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Opposite role of changes in mitochondrial membrane potential in different apoptotic processes

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Abstract We have studied the role of changes in mitochondrial membrane potential ($\Delta\Psi$) in two widely-used models of apoptosis, such as dexamethasone-treated rat thymocytes and U937 human cells treated with tumor necrosis factor-α and cycloheximide. To dissipate $\Delta\Psi$, we used low concentrations of valinomycin, unable per se to induce apoptosis, and demonstrated that the decline in $\Delta\Psi$ exerts opposite effects in the two models. Indeed, in U937 cells, depolarization of mitochondria increased apoptosis, which decreased in rat thymocytes. This leads to the suggestion that disruption of $\Delta\Psi$ plays opposite roles depending on the experimental model. In U937 cells, the drop of $\Delta\Psi$ is a possible contributory cause for the apoptotic process; in rat thymocytes, it could be a limiting factor. We propose that these opposite effects could be due to the different ATP requirement of each apoptotic pathway.

Key words: Mitochondrion; Mitochondrial membrane potential; Apoptosis; Flow cytometry

1. Introduction

Mitochondria, the main cellular source of ATP, can play a crucial role during apoptotic cell death, being involved in several mechanisms, such as production of oxygen free radicals [1,2], control of Ca^{2+} ions [3], extrusion of apoptogenic molecules such as cytochrome *c* (cyt *c*) [4], or the so called apoptosis inducing factor [5]. As apoptosis is an active process, it is reasonable that, at least during the earliest phases, cells maintain a significant driving force for ATP synthesis and, consequently, a high mitochondrial membrane potential ($\Delta\Psi$). Indeed, using different experimental models, many authors found no early alterations of $\Delta\Psi$ [6–9]. Moreover, the ATP/ADP ratio is considered crucial for the decision of cell fate [10,11], and it seems that when the ATP/ADP ratio falls below the critical value of 0.2, cells undergo apoptosis [12]. Only when a severe drop of ATP occurs, apoptosis ceases and ushers in necrosis. In this perspective, prolonged mitochondrial depolarization would cause necrotic cell death.

A new role has been proposed for mitochondria during the induction of apoptosis: these organelles act as effectors of the central phase of apoptotic pathway, after which cells are irreversibly committed to die [13]. This suggests that the impairment of mitochondrial function and the consequent collapse in $\Delta\Psi$ represent the main stimulus for apoptosis to occur [14].

To give further insights into the role of mitochondria during apoptosis, we analyzed $\Delta\Psi$ changes in two widely used models: dexamethasone (DEX)-treated rat thymocytes or tumor necrosis factor-α (TNF-α)-treated U937 human cells. Using low concentrations of valinomycin (Val) to dissipate $\Delta\Psi$ [15], we observed opposite effects, as $\Delta\Psi$ collapse decreased apoptosis in thymocytes but increased it in U937 cells.

2. Materials and methods

2.1. Cell cultures and experimental procedures

U937 cells were cultured in complete medium (RPMI 1640 with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin) and kept at 37°C in a humidified atmosphere (5% CO_2 in air). Cells were collected during the log phase of growth, washed in phosphate-buffered saline (PBS), counted and adjusted at a density of 200 000/ml in complete medium. Cells were seeded in Petri dishes and kept at 37°C in a humidified atmosphere (5% CO_2 in air) for different periods. Time-course (3, 8 and 24 h) and dose-response experiments were performed using 100 nM, 1 μM and 10 μM Val (Sigma, St. Louis, MO, USA). In a further set of experiments, U937 cells were pre-treated with 4 μM cycloheximide (CHX) for 2 h and then with 50 IU/ml TNF-α for 6 h. In some experiments, after 1 h of incubation with TNF-α, U937 cells were added with 1 μM cyclosporin A (CsA, Sandoz, Basel, Switzerland).

Three week old Sprague–Dawley rats were killed by decapitation and thymus was rapidly excised. Thymocytes were prepared by gently pressing the organ against fine stainless steel screens, counted and adjusted at 5×10^6 cells/ml in complete medium; then were treated with 100 nM DEX (Sigma) for 18 h in the presence or not of 100 nM Val.

2.2. Flow cytometry

Cytofluorometric analyses were performed using a FACScan (Becton Dickinson, San José, CA, USA), on a minimum of 10 000 cells per sample. We assessed: (1) viability, by the propidium iodide (PI) exclusion test. Cells were collected, resuspended in 400 μl PBS, stained with 5 μg/ml PI and analyzed. Those with an intact plasma membrane (viable or apoptotic, but not necrotic) were negative for PI; (2) early

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Abbreviations: $\Delta\Psi$, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; Val, valinomycin; TNF-α, tumor necrosis factor-α; PI, propidium iodide; CsA, cyclosporin A; MPT, mitochondrial permeability transition

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apoptosis, by the exposure of phosphatidylserine, using FITC-conjugated annexin-V (ANX-V, Bender MedSystem, Wien, Austria). Briefly, 4×10^5 cells were resuspended in 200 μl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated in the dark with 5 μl of ANX-V for 10 min at room temperature. Samples were washed with binding buffer, resuspended in PBS, counterstained with 5 μg/ml PI to identify dead cells and analyzed; (3) late apoptosis, by the classic test of DNA hypodiploid peak revealed by PI. Cells were resuspended in hypotonic solution (0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml PI) and kept for 30 min at 4°C before the analysis; (4) $\Delta\Psi$, by the lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Molecular Probes, Eugene, OR, USA) [15]. Cells were stained with 2.5 μg/ml JC-1 and kept at room temperature for 10 min, washed twice, resuspended in 400 μl PBS and analyzed.

2.3. Confocal microscopy

To study the release of cyt *c* from mitochondria, U937 cells were stained with 50 nM MitoTracker Red CMX-Ros (MT, Molecular Probes) for the detection of $\Delta\Psi$ and incubated for 30 min at 37°C. Cells were then washed with cold PBS and fixed in 2% paraformaldehyde PBS, then permeabilized with ice-cold methanol. They were then labeled with monoclonal antibody (mAb) against cyt *c* (Pharmingen, San Diego, CA, USA) following standard procedures for intracellular antigen staining, and spun down on a glass slide. Confocal analysis was performed with a TCS 4D confocal microscope (Leica, Germany) equipped with an argon laser and managed with Scanware software. Images were obtained with a 63× magnification and further enlarged with an electronic zoom. Image analysis was performed using Confocal Assistant 4.02 (from Bio-Rad) and LView Pro 1.D2/32 software.

2.4. Western blot analysis

After 6 h of treatment, U937 cells were harvested and washed twice in PBS and then resuspended in 80–140 μl of RIPA buffer (Tris buffer pH 8 50 mM, NaCl 150 mM, sodium deoxycholate 0.5%, sodium dodecyl sulfate (SDS) 0.1%, Triton X-100 1%, EDTA 5 mM, PMSF 100 μg/ml, leupeptin, aprotinin and pepstatin 5 μg/ml) for protein extraction. Protein concentration was determined according to Bradford [16]. Protein extracts (40 μg) were subjected to electrophoresis in an SDS–polyacrylamide gel (8% for PARP, 12% for CPP32) and then transferred onto a nitrocellulose paper by electrophoresis in a Transblot chamber (Mini Protean II, Bio-Rad, Hercules, CA, USA). Proteins were identified using mAbs against PARP (Biomol Laboratories, Plymouth Meeting, PA, USA) and CPP32 (Transduction Laboratories, Lexington, KY, USA) and ECL-Western reagents (Amersham Corp.).

2.5. Intracellular ATP content determination

Cells were collected after 2 h of incubation, washed, counted and resuspended at the concentration of 5×10^6/ml in distilled water. Cells were then boiled for 1 min and spun down to eliminate membranes and DNA. ATP was measured in the supernatant using ATP Bioluminescence Assay kit CLS II (Boehringer Mannheim, Germany) and LKB 1250 Wallac luminometer (Finland).

2.6. Statistical analysis

Statistical analysis was performed using the paired Student's *t*-test.

3. Results

3.1. Low doses of Val provoke a prolonged mitochondrial depolarization but do not induce apoptosis, that was caused by high doses of Val, unable however to activate caspases

To study the effects of Val, we performed dose- and time-response analyses (Fig. 1, upper part). U937 cells were treated with increasing doses of Val for different periods of time, and the dose of Val we used to depolarize mitochondria (100 nM up to 1 μM) did not induce apoptosis per se even after 24 h of incubation, as assessed by confocal microscopy (not shown) and flow cytometry. However, a 100-fold higher dose (10 μM) induced cell death.

To ascertain whether such a proapoptotic effect of high

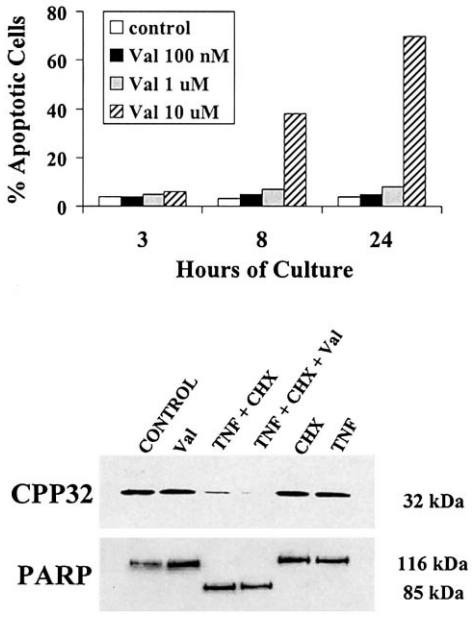

Fig. 1. Induction of apoptosis and caspase activity in Val-treated U937 cells. Upper part: analysis of U937 cells after 8 h of culture in the presence of different doses of Val. A dose-response study was performed using doses of Val ranging from 100 nM to 10 μM. Only the highest dose induces apoptosis. The histogram shows a typical experiment out of five, indicating the percentage of apoptotic cells, as assessed by flow cytometry after PI staining. Lower part: Western blot shows procaspase 3 (CPP32) and PARP cleavage after 6 h of incubation with the indicated substances.

doses of Val was caused by a direct action on ICE or other ICE-related proteases, we investigated the activation of the apoptosis-related caspase CPP32 and the degradation of a typical substrate for proteases such as PARP. We studied the cleavage of 32 kDa procaspase 3 and of 116 kDa PARP to the 85 kDa form by Western blot analysis in U937 cells (Fig. 1, lower part). Val was not able per se to induce CPP32 maturation and PARP cleavage, and the same was observed for TNF-α or for CHX alone. On the contrary, TNF-α plus CHX, and TNF-α plus CHX plus Val induced massive CPP32 maturation and PARP cleavage. Thus, an involvement of Val on the induction/processing of such apoptosis-related proteins can be excluded.

3.2. Effects of Val on apoptosis induced by TNF-α in U937 cells or by DEX in rat thymocytes

Table 1 reports the results concerning changes in $\Delta\Psi$, apoptosis and alteration of plasma membrane integrity in U937 cells or rat thymocytes. According to [17], CHX alone or TNF-α alone did not induce a notable cell death (data not shown). After 6 h of incubation, the percentage of apoptotic cells was significantly lower in samples treated with TNF-α plus CHX (26.5%) with respect to those treated also with 100 nM Val (42.8%, $P<0.01$). Consequently, an increase in the percentage of cells undergone secondary necrosis (permeant to PI) occurred. Since Val alone did not induce apoptosis (like TNF-α alone), we checked whether a possible synergistic effect of CHX and Val could exist, but together they were unable to induce apoptosis (not shown).

Concerning rat thymocytes, spontaneous $\Delta\Psi$ decrease and appearance of apoptotic cells were present after 18 h of cul-

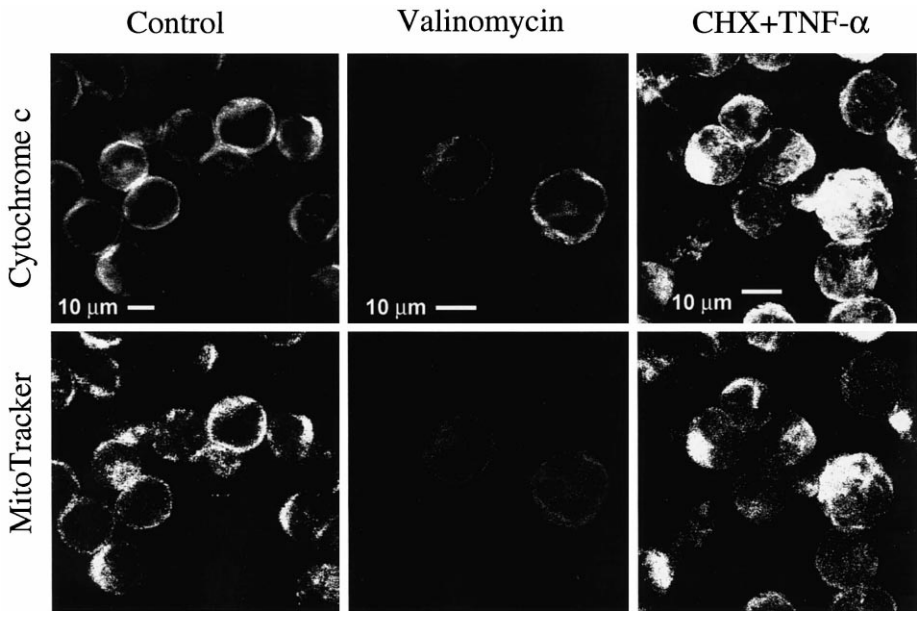

Fig. 2. Confocal analysis of cyt *c* release. Upper panels: U937 cells stained with anti-cyt *c* mAb, as revealed by a FITC-conjugate secondary antibody, showed a green fluorescence, which was collected by a 520 nm band-pass filter. Lower panels: the same cells counterstained with a $\Delta\Psi$-sensitive fluorescent probe, MT, showed a red fluorescence, related to $\Delta\Psi$, revealed by 599 nm band-pass filter. All images have been transformed in black and white using CAS software. Left panels: control, untreated cells. Central panels: cells treated with 100 nM Val. Right panels: cells treated with TNF-α plus CHX. Note that the fluorescence is widely spread into the cytoplasm of some cells (arrows), and markedly different from that of control or Val-treated cells. Also note that a marked decrease in $\Delta\Psi$ occurs only in cells treated with Val.

ture. Adding Val for such a period caused abundant cell death, even if we used low doses (100 nM). Nevertheless, for a shorter period of time (6 h), Val did not induce cell death (not shown). When used in combination with DEX for 18 h, Val partially decreased glucocorticoid-induced apoptosis. As reported [6], DEX induced a time-dependent increase in the percentage of cells with low $\Delta\Psi$, reaching the same level of Val after 18 h, i.e. only when a massive cell death is present. We analyzed the effect of a shorter exposure to Val (6 h) measuring ANX-V binding. Our results confirmed that Val protected thymocytes from apoptosis since the earliest phases of such process. The percentage of control, untreated cells that bound ANX-V was 5.2 ± 0.4 (mean ± S.E.M. of four experiments), that of cells treated with Val alone 6.8 ± 0.6 (P = NS), that incubated with DEX 27.3 ± 1.8 ($P < 0.01$ vs. control), that of cells incubated with DEX and Val 8.0 ± 0.4 ($P < 0.005$ vs. DEX; $P < 0.01$ vs. control).

Table 1
Effects of collapse in $\Delta\Psi$ on apoptosis in U937 cells or rat thymocytes

Treatment	Low $\Delta\Psi$	Apoptotic	PI+
Control U937	3.4 ± 0.6	2.2 ± 0.3	1.0 ± 0.3
TNF-α+CHX	26.7 ± 2.3*	26.5 ± 2.3*#	9.4 ± 1.7*#
Val	73.7 ± 6.4*°	3.0 ± 0.5	1.3 ± 0.4
TNF-α+CHX+Val	82.7 ± 1.3*°#	42.8 ± 5.4*°#	17.9 ± 4.3*#
Control thymocytes	15.9 ± 1.4	14.6 ± 3.7	12.0 ± 2.4
DEX	86.9 ± 3.1*	90.2 ± 1.9*	48.1 ± 5.9*
Val	84.5 ± 3.7*	56.2 ± 3.6•#	28.7 ± 5.8*§
DEX+Val	88.4 ± 2.4*	69.1 ± 2.8•#–	37.9 ± 4.8*″

Data refer to the percentage of cells with low $\Delta\Psi$, apoptotic, and with impaired membrane integrity (permeant to PI). Incubation was 6 h for U937 cells, 18 h for rat thymocytes. Data are expressed as mean ± S.E.M. of six experiments.
Statistical analysis was performed using the paired Student's *t*-test: U937: P always < 0.01, * = vs. control; ° = vs. TNF-α+CHX; # = vs. Val.
Rat thymocytes: * = vs. control, $P < 0.01$; • = vs. control, $P < 0.05$; # = vs. DEX, $P < 0.01$; § = vs. DEX, $P < 0.05$; – = vs. Val, $P < 0.01$; ″ = vs. Val, $P < 0.05$.

3.3. Effects of Val on cyt c release

We tested the possibility that $\Delta\Psi$ collapse provoked a release of cyt *c* in the cytoplasm from the mitochondrial intermembrane space. Fig. 2 shows confocal analysis of U937 cells labeled with anti-cyt *c* mAbs (upper panels) and counterstained with MT, used to identify mitochondria (lower panels). Left panels show control, untreated cells, in which fluorescences from anti-cyt *c* mAb and from mitochondria display the same distribution pattern. Central panels show U937 cells after 4 h of Val treatment. Fluorescence from anti-cyt *c* mAb is still well localized, while that from mitochondria is dropped, indicating a fall in $\Delta\Psi$. Right panels show U937 cells after 3 h of treatment with TNF-α plus CHX. Cyt *c* fluorescence is widely spread in the cytoplasm of many cells, while that of MT is partially dropped. This clearly suggests that depolarization of mitochondria induced by Val per se does not provoke cyt *c* efflux.

In parallel experiments, to functionally test the hypothesis that TNF-α induces apoptosis by releasing cyt *c*, we tried to block apoptosis using 1 μM CsA, but observed no effect. Indeed, apoptosis was 52.7 ± 1.3% in TNF-α plus CHX-treated cultures, and 50.1 ± 4.5% in the presence of CsA (P = NS). The difference between the percentage of apoptotic cells revealed by this method and that of PI staining (26.5%, Table 1) was likely due to the fact that they analyze two different phases of the apoptotic process.

3.4. Intracellular ATP content analysis

We finally measured the intracellular ATP content in U937

Table 2
Intracellular ATP content in U937 cells and in rat thymocytes during apoptosis

U937 cells		Rat thymocytes	
Treatment	ATP content	Treatment	ATP content
Control	3.056 ± 0.16	Control	1.240 ± 0.29
TNF-α+CHX	1.987 ± 0.12*	DEX	1.210 ± 0.36
Val	0.930 ± 0.07*#	Val	0.167 ± 0.06*§
TNF-α+CHX+Val	0.993 ± 0.13*#	DEX+Val	0.090 ± 0.03*§

The table shows the intracellular ATP content (expressed in mmol/million cells) in U937 cells and rat thymocytes after 2 h of culture in the presence of TNF-α+CHX and/or Val, and DEX and/or Val, respectively.
Data are expressed as mean ± S.E.M. of three separate experiments for each cell type. Statistical analysis was performed using the paired Student's *t*-test: * = vs. control, $P < 0.05$; # = vs. TNF-α+CHX, $P < 0.05$; § = vs. DEX, $P < 0.05$.

cells and rat thymocytes after 2 h of incubation with TNF-α plus CHX or with DEX, respectively, in the presence or absence of Val (Table 2). TNF-α plus CHX treatment caused a significant decrease in ATP content in U937 cells, while DEX did not exert a similar effect in rat thymocytes. As expected, Val caused a dramatic decrease of ATP content in both cell types.

4. Discussion

We first investigated the role of $\Delta\Psi$ in causing apoptosis, and, using different techniques, found that in U937 cells, mitochondrial depolarization induced by low doses of the K^+ ionophore Val is per se not able to trigger apoptosis. Thus, in these cells, the drop in $\Delta\Psi$ is not linked to the release of apoptogenic compounds from mitochondria. This is in agreement with recent data obtained in human osteosarcoma cells that may undergo mitochondrial permeability transition (MPT) and swelling in a reversible manner, without any sign of cell death [18].

High doses of Val induce apoptosis in different cells [19,20]. Nevertheless, it is quite unlikely that this effect could be due to the $\Delta\Psi$-dissipating action of Val, since a dose capable of collapsing $\Delta\Psi$ is unable to induce apoptosis in our model (i.e. U937 cells). It is more likely that high doses of the ionophore can induce apoptosis by non-specific Ca^{2+} release due to hyperpolarization of the plasma membrane [19], or by other phenomena involving changes in ion osmolarity. It is noteworthy that low doses of Val can induce cell death in cells that are already programmed to die like rat thymocytes, but only after a relatively long period of time (18 h). This phenomenon could be due to the collapse of $\Delta\Psi$, which in turn provokes a severe drop in ATP (whose levels were about 13% of control cells, see Table 2), much marked than that observed in U937 cells (about 30%).

TNF-α induces CPP32 activation in ML-1a cells, and this activation requires mitochondrial respiratory functions [21]. On the contrary, we observed that TNF-α alone was unable to activate CPP32 on U937 cells. Also CHX failed to activate the protease cascade. This is in agreement with previous reports [22], suggesting that the sensitizing effect of CHX to TNF-α is due to its ability to increase cyclin A-associated kinase. Only when CHX and TNF-α are both present in the same culture, the activation of CPP32 and PARP cleavage can occur. We can reasonably exclude that Val increases TNF-α-induced apoptosis by activating proteases, since in Val-treated U937 cells, neither cleavage of PARP nor of CPP32 caspase occurs. Even if the involvement of other ICE-like proteases triggered by the depletion of cytoplasmic K^+ is theoretically possible, as already suggested [23], it is likely that the different effects of Val in the two models (i.e. increase or decrease of apoptosis) should be due to its action on $\Delta\Psi$.

In thymocytes, glucocorticoid-induced apoptosis needs protein neosynthesis [24]. If energy is in some way dissipated, it is likely that this form of death does not occur. Thus, if $\Delta\Psi$ decreases and ATP is no longer available for protein synthesis, apoptosis could be inhibited, at least partially and until the drop of ATP itself causes cell death. This is confirmed by the analysis of ATP content, which showed no significant changes after 2 h of culture with DEX in rat thymocytes. This is in agreement with previous data showing no early loss of ATP [25]. When Val was added, ATP decreased and apoptosis was inhibited. On the other hand, in a different system which does not need full energy supply, such as TNF-α plus CHX-treated U937 cells [26], Val treatment and the consequent disruption of $\Delta\Psi$ did not confer any protection. A rapid (2 h) ATP decrease was present in TNF-α plus CHX-treated cells, although the $\Delta\Psi$ impairment occurred later (6 h). Since Val alone does not induce apoptosis, the fall of ATP content should not be considered the cause, but rather a consequence of the apoptotic process. Together with the data on cyt *c* release, this indicates a possible mitochondrial involvement in the absence of $\Delta\Psi$ fall during TNF-α-induced apoptosis. Nevertheless, this death process seems not to be inhibited by low doses of CsA. This suggests that the pathway activated by TNF-α could by-pass the MPT step. However, it is known that CsA has only a transient effect on MPT [27], and this could account for the loss of any protective effect. The use of a more stable inhibitor of MPT would clarify this hypothesis.

In conclusion, our data suggest that at least two different families of apoptotic pathways can exist, based on the sensitivity to Val and thus to the collapse in $\Delta\Psi$: the first in which Val increases apoptosis, the second in which Val protects from apoptosis. This leads to the suggestion that disruption of $\Delta\Psi$ plays opposite roles depending on the experimental model which is used, e.g. signal transduction-induced apoptosis (like in U937 cells) and programmed cell death (like in rat thymocytes). In the first model, the drop of $\Delta\Psi$, and thus of ATP, is a possible contributory cause for the apoptotic process; in the latter, it could be a limiting factor for apoptosis to occur.

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