Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis

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Abstract **In a number of experimental systems, the early stage of the apoptotic process, i.e. the stage which precedes nuclear disintegration, is characterized by the breakdown of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$). Here we address the question as to whether mitochondrial permeability transition (PT) pores may account for the $\Delta\Psi_m$ dissipation in lymphocyte apoptosis. Drugs known for their PT-inhibitory potential (bongkrekic acid, cyclosporin A, and the non-immunosuppressive cyclosporin A analogue *N*-methyl-Val-4-cyclosporin A) are capable of preventing the apoptotic $\Delta\Psi_m$ disruption. Moreover, pharmacological modulation of PT-mediated $\Delta\Psi_m$ dissipation can prevent apoptosis. Thus, while suppressing the $\Delta\Psi_m$ disruption, bongkrekic acid also inhibits the apoptotic chromatinolysis. In conclusion, these data are compatible with the hypothesis that apoptotic $\Delta\Psi_m$ disruption is mediated by the formation of PT pores and that PT-mediated $\Delta\Psi_m$ disruption is a critical event of the apoptotic cascade.**

Key words: T lymphocyte; Apoptosis; Mitochondrial transmembrane potential; Permeability transition; Programmed cell death

1. Introduction

Cells undergoing apoptosis exhibit a decrease in $\Delta\Psi_m$ which precedes nuclear signs of apoptosis [1–7]. This applies to nerve cell growth factor-deprived sympathetic neurons [1], p53-mediated cell death of fibroblasts [2], lymphocytes exposed to glucocorticoids or lethal antigen receptor-mediated activation signals [3,4,6,8], and tumor necrosis factor-stimulated U937 cells [5,8]. Purification of cells with an abnormally low $\Delta\Psi_m$ ($\Delta\Psi_m^{low}$ cells) but still intact nuclei reveals that a reduction in $\Delta\Psi_m$ accompanies an already irreversible step of apoptosis. Thus, $\Delta\Psi_m^{low}$ cells but not $\Delta\Psi_m^{high}$ controls proceed to DNA fragmentation within 1 h of in vitro culture and later lose viability, even when the apoptosis inducing stimulus is withdrawn [3]. Apoptotic $\Delta\Psi_m$ disruption is accompanied by an immediate shutdown of mitochondrial biogenesis [9]. Accordingly, both the transcription of the mitochondrial genome [10] and the synthesis of mitochondrial proteins [2] are perturbed early during the apoptotic process. Another consequence of $\Delta\Psi_m$ disruption is the uncoupling of oxidative phosphorylation [2] and the generation of superoxide anion on the uncoupled respiratory chain [8]. Loss of mitochondrial function is also observed in anucleate cells induced to undergoprogrammed cell death [11,12], indicating that apoptotic alterations of mitochondrial function can occur in complete independence of the nucleus.

Taken together, these observations suggest that early alterations of mitochondrial function may be important for the apoptotic process. In accord with this speculation, mitochondria are required in some cell-free systems to induce nuclear apoptosis [13]. To maintain the $\Delta\Psi_m$, the inner mitochondrial membrane must be essentially impermeable for electrolytes. An abundant literature (reviewed in [14,15]) has established that experimental manipulation of isolated mitochondria can result in the formation of protaceous pores in the inner mitochondrial membrane that allow for the free distribution of solutes <1500 Da, thereby leading to the immediate dissipation of the $\Delta\Psi_m$. These pores are referred to as mitochondrial 'megachannels' or 'permeability transition' (PT) pores [14,15]. Recently, formation of PT pores has been involved in the post-ischemic or toxin-mediated death of myocardial cells and hepatocytes [16–22]. Prompted by these studies, we addressed the question of whether PT might be responsible for the apoptotic $\Delta\Psi_m$ disruption. To address this problem we have used two well-studied inhibitors of PT: cyclosporin A and bongkrekic acid, a ligand of the adenine nucleotide translocator. We show here that these PT inhibitors prevent apoptotic $\Delta\Psi_m$ dissipation. Moreover, we show that prevention of apoptotic $\Delta\Psi_m$ dissipation can actually inhibit apoptotic chromatinolysis. These data suggest that PT may be involved in the apoptotic cascade.

2. Materials and methods

2.1. Culture conditions and apoptosis induction

Female BALB/c mice (6–10 weeks) received intraperitoneal (i.p.) injections of dexamethasone (DEX; 1 mg; Sigma, St. Louis, MO) or PBS as a vehicle control (200 μl) [23]. Splenocytes were prepared on ice, depleted from erythrocytes by hypotonic lysis [24], and maintained in complete culture medium (RPMI 1640 medium supplemented with 10% FCS, L-glutamine, HEPES and antibiotics) at 0 to 4°C until labeling and analysis. Proliferation assays were performed on concanavalin A-stimulated (2 μg/ml; Sigma) BALB/c splenocytes (2×10^5 in 200 μl medium, triplicates) cultured at 37°C for 72 h in Nunc flat bottom 96 microtiter plates. For the assessment of DNA synthesis, cells were harvested after an 18 h pulse label with 1 μCi of [^{3}H]thymidine (Amersham, London, UK).

2.2. Cytofluorometric analysis of mitochondrial transmembrane potentials, ROS generation, and nuclear DNA loss

To evaluate mitochondrial transmembrane potentials ($\Delta\psi_m$) and superoxide anion generation, cells (5×10^5/ml) were incubated with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$; Molecular Probes; 40 nM in PBS) [3] and dihydroethidine (HE, 2 μM) [8] for 15 min at 37°C followed by analysis on an Epics cytofluorometer (Coulter, Miami, FL). Forward and side scatters were gated on the major

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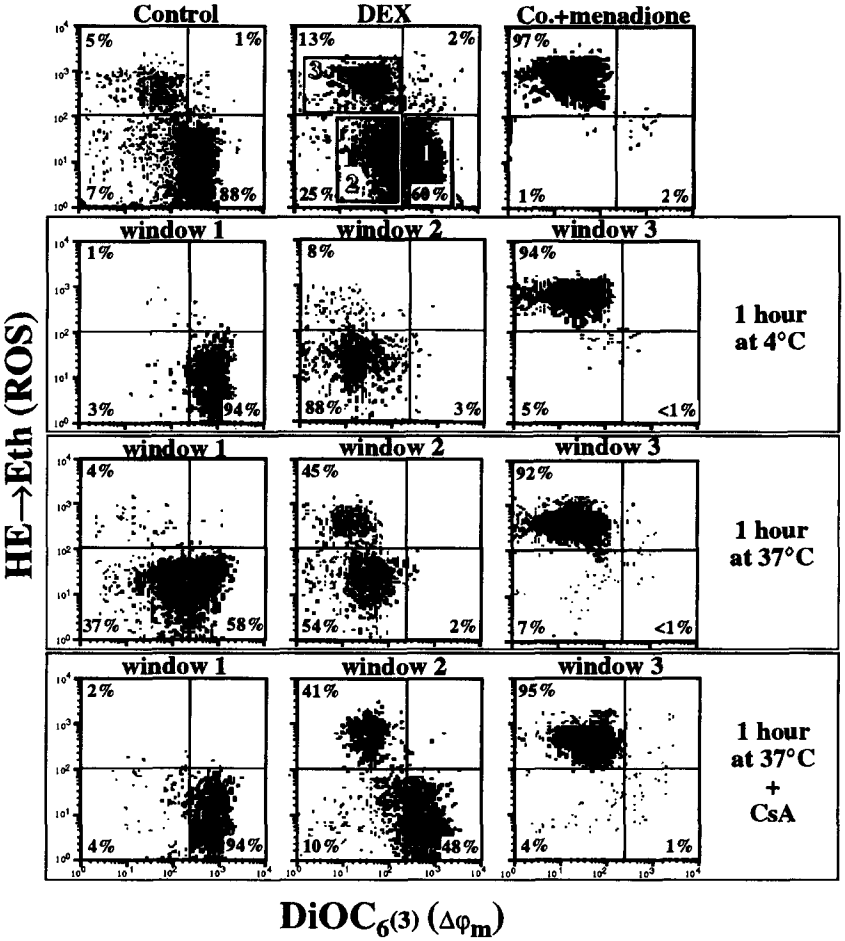


Fig. 1. Effect of CsA on the $\Delta\Psi_m$ reduction accompanying early lymphocyte apoptosis. Splenocytes from DEX-primed BALB/c mice (1 mg i.p., 12 h before analysis) or vehicle controls were labelled with a mixture of $DiOC_6(3)$ (that measures $\Delta\Psi_m$) and HE (that measures superoxide radical generation) for 15 min at 37°C immediately before cytofluorometric analysis ($DiOC_6(3)$ as green fluorescence and HE→Eth conversion as red fluorescence). Purified cells from windows 1–3 were kept for 60 min at 4 or 37°C, in the presence or absence of CsA (10 μM), followed by relabeling and reanalysis under identical conditions. Control cells were cultured during 30 min with menadione (1 mM at 37°C) followed by $DiOC_6(3)$/HE labelling (upper right quadrant). Results are representative of four independent experiments.

population of normal-sized lymphoid cells. In control experiments, cells were incubated with menadione (1 mM, Sigma), a drug that generates superoxide anion and disrupt the $\Delta\psi_m$. The frequency of cells having undergone chromatinolysis (subdiploid cells) was determined by ethanol permeabilization, followed by propidium iodine (PI) staining [25].

2.3. *Functional studies of purified cell populations*

For cytofluorometric purification, splenocytes were first depleted from cellular debris by density gradient (Ficoll Hypaque, Pharmacia, Uppsala, Sweden) centrifugation, then labelled with $DiOC_6(3)$ and HE. Lymphocytes with normal size were gated on via the forward light scatter and cell populations were sorted on an Elite cytofluorometer (Becton and Dickinson, San José, CA; windows as in Fig. 1) to be recovered in complete medium on ice. Cells were incubated in the presence or absence of cyclosporin A (1–10 μM, Sandoz), *N*-methyl-Val-4-cyclosporin A (SDZ 220-384, kindly provided by Dr. Roland Wenger, Sandoz AG, Basel, Switzerland) and/or bongkrekic acid (BA; purified as described in [26] and kindly provided by Dr. Duine; Delft University of Technology, Delft, Netherlands) for 15 min at 4°C, followed by a brief culture period (1 h at 4 or 37°C), relabeling with $DiOC_6(3)$ and HE (see above) and reanalysis on the Elite cytofluorometer. Alternatively, cells were cultured for 4 h in the absence or presence of 50 μM BA, followed by ethanol fixation and PI staining.

3. Results and discussion

3.1. *A cyclosporin A-inhibitable step of $\Delta\Psi_m$ reduction accompanies apoptosis*

12 h after injection of a lymphocyte-depleting dose of the glucocorticoid receptor agonist dexamethasone (DEX), splenic cellularity decreases. One particularity of this in vivo system of apoptosis induction is that DEX-primed splenocytes fail to exhibit alterations associated with late apoptosis such as chromatinolysis and DNA fragmentation. This can be explained by the effective elimination of dying lymphocytes before end-stage apoptosis is attained [23]. When compared to sham-treated controls, a relatively high percentage of DEX-primed splenocytes exhibits a low $\Delta\Psi_m$, as measured by means of the cationic lipophilic fluorochrome $DiOC_6(3)$ ([3] and Fig. 1). Such $\Delta\Psi_m^{low}$ cells can be subdivided into two subpopulations, one subpopulation which behaves like $\Delta\Psi_m^{high}$ cells in the sense that it is incapable of oxidizing hydroethidine (HE) to the fluorescent product ethidium (Eth), and another which is characterized by even lower $\Delta\Psi_m$ levels and an enhanced capacity of HE→Eth conversion (HE^+ cells). This HE→Eth conversion reflects mitochondrial superoxide anion generation

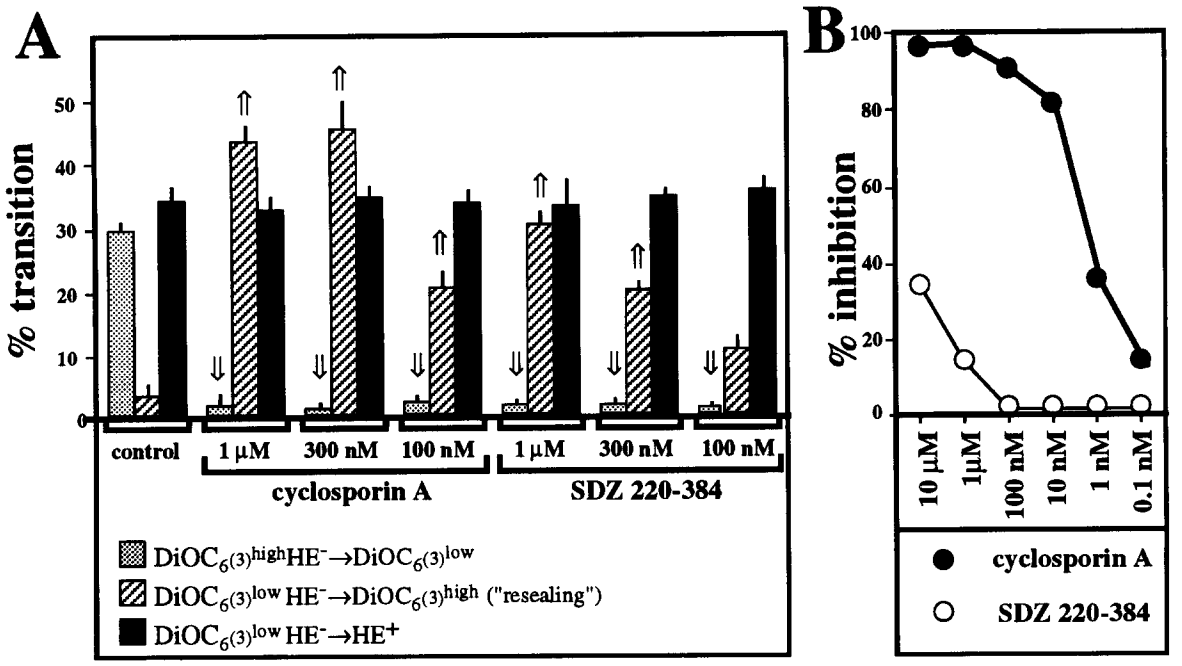


Fig. 2. Comparison of the $\Delta\Psi_m$-modulatory and immunosuppressive effects of CsA and of *N*-methyl-Val-4-CsA. (A) Effect of CsA and *N*-methyl-Val-4-CsA (SDZ 220-384) on the $\Delta\Psi_m$ reduction accompanying DEX-induced apoptosis. DEX-primed splenocytes were sorted cytofluorometrically into three normal-sized populations: $DiOC_6(3)^{high}HE^-$, $DiOC_6(3)^{low}HE^-$, and $DiOC_6(3)^{low}HE^+$ cells, followed by in vitro culture (1 h, 37°C), relabeling and reanalysis (manipulation similar to that in Fig. 1). The transition to the immediately posterior stage of pre-apoptosis is indicated ($X \pm$ S.E.M. of triplicates). Moreover, the 'resealing effect' (restablishment of a normal $DiOC_6(3)$ incorporation among $DiOC_6(3)^{low}HE^-$ cells) is quantitated (hatched columns). Background values obtained in control cultures kept at 4°C (Fig. 1A) were subtracted from experimental values. (B) Dose response of proliferation-inhibitory effects of CsA and *N*-methyl-Val-4-CsA. Mouse splenocytes were stimulated with the T cell mitogen concanavalin A (2 μg/ml) in the presence of the indicated amount of CsA or *N*-methyl-Val-4-CsA. Results are expressed as % inhibition of [^{3}H]thymidine uptake. Unsuppressed control values were $44 \pm 5 \times 10^4$ cpm.

[27], since inhibition of the respiratory chain abolishes HE-→Eth conversion [3,8]. Purification of these cell populations in a fluorescence-activated cell sorter, followed by short-term in vitro culture at 37°C (not at 4°C), relabeling and reanalysis, reveals the following developmental sequence: $DiOC_6(3)^{high}HE^- \rightarrow DiOC_6(3)^{low}HE^- \rightarrow DiOC_6(3)^{low}HE^+$. We have tested the effect of cyclosporin A (CsA) on this series of events and observed that CsA markedly inhibits the advancement of $DiOC_6(3)^{high}HE^-$ to the $DiOC_6(3)^{low}HE^-$ stage. In contrast, CsA fails to affect the transition from the $DiOC_6(3)^{low}HE^-$ to the $DiOC_6(3)^{low}HE^+$ stage. However, it does cause a $\Delta\Psi_m$ increase in a significant percentage of $DiOC_6(3)^{low}HE^-$ cells, which thus re-acquire a normal, $DiOC_6(3)^{high}HE^-$ phenotype (Fig. 1).

These observations recall the known effect of CsA on 'permeability transition' (PT) of isolated mitochondria, as well as of mitochondria in anoxia-damaged cells. CsA is considered as a prototypical inhibitor of mitochondrial PT [14,28–30]. Moreover, CsA causes a phenomenon of mitochondrial membrane hyperpolarisation [31] and 'resealing' [30], i.e. it closes already open PT pores of isolated mitochondria and thus allows for the restablishment of an intact electrochemical gradient.

3.2. *A non-immunosuppressive cyclosporin analog inhibits the apoptosis-associated* $\Delta\Psi_m$ *loss*

The PT-inhibitory effect of CsA is not mediated via calcineurin [14,28–30,32], one of the principal target molecules responsible for immunosuppressive CsA effects [33]. Thus, a

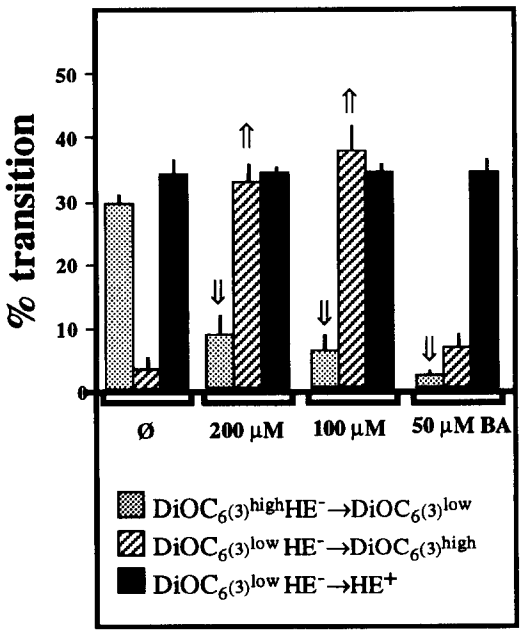


Fig. 3. Effect of bongkrekic acid (BA) on the $\Delta\Psi_m$ reduction accompanying DEX-induced apoptosis. DiOC6(3)/HE-labelled splenocytes from DEX-primed mice belonging to three different subpopulations were purified and cultured for 1 h at 37°C in the presence or absence of the indicated doses of BA.

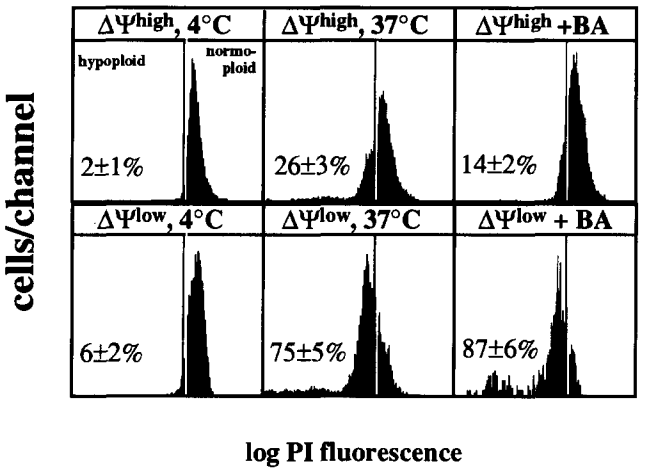


Fig. 4. Effect of BA on purified cells with different $\Delta\Psi_m$ values. $DiOC_6(3)^{high}HE^-$ and $DiOC_6(3)^{low}HE^-$ splenocytes from a DEX-primed mouse were purified as in Fig. 1, followed by culture of cells at 4 or 37°C for 4 h, ethanol fixation and determination of DNA content after labelling with propidium iodine. During this period of culture, cells were kept in the presence or absence of BA. Percentages refer to the portion of hypoploid cells in each preparation (range of duplicates). Results are representative of three independent experiments.

synthetic CsA derivative, *N*-methyl-Val-4-cyclosporin A (SDZ 220-384), which binds cyclophilin and inhibits PT [30] but has rather limited calcineurin-dependent effects [34], does inhibit the DEX-induced $\Delta\Psi_m$ loss of T lymphocytes and stimulates a resealing-like effect in $DiOC_6(3)^{low}HE^-$ cells (Fig. 2A). At the dose at which *N*-methyl-Val-4-CsA exerts these effects (300 nM), it does not inhibit T cell proliferation in vitro (Fig. 2B). In conclusion, the immunosuppressive and $\Delta\Psi_m$-modulatory effects of CsA can be dissociated. These observations are compatible with the idea that PT could constitute the mechanism by which pre-apoptotic cells lose their $\Delta\Psi_m$. Although CsA and its analogue are efficient inhibitors of apoptotic $\Delta\Psi_m$ reduction in short-term experiments (30 to maximally 60 min), it fails to maintain the $\Delta\Psi_m$ during longer incubation periods (not shown). This finding is again in accord with several studies [14,20,28] reporting that the pharmacological effects of CsA on PT are transitory. To demonstrate the involvement of PT in apoptosis, we thus were forced to employ an alternative inhibitor of PT, bongkrekic acid (BA) [35].

3.3. *Bongkrekic acid, a specific ligand of the adenine nucleotide translocator, inhibits the apoptotic $\Delta\Psi_m$ dissipation and inhibits the loss of nuclear DNA in $\Delta\Psi_m^{low}$ cells*

BA is known to affect the molecular conformation of the ANT, thereby reducing the probability of PT pore gating [36–40]. BA prevents the $\Delta\Psi_m$ reduction, as do CsA and *N*-methyl-Val-4-CsA (Fig. 3). As do the two other PT inhibitors, CsA and *N*-methyl-Val-4-CsA, BA induces a resealing-like phenomenon, i.e. it enhances the $\Delta\Psi_m$ of $\Delta\Psi_m^{low}$ cells, provided that these have still a normal HE^- phenotype (Fig. 3). This latter effect is only elicited at relatively high concentrations of BA ($\geq$100 μM), whereas the $\Delta\Psi_m$-stabilizing effect is observed at 50 μM. None of the PT inhibitors is capable of normalizing the $\Delta\Psi_m$ of HE^+ cells, nor do they impede mitochondrial ROS generation (Figs. 2A,3). In synthesis, this set of data is compatible with the hypothesis that opening of PT pores might account for the apoptotic $\Delta\Psi_m$ disruption.

If $\Delta\Psi_m^{low}$ DEX-primed splenocytes are purified cytofluorometrically (as described in Fig. 1, window 2) and cultured in vitro for 4 h at 37°C, they rapidly undergo apoptotic chromatinolysis, and this phenomenon is not inhibited by BA (Fig. 4). In contrast, BA partially inhibits the DNA fragmentation of purified $\Delta\Psi_m^{high}$ cells (Fig. 4). Thus, a $\Delta\Psi_m$-stabilizing dose of BA prevents nucleolysis in cells in which the $\Delta\Psi_m$ reduction has not yet occurred (Fig. 4).

3.4. *Concluding remarks*

As pointed out in section 1, $\Delta\Psi_m$ disruption appears to be the earliest alteration of cellular biochemistry that constitutes a constant feature of pre-apoptotic cells [1–7]. Here, we provide evidence suggesting that this $\Delta\Psi_m$ reduction is a consequence of mitochondrial PT, a phenomenon that has been studied for decades in isolated mitochondria without its physiological function having become clear [14,15]. The hypothesis that apoptotic $\Delta\Psi_m$ reduction would result from PT is supported by the finding that both CsA and bongkrekic acid (BA), two well-known inhibitors of PT, efficiently prevent the apoptosis-associated fall in $\Delta\Psi_m$. BA and cyclophilin ligands (CsA and *N*-methyl-Val-4-CsA) also restablish the $\Delta\Psi_m$ in pre-apoptotic cells, provided that they are still HE^-. In contrast, they fail to restore the $\Delta\Psi_m$ of HE^+ cells. The reasons for this selective effect are not clear. We have previously shown that HE-detectable generation of superoxide anion causes the oxidation of inner mitochondrial membrane cardiolipin [8], which, in turn, is essential for the function of at least one of the PT pore components, the ANT [41]. Thus, the HE^+ stage marks a truly irreversible stage of self-inflicted mitochondrial destruction.

As to the localization of the point-of-no-return of the apoptotic cascade, a number of observations indicate that this point coincidates with $\Delta\Psi_m$ disruption. This is suggested by the fact that mitochondria which are undergoing PT in vitro release a protein capable of inducing chromatin condensation and DNA fragmentation in isolated nuclei [42]. Moreover, $\Delta\Psi_m^{high}$ cells can be rescued from apoptosis by withdrawal of the apoptosis-inducing stimulus [3] or inhibition of $\Delta\Psi_m$ disruption (this paper), whereas the same manipulations do not delay the death of $\Delta\Psi_m^{low}$ cells. In conclusion, our data suggest that apoptotic $\Delta\Psi_m$ dissipation is mediated by PT and that interventions on PT can modulate apoptosis.

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