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Mechanisms of the resistance to the mitochondrial permeability transition in tumour cells

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Abstract

Tumour cell mitochondria often have a high Ca^{2+} threshold for induction of the mitochondrial permeability transition (MPT). The mechanisms of inhibited MPT are briefly reviewed. MPT stimulates the release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria. This is central in the signal pathways leading to apoptosis. Inhibited apoptosis may lead to excessive cell proliferation while induced apoptosis is the aim of cancer therapies. Also cell death by necrosis may result from impaired mitochondrial function due to MPT and the resulting decrease in cellular ATP levels. Many prooxidants trigger cell death by inducing MPT. Tumour cells may have greater resistance to prooxidants and free radicals formed also in radiation therapy. One mechanism for the inhibition of MPT could be increased expression of the Bcl-2 protein in tumour cells. We found this to be the case in Zajdela hepatoma mitochondria. Another mechanism could be the increased contents of Mg^{2+} in tumour mitochondria, since Mg^{2+} is an inhibitor of MPT. This was found to be the case in Ehrlich ascites tumour cell mitochondria. Other contributing factors could be inhibition of phospholipase A_2 by the Mg^{2+}, changes in the amounts and properties of the adenine nucleotide translocator and/or mitochondrial ATP synthase.

Keywords: Adenine nucleotide translocator; Apoptosis; ATP synthase; Bcl-2; Ca^{2+}; Cell death; Mg^{2+}; Mitochondria; Permeability transition; Phospholipase A_2; Prooxidants

Malign transformations may arise through mutations in genes coding for proteins that are involved in signal pathways for regulation of cell proliferation or programmed cell death. If a signal for cell proliferation is not turned off or an apoptotic signal is not carried out, we have an inappropriate cell proliferation [1–3]. It is of interest that tumour cells often have significant alterations in their energy metabolism. Glycolysis is often enhanced, and some mitochondrial functions may also have been modulated [4–8]. Recently it has been established that mitochondria are intimately involved in the signal pathways for apoptosis by being able to release apoptotic factors, cytochrome c and AIF (apoptosis-inducing factor) that activate the proteolytic caspase cascade together with other factors [9,10]. Also the release of Ca^{2+} from mitochondria may be a contributing factor [11,12]. The release of the apoptotic factors are associated with the mitochondrial permeability transition (MPT). In MPT a large pore in the inner membrane is opened causing loss of $\Delta\Psi$ and swelling [9,13]. However, release has also been observed without loss of $\Delta\Psi$ [14,15].

Inhibition of MPT enables the mitochondria to accumulate more Ca^{2+} before MPT is triggered. In order to understand its regulation and relation to apoptosis, it would be essential to know the components of the MPT pore. These have not been identified with certainty but the adenine nucleotide translocator has been implicated

Abbreviations: AIF, apoptosis-inducing factor; CsA, cyclosporin A; $\Delta\Psi$, mitochondrial membrane potential; EATC, Ehrlich ascites tumor cells; EATCM, EATC mitochondria; IF1, ATP synthase inhibitory factor 1; MPT, mitochondrial permeability transition; PLA_2, phospholipase A_2; RLM, rat liver mitochondria; ROS, reactive oxygen species; VDAC, mitochondrial outer membrane porin, voltage-dependent anion channel; ZHM, zajdela hepatoma cell mitochondria.

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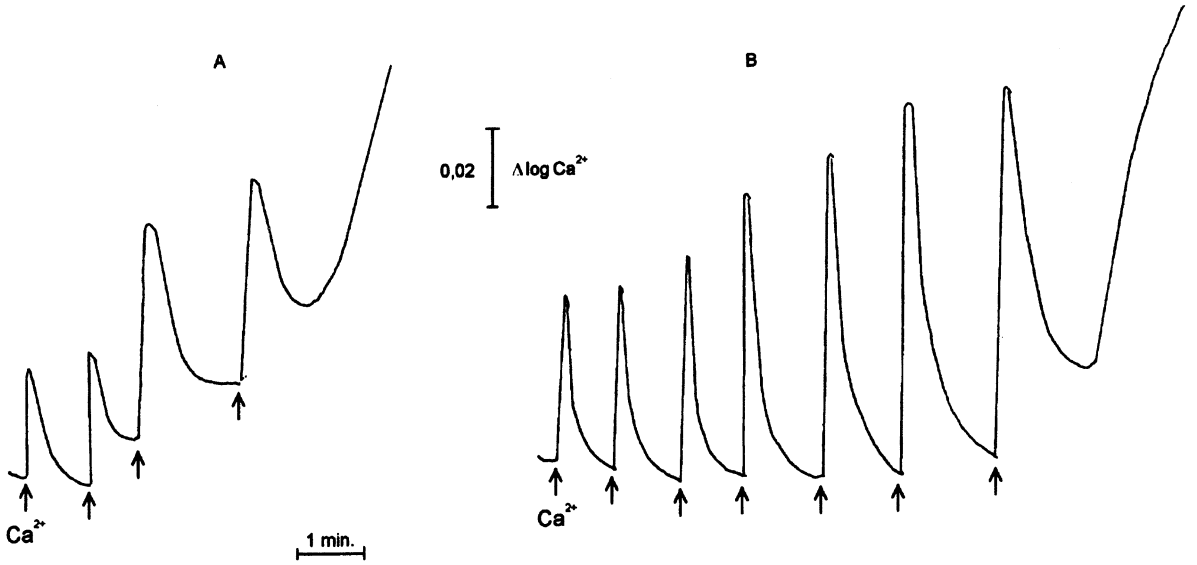

Fig. 1. The difference in the Ca^{2+} threshold for RLM and ZHM. A, RLM; B, ZHM. The medium contained 100 mM KCl, 50 mM sucrose, 2 mM KH_2PO_4, 0.5 mM $MgCl_2$ and 5 mM Hepes, pH 7.5, mitochondria 1.2 mg protein/ml. Additions of 30 μM Ca^{2+} are indicated by arrows. Medium [Ca^{2+}] was measured with a calcium-sensitive electrode (Orion) [82]. Reproduced from Ref. [71] with the permission of the Publisher.

as the main component [16,17]. There may be other channel-forming components associated with the pore, such as the so-called VDAC channel (voltage-dependent anion channel) present in the outer membrane and membrane contact sites [18,19]. Of interest are also hydrophobic small molecules that may be associated with the pore components, such as the reaction products of phospholipase A_2 (PLA_2) [20,21]. Thus, inhibitors of the enzyme frequently inhibit MPT [21,22]. These reaction products themselves may also increase the permeability of lipid bilayers to Ca^{2+}, K^+ and H^+ [20] and may exhibit channel activity [23]. This may contribute to the release of Ca^{2+} from mitochondria [24,25].

Here we briefly review the mechanisms by which MPT may have become suppressed in tumour cells, focusing on Ehrlich ascites tumour cells (EATC) and Zajdela hepatoma cells that we and others have studied in detail.

1. MPT regulation in normal and tumour cells

It was early observed that uptake of Ca^{2+} by mitochondria causes oxidation of pyridine nucleotides, hydrolysis of ATP, mitochondrial swelling and release of accumulated Ca^{2+} [26]. Today it is evident that these events result from MPT.

MPT is stimulated by a number of factors, of which the most important ones are Ca^{2+}, prooxidants and substances that react with vicinal thiols, while Cyclosporin A (CsA), ADP, Mg^{2+}, and other factors like lowered pH inhibit it [27–30]. MPT is also modulated by the electron flux through respiratory chain complexes [31] and $\Delta\Psi$ itself [32–34]. The pore opening may be reversible when of short duration [26,35], but finally becomes irreversible [35,36], due to protein oxidation and cross-linking by S–S bonds [37]. Mitochondrial reactive oxygen species (ROS) production promotes MPT and the cross-linking [38]. Since MPT promotes mitochondrial ROS production [38], a vicious cycle is created. GSH plays a central role in eliminating free radicals and preventing oxidation of protein sulfhydryl groups. Reduced pyridine nucleotides again are needed in order to regenerate GSH from GSSG [39].

It is frequently observed that tumour mitochondria are able to accumulate substantial amounts of Ca^{2+} before MPT is induced [40,41]. Fig. 1 shows an example of this. Three additions of Ca^{2+} were sufficient to trigger MPT in rat liver mitochondria (RLM), seen as a permanent loss of accumulated Ca^{2+}. In Zajdela hepatoma mitochondria (ZHM) seven additions were required. Note also that RLM were unable to lower medium [Ca^{2+}] to pre-addition levels after each addition of a pulse of Ca^{2+} while ZHM were able to do so.

The high Ca^{2+} threshold for both reversible and irreversible MPT in tumour mitochondria makes it possible to observe oscillations in various parameters (respiratory rate, $\Delta\Psi$, pH and Ca^{2+} fluxes) following the additions of substantial amounts of Ca^{2+} [35]. This in RLM would cause irreversible MPT. Fig. 2 shows inhibition of the oscillations in permeabilized EATC by CsA, demonstrating the essential role of reversible MPT opening in these oscillations.

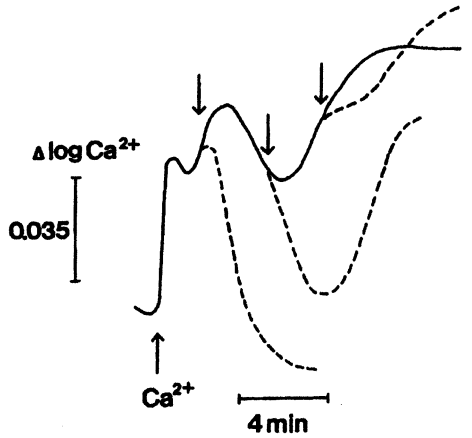

Fig. 2. The MTP pore opening is involved in Ca^{2+}-induced oscillations of Ca^{2+} fluxes in EACTM. Continuous trace, control, 100 μM Ca^{2+} added at arrow; Broken lines, Ca^{2+}-levels measured with a calcium-sensitive electrode after addition of 5 nM CsA at indicated points of the Ca^{2+}-induced oscillation. The medium contained 100 mM mannitol, 100 mM NaCl, 4 mM NaH_2PO_4, 2 mM KCl, 2 mM Hepes, pH adjusted to 7.4, 4 mM succinate, 6 μM rotenone and cells corresponding to 1.5 mg protein/ml. The cells were permeabilized by addition of 37 μM digitonin. Reproduced from Ref. [35] with the permission of Harcourt Brace & Co, UK.

There are thus a number of possible mechanisms for the inhibition of MPT and increased Ca^{2+} uptake capacity in tumour cell mitochondria. One central aspect is the prooxidant/antioxidant balance. Tumour cells may have a high expression of the oncogenic protein Bcl-2 which also potently inhibits MPT and apoptosis [9,42,43]. Tumour mitochondria frequently contain relatively large amounts of Mg^{2+} [41,44] and decreased PLA_2 activity [45], which may contribute to the resistance to MPT.

2. Stimulation of MPT pore opening and cell death by prooxidants

Added or endogenously produced prooxidants from oxygen can induce mitochondrial damage and MPT [46,47]. ROS may be formed by mitochondria, from xanthine by xanthine oxidase, by NADPH oxidase, or by other mechanisms such as activation of lipoxygenase [48]. Which of these mechanism is most important depends on the tissue and the experimental conditions. Prooxidant formation in ischemia-reperfusion injury is believed to be a major factor triggering MPT pore opening [49,50]. Prolonged pore opening then contributes to cell death. Whether that occurs by necrosis as in ischemia-reperfusion or in an orderly programmed manner as in apoptosis depends on the ATP status. If ATP becomes depleted, necrosis rather than apoptosis ensues [13,51]. This is of interest in cancer therapy where the aim is to induce apoptosis in tumor cells by radiation or chemotherapy [3].

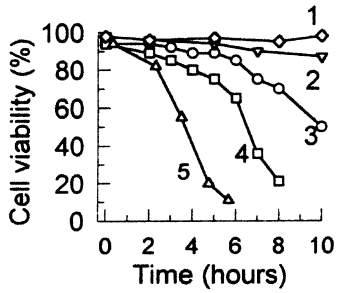

Fig. 3. Prooxidant-induced cell death in EATC. The cells (5×10^5 cells/ml) were incubated in vitro in the medium of Fig. 1 in the presence of prooxidants. Cell viability was determined by following the penetration of the dye Trypane blue into cells. Trace 1, control; Trace 2, 10 μM *t*-butylhydroperoxide; trace 3, 5 μM cumenehydroperoxide; Trace 4, 5 μM menadione; Trace 5, 10 μM menadione. Reproduced from Ref. [53] with the permission of Harcourt Brace & Co, AU.

Addition of prooxidants to cell culture is frequently used as a model for studying the involvement of MPT in cell death [52]. We have used this model in EATC [53]. Low concentrations (1–20 μM) of the prooxidants menadione, cumenehydroperoxide or butylhydroperoxide lowered the Ca^{2+} threshold and induced MPT, cell cycle disturbances and cell death. Fig. 3 shows that menadione — a quinone — potently induced cell death after a few hours, as did cumenehydroperoxide, and somewhat less potently, *t*-butylhydroperoxide, while hydrogen peroxide had at most a very slight effect. The cell death was preceded by a drop in the ΔΨ and in the Ca^{2+}-uptake capacity of mitochondria in permeabilised cells, as shown for menadione in Fig. 4. This is analogous to what was observed in hepatocytes [54].

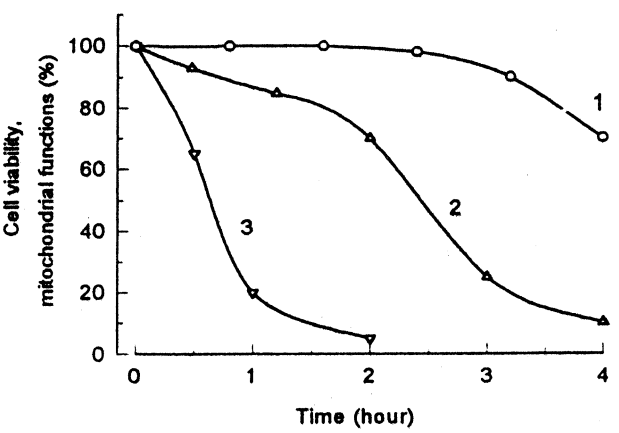

Fig. 4. EATC death induced by menadione is preceded by a drop in ΔΨ and Ca^{2+}-uptake capacity indicating MPT. Cells (5×10^6) were incubated as in Fig. 2 in the presence of 5 μM menadione, ΔΨ was measured with the aid of teramethylrhodaminemethylester as in [83], aliquots were taken at indicated times, washed and permeabilized with 30 μM digitonin and Ca^{2+} uptake capacity measured as in Fig. 1. Trace 1, cell viability; Trace 2, ΔΨ; Trace 3, mitochondrial Ca^{2+} uptake capacity with 5 mM glutamate and 5 mM malate as substrate. Reproduced from [53] with the permission of Harcourt Brace & Co, AU.

It is thus possible that changes in the prooxidant/antioxidant balance account for the high resistance of tumour mitochondria to MPT. This could be achieved by suppression of ROS production or by strengthening the antioxidant defence systems. However, ZHM had more than one order of magnitude higher rates of superoxide generation than RLM [55] which supports the latter mechanism.

3. Proteins of the Bcl-2 family and MPT

Bcl-2 and related proteins, i.e. Bcl-X_L, have received increasing attention as antiapoptotic proteins, while other proteins of this family such as Bax promote apoptosis [9,43]. These proteins are bound to various intracellular membranes, substantial amounts to mitochondria. The localisation was reported to be mainly on the outer membrane [56], but also to the inner membrane [57,58]. Of interest is the association of Bcl-2 with the adenine nucleotide translocator that is a likely candidate as a component of the MPT pore [17,30].

Inhibition of MPT by Bcl-2 has been suggested to be mainly responsible for its antiapoptotic effect [59]. Several mechanisms for this inhibition have been suggested. One is the strengthening of antioxidant defence [60,61] possibly mediated by prevention of cytochrome c release that induces production of superoxide by cytochrome oxidase [62]. Bcl-2 may bind to Bax and thereby inhibit its MPT-stimulating activity [61]. Also the channel-forming capability of proteins belonging to the Bcl-2 family may be involved [63,64]. Phosphorylation of Bcl-2 [65,66] may also be one regulatory step as is the phosphorylation of procaspase-9 [67].

Overexpression of Bcl-2 or Bcl-X_L makes mitochondria more resistant to effectors of MPT and cytochrome c release [42,68–70]. It was therefore of interest to see whether this was the mechanism in the cell lines studied by us. We found an increased amount of Bcl-2 in ZHM [71] but not in EATC [72] (Fig. 5). Both in ZHM, EATCM and RLM, there was also a protein with a lower M_R, ca. 20 kDa, that reacted with Bcl-2 antibodies. Its identity is not clear but it could be Bax or Bcl-X_S. The increased transcription of Bcl-2 was verified by Northern blotting of the corresponding mRNA in ZHM [71]. These findings identify increased amounts of Bcl-2 as a major factor in the resistance to MPT in ZHM, but it is not the mechanism in EATCM.

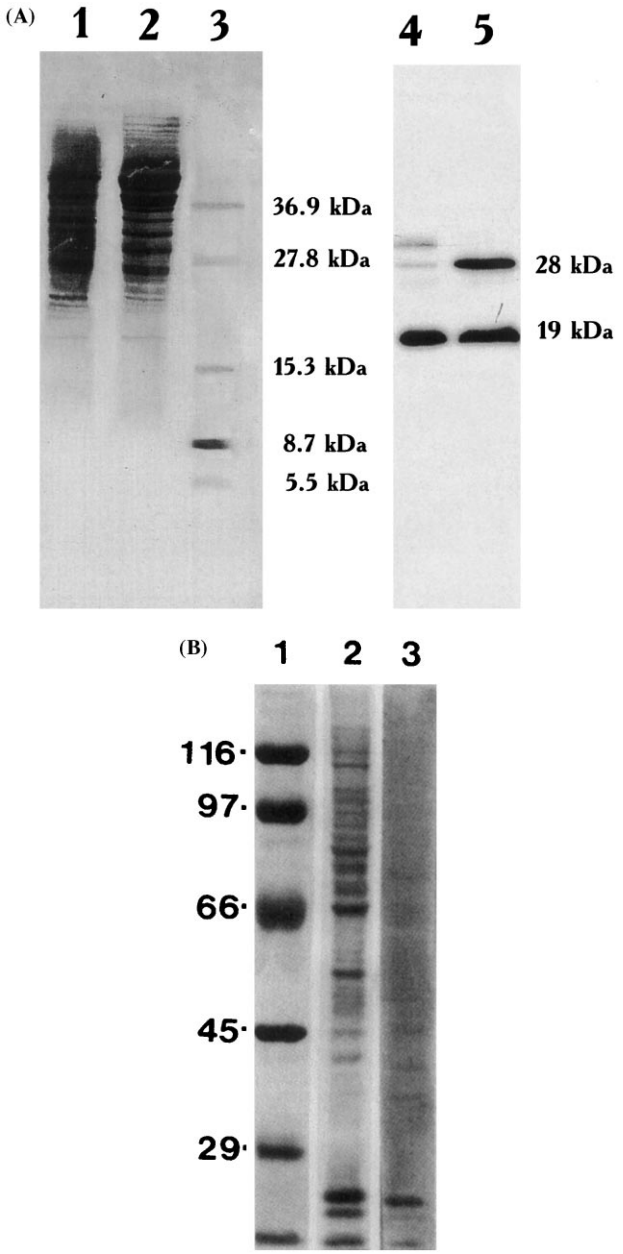

Fig. 5. ZHM but not EATCM have increased amounts of Bcl-2. A Bcl-2 -specific antibody (C21, Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used in Western blotting. A, Protein patterns in SDS-PAGE of ZHM (Lane 1) and RLM (Lane 2), protein standards (Lane 3) and Western blotting of RLM (Lane 4) and ZHM (Lane 5). Reproduced from [71] with the permission of the Kluwer Academic Publishers. B, Protein pattern in EATCM (Lane 2, lane 1 shows protein standards) and corresponding Western blotting (Lane 3). Reproduced from [72] with the permission of John Libbey & Co, LTD.

4. Mg^{2+} as a suppressor of MPT

Tumour mitochondria often contain relatively high amounts of Mg^{2+}, with a higher Mg^{2+}/Ca^{2+} [5,41,44], which may inhibit MPT. It was earlier believed that the high Ca^{2+} uptake capacity of EATCM was due to the inhibition of phospholipase A_2 (PLA_2) activity [40,41,45] which was thought to be the cause of the increased mitochondrial permeability following excessive Ca^{2+} uptake. On reexamination of this question it was found that Mg^{2+} prevented Ca^{2+}-binding to the inner membrane (Fig. 6), which may explain the inhibition of MPT and of PLA_2 [22,72]. The Mg^{2+}/Ca^{2+}

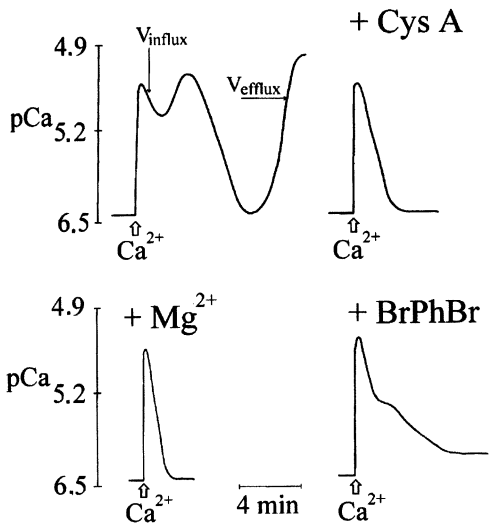

Fig. 6. Inhibition of MPT in EATCM by added Mg^{2+} or bromophenacylbromide (BrPhBr). The medium contained 100 mM KCl, 25 mM NaCl, 4 mM KH_2PO_4, 5 mM succinate, 10 M rotenone, 10 mM Hepes, pH 7.4, and mitochondria (3.2 mg protein/ml). Addition of 125 μM Ca^{2+} triggered an oscillatory state, ending in release of accumulated Ca^{2+}, indicating MPT (Upper, left trace), as shown by effect of CsA (Cys A, upper right trace). In the presence of 2 mM Mg^{2+} (lower left trace) EATCM were able to accumulate all the Ca^{2+} (downward deflection), and in the presence of 1 (M BrPhBr most of it without induction of MPT (lower right trace). Reproduced from Ref. [72] with the permission of John Libbey & Co, LTD.

ratio is clearly of importance in tumour cells in the competition between these cations for binding sites in the inner membrane, presumably mainly cardiolipin. The inhibitor of PLA_2, bromophenacyl bromide, also inhibited MPT [22,72] (Fig. 7). Local anesthetics often act as inhibitors of PLA_2 [73] and also as competitive inhibitors of Ca^{2+}-binding [74,75]. The effect of Mg^{2+} thus seems to be mainly by inhibiting MTP, either by binding to a regulatory site in the pore complex or/and by displacing Ca^{2+} from its activating site [29], though there might also be a contribution due to inhibition of

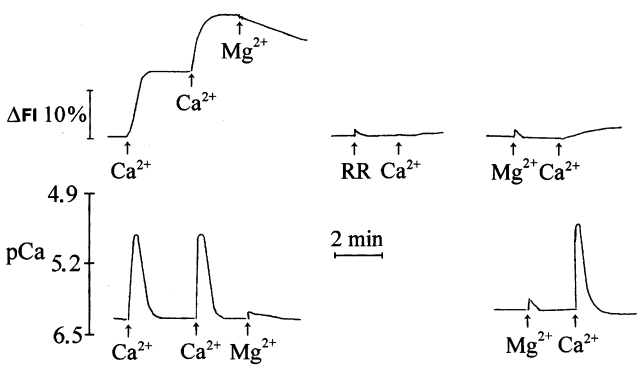

Fig. 7. The effect of Mg^{2+} on the binding of Ca^{2+} to the inner mitochondrial membrane. Tetracyclin 20 μM was used as a fluorescent probe of binding, upper traces. The lower traces shows the Ca^{2+} signals, recorded with a calcium-sensitive electrode. Reproduced from Ref. [72] with the permission of John Libbey & Co, LTD.

PLA_2 and the formation of its reaction products that may enhance MPT [76]. Mg^{2+} may also have antioxidant effects [77].

5. Other factors influencing MPT in tumour cells

The energy metabolism was early found to be changed in tumour cells which, for providing energy for their rapid proliferation, rely more on the fast ATP synthesis in glycolysis even under aerobic conditions than on oxidative phosphorylation [6]. Their hexokinase often is hyperactive and glycolytic phosphate intermediates are produced in significant amounts [8]. The expression of mitochondrial proteins is also altered with less transcription of ATP synthase gene while the inhibitory factor IF1 may be overexpressed which also favours glycolysis [7]. Thus in Morris hepatoma mitochondria and EATCM this is seen as lowered rates of uncoupler-stimulated ATPase [4]. We could also see an inhibition both of ATPase and oxidative phosphorylation in EATCM [78], especially after preloading with Ca^{2+} [79]. ADP caused a smaller drop in the $\Delta\Psi$ in ZHM than in RLM [71] which is to be expected if oxidative phosphorylation is suppressed. Indeed, this was found to be the case. Thus, in RLM the rates of oxidative phosphorylation were four times higher than in ZHM (2.8 and 0.7 nmoles $ATP \times s^{-1} \times mg^{-1}$ protein, respectively) though the Ca^{2+} uptake rates were higher in ZHM [71]. This oligomycin-like effect in tumor mitochondria would promote a high $\Delta\Psi$ and high level of NAD(P)H which would counteract opening of the MPT pore.

The lowered rate of oxidative phosphorylation and lower activity of uncoupler-stimulated ATPase activity could also be due to smaller amounts of ANT. This is of special interest since ANT is a likely candidate for the integral part of the MTP pore [16,17]. Thus, it has long been known that the Ca^{2+} efflux from mitochondria was low in hypothyroidism [80]. Recently it was reported that the amount of ANT was low and the Ca^{2+} threshold to MPT was high in hypothyroidism. Also MPT was insensitive to the adenine nucleotide inhibitor atractyloside that normally stimulates MPT [81]. This may also be true in tumour cells.

6. Conclusions

Tumour mitochondria are often resistant to induction of MPT which may be a means to resist apoptosis. In MPT apoptotic factors are released from mitochondria and ATP synthesis is inhibited and its hydrolysis stimulated. More uptake of Ca^{2+} by tumour mitochondria is needed for triggering MPT. There is thus an alteration of the regulation of the opening of the pore

responsible for MPT. In many tumour cells this factor is an increased synthesis of the antiapoptotic protein Bcl-2. This was found to be the case in Zajdela hepatoma but not in Ehrlich ascites cells. In the latter the increased content of Mg^{2+} in mitochondria seems to be the responsible factor. Also other factors may be involved, such as the prooxidant/antioxidant balance, since the oxidation state of thiols in the MPT pore strongly influence the pore opening. Bcl-2 may act that way or by direct binding to pore components, while Mg^{2+} is known to bind to a regulatory site of the pore complex and to compete with Ca^{2+} binding.

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