the functional impact of K⁺ channels, K⁺ depletion, and $\Delta\Psi_p$ breakdown on thymocyte apoptosis. Our results suggest that gross perturbations of K⁺ currents leading to $\Delta\Psi_p$ collapse are only observed at a relatively late, postmitochondrial stage of apoptosis. However, inhibition of K⁺ channels may prevent the apoptosis induced by some but not all inducers at a premitochondrial stage. These data underscore the physiological importance of plasma membrane ion gradients in the control of apoptosis.

Materials and Methods

**Induction and inhibition of apoptosis**

Thymocytes from female 4- to 8-wk-old BALB/c mice were cultured at 37°C with 5% CO₂ in RPMI 1640 supplemented with 10% FCS, t-glutamine, antibiotics, and 2-ME (50 μM; Sigma, St. Louis, MO). The following cell death inducers were employed: the glucocorticoid receptor agonist dexamethasone (DEX) (final concentration 1 μM; Sigma), etoposide (10 μM; Sigma), irradiation (10 Gy; RX30555 irradiator, Gravaton Industries, Gosport, U.K.), C₂ ceramide (50 μM; Sigma), valinomycin (100 μM; Sigma), PK11195 (200 μM; Sigma), or an Ab specific for CD95/Apo-1/ Fas (clone 154000D, 500 ng/ml; PharMingen, San Diego, CA). The following apoptosis inhibitors were tested: the K⁺ channel blockers tetraethylammonium (TEA) and tetrapentylammonium (TPA) (200-μM standard dose; Sigma), the glucocorticoid receptor antagonist RU38486 (10 μM; kindly provided by Roussel-Hoechst-Marion), the broad-spectrum caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylethketone (Z-VAD-fmk) (100-μM final concentration; stock 10 mM in ethanol; Bachem, Basel, Switzerland), or the ligand of the adenine nucleotide translocator bongkrekic acid (50-μM final concentration; kindly provided by Dr. Duine, Delft University, Delft, The Netherlands).

**Cytofluorometric determination of $\Delta\Psi_p$, viability, phosphatidylserine exposure, reactive oxygen species (ROS), cytotoxic K⁺, and DNA loss**

For the determination of the $\Delta\Psi_p$, DiOC₃(3) (at a final concentration of 40 nM, stock 40 μM in ethanol, and an excitation wavelength of 488 nm (emission 529 nm); Molecular Probes, Eugene, OR) was employed (32). Alternatively, we used 20 nM of chloromethyl-X-rosamine (CMXRos) (fluorescence at 600 nm), as described previously (33). The fluorescence intensity or emission spectrum of these dyes is not influenced by variations in the pH (data not shown), within a pH range relevant for apoptosis regulation (pH 6.0–8.2) (9). To determine the contribution of the $\Delta\Psi_p$, DiOC₃(3) staining was performed in either complete medium, 140 mM NaCl supplemented with 2 mM glucose and 10 mM HEPES (pH 7.4), or 140 mM KCl plus 2 mM glucose plus 10 mM HEPES (pH 7.4), as indicated. This latter procedure leads to the collapse of the $\Delta\Psi_p$ (34). Nons viable cells were excluded by simultaneous staining with propidium iodide (PI) (final concentration of 5 μM; stock 10 mM in DMSO; excitation at 488 nm, emission 620 nm; Molecular Probes). Loss of membrane integrity was determined by means of the vital dye ethidium (Eth) bromide (200 ng/ml; 5 min at room temperature, excitation at 480 nm, emission 600 nm). An FITC- annexin V conjugate (1/400 dilution; 1 μg/ml, 15 min at 4°C; Brand Applications, Maastricht, The Netherlands; 525 nm) with a high affinity for PS (35, 36) was used for the assessment of aberrant PS exposure. Labeling with FITC-annexin V was performed after the removal of FCS by washing cells twice in HEPES buffer (10 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 1 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂). The generation of ROS was monitored with hydroethidine (HE) (final concentration of 2 μM; stock 10 mM in DMSO, excitation at 488 nm, emission 620 nm; Molecular Probes). For the determination of intracellular K⁺ levels, cells were loaded for 15–30 min with cell-permeant benzofuran isophtalate (PBFI)-ace toxyethyl ester (final concentration of 2.5 μM; stock 500 μM in DMSO). The resulting PBFI fluorescence was elicited at 485 nm and measured at 653 nm as described previously (29, 37). The frequency of hypoploid cells was determined by ethanol fixation followed by staining with PI as described previously (38) using an Epics Profile II Analyzer (Coulter, Hialeah, FL). All other stainings were analyzed using a FACScanSante cyto fluorometer (Becton Dickinson, Mountain View, CA), which was also used for cell sorting.

**Confocal microscopy**

Cells were stained with DiOC₃(3) as described above and were examined with a Leica TCS NT confocal microscope (Leica Microsystems, Rueil Malmaison, France) equipped with a 15-mW argon-krypton laser configured with an inverted Leica DM IRBE. The 488-nm line was used to excite DIOC₃(3). Each image consisted of the projection of 50 optical sections performed at intervals of 200 nm in the z-axis. xy fields of 1024 × 1024 pixels were scanned using an oil PI Apo 100× (not applicable = 1.4) objective. Chats (0.2 × 0.2-μm size) were quantified, and images were processed using the Leica TCS NT software (PowerScan module) and printed on a Sony HD-8800 color printer.
DNA fragmentation analysis and electron microscopy

DNA fragmentation (5 × 10^5 cells/lane) was determined by agarose gel electrophoresis (32). Electron microscopy was performed on ultrathin sections of glutaraldehyde/osmiumtetroxide-fixed, Epon-embedded cells after uranyl acetate and lead citrate staining as described previously (41).

Results and Discussion

Sequential loss of \( \Delta \Psi_m \) and \( \Delta \Psi_p \) in thymocyte apoptosis

Lipophilic cations such as DiOC$_6$(3) accumulate in the mitochondrial matrix, driven by the electrochemical gradient following the Nernst equation. Therefore, the concentration of such cations is 2–3 logs higher in the mitochondrial matrix than in the CS (11). Given that the resting \( \Delta \Psi_p \) of lymphocytes is usually 60–70 mV, the cytosolic concentration is ~10-fold higher than in the incubation medium. This implies that two potentials (\( \Delta \Psi_m \) plus \( \Delta \Psi_p \)) influence the cellular uptake of DiOC$_6$(3). To distinguish the relative contribution of \( \Delta \Psi_m \) and \( \Delta \Psi_p \) to DiOC$_6$(3) uptake, thymocytes were stained with DiOC$_6$(3) in the presence of either 140 mM NaCl (which is compatible with the maintenance of \( \Delta \Psi_p \)) or 140 mM KCl (which abolishes \( \Delta \Psi_p \)). As expected, staining thymocytes in the presence of high EC K$^+$, which causes a \( \Delta \Psi_p \) depolarization, induces a reduction in DiOC$_6$(3) fluorescence (Fig. 1A). This \( K^+ \)-dependent reduction is not seen when cells are treated with the \( K^+ \)-channel blocker TPA, indicating that TPA-inhibitable, voltage-gated \( K^+ \) channels maintain the \( \Delta \Psi_p \) in this cell type, as reported previously (42). Moreover, no \( K^+ \)-mediated reduction is found for DiOC$_6$(3)$_{low}$ thymocytes stimulated with DEX, indicating that such cells do not have \( \Delta \Psi_p \) or \( \Delta \Psi_m \) (Fig. 1A), a finding which is in line with our previous observation that such cells have undergone massive \( K^+ \) efflux (29) and thus must be unable to generate \( \Delta \Psi_p \). As a result, it appears that both TPA and DEX cause a \( \Delta \Psi_p \) collapse, although both reagents affect \( \Delta \Psi_m \) in a differential fashion.

This interpretation was confirmed using confocal analysis of DiOC$_6$(3)-stained control, DEX-, or TPA-treated cells. TPA selectively affects the cytosolic (but not the mitochondrial) accumulation of DiOC$_6$(3). The gradient between the EC and the cellular DiOC$_6$(3)-dependent fluorescence disappears after preincubation of cells with TPA (Fig. 1C). In contrast, DEX reduces both the plasma membrane and the mitochondrial DiOC$_6$(3) concentration gradients (Figs. 1, B and C).

Previously, we have reported the existence of a minor (≤5%) DiOC$_6$(3)$_{intermediate}$ population among preapoptotic thymocytes (4, 43). These cells represent an intermediate stage of the apoptotic process because, once purified, they quickly become DiOC$_6$(3)$_{low}$ (44). To clarify the \( \Delta \Psi_m/\Delta \Psi_p \) status of these cells, DEX-treated thymocytes were stained with DiOC$_6$(3), followed by cytometric separation of cells into DiOC$_6$(3)$_{high}$ (I), DiOC$_6$(3)$_{intermediate}$ (II), and DiOC$_6$(3)$_{low}$ (III) cells (Fig. 2B). These cells were then restained for 15 min in NaCl (which would maintain the \( \Delta \Psi_p \)) or KCl (which would depolarize the \( \Delta \Psi_p \)) with either DiOC$_6$(3) (Fig. 2C) or CMXRos (Fig. 2D), a \( \Delta \Psi \)-sensitive dye emitting a fainter, more intense red fluorescence than DiOC$_6$(3) (33). As expected, DiOC$_6$(3)$_{high}$ cells possess \( \Delta \Psi_p \) whereas DiOC$_6$(3)$_{low}$ cells have a greatly reduced, if any, \( \Delta \Psi_p \). DiOC$_6$(3)$_{intermediate}$ cells treated with 140 mM KCl lower their fluorescence upon staining with DiOC$_6$(3) or DEX or de novo staining with CMXRos (Figs. 2, C and D), indicating that the cells have undergone an at least partial \( \Delta \Psi_m \) collapse; however, these cells retain their \( \Delta \Psi_p \). Electron microscopy revealed that this DiOC$_6$(3)$_{intermediate}$ population lacks advanced chromatin condensation and cell shrinkage (Fig. 2F), yet manifests mitochondrial matrix swelling compared with DiOC$_6$(3)$_{high}$ cells, which have a normal phenotype (Fig. 2E). The mitochondrial swelling of DiOC$_6$(3)$_{intermediate}$ cells must be transient, because DiOC$_6$(3)$_{low}$ cells, which represent a later stage of the process with full nuclear apoptosis, have undergone shrinkage both of the cytoplasm and of the mitochondria (Refs. 13, 33, and 44 and data not shown). In conclusion, thymocytes undergoing apoptosis dissipate their \( \Delta \Psi_m \) and manifest a transient mitochondrial swelling before they lose \( \Delta \Psi_p \). This interpretation was confirmed using confocal analysis of DiOC$_6$(3)-stained control, DEX-, or TPA-treated cells. TPA selectively affects the cytosolic (but not the mitochondrial) accumulation of DiOC$_6$(3). The gradient between the EC and the cellular DiOC$_6$(3)-dependent fluorescence disappears after preincubation of cells with TPA (Fig. 1C). In contrast, DEX reduces both the plasma membrane and the mitochondrial DiOC$_6$(3) concentration gradients (Figs. 1, B and C).

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The specific K$^+$ channel blocker TPA causes a dose-dependent loss of \( \Delta \Psi_p \) without affecting \( \Delta \Psi_m \), which can be measured independently from \( \Delta \Psi_p \) by assessing the ratio between cytosolic and mitochondrial DiOC$_6$(3) retention (Figs. 1 and 3A). This has been shown by comparative DiOC$_6$(3) staining in the presence or absence of 140 mM NaCl or KCl (Figs. 1A and 3A) or by confocal quantification of the DiOC$_6$(3) concentration gradients on the plasma and mitochondrial membranes (Figs. 1, B and C and Fig. 3C). The \( \Delta \Psi_p \) decrease induced by TPA appears to be almost total.
because the DiOC$_6$ (3) retention of TPA-treated cells is not reduced by treatment with 2 mM ouabain (data not shown), an inhibitor of the Na/K ATPase that is specifically involved in the maintenance of $\Delta \Psi_m$ yet dispensable for that of the inner mitochondrial membrane (45). Even upon prolonged incubation (4 h in Fig. 3A), TPA does not cause thymocyte apoptosis, as assessed by measuring nuclear hypoploidy (numbers in cycles in Fig. 3A). In contrast, incubating cells with DEX during the same period readily

**FIGURE 2.** Temporal sequence of $\Delta \Psi_m$ and $\Delta \Psi_p$ collapse induced by DEX. Untreated cells (A) or DEX-treated (1 $\mu$M, 4 h) thymocytes (B) were stained with DiOC$_6$(3), followed by cell sorting into DiOC$_6$(3)$^{\text{high}}$ (I, 41% of total), DiOC$_6$(3)$^{\text{intermediate}}$ (II, 3% of total), or DiOC$_6$(3)$^{\text{low}}$ (III, 23% of total) populations, as indicated by the windows in B. After sorting, cells were washed in 140 mM NaCl or 140 mM KCl, followed by restaining with DiOC$_6$(3) (C) or de novo staining with CMXRos (D) and reanalysis. This experiment was repeated three times, yielding similar results. In addition, DiOC$_6$(3)$^{\text{high}}$ (E) or DiOC$_6$(3)$^{\text{intermediate}}$ (F) cells were fixed and subjected to electron microscopy. Mitochondria-rich areas of representative cells are shown.

**FIGURE 3.** Effect of TPA on DiOC$_6$(3) uptake. Thymocytes were cultured in the absence (A) or presence (B) of DEX. In addition, the indicated amount of TPA or TEA was added to the culture medium. After 4 h of incubation, cells were stained with DiOC$_6$(3) in complete culture medium (A and B). The DiOC$_6$(3) incorporation profiles obtained in the absence of TPA are shown as an internal control in each individual graph. Alternatively, the concentration of DiOC$_6$(3) within the mitochondrion-rich and mitochondria-free cytoplasm was measured by confocal microscopy as described in the legend to Fig. 1C. The fluorescence intensity was measured for each subcellular region of 10 different cells. Data are expressed as mean values ± SEM ($n = 10$).
induced a total collapse in DiOC$_6$ (3) retention and nuclear apoptosis in ~40% of the cells. Unexpectedly, when thymocytes were kept in culture in the continuous presence of both DEX and TPA (Fig. 3B), we found that TPA treatment prevented the cells from responding to DEX by advancing to the DiOC$_6$ (3)$^{low}$ phenotype and caused them to lose their $\Delta \Psi_m$ (Fig. 3, B and C). Dose-response analyses revealed a strong correlation between the TPA-mediated collapse of $\Delta \Psi_m$ and the prevention of DEX-induced $\Delta \Psi_m$ dissipation (Fig. 3, A and B). Whereas TPA was an efficient inhibitor of the DEX-triggered $\Delta \Psi_m$ collapse at doses as low as 20 $\mu$M, a dose also reported to prevent UV-induced cell shrinkage of HL60 cells (28), another quaternary ammonium, TEA, which has a lower affinity for K$_1$ channels than TPA (46), had to be employed at millimolar concentrations to obtain a similar effect (Fig. 3, A and B). Again, this effect correlated with the induction of a $\Delta \Psi_m$ collapse (Fig. 3, A and B).

In a further series of experiments, we addressed the question of whether TPA would selectively affect some features of DEX-induced apoptosis or rather block the entire apoptotic program. As shown in Fig. 4, we found that TPA prevented all characteristics of apoptosis, including the mitochondrial hypergeneration of ROS (measured by determining the oxidation of HE to Eth, Fig. 4A), the exposure of phosphatidylserine on the plasma membrane surface (measured by FITC-annexin V conjugates, Fig. 4B), cytosolic K$^+$ efflux (measured with PBFI, Fig. 4C), and nuclear DNA loss (quantitated by PI staining of ethanol-fixed cells, Fig. 4D). TPA also prevents the activation of caspases capable of cleaving the substrate DEVD (Fig. 5A), as well as oligonucleosomal DNA fragmentation (Fig. 5B). Electron microscopy confirmed that thymocytes treated with both TPA and DEX exhibited a normal morphology (Fig. 6). Similar data were obtained when the topoisomerase type II inhibitor etoposide was used instead of DEX (Fig. 4). The dose of TPA necessary for the blockade of nuclear apoptosis was found to correlate with that causing a $\Delta \Psi_m$ collapse (numbers in circles in Fig. 3, A and B). Thus, TPA can block the thymocyte apoptosis induced by the glucocorticoid agonist DEX or by the genotoxic agent etoposide.

**FIGURE 4.** Cytofluorometric assessment of various apoptosis-associated parameters and their modulation by TPA. Thymocytes were cultured for 4 h with DEX, etoposide, or a mAb specific for Fas/CD95 and/or TPA, followed by staining with DiOC$_6$ (3) plus HE (A), Eth bromide plus FITC-annexin V (B), or PBFI (C). Alternatively, cells were permeabilized with ethanol, and the nuclear DNA content was determined with PI (D). Results are representative of four independent experiments.

**FIGURE 5.** Effect of TPA on caspase and endonuclease activation. Thymocytes treated as described in the legend to Fig. 4 were subjected to lysis and determination of DEVD-aminotrifluoromethylcoumarin cleavage (A). Alternatively, DNA was extracted (5 $\times$ 10$^5$ cells/lane) and subjected to agarose gel electrophoresis for the detection of oligonucleosomal DNA fragmentation (B). Cells were cultured for 4 h with the indicated combination of DEX, etoposide, anti-CD95, and/or TPA, followed by analysis of the indicated parameter. Shaded boxes indicate addition of the corresponding substance.
K⁺ channel blockade blocks a specific rather than general apoptosis pathway

To determine whether TPA would block DEX-induced apoptosis at an early or a late stage, we performed time-course inhibition experiments. RU38486, a glucocorticoid receptor antagonist, can block DEX-induced thymocyte apoptosis (12, 41) when added early after DEX (Fig. 7a). In contrast, the caspase inhibitor Z-VAD.fmk prevents the advent of nuclear apoptosis at a later stage, in accordance with the idea that caspase-dependent endonuclease activation occurs after the commitment to apoptosis triggered via glucocorticoid receptor occupation (4). Compared with Z-VAD.fmk, TPA has to be added at a relatively early stage to obtain a suppression of DEX-induced apoptosis (Fig. 7a); this finding is in line with the fact that TPA prevents all signs of DEX-induced apoptosis, including the early mitochondrial changes (Figs. 3 and 4), whereas Z-VAD.fmk blocks the process leading to DNA fragmentation after Δψₘ dissipation and mitochondrial swelling (4, 43).

The above findings indicate that TPA acts at a relatively early stage of the DEX-induced apoptotic process, suggesting that it could interfere with the activation of the apoptotic machinery rather than with the machinery itself. Therefore, we determined whether TPA would have a vast spectrum of antiapoptotic action or instead whether it would fail to prevent apoptosis induction by some apoptosis triggers. We explored the effects of TPA on apoptosis induction by 1) ceramide, a proapoptotic second messenger elicited by glucocorticoids and genotoxic stress (47, 48), 2) valinomycin, a K⁺ ionophore that dissipates cellular and mitochondrial ion gradients (49), 3) PK11195, a ligand of the peripheral benzodiazepine receptor that facilitates the opening of the PT pore (50), and 4) Fas/CD95 cross-linking, a manipulation leading to primary caspase activation (51). TPA prevents the apoptosis induced by ceramide but has no inhibitory effect on the apoptosis caused by valinomycin, PK11195 (Fig. 7b), or anti-Fas (Figs. 4, 5, and 7b). Thus, TPA can prevent the cell death induced by DEX and DNA damage after the stage of ceramide generation, yet remains ineffective when apoptosis is enforced by direct K⁺ depletion, PT pore opening, or caspase activation.

Concluding remarks

The present work demonstrates that, during thymocyte apoptosis, gross alterations of plasma membrane function and structure occur after signs of mitochondrial dysfunction have manifested (Fig. 8). Thus, dissolution of the Δψₘ and transient mitochondrial swelling are observed before several changes affect the plasma membrane: loss of the Δψₘ (Figs. 1 and 2), reduction of intracellular K⁺ (29), cell shrinkage, and loss of membrane asymmetry with aberrant exposure of phosphatidylserine residues on the plasma membrane surface (33). TPA collapses the Δψₘ of thymocytes, indicating that TPA-inhibitible, voltage-gated K⁺ channels generate this Δψₘ. However, TPA has no major toxic effects on thymocytes (Fig. 6), does not cause a nonspecific permeabilization of membranes with Ca²⁺ influx (data not shown), and does not cause PS exposure (Fig. 4B), indicating that Δψₘ collapse by itself does not perturb the distribution of lipids in the plasma membrane. Unexpectedly, we found that the prevention of K⁺ efflux by TPA fully inhibits the thymocyte apoptosis induced by a variety of stimuli that may be p53-dependent (etoposide, γ-irradiation) or p53-independent (DEX, ceramide). These pathways have in common that they require de novo mRNA and protein synthesis (52) and that they are inhibited by PT pore blockade (26, 41, 53) or the knock-out of

FIGURE 6. Ultrastructure of thymocytes cultured with DEX and/or TPA. Cells treated as described in the legend to Fig. 4 or Fig. 5 were examined by electron microscopy.
Apaf-1 or caspase-9 (54, 55), two molecules which link mitochondrial damage to downstream events of apoptosis. TPA intercepts the apoptotic pathway at the early, premitochondrial level, because 1) it prevents the early ΔΨm dissipation and later mitochondrial ROS generation induced by DEX or etoposide (Figs. 3A and 4A), 2) has no effect when added late (Fig. 7a) or when added to DiOC6(3)intermediate cells that have already disrupted of their ΔΨm (data not shown), and 3) has no inhibitory effect when apoptosis is enforced by PK11105 (Fig. 7b), an agent that acts directly on the PT pore. Moreover, TPA fails to prevent the apoptosis induced by valinomycin, a K+specific ionophore (Fig. 7a). This latter negative result suggests that TPA exerts its antiapoptotic effect by virtue of is modulatory effect on K+ fluxes rather than via a yet-to-be discovered nonspecific effect. TPA does not suppress the apoptosis induced by anti-Fas/CD95 (Figs. 4 and 5), a pathway that causes direct caspase activation without any need of protein synthesis, Apaf-1, or caspase-9 (51, 54–56). Hence, TPA acts on a specific rather than on a general pathway of apoptosis (Fig. 8). Of note, TPA does not prevent the K+ efflux triggered by anti-Fas/CD95 (Fig. 4C), indicating that other TPA-resistant mechanisms of K+ efflux can intervene in a late stage of apoptosis, at least in the Fas/CD95 pathway. The exact mechanisms by which TPA prevents the early mitochondrial changes triggered by DEX, DNA damage, or ceramide remain elusive. Potassium channel blockers have been shown previously to inhibit lymphocyte activation processes (31, 57), and it may be possible that an analogous effect underlies the apoptosis-inhibitory effect of TPA.

Several published reports suggest that the regulation of K+ fluxes has a major impact on apoptosis regulation. Valinomycin, a K+-specific ionophore, is a potent inducer of apoptosis in many cell types, including neurons (58), hepatoma cells (59), pre-B cells (49), and thymocytes (Refs. 60 and 61 and Fig. 7b). K+ depletion suffices to cause apoptosis in neurons (3, 62, 63), and K+ efflux underlies a murine model of cerebellar cell death in which the weaver mutation causes the constitutive activation of the G protein-regulated inward rectifier 2 K+ channel (64). In neocortical cells, attenuating outward K+ current with TEA or elevated EC K+ rescues the apoptosis induced by staurosporine, serum withdrawal (58), or β-amyloid (65, 66). Plasma membrane channels mediating a K+ efflux can cause apoptosis in a variety of different cell types. Thus, ATP-activated P2Z/P2X purinergic receptors in macrophages simultaneously mediate pore formation, ΔΨp collapse, and apoptosis (67). Similarly, β-amyloid (68) and the HIV-encoded Vpr protein (69) can incorporate into plasma membranes, thereby perturbing ion homeostasis and causing cell death. In strict contrast to these data, several apoptosis-inducing agents have been reported to inhibit K+ channels. This applies to ceramide, which inhibits the voltage-gated potassium channels of T lymphocytes via tyrosine kinases (70), 4-aminopuridine, which blocks an outward rectifier K+ channel (71), or the apoptogenic peptides Reaper and Grim (72). It remains to be confirmed that these effects are truly responsible for apoptosis induction by these agents.

At first glance, the relationship between the mitochondrial PT pore opening and K+ efflux appears paradoxical. On one hand, ΔΨm breaks down before a major CS K+ loss and concomitant ΔΨp loss occur (Figs. 1 and 2 and Ref. 29). On the other hand, a blockade of K+ channels can prevent the mitochondrial dysfunction required for DEX- or etoposide-mediated apoptosis (Figs. 3 and 4). As a possibility, subtle changes in K+ homeostasis occurring together with the PT pore opening may participate in a positive feed-forward loop, facilitating the apoptosis-related mitochondrial dysfunction. This possibility is suggested by the recent finding that the PT pore possesses a low conductance mode of opening that is K+-selective and thus may cause selective alterations of local K+ concentrations (73). Alternatively, K+ fluxes might play a pleiotropic role at several levels of the apoptotic cascade, in analogy to cytosolic free Ca2+, which can function, in the low micromolar range, as a facultative premitochondrial second messenger-signaling PT pore opening (74) and increase as an obligate consequence of the PT pore opening to levels of >200 μM at a late, postmitochondrial stage of the apoptotic process (75, 76). Thus, subtle changes in K+ fluxes could be involved in the early apoptotic induction phase, whereas a major K+ loss would only occur during the late degradation phase of apoptosis. Irrespective of these theoretical possibilities, the present data underline the importance of K+ as an endogenous apoptosis modulator.

FIGURE 7. Time course and spectrum of apoptosis-inhibitory action of TPA. A, Time course of apoptosis inhibition mediated by RU38480, TPA, or Z-VAD.fmk. Thymocytes were cultured for 5 h in the presence of DEX. The indicated agents were added 30, 60, 90, 120, 180, or 240 min after initiation of the culture. Hypoploidy was assessed after 5 h, and the percentage of inhibition of this parameter was determined. Control values of untreated thymocytes were 15 ± 2%; values of thymocytes treated with DEX only were 52 ± 3%. B, TPA-mediated inhibition of apoptosis induced by a panel of different inducers. Thymocytes were cultured for 4 h with the indicated apoptosis inducer in the presence or absence of TPA (200 μM), and the frequency of subdiploid cells was measured. Results are mean values of three independent determinations ± SEM.
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References
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