

## **Mitochondrial Permeability Transition Is a Central Coordinating Event of Apoptosis**

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### **Summary**

In a number of experimental systems, the early stage of the apoptotic process, i.e., the stage that precedes nuclear disintegration, is characterized by the breakdown of the inner mitochondrial transmembrane potential ( $\Delta\Psi_m$ ). This  $\Delta\Psi_m$  disruption is mediated by the opening of permeability transition (PT) pores and appears to be critical for the apoptotic cascade, since it is directly regulated by Bcl-2 and since mitochondria induced to undergo PT *in vitro* become capable of inducing nuclear chromatinolysis in a cell-free system of apoptosis. Here, we addressed the question of which apoptotic events are secondary to mitochondrial PT. We tested the effect of a specific inhibitor of PT, bongkreikic acid (BA), a ligand of the mitochondrial adenine nucleotide translocator, on a prototypic model of apoptosis: glucocorticoid-induced thymocyte death. In addition to abolishing the apoptotic  $\Delta\Psi_m$  disruption, BA prevents a number of phenomena linked to apoptosis: depletion of nonoxidized glutathione, generation of reactive oxygen species, translocation of NF $\kappa$ B, exposure of phosphatidylserine residues on the outer plasma membrane, cytoplasmic vacuolization, chromatin condensation, and oligonucleosomal DNA fragmentation. BA is also an efficient inhibitor of p53-dependent thymocyte apoptosis induced by DNA damage. These data suggest that a number of apoptotic phenomena are secondary to PT. In addition, we present data indicating that apoptotic  $\Delta\Psi_m$  disruption is secondary to transcriptional events. These data connect the PT control point to the p53- and ICE/Ced 3-regulated control points of apoptosis and place PT upstream of nuclear and plasma membrane features of PCD.

In many models of apoptosis induction, disruption of the mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) is observed shortly before other features of apoptosis (generation of reactive oxygen species, plasma membrane perturbations, chromatin condensation, and DNA fragmentation) become detectable. This applies to very different cell types and protocols of apoptosis induction (1–5).  $\Delta\Psi_m$  disruption marks a point-of-no-return of the apoptotic cascade (3), and mitochondria or mitochondrial products induce chromatin condensation and DNA fragmentation in cell-free systems of apoptosis (5–7). We have recently shown that in a cell-free system, mitochondria only become proapoptotic when they undergo permeability transition (PT; 5), a phenomenon that is also thought to account for  $\Delta\Psi_m$  disruption in intact cells (8). Intriguingly, Bcl-2 is an inhibitor of PT, both in isolated mitochondria and in cells (5, 8), underlin-

ing that PT may indeed constitute a central checkpoint of the apoptotic cascade. In addition to the putative PT/Bcl-2 checkpoint, it is currently assumed that the ICE/Ced3 family of proteases controls a further apoptotic checkpoint (reviewed in 9). Thus far, the hierarchy between these two hypothetical control points has remained obscure (10).

Although published data establish that end-stage apoptosis is chronologically secondary to PT-mediated  $\Delta\Psi_m$  disruption (1–4), they do not clarify which features of apoptosis are linked to PT in a cause-effect relationship. To resolve this problem, we have studied the effects of PT inhibition on the apoptotic cascade in a prototypic model of apoptosis, namely the dexamethasone (DEX)-induced death of thymocytes (11). Recently, we have observed that the substance that most closely mimics the PT-inhibitory effect of Bcl-2, both in cells and in isolated mitochondria, is bongkreikic acid (BA; 5). BA is a specific ligand of a protein located in the inner mitochondrial membrane, the adenine

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nucleotide translocator (ANT; 12, 13). The ANT belongs to the structures of the mitochondrial membrane that may participate in the formation of PT pores, probably via interaction with proteins from the outer membrane (13), and BA is known to affect the molecular conformation of the ANT, thereby reducing the probability of PT pore gating (12, 13). As does Bcl-2, BA inhibits PT pore opening in response to prooxidants, protonophores, and the ANT ligand atractyloside, but fails to interfere with PT induction by  $\text{Ca}^{2+}$  and chemical thiol cross-linking (5). While inhibiting PT, both BA and Bcl-2 prevent the release of (a) proapoptotic protein(s) from mitochondria (5). Thus, BA mimics the mitochondrial effects of Bcl-2.

Here, we tested the effect of BA on cells undergoing apoptosis rather than on isolated mitochondria. We show that via its PT-inhibitory ( $=\Delta\Psi_m$ -stabilizing) effect, BA inhibits all major cytoplasmic, plasma membrane, and nuclear features of apoptosis. These data establish PT as a central event of the apoptotic cascade. In addition, we addressed the question as to whether proteases from the ICE/Ced3 family act upstream of mitochondria. Indeed, inhibition of such proteases prevents the apoptotic  $\Delta\Psi_m$  dissipation, suggesting that they act on a level that is hierarchically superior to the Bcl-2/PT control point of apoptosis.

## Materials and Methods

**Culture Conditions and Apoptosis Induction.** Thymocytes were obtained from female BALB/c mice (6–10 wk old) and were cultured in the absence or presence of dexamethasone (DEX; 1  $\mu\text{M}$ ), protoporphyrin IX (PPIX; 30  $\mu\text{M}$ ), cycloheximide (35  $\mu\text{M}$ ), actinomycin D (3  $\mu\text{M}$ ), the protease inhibitor *N*-tosyl-L-lysyl chloromethylketone ([TLCK] 50  $\mu\text{M}$ ; all reagents from Sigma Immunochemicals, St. Louis, MO), the inhibitor of interleukin-1-converting enzyme (ICE)-like proteases *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk; Enzyme Systems Products, Dublin, CA), RU-38486 (10  $\mu\text{M}$ ; kindly provided by Roussel Uclaf, Romainville, France), and/or bongkreik acid (BA), purified as described (14) and kindly provided by Dr. Duine (Delft University of Technology, Delft, The Netherlands). The standard dose of BA was 50  $\mu\text{M}$  during 4–12 h, followed by assessment of mitochondrial or nuclear parameters of apoptosis. Alternatively, apoptosis was induced in thymocytes from C57Bl/6 mice that were rendered deficient for p53 by homologous recombination (kindly provided by F. Radvanyi, Institut Curie, Paris, France). Thymocytes from p53<sup>-/-</sup> animals (15) or p53<sup>+/+</sup> littermates were irradiated (10 Gy) or cultured in the presence of etoposide (10  $\mu\text{M}$ ; Sigma) or DEX (10  $\mu\text{M}$ ).

**Cytofluorometric Analysis of Mitochondrial Transmembrane Potentials, Reactive Oxygen Species, and Nonoxidized Glutathione.** To measure  $\Delta\Psi_m$  and superoxide anion generation, cells ( $5 \times 10^5/\text{ml}$ ) were incubated with 3,3'-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>(3), 40 nM in PBS; Molecular Probes, Inc., Eugene, OR) (3) and dihydroethidine (HE; 2  $\mu\text{M}$ ) (8) for 15 min at 37°C followed by analysis on an Epics cytofluorometer (Coulter Corp., Hialeah, FL) while gating on all cells. Levels of intracellular thiols (mainly nonoxidized glutathione) were quantitated using monochlorobimane (MCB, 40  $\mu\text{M}$ ; Molecular Probes) as a specific probe (16). As a negative control, cells were incubated during 30 min with the glutathione-depleting agent *N*-ethylmaleimide (50  $\mu\text{M}$ ; Sigma).

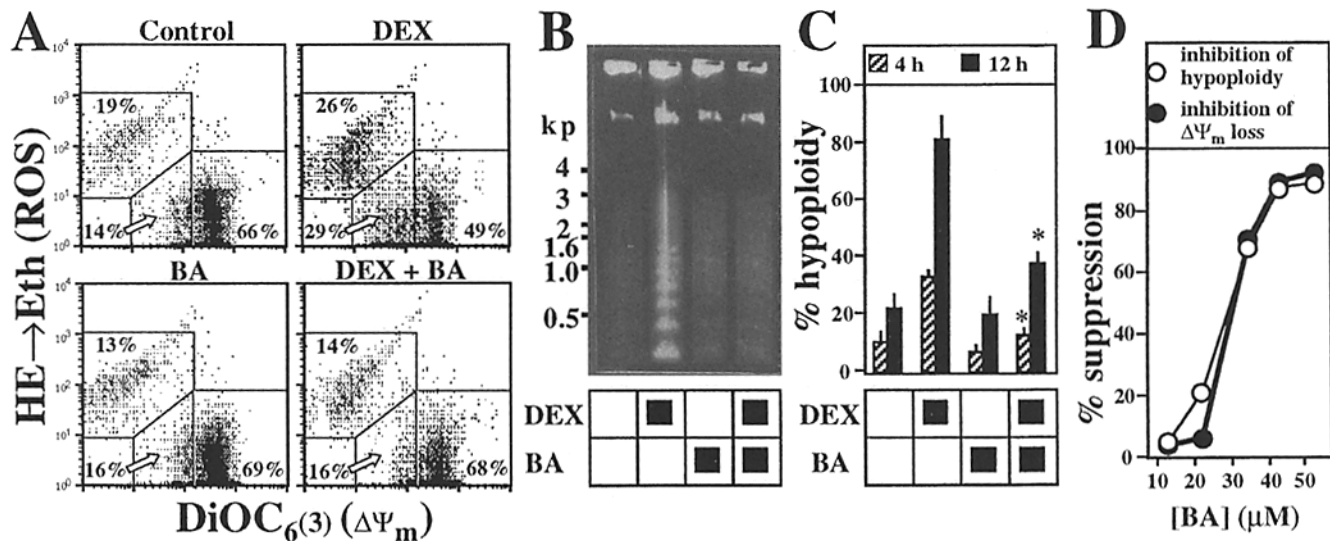
**Analysis of Nuclear DNA Loss, Cell Viability, Phosphatidylserine Exposure, and Electron Microscopy.** The frequency of subdiploid cells was determined by propidium iodide (PI) staining of ethanol-permeabilized cells. The apoptosis-associated increase in membrane permeability for propidium iodide was assessed as described (3). An FITC-annexin V conjugate (1/400 dilution, 1  $\mu\text{g}/\text{ml}$ , 15 min at 4°C; Brand Applications, Maastricht, The Netherlands) with high affinity for PS was used for the assessment of aberrant PS exposure (17). Cells were labeled simultaneously with ethidium bromide (EthBr; 200 ng/ml) and FITC-annexin V, and data are shown for viable (EthBr<sup>-</sup>) cells. DNA fragmentation ( $10^6$  cells/lane) was determined by horizontal agarose gel electrophoresis (3). For electron microscopy, cells were prepared by glutaraldehyde fixation (2.5% in 0.1 M cacodylate buffer, pH 7.2, 2 h at room temperature), washed twice in cacodylate buffer, fixed in 2% OsO<sub>4</sub>, encapsulated in agar, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were floated successively on uranyl acetate and lead citrate solutions before being observed in an AEI6B electron microscope at 60 kV.

**Determination of NF $\kappa$ B Translocation.** Nuclear extracts were prepared according to standard protocols (18). For the gel retardation assay, nuclear proteins (30  $\mu\text{g}$ ) were preincubated with 10  $\mu\text{g}$  salmon sperm DNA (GIBCO BRL, Cergy, France) for 15 min on ice before addition of the <sup>32</sup>P-labeled NF $\kappa$ B oligonucleotide probe (5' ACAAGGGACTTTCCGCTGGGGACTTTCCAG 3'; 2–10  $\times 10^5$  dpm/ng). Protein-bound oligonucleotide was separated from the free probe using a spin column (GIBCO BRL). 2  $\mu\text{l}$  of 0.1% bromophenol blue and 25  $\mu\text{l}$  of the mixture were loaded onto 6% polyacrylamide gel. Electrophoresis was performed during 100 min at 150 V in tris-borate EDTA buffer, followed by autoradiography. Nuclear extracts from THP1 cells that were stimulated during 60 min with 20 ng/ml of LPS served as positive controls. As a specificity control, nuclear extracts were incubated in a 1,000-fold excess of unlabeled NF $\kappa$ B oligonucleotide probe.

## Results and Discussion

*BA, an Inhibitor of Mitochondrial Permeability Transition, Suppresses the DEX-induced  $\Delta\Psi_m$  Reduction and Apoptosis.* BA is known to affect the molecular conformation of the ANT, thereby reducing the probability of PT pore gating (12, 13). We have used this PT inhibitor to address the significance of PT in thymocyte apoptosis. As shown in Fig. 1 A, BA inhibits the DEX-induced  $\Delta\Psi_m$  loss. Concomitantly, BA prevents the DEX-induced oligonucleosomal DNA fragmentation (Fig. 1 B) and nuclear DNA loss (Fig. 1 C), indicating that inhibition of PT can prevent the nuclear manifestations of apoptosis in intact cells. Transmission electron microscopy revealed that BA, when added together with DEX, reduced significantly the number of cells showing chromatin condensation, as compared to cells treated with DEX alone (Fig. 1 E). The dose of BA required to inhibit chromatinolysis is the same as that which is necessary to prevent the DEX-induced  $\Delta\Psi_m$  reduction (Fig. 1 D), underscoring the functional linkage between both phenomena. In conclusion, BA inhibits the DEX-induced manifestations of apoptosis, both at the level of mitochondria and at the level of chromatin structure.

*Effect of BA on Metabolic and Plasma Membrane Manifestations of Apoptosis.* In addition to inhibiting nuclear apop-

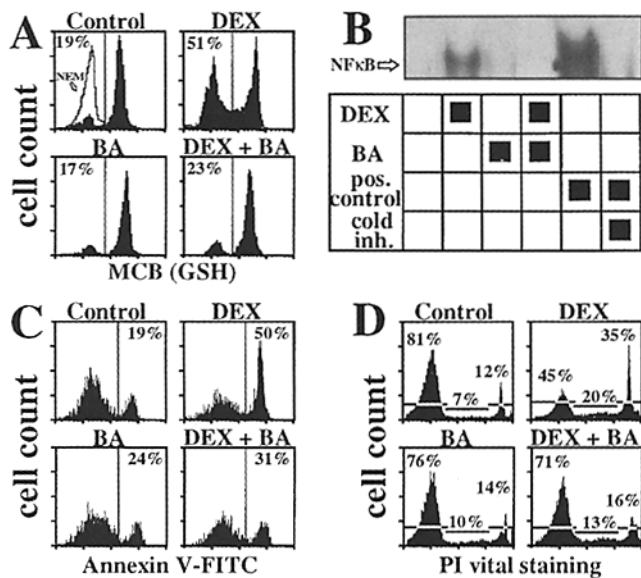


**Figure 1.** BA inhibits the DEX-induced  $\Delta\Psi_m$  disruption and nuclear apoptosis of thymocytes. Thymocytes were cultured in the presence or absence of DEX (1  $\mu\text{M}$ ) and/or BA (standard dose = 50  $\mu\text{M}$ ), and the following parameters were assessed after 4 h of culture: DiOC<sub>6</sub>(3)/HE staining for the simultaneous assessment of  $\Delta\Psi_m$  and superoxide anion generation (A), DNA fragmentation (B), DNA hypoploidy ( $x \pm \text{SEM}$ ,  $n = 3$ ) (C), and apoptotic ultrastructure (E). DNA hypoploidy (C) was also measured 12 h after initiation of cultures. In addition, the BA dose necessary to antagonize the DEX-triggered  $\Delta\Psi_m$  loss, and chromatinolysis was determined (D). The dose-response curve quantifies the effects of BA on DEX-induced  $\Delta\Psi_m$  reduction and hypoploidy (background values subtracted, values for 4 h as in A and C). Results are representative for five independent experiments. Arrows in E indicate vacuolization of the cytoplasm induced by DEX treatment. Bars, 10  $\mu\text{M}$ .

tosis, BA antagonizes the DEX-induced depletion of intracellular glutathione levels (Fig. 2 A) (16), as well as the DEX-triggered hypergeneration of superoxide anions (Fig. 1 A). This latter result is coherent with the  $\Delta\Psi_m$ -stabilizing effect of BA, since superoxide radicals are formed on the uncoupled respiratory chain, after PT has occurred (8). In accord with its effect on the cellular redox potential, BA prevents the DEX-stimulated NF $\kappa$ B translocation (Fig. 2 B). BA also abolishes the membrane exposure of phosphatidylserine residues (Fig. 2 C) provoked by DEX, and thus prevents one of the principal membrane features of early apoptosis (17, 19). BA also prevents the later loss of cell vi-

ability affecting DEX-treated thymocytes (Fig. 2 D) and abolishes cytoplasmic features of apoptosis such as vacuolization (Fig. 1 E). In conclusion, prevention of apoptosis-associated PT by BA does not only impede the  $\Delta\Psi_m$  reduction, but also prevents apoptotic changes at the level of the cytoplasm and the plasma membrane.

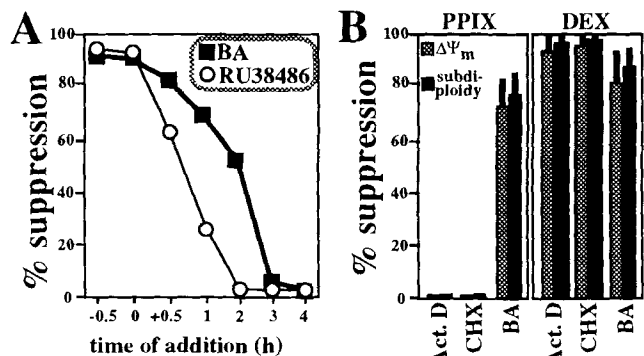
*BA Does Not Act by Quenching Early Activation Signals.* BA is effective in inhibiting chromatinolysis, even when it is added up to 2 h after DEX addition (Fig. 3 A), i.e., at a time point at which the glucocorticoid receptor antagonist RU38486 fails to interfere with apoptosis, indicating that BA does not act by quenching of early glucocorticoid re-



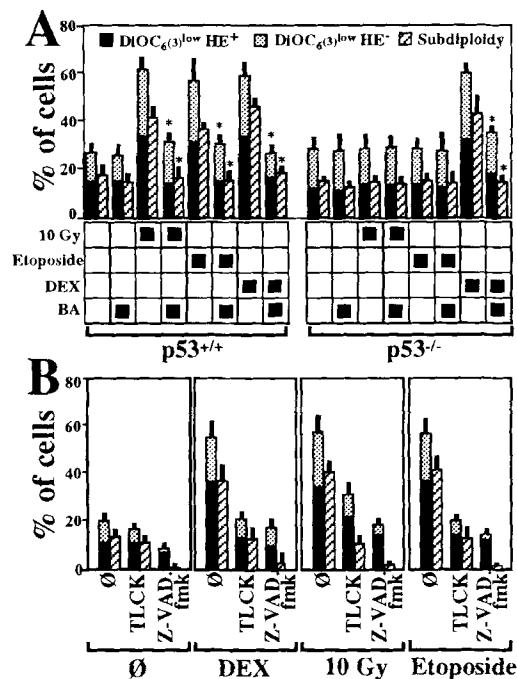
**Figure 2.** BA prevents the biochemical features of DEX-induced apoptosis. Cells were cultured as in Fig. 1 and the following parameters were assessed: cellular content of MCB-reactive thiols (A), NFκB translocation (B), exposure of phosphatidylserine residues on the cell surface at 4 h (C), and cell viability at 12 h (D). For determination of MCB-reactive thiols (A), cells were stained with MCB and analyzed by cytofluometry. Percentages refer to the frequency of MCB<sup>low</sup> cells. Thymocytes treated with the glutathione-depleting agent N-ethylmaleimide served as negative controls. NFκB translocation (B) was determined by gel retardation assays of nuclear extracts incubated with a radiolabeled oligonucleotide binding to NFκB. Nuclear extract from LPS-activated THP1 cells served as a positive control, and cold inhibition by a 1,000-fold excess of cold oligonucleotide as a specificity control. Phosphatidylserine exposure (C) was determined by staining of viable (EthBr<sup>-</sup>) cells with FITC-labeled recombinant annexin V. Percentages refer to the frequency of annexin V staining cells among the EthBr<sup>-</sup> population. Cell viability (D) was determined by staining with PI. The percentages of PI<sup>low</sup>, PI<sup>intermediate</sup>, and PI<sup>high</sup> cells are given.

ceptor-mediated activation signals. Moreover, BA does not inhibit apoptosis via inhibition with macromolecular synthesis. This is suggested by the fact that BA effectively prevents the  $\Delta\Psi_m$  disruption and chromatinolysis of thymocytes responding to PPIX (Fig. 3 B), a substance that induces PT directly by acting on a mitochondrial receptor (20). PPIX-induced apoptosis is not affected by inhibitors of RNA or protein synthesis, yet is fully antagonized by BA (Fig. 3 B). Thus, a mode of apoptosis induction that does not require macromolecule synthesis is efficiently antagonized by BA.

**Dissociation between BA-modulated and p53-mediated Apoptotic Control.** To determine the putative relationship between the p53-mediated control and the mode of action of BA, we studied whether BA would conserve its apoptosis-inhibitory effect in thymocytes from mice homozygous for deletion mutations of the antioncogene *p53* (*p53*<sup>-/-</sup>). As shown in Fig. 4 A, thymocytes from normal C57Bl/6 mice irradiated with 10 Gy exhibit chromatinolysis associated with  $\Delta\Psi_m$  reduction. Control experiments confirmed that thymocytes from mice homozygous for deletion mutations of the antioncogene *p53* (*p53*<sup>-/-</sup>) fail to undergo apoptosis



**Figure 3.** Mechanisms of BA-mediated inhibition of DEX-induced thymocyte apoptosis. (A) Time dependency of the apoptosis-inhibitory BA effect. Thymocytes were cultured during 30 min in medium alone. Thereafter, DEX (1  $\mu$ M) was added to cultures (time point 0), and hypoploidy was determined 6 h later. At different time points, BA (50  $\mu$ M) or RU38486 were added to cultures. Control cultures of thymocytes maintained in the presence or absence of BA and/or RU38486 displayed hypoploidy values ranging between 10 and 16%. Cells that were kept with DEX only exhibited hypoploidy values of  $53 \pm 4\%$ . (B) Effect of BA on PPIX-induced apoptosis. Thymocytes were cultured with PPIX (30  $\mu$ M) or DEX (1  $\mu$ M) in the presence of actinomycin D (3  $\mu$ M), cycloheximide (35  $\mu$ M), or BA (50  $\mu$ M), followed by the determination of mitochondrial and nuclear parameters of apoptosis after 4 h of culture. PPIX induced a significant increase in the  $\Delta\Psi_m^{\text{low}}$  cells ( $39 \pm 4\%$  above control values) and hypoploid cells ( $28 \pm 4\%$ ).



**Figure 4.** Relationship between  $\Delta\Psi_m$  and the p53 or protease check points of apoptotic regulation. (A) Effects of BA on p53-dependent apoptosis. Thymocytes recovered from *p53*<sup>-/-</sup> and control mice (*p53*<sup>+/+</sup>) were irradiated (10 Gy) as indicated and cultured during 4 h in the presence of etoposide (10  $\mu$ M), DEX (1  $\mu$ M), and/or BA (50  $\mu$ M), followed by determination of mitochondrial and nuclear signs of PCID. Results are representative for three different experiments. (B) Effect of protease inhibitors on mitochondrial and nuclear features of apoptosis. Thymocytes from *p53*<sup>+/+</sup> BALB/c mice were cultured during 4 h after irradiation, addition of DEX or etoposide, in the presence of the serine protease inhibitor TLCK (50  $\mu$ M) or Z-VAD.fmk (50  $\mu$ M), followed by determination of  $\Delta\Psi_m$ , ROS generation, or hypoploidy.

in response to irradiation and the topoisomerase II inhibitor etoposide (21, 22). Such  $p53^{-/-}$  thymocytes also fail to exhibit signs of irradiation- or etoposide-induced  $\Delta\Psi_m$  reduction, suggesting that p53 controls a common event upstream of both apoptotic  $\Delta\Psi_m$  dissipation and of nuclear apoptosis. Addition of BA to  $p53^{+/+}$  thymocytes prevents both the  $\Delta\Psi_m$  reduction and the nuclear signs of apoptosis induced by irradiation or etoposide (Fig. 4 A). Moreover, BA prevents the DEX-induced death both in  $p53^{-/-}$  and in  $p53^{+/+}$  thymocytes, indicating a complete dissociation between the BA- and the p53-regulated checkpoints of apoptosis.

**Relationship between Apoptotic  $\Delta\Psi_m$  Disruption and Proteases.** Different types of proteases have been implicated in the control of apoptosis, at the level of the initial triggering event, as well as at the level of the late effector phase (9). To determine whether ICE-like proteases might act upstream of PT-mediated  $\Delta\Psi_m$  disruption, thymocytes were exposed to apoptosis inducers such as DEX, etoposide or  $\gamma$ -irradiation, in the presence or absence of protease inhibitors. The cysteine protease inhibitor Z-VAD.fmk, which acts on ICE-like proteases (23, 24), prevents both the  $\Delta\Psi_m$  reduction and nuclear apoptosis that are induced by these stimuli (Fig. 4 B). Similarly, the serine protease inhibitor TLCK inhibits thymocyte apoptosis, as described (25), and also prevents  $\Delta\Psi_m$  reduction (Fig. 4 B). Since TLCK and specific inhibitors of ICE-like proteases and CPP32 do not prevent the mitochondrial release of a proapoptotic factor nor its action on isolated nuclei in a cell-free system (5), it appears that such proteases act upstream rather than downstream of the PT/Bcl-2 control point of apoptosis.

**Concluding Remarks.** In the present paper, we demon-

strate that the PT-inhibitory substance BA does not only prevent the apoptotic  $\Delta\Psi_m$  disruption, but that it also abolishes a number of nuclear, cytoplasmic, and plasma membrane changes that characterize apoptosis. Thus, much as the mitochondrial oncoprotein Bcl-2, BA has an antioxidant effect (in the sense that it prevents glutathione depletion, superoxide anion generation, and NF $\kappa$ B translocation; 26) and inhibits PS exposure on the plasma membrane (19). These data underline the probable involvement of PT as a critical coordinating event of the apoptotic cascade. Our data also suggest that the BA-inhibitible PT control point is completely independent of p53 and that PT occurs secondary to the activation of certain cysteine and serine proteases. This does not exclude that some proteases are activated downstream of PT. In particular, it appears that the mitochondrial intermembrane factor(s) released upon PT, which cause(s) apoptosis in isolated nuclei, is/are endowed with some protease activity or can activate nuclear proteases. This assumption is based on the observation that several unidentified nuclear proteins become degraded after incubation of isolated nuclei with mitochondrial factor(s) (our unpublished observation). However, it is clear that some critical proteases control a checkpoint upstream of PT, since this is also suggested by the very rapid kinetics of protease activation in some models of apoptosis (27). At the present time, little is known about the mechanisms by which biochemical pathways PT is induced in cells that are undergoing apoptosis. Nevertheless, the present study indicates that in addition to protease cascades, mitochondrial PT may fulfill a major role in coordinating the death pathways that invariably accompany late-stage apoptosis.

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