Personal review

RELATIONSHIP BETWEEN MEMBRANE POTENTIAL, CALCIUM FLUXES AND MATRIX SWELLING IN RAT LIVER MITOCHONDRIA; EFFECT OF ETHANOL FEEDING

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Summary

The main objective of the present work was to establish the exact temporal relationship between membrane potential collapse, calcium release and mitochondrial matrix swelling during a metabolic stress. For this purpose, liver mitochondria were prepared from normal and ethanol-fed rats and incubated in different media placed in the cuvettes of a diode array spectrophotometer, with succinate as substrate. The media used were supplemented with an appropriate probe for either membrane potential or calcium fluxes and matrix swelling was monitored in parallel with each of the two parameters. A series of discrete CaCl₂ pulses were used to alter the three respiration-dependent parameters and to establish the mitochondrial sensitivity to metabolic stress. From the comparison of the spectrophotometric recordings, a correlation could be established between the type of medium used and/or the concentration of calcium added to the mitochondrial suspension and the moments of initiation of swelling, ∆Ψ collapse and calcium release (occurring in this order). Significant differences in this regard were also noticed between the mitochondria of the control and of the ethanol-fed rats. Based on the present results, certain situations reported in the literature, in which membrane potential is apparently preserved after the occurrence of the mitochondrial permeability transition, could be explained if the mechanisms of response of the most frequently used membrane potential sensitive dyes were seriously taken into consideration.

Key words: Membrane potential; calcium fluxes; matrix swelling; mitochondrial permeability transition; ethanol-fed rats

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Introduction

Mitochondrial permeability transition (MPT) represents a drastic change in the permeability of the mitochondrial inner membrane, induced by different agents or conditions, which is achieved through the opening of a membrane structure known as the permeability transition pore (PTP). The opening of this pore is accompanied by membrane potential (∆Ψ) collapse and calcium release, uptake of electrolytes and water, matrix swelling and ruptures of the mitochondrial outer membrane. As a consequence, several factors present in the intermembrane/intercrystal space, among which cytochrome c (cyt c), are liberated into the cytosol, triggering the apoptotic death of the cell (for a recent review on this subject, see Garrido et al., 2006).

Ever since the discovery that MPT is involved in the process of apoptotic cell death, the history of mitochondria seems to be rewritten. Many physical or chemical agents (hereafter called stress factors), previously known to have uncoupling effects, due to their stimulation of the mitochondrial state-4 respiration and their negative effect on the phosphorylation efficiency, are now practically rebaptized as MPT-inducing agents or even apoptotic agents, while agents that prevent uncoupling and associated phenomena (notably, MPT) are termed antiapoptotic.
However, whether MPT-inducing agents should be called apoptotic is a matter of debate, since many MPT-inducing agents have been shown to produce different types of death, depending on the origin of the biological material, experimental setup and circumstances, energy availability etc. (Kroemer et al., 1998; Lemasters et al., 1998; Leist et al., 1999; Kim et al., 2003). Some of the best known examples of naturally occurring MPT-inducing agents in the cell are indeed uncoupling agents. These include excess calcium ions, strong oxidant agents, such as reactive oxygen species and other free radicals, resulting from cell metabolism (particularly that of mitochondria), and free fatty acids, resulting from the action of membrane phospholipases (see Szewczyk and Wojtczak, 2002, for a good introduction to this problem). Even though the apoptotic effects of these agents are well documented, their necrotic effect, especially that of calcium ions, is also known (Zhu et al., 2000; Smaili et al., 2003; Orrenius et al., 2003; Halestrap, 2006; Bernardi et al., 2006). Indeed, the presence of excessive calcium leads to swelling, i.e., oncosis, which is a more appropriate term than necrosis for the death resulting from calcium overload (necrosis is just the post-mortem alteration following oncosis). Without getting involved in the so-called apoptosis/necrosis paradox, we should nevertheless stress the fact that MPT is implicated in both apoptosis and necrosis, sometimes even being considered “the point of no return” in either type of cell death. Therefore, in the present work, we concentrated on the matrix swelling as an expression of MPT and studied the occurrence of other two important events ($\Delta\Psi$ collapse and calcium release) in connection to this phenomenon, following the application of a calcium load, under variable conditions.

Even though it has been observed that MPT is an almost “all or nothing” phenomenon at the level of a single organelle (Huser et al., 1998; Huser and Blatter, 1999), since it does not occur concomitantly in all mitochondria, the permeability transition of a population of organelles should follow a sigmoidal curve (characteristic of the so-called cooperative phenomena). It has been indeed observed that the matrix swelling of a suspension of mitochondria follows such a sigmoidal curve (for ex., Jung and Brierly, 1999; Vyssokikh and Brdiczka, 2003), leading shortly to MPT, i.e. the cause-effect relationship or at least the tight correlation between swelling and MPT is unambiguous. This is in fact the reason why, in most of the cases, MPT is monitored as matrix swelling. In agreement with the chemiosmotic theory, it is also generally accepted that the uncoupling of oxidative phosphorylation is a consequence of the membrane potential collapse, associated with the dissipation of the transmembrane proton electrochemical gradient generated by respiration. However, some literature data seem to question the relationship between the membrane potential collapse (or uncoupling) and MPT and, even when this is accepted, it is not perfectly clear which is the cause and which is the consequence or the effect. Thus, although many conditions/situations leading to $\Delta\Psi$ collapse and/or MPT and finally to the liberation of the apoptogenic factors from the intermembrane space have been amply studied in cellular and mitochondrial suspensions, a consensus with regard to the order of occurrence, the way of interdependence of these events or even their strict association has not been achieved, as can be demonstrated by the diverse conclusions and opinions present in the literature (cf., for ex., Scorrano et al., 1997; Finucaine et al., 1999; Aronis et al., 2002; Ly et al., 2003; Galindo et al., 2003; Krasnikov et al., 2005).

We are aware that some of the differences observed can be explained by the diversities of the experimental conditions and death mechanisms, but a close analysis reveals that many uncertainties still arise primarily from
methodological limitations, as admirably argued by Bernardi et al. (1999). Despite some technical progress made since that review was published, the problem has not been entirely clarified. One reason is the complexity of the biological system itself, where processes are usually regulated by feed-back loops of several interdependent mechanisms, in which the effect can either inhibit or stimulate the cause. Put it in simpler terms, in the case discussed, membrane potential collapse could be concomitantly both the cause and the effect of MPT.

The purpose of the present work was to establish the exact relationship between the 3 parameters discussed above: membrane potential, calcium release and matrix swelling. In order to achieve this, we took advantage of our previous experience in working with membrane potential and calcium probes for assessing drug effects at the level of hepatic mitochondria (Tarba and Orbai, 1986; Tarba and Crăciun, 1990; Petrescu and Tarba, 1997), when we observed that treatments with certain chemical agents seemed to lead to a phenomenon of partial “decoupling” of membrane potential collapse from swelling (the term decoupling is used here to avoid confusion with the classical phenomenon of mitochondrial uncoupling). Due to technical limitations, however, we could not ascertain the reality and the origin of this phenomenon. The latter developments in the field and the recent presence of a diode array spectrophotometer in our laboratory, which can be used for parallel (simultaneous) measurements of two or more parameters, incited us to a reassessment of this problem.

Among the agents studied by us, several alcohols used for acute or chronic treatment of rats seemed to yield mitochondria displaying both uncoupling effects, as measured oxygraphically, and decoupling effects, as measured spectrophotometrically (for ex., Tarba and Orbai, 1986, and also unpublished observations). Of direct importance for our work is also the observation that either an in-vitro or an in-vivo treatment with ethanol leads to mitochondria exhibiting an increased tendency to matrix swelling and a higher sensitivity to calcium ions and/or reactive oxygen species than the control mitochondria (Pastorino et al., 1999; Pastorino and Hoek, 2000; Sakurai et al., 2000; Tarba and Deaciuc, 2002; Hajioczy et al., 2005). For the reasons presented above, in trying to clarify the temporal sequence of events leading to MPT, we also combined chronic ethanol feeding, as a natural model of sensitizing hepatic mitochondria to MPT-inducing agents, with the use of suspending media of variable ionic content and the addition of calcium as the most effective MPT-inducer, while mitochondrial membrane potential and calcium release were monitored in parallel with matrix swelling by the aid of a diode array spectrophotometer. A preliminary report of this work has already appeared (Tarba and Suârășan, 2004).

Material and methods

Male white Wistar rats were kept under standard conditions in our animal facility for 14-18 weeks, starting from an average weight of 130 g/individual, and the evolution of their weight was assessed periodically. The rats were given a premix containing all the ingredients of the Larsen diet and allowed free access to water. One group served as control (C), while in another group (E) each rat was supplemented daily with 1.5 ml of 48% ethanol/100 g body weight (equivalent to 5.7 g ethanol/kg body weight), administered in the morning on a little piece of dry bread, before the animals were allowed access to water. At the end of the period, the rats were fasted for 24 hrs and sacrificed by decapitation after a slight anaesthesia. The body was cooled under running water and the liver extracted immediately and used for the preparation of mitochondria, according to standard differential centrifugation procedures, in a medium containing 200
mM mannitol, 70 mM sucrose, 5 mM Hepes-KOH (pH 7.37) and 0.5 mM Na-EDTA. The washing and preserving medium lacked the chelating agent (EDTA).

A diode-array spectrophotometer (Specord S 100B, Analytik Jena, Germany), which permits concomitant measurements at different wavelengths, was used to monitor membrane potential or calcium fluxes in parallel with the matrix swelling. Membrane potential was estimated as the absorbance difference between 670 and 700 nm, using the cyanine dye diS-C$_2$-(5) (2.5 µM) as a voltage sensitive probe (Tarba, 1978; 1883a; 1983b; Tarba and Crăciun, 1990), while calcium was measured by the use of 30 µM arsenazo-III (650–700 nm) as a calcium specific indicator (Scarpa, 1979). The change in absorbance at 700 nm (which represents an almost isobestic point for both dyes) was used for assessing the extent of swelling. Since swelling is usually reported in the literature as being measured at 540 nm, we also made some measurements at this wavelength, initiating the recordings by the addition of mitochondria to the complete reaction medium (see the composition of the media below). As expected, although the absorbance changes were larger, the kinetics of the recordings were similar at the two wavelengths. For parallel measurements, 1 mg/ml of mitochondrial protein was usually incubated directly in the spectrophotometer cuvettes (in a final volume of 2 ml), at room temperature (around 24°C), in 3 different media, labelled as MS (mannitol-sucrose), MKS (mannitol-potassium-sucrose) and MKS–Mg (MKS without Mg). The MS medium contained 210 mM mannitol, 70 mM sucrose, 1.5 mM MgCl$_2$, 1 mM KP$_i$, and 5 mM Hepes-KOH buffer (pH 7.37), MKS was composed of 110 mM mannitol, 65 mM KCl, 40 mM sucrose, 1.5 mM MgCl$_2$, 1 mM KP$_i$, and 5 mM Hepes-KOH buffer, while MKS–Mg lacked Mg ions. In addition, a special swelling medium (KSW) was also used. This fourth medium contained 100 mM KCl, 50 mM sucrose, 5 mM KP$_i$ and 10 mM Hepes (pH 7.37). The respiration and the associated phenomena monitored by us (∆Ψ, Ca$^{2+}$ fluxes and swelling) were triggered by the addition of Na$^+$-succinate (5 mM) in the presence of 8 µM rotenone. Different amounts of CaCl$_2$ were usually added gradually, in several pulses, up to the desired final concentration or until a certain effect was obtained. When necessary, ∆Ψ magnitude was estimated using standard membrane potentials generated by K$^+$ gradients and valinomycin, according to a methodology described previously (Tarba, 1978; 1983a; 1983b), while calcium concentrations could be assessed by adding standard calcium pulses in the presence of ruthenium red (RR), an inhibitor of the mitochondrial calcium uptake. The most important quantitative result, however, was obtained by counting the number of calcium pulses of a known concentration (usually, 12.5 µM each) necessary to be added to the mitochondrial suspension for inducing calcium release. We have used this number (Tarba and Suărășan, 2004) as a measure of the calcium retention capacity (CRC), in the same way as used and discussed for example by Nogueira et al. (2005). This phenomenon of massive calcium efflux is associated with the permeability transition (as monitored by matrix swelling) and with the ∆Ψ collapse. The addition of calcium in discrete pulses was chosen for both practical and fundamental reasons. Practically, this is the fastest way to determine the sensitivity threshold to calcium with a reasonable accuracy (time is a very important factor in working with these organelles that are known to suffer age-associated damages rather rapidly). Fundamentally, it has been shown that in the living cell the mitochondria are subjected to calcium oscillations and even to calcium “sparks” originating in the nearby regions of the endoplasmic reticulum and that this is significant for the role of the mitochondrion in cell calcium regulation and even for the mitochondrial metabolism (Sparagna et al., 1995; Hoek et al., 1995; Robb-Gaspers et
al., 1998; Duchen et al., 1998; Szalai et al., 1998; Bernardi, 1999; Rizzuto et al., 2000; Ishii et al. 2006). For this reason, the addition of calcium in pulses has been in fact used as a study method in some of the above mentioned papers as well as in others (Chalmers and Nicholls, 2003; Nakagawa et al., 2005; Nogueira et al., 2005).

The respiratory rates were measured polarographically, essentially as previously described (Petrescu and Tarba, 1997), but using MKS as the basic suspending medium, in which KPi was increased to 5 mM and 0.5 mM EDTA was added. Respiratory control ratio (RCR) was calculated as the ratio between state 3 (respiration rate in the presence of ADP) and state 4 (respiration rate after ADP exhaustion).

The chemicals used were of analytical grade. Succinate, rotenone, valinomycin, arsenazo-III and ruthenium red were from Sigma, while diS-C2-(5) was from Eastman-Kodak. All the aqueous solutions were prepared with double distilled water. Calcium content (residual calcium) of this water was estimated by atomic absorption spectroscopy as being around 1 mM. The statistical significance of the differences between the two groups of rats with regard to the capacity of mitochondrial calcium retention was computed by the (unpaired) Student t test

**Results**

**Spectrophotometric measurements in mitochondria of the control rats.** The absorption spectra of the ΔΨ probe added to a control mitochondrial suspension, in the absence (curve 1) or presence (curve 2) of succinate are shown in Fig.1.

![Fig.1](left): Effect of mitochondrial membrane potential on the spectrum of diS-C2-(5). Each cuvette contains 2 mg mitochondrial protein suspended in 2 ml MKS medium to which 8 μM rotenone is added. Curve 1 is recorded upon addition of 2.5 μM diS-C2-(5) to the sample cuvette (stock solution: 0.5 mM in ethanol). Membrane potential is generated by concomitant addition of 5 mM Na-succinate to both cuvettes) and curve 2 is recorded 1 min later.

![Fig.2](right): The spectra of arsenazo-III in absence (curve 1) and presence of calcium (curve 2). The same general procedure and conditions are used as in Fig.1, but curve 1 results upon addition of 30 μM arsenazo-III to the sample cuvette and curve 2 upon further addition of 175 μM CaCl2 to both cuvettes. Succinate is also present in both cuvettes at a concentration of 5 mM.
As can be seen, the generation of membrane potential by succinate-dependent respiration is associated with an evident absorbance decrease at 670 nm. Analogous recordings are shown in Fig.2 for the calcium probe in the absence (curve 1) or presence of calcium (curve 2). It can be observed that the addition of calcium is associated with large increases at 600 and 650 nm. As can also be seen from both figures, the spectra of the two dyes have an isobestic (or quasi-isobestic) point around 700 nm, a reason for which this wavelength was selected for monitoring swelling as well as a neutral reference in the kinetic studies of membrane potential and calcium fluxes, for which 670 and 650 nm, respectively, were selected as active wavelengths.

The behaviour of the control mitochondria (C-mitochondria) in three of the four different incubation media used for kinetic studies of membrane potential, calcium fluxes and swelling, under stress conditions induced by calcium, are presented in Figs. 3-8.

**Fig.3** (left). **Effect of repetitive calcium addition on membrane potential and matrix swelling of control mitochondria suspended in MS medium.** The same general procedure and conditions are used as in Fig.1, but the incubation medium is MS and the spectrophotometer is operated in a differential kinetic mode using the wavelength pair 670 and 700 nm. This allows the recording of the absorbance changes vs. time at the two wavelengths as well as the difference between them. The upper trace is recorded at 670 nm and represents a composite behaviour of both membrane potential and swelling, while the lower trace is recorded at the isobestic point (700 nm) and therefore represents the absorbance decrease associated only with the matrix swelling. The middle curve is the result of the differential recording (670–700 nm), reflecting the absorbance changes associated with variations in membrane potential. The addition artefacts can also be observed on the upper and lower traces, marking the moments of addition to the sample cuvette of 5 mM Na-succinate (0), eight 12.5-µM calcium pulses (1–8) and one pulse of 100 µM calcium (9).

**Fig.4** (right). **Calcium fluxes and matrix swelling of control mitochondria** This figure is a counterpart of Fig.3 in which the ΔΨ probe has been replaced by the calcium probe (30 µM arsenazo-III) and the composite curve has been eliminated for clarity. The upper trace represents now the differential recording (650 – 700 nm), reflecting changes associated with the calcium fluxes, while the lower trace (recorded at 700 nm) reflects the absorption decrease associated with the matrix swelling. The first small decrease in absorbance (on the upper curve) is associated with calcium uptake upon the addition of 5 mM Na-succinate to the sample cuvette (0) while the rest of the changes are determined by the addition of eight 12.5-µM calcium pulses (1–8), one pulse of 25 µM (9) and one of 75 µM calcium (10).
The short abrupt changes in absorption (spikes or artefacts) observed in Fig.3 can be used for establishing the exact moments of various additions as well as the moments of other changes. Thus, one can observe a certain correlation between the parameters reflected by the middle and the lower curve (i.e., \( \Delta \Psi \) and swelling). As long as the mitochondria do not swell, \( \Delta \Psi \) preserves its steady-state value. However, small transient decreases in \( \Delta \Psi \) (absorbance increases on the middle trace) can be observed at the 6th and 7th calcium pulses, where the absorbance on the lower curve begins to decrease (reflecting the initiation of swelling). A more visible decrease in membrane potential (absorbance increase on the middle trace) occurs after the 8th pulse of calcium and it is associated with a stronger swelling (absorbance decrease on the lower trace).

Fig.4 is a counterpart of Fig.3 in which the \( \Delta \Psi \) probe has been replaced by the calcium probe (arsenazo-III) and the composite curve has been eliminated for more clarity. Here, the upper trace represents the differential recording (650 – 700 nm) and it reflects changes associated with the calcium fluxes. The first decrease in absorbance is associated with the addition of succinate (0) and the consecutive uptake of the residual calcium, and it is followed by a series of abrupt increases and exponential decays (saw-tooth kinetics), reflecting the addition and consecutive uptake of several pulses of calcium. Like in Fig.3, the lower trace (recorded at 700 nm) reflects the absorption decrease associated with the matrix swelling (the majority of the addition artefacts can also be observed). This time, a correlation between swelling and calcium release can be observed, similar to that seen in the case of \( \Delta \Psi \). Thus, the swelling begins after the 6th pulse of calcium, which is still taken up completely, the 7th calcium pulse is incompletely taken up, while after the 8th pulse there is a massive calcium efflux (release) coincidental with an ampler swelling.

Fig.5. Membrane potential and matrix swelling in control mitochondria suspended in MKS medium. Except for the suspending medium, the same general procedure and conditions are used as in Fig.3. However, as in Fig.4, the composite curve has been eliminated for more clarity. The upper trace reflects the behaviour of membrane potential, while the lower one represents the swelling. The spikes on the lower trace are associated with the addition to the sample cuvette of 5 mM Na-succinate (0), six pulses of 12.5 \( \mu \)M calcium (1–6) and one of 125 \( \mu \)M calcium (7).

The recordings in Figs.5-7 illustrate the behaviour of C-mitochondria in the MKS medium. It can be seen that mitochondria are more sensitive to calcium addition than in the MS medium, reflecting the richer ionic composition of the medium, especially the presence of K\(^+\) (approximately 72 mM). Thus, from Fig.5, one can observe that the swelling (decrease on the lower curve) begins after the 2nd or 3rd pulse of calcium and becomes very strong after pulses 5 and 6. The second observation is that small transient decreases in \( \Delta \Psi \) occur only after the 4th and 5th calcium pulses (upper curve), while an obvious larger decrease occurs after the 6th pulse. This partial decoupling between the swelling and the membrane potential collapse in an ionic medium is even more
evident if a larger concentration of calcium is added in one pulse, as shown in Fig.6. Here, instead of a series of 12.5-µM Ca\(^{2+}\) pulses, two pulses of 50 µM Ca\(^{2+}\) each are used. It can be seen that although the swelling begins in less than 30 sec after the addition of the first pulse of calcium (lower trace), it takes around 4 min for the beginning of ΔΨ collapse (upper trace). Fig.7 is a counterpart of Fig.5, in which (instead of ΔΨ) calcium fluxes are studied in relation to matrix swelling. However, similar observations can be made as in Fig.5, i.e., a greater sensitivity of mitochondria to calcium (i.e., a lower CRC) and a certain degree of decoupling between the initiation of swelling and of the calcium release. In the absence of magnesium (MKS–Mg medium), an even greater sensitivity of mitochondria can be observed (not presented here).

**Fig.6.** Effect of high concentration calcium pulses on the membrane potential and matrix swelling of control mitochondria suspended in MKS medium. The same conditions are used as in Fig.5, but calcium is added in 50-µM pulses. The spikes on the lower trace are associated with the addition of 5 mM Na-succinate (S) and two 50-µM calcium pulses to the sample cuvette.

**Fig.7.** Calcium fluxes and matrix swelling in control mitochondria suspended in MKS medium. Except for the incubation medium, the conditions are identical to those in Fig.4. The upper trace reflects the changes associated with the calcium fluxes, while the lower trace reflects the absorption decrease associated with the matrix swelling. The first small decrease in absorbance on the upper trace reflects the addition of 5 mM Na-succinate to the sample cuvette (0), while the rest of the changes are due to the addition of six 12.5-µM calcium pulses (1–6), one of 25 µM (7) and two of 50 µM calcium (8,9).

If, in addition, the concentrations of K\(^+\) and P\(_i\) are further increased, as in the swelling medium (KSW), the swelling usually begins after the second pulse of calcium, whereas ΔΨ collapse and calcium release begin after the third calcium pulse (see, for illustration, Fig.8, A and B).

Considering this kind of pair-recordings (A and B), we can use swelling kinetics as a reference and compare indirectly the behaviour of membrane potential and calcium fluxes through their relation to swelling. The result is that, irrespective of the conditions used, there is always a strong correlation (parallelism) between the moments of ΔΨ collapse and calcium release. This result is in fact in agreement with what is known from literature reports about the
dependence of calcium fluxes on $\Delta \Psi$ (Rottenberg and Scarpa, 1974; Nicholls, 1978; Beatrice et al., 1980; Bernardi and Azzone, 1983; Wingrove and Gunther, 1986a; 1986b; Gunther and Pfeiffer, 1990; Bernardi, 1999).

Fig.8. Membrane potential and matrix swelling (A) and calcium fluxes and matrix swelling (B) in control mitochondria suspended in KSW medium. Except for the incubation medium, the conditions are identical to those in Fig.5 (for A) or in Fig.7 (for B). The spikes on the lower traces in both panels are associated with the addition to the sample cuvette of 5 mM Na-succinate (0) and three pulses of 12.5 µM calcium (1–3). The rest of the additions are: in A, one pulse of 150 µM calcium (4); in B, one pulse of 12.5 µM (4), one of 25 µM (5), one of 50 µM (6) and one of 75 µM calcium (7).

A certain degree of decoupling between either $\Delta \Psi$ collapse or calcium release and matrix swelling can also be observed, which seems to be slightly higher for $\Delta \Psi$ than for calcium. However, an inspection of the curves in all the figures presented demonstrates that regardless of how large the decoupling effect may be, $\Delta \Psi$ collapse and calcium release occur when the swelling curve passes its main inflection point. As mentioned in the introduction, the swelling is the consequence of the opening of the permeability transition pore. Thus, the time of (or around) the inflection point of the swelling curve could be considered as the average moment of MPT, and one can say that $\Delta \Psi$ collapse and calcium release proceed almost in parallel with MPT. Nevertheless, since the initiation of swelling precedes the initiation of both $\Delta \Psi$ collapse and calcium release, it is reasonably to conclude that MPT is the cause of $\Delta \Psi$ collapse and not the other way around. Given the interdependence of these phenomena, however, we think that this conclusion does not exclude certain ion movements that preceded the MPT, as suggested by the work of Krasnikov et al. (2005). Moreover, a closer analysis of a series of swelling curves obtained at 540 nm with different calcium concentrations, as those shown in Fig.9, where mitochondria are added last reveals that the swelling curves do not have a simple sigmoidal kinetics.

As can be seen from the upper curve (a), where only the residual calcium is present, i.e., a low concentration which is physiologically more relevant for our study, the absorbance decrease associated with swelling has 3 distinct phases: an initial small exponential decrease, a middle steady-state period and a final sigmoidal decrease. It is clear that $\Delta \Psi$ collapse and calcium release correlate with this last phase. The initial
phase is very likely due to a small proportion of mitochondria (the most damaged and/or most sensitive ones) undergoing the permeability transition and losing their $\Delta \Psi$ and calcium. The lost calcium, however, increases the total calcium load of the rest of the mitochondria and makes them more prone to swelling and MPT.

The initial phase of swelling even less conspicuous. Depending on the mitochondrial sensitivity, the suspending conditions and the initial calcium load, this chain reaction leads, after a longer or a shorter intermediate phase (see curves $b$ and $c$ in Fig.9), to the permeability transition of the majority of mitochondria. This type of interdependence, in which calcium efflux may be accompanied by other factors released from the intermembrane space or from the broken membranes of a limited number of mitochondria, could affect the permeability of the intact membranes by several mechanisms (not only MPT) and complicate the relationship between swelling/MPT and the other two parameters studied. Still, the present results point to the fact that, in experiments similar to ours, MPT induced by calcium is (directly and/or indirectly) the main factor responsible for triggering the chain of other closely related events ($\Delta \Psi$ collapse, calcium and cyt c release, etc.).

The fact that calcium is responsible for triggering the whole chain of events in our experiments is also demonstrated by the illustration in Fig.10A, where 1 $\mu$M ruthenium red (RR) added to the mitochondrial suspension prior to calcium completely blocks $\Delta \Psi$ collapse and drastically limits the swelling. Moreover, under these conditions, even the additional presence of 1 mM t-butyl hydroperoxide (t-BHP) is not able to induce $\Delta \Psi$ collapse or swelling, although the ability of this compound to produce MPT and other associated phenomena is well known even at lower concentrations (see, for ex., Beatrice et al., 1982, and Bellomo et al., 1984). Indeed, as can be seen from Fig.10B, in the absence of RR, this strong peroxidant agent induces swelling and $\Delta \Psi$ collapse even if only traces of residual calcium are present, although further addition of calcium produces a new round of swelling. A similar effect is obtained if 1 mM atractyloside (another proapoptotic and MPT-inducing agent) is used instead of t-BHP (not presented here).

**Fig.9. Kinetics of matrix swelling at different calcium concentrations.** From a mixture of 4 ml KSW medium supplemented with 8 $\mu$M rotenone and 5 mM Na-succinate, 2 ml are added to the reference and 1.95 ml to the sample cuvette. Different concentrations of calcium are also added and after the run has been started, 2 mg mitochondrial protein (as a 50-µl aliquot) are injected into the sample cuvette: (a), no calcium added; (b), 25 $\mu$M calcium; (c), 200 $\mu$M calcium.

This scenario has been in fact discussed in the literature (see, for example, the reviews by Bernardi et al., 1999; 2006), although, to our knowledge, the existence of the small absorbance decrease (slight initial swelling) has not been explicitly given any consideration. This is probably due to the fact that most of the experiments are usually performed by the addition of calcium (or other stress factors) to mitochondria which are already respiring in the presence of succinate (or other substrates) and some residual or lost calcium, a fact which makes
Figure 10. Effect of ruthenium red (A) and t-butyl hydroperoxide (B) on membrane potential and matrix swelling. In A, the incubation medium (MKS) contains 1 µM ruthenium red whereas in B it contains 1 mM t-BHP. In both cases, diS-C2-(5) is present only in the sample cuvette at a concentration of 2.5 µM. In A, trace a represents the membrane potential recording Succinate (5 mM) is added to both cuvettes at the moment indicated (S), while the rest of the additions are made to the sample cuvette at the points indicated by the spikes on trace b (matrix swelling). In B, as usual, the upper trace is the membrane potential and the lower one is the swelling. The two pulses of calcium added are: 25 µM (1) and 75 µM (2).

It is clear that the situation in the cell is even more complicated than described above, because mitochondria are under the influence of many more effectors. However, we hope to have been reasonably close to the basic conditions of the MPT occurrence in vivo. This expectation is supported not only by the appropriate ionic conditions offered by the most important medium employed (MKS), but also by the fact that we used very well prepared (i.e., intact) mitochondria, as demonstrated by the high respiratory control ratios measured with succinate (around 4) and by our electron microscopic images published elsewhere (Tarba and Florea, 2006).

Spectrophotometric measurements in mitochondria of ethanol-fed rats. Other clues supporting the assertion above and expanding the explanation given by us to the so-called decoupling effect come from our studies on mitochondria from ethanol-fed rats (E-mitochondria). In most of the cases, the functional behaviour and ultrastructural appearance of E-mitochondria (see Tarba and Florea, 2006, for ultrastructural aspects) were deficient as compared to the control, suggesting that adverse conditions present either in vivo (such as alcohol intake) or in vitro (such as an inappropriate composition of the incubation medium) sensitize mitochondria and make them more prone to MPT, regardless of the origin of the condition (i.e., occurring in vivo or in vitro).

Part of the results obtained with E-mitochondria are illustrated in Figs.11 and 12 and some statistical data are presented comparatively (with the control) in Table I. Fig.11 illustrates the behaviour of mitochondria with regard to ΔΨ (panel A)
and calcium fluxes (panel B) in the MKS medium. As can be seen from Fig.11A, swelling begins after the first or second pulse of calcium (trace b), while membrane potential decreases after the second pulse and collapses after the third one (trace a). A certain instability of membrane potential can be observed already after the first calcium pulse, expressed as a small abrupt absorbance increase ($\Delta \Psi$ decrease) followed by a slow absorbance decrease. This type of behaviour (also present, but less conspicuous in C-mitochondria) can be easily explained if one takes into consideration the mechanism of diS-C$_{2}$-(5) response (Tarba, 1978; 1883a; 1983b). Simply, the sensitive/sensitised mitochondria (expected to be present here in a larger number than in the case of the control), upon the collapse of their $\Delta \Psi$, can no longer keep the cationic dye concentrated on the inner (negative) surface of their internal membrane (this is reflected by the rapid but small increase in absorbance seen following the first addition of calcium) and after the dye redistribution (equilibration) between the two membranes of these inert mitochondria, some of the dye is gradually lost to the intact (active) mitochondria which continue to accumulate and concentrate it, resulting in an apparent increase in $\Delta \Psi$. In fact, due to this phenomenon of secondary reassociation of the dye with intact mitochondria, $\Delta \Psi$ of E-mitochondria seems larger than that of C-mitochondria. However, the standardization procedure with K$^+$ gradients and valinomycin (not shown here) demonstrated that $\Delta \Psi$ amplitude is practically identical in both cases (around 190 mV).

As can be seen from Fig.11B, calcium fluxes follow a pattern similar to that observed in Fig.11A for $\Delta \Psi$. Thus, the first calcium pulse is taken up completely, the second one is only partially taken up, while after the third pulse the mitochondria loose all the accumulated calcium.

Fig.11. Membrane potential and matrix swelling (A) and calcium fluxes and matrix swelling (B) in mitochondria of ethanol-fed rats suspended in MKS medium. E-mitochondria are suspended under conditions identical to those in Fig.5 (for A) or Fig.7 (for B). In A, trace a represents the membrane potential and trace b the matrix swelling. In B, the upper trace represents the calcium fluxes and the lower trace is the swelling. In both panels, the changes are recorded following the addition of 5 mM Na-succinate to the sample cuvette (0). The rest of the additions are: two 12.5-µM calcium pulses (1, 2), one pulse of 25 µM (3) and one of 150 µM calcium (4).
The lower capacity of E-mitochondria to resist to metabolic stress (lower CRC) is even more obvious if the special swelling medium (KSW) is used. It can be seen from Fig.12A that under these conditions ΔΨ starts to collapse practically without any addition of calcium. Calcium release also occurs very easily, as can be seen from Fig.12B, where one pulse of calcium (Ca) is enough to induce calcium efflux, while the swelling begins already from the moment of succinate addition (S).

**Fig.12.** Membrane potential and matrix swelling (A) and calcium fluxes and matrix swelling (B) of E-mitochondria suspended in KSW medium. Except for the origin of mitochondria, the conditions are identical to those in Fig.8. In both panels, the absorbance changes are initiated by the addition of 5 mM Na-succinate to the sample cuvette (S). In A, where trace a represents the membrane potential and trace b the matrix swelling, only residual calcium is present, whereas in B one pulse of 12.5-µM calcium is added when indicated (Ca).

Quantitative comparison of mitochondrial sensitivity to calcium. As mentioned before, if we take into consideration the number of calcium pulses (12.5 µM each or 12.5 nmols/mg protein) necessary to induce calcium release, the sensitivity of mitochondria can be readily quantified using integers and fractions of 0.5 (when the moment of release is appreciated to be in-between two pulses). Calcium retention capacity (CRC), expressed as the mean values of these numbers (x̄), can be compared by the use of the Student t test or other more sophisticated tests. The comparison presented in Table I demonstrates that E-mitochondria respond statistically different from C-mitochondria. It is clear that even in the MS medium, if the number of recordings were higher the differences would have been statistically significant (i.e., p < 0.05). This statistical analysis confirms the observations resulted from comparing the individual spectrophotometric kinetic recordings, i.e., the fact that mitochondria of alcoholic rats are characterized by a higher sensitivity to metabolic stress, specifically to that exerted by ion movements and an increased calcium load. In addition, one can see that the lack of Mg enhances even more this sensitivity, a fact which is in accord with what is known from the literature regarding the involvement of Mg²⁺ in the regulation of calcium fluxes and, more generally, in calcium-dependent events (Hunter et al., 1976; Zoccarato et al., 1981; Bernardi and Pietrobon, 1982; Kowaltowski et al., 1998; Bernardi, 1999; Hagen et al., 2003; Nogueira et al., 2005).
### Table I. Comparison of the calcium retention capacity by mitochondria of the two groups of rats

<table>
<thead>
<tr>
<th>Statistical parameter</th>
<th>Group</th>
<th>Mean ± SE</th>
<th>n</th>
<th>Mean ± SE</th>
<th>n</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td></td>
<td>7.750 ± 0.250</td>
<td>2</td>
<td>4.750 ± 0.750</td>
<td>2</td>
<td>3.795</td>
<td>≥0.05</td>
</tr>
<tr>
<td>MKS</td>
<td></td>
<td>5.214 ± 0.343</td>
<td>8</td>
<td>2.333 ± 0.105</td>
<td>5</td>
<td>7.487</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MKS–Mg</td>
<td></td>
<td>3.286 ± 0.184</td>
<td>8</td>
<td>2.000 ± 0.129</td>
<td>5</td>
<td>5.520</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td>KSW</td>
<td></td>
<td>3.143 ± 0.143</td>
<td>6</td>
<td>1.000 ± 0.000</td>
<td>4</td>
<td>9.488</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

**Note.** The meaning of the symbols is as follows: C = control mitochondria; E = mitochondria of ethanol-fed rats; \( \bar{x} \) = mean number of 12.5-µM calcium pulses needed for calcium release; SE = standard error of mean; n = number of recordings (each typical of a different mitochondrial preparation).

### Discussion

A few words are probably necessary regarding the methodology employed. In general, we have tried to use conditions that were reasonably close to the natural ones, so as the results obtained to have a certain biological relevance. Although several media and conditions were employed, as imposed by the research strategy, the incubation medium most extensively used for experiments had an ionic composition close to that of the cytosol. This includes the concentration of phosphate and magnesium, two of the most important modulators of the calcium fluxes in mitochondria (Bernardi and Pietrobon, 1982; Kowaltowski et al., 1996; 1998; Bravo et al., 1997; Chavez et al., 1997; Hagen et al., 2003; Nogueira et al., 2005), as well as a K⁺/Na⁺ ratio not far from that present in the cytosol. In addition to the 1 µM calcium brought by the distilled water, it appears that other sources (including mitochondria themselves) may have also contributed to the presence of a higher “residual calcium” in the incubation medium, which apparently varied roughly between 1.5 and 6 µM, as can be deduced from the absorption changes following the addition of succinate and calcium to mitochondria suspended in the presence of arsenazo-III (see Figs.4, 7, 8B, 11B and 12B). This is definitely higher than the normal cytosolic concentration of calcium, but, as long as we take this fact into account, it no longer represents a problem for our study, where usually much higher concentrations of calcium are used to produce metabolic stress, in line with other studies of this type (see for example Nogueira et al., 2005).

A certain question may be raised with regard to the use of rotenone for inhibiting the NADH-linked respiration, since it has been reported that in certain systems rotenone may be an inducer of the permeability transition, apparently through production of reactive oxygen species as a consequence of the respiratory chain inhibition (Wolvetang et al., 1994; Matsunaga et al., 1996; Isenberg and Klauning, 2000; Li et al., 2003). Other reports, however, seem to arrive at a different conclusion or interpretation. It has been shown (e.g., by Fontaine et al., 1998) that the electron flow itself through the complex I of muscle mitochondria induces MPT through the production of reactive oxygen species. Moreover, the addition of rotenone to two permeabilized carcinoma cell lines actually
inhibited the permeability transition (Chauvin et al., 2001). In our case, however, the respiration is resumed by the addition of succinate, approximately 1 min after the addition of rotenone. In fact, under these conditions, it has been shown that rotenone inhibits superoxide production through reverse electron transport (see, for ex., Lambert and Brand, 2004), and thus it should limit MPT and not enhance it. It is worth mentioning that we started working without the use of rotenone and the results were basically the same, but, in the absence of rotenone, they tended to be more variable, especially in the case of membrane potential recordings. In fact, the main reason of using rotenone in this type of experiments is to allow the visualization of the spectral changes associated with membrane potential generation by succinate addition, changes that are otherwise obscured by the residual NADH respiration and the associated membrane potential, variable from preparation to preparation (situation eliminated by the action of rotenone).

Our results have clearly pointed out that even though the swelling (and thus MPT) is initiated before ΔΨ collapse, the latter follows rather close and occurs in parallel with the extensive swelling (the time when most of the mitochondria suffer the permeability transition). However, when working with a population of mitochondria (even with the population of a single cell), a certain degree of decoupling of the two phenomena (ΔΨ collapse starting later than the initiation of swelling) is to be expected due to methodological reasons. This possibility has been discussed by Bernardi et al. (1999) and it is also suggested by the present experiments. In fact, we have previously demonstrated (Tarba, 1978) that this behaviour is indeed characteristic for the ΔΨ monitored by the same cyanine dye as employed in the present study. This was done by adding a mixture of inert liposomes (lipid vesicles that lack an ion gradient) to active liposomes (i.e., possessing a ΔΨ as a consequence of an ion gradient and the presence of an appropriate ionophore). The apparent increase in membrane potential upon reassociation of the dye lost by the inert liposomes with the active liposomes was very similar to what we observed in the present study, especially when working with mitochondria of ethanol-fed rats, where the proportion of sensitive mitochondria loosing their dye (and calcium) as a consequence of ΔΨ collapse is clearly higher than in the control mitochondria (see the presentation of Fig.11A). An increased sensitivity is most likely also induced in single cell experiments due to harsh technical conditions (for ex., the strong illumination needed by confocal microscopy). Thus, it is reasonably to consider that many observations in which cyt c appears to be released before ΔΨ collapse are due to methodological reasons. It is worth mentioning that we have occasionally seen in some mitochondria decoupling times of up to 5 minutes, under certain conditions, a period that may be enhanced in experiments performed in intact cells. Nevertheless, in making this statement we do not imply that in other types of experiments cyt c release could not occur without MPT and ΔΨ collapse, since it has been proposed that there are two basic mechanisms of cyt c release and only one of them is directly dependent on calcium and MPT (see, for ex., Gogvadze et al., 2001).

The fact that calcium release seems to slightly precede ΔΨ collapse can be explained by the fact that arsenazo-III has a different mechanism of response than diS-C2-(5), i.e., it does not have such a high affinity for the mitochondria as diS-C2-(5) (does not reaccumulate so easily) and thus it is more responsive to the presence of calcium in the external medium. If this fact is taken into consideration, then the sequence of events resulting from the present study is “swelling ↔ ΔΨ collapse → calcium release”, with certain elements of interdependence, as discussed above.
Conclusions

In conclusion to our own work, we can say that the methodology employed satisfactorily answers the question of the relationship between swelling, membrane potential collapse and calcium fluxes, thus establishing the most likely sequence of events leading to permeability transition and implicitly to the release of cytochrome c (and other apoptogenic or necrotic factors), at least in situations in which calcium is the main triggering agent. The use of sensitized mitochondria isolated from rats fed ethanol under controlled natural conditions also contributed to establishing the above-mentioned sequence. In addition, our work suggests that the present methodology could be successfully used for studying the functional alterations of mitochondria isolated following different treatments, involuntary and voluntary intoxications (such as drug abuse), etc. It constitutes in fact a new argument that, indeed, MPT should not be considered any longer as an “in vitro artifact” but as a “disease target”, as suggestively expressed by Bernardi et al. (2006) in their recent review of the problem.

References


Kowaltowski, A.J., Naia-da-Silva, E.S., Castilho, R.F., Vercesi, A.E.: Ca\(^{2+}\)-stimulated mitochondrial oxygen species generation and permeability transition are inhibited by dibucaine or Mg\(^{2+}\), Arch. Biochem. Biophys. 359, 77-81, 1998.


