The mitochondrial permeability transition: the brain’s point of view

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Abstract

The mitochondrial permeability transition (mPT) has been implicated in both central nervous system ischaemia/reperfusion injury and excitotoxic neuronal death. To characterize the mPT of brain mitochondria, fluorescent mitochondrial dyes were applied to cultured neurons and astrocytes and isolated brain mitochondria were prepared. In astrocytes, mPT induction was observed as calcium-induced mitochondrial swelling following permeabilization by digitonin or introduction of a calcium ionophore. In hippocampal neurons, mPT induction was observed upon introduction of calcium and ionophore or application of toxic doses of glutamate. In isolated brain mitochondria, calcium dose-dependently produced calcium accumulation and mitochondrial swelling that was prevented by pretreatment with ADP or cyclosporin A. Additionally, when mitochondrial substrates were limited, calcium dose-dependently produced mitochondrial depolarization without swelling or calcium accumulation that was reversed by ADP, cyclosporin A or Ruthenium Red. The degree of mitochondrial depolarization was modulated by free fatty acids, magnesium, calcium concentration and protonophore Repolarization of mitochondria and closure of this low-conductance manifestation of the mPT pore by cyclosporin A was modulated by the degree of depolarization.

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Mitochondrial dysfunction features prominently in the biochemical pathways associated with both necrosis and apoptosis, as exemplified in both neuronal excitotoxicity and central nervous system (CNS) ischaemia respectively [1–4]. Such dysfunction appears to stem from activation of the mitochondrial permeability transition (mPT), opening of a non-selective pore in the inner mitochondrial membrane with consequent mitochondrial swelling [5–7]. In cultured neurons, an antagonist of the mPT, cyclosporin A (CsA), was shown to partially protect against a single excitotoxic dose of glutamate or N-methyl-D-aspartate and against glutamate-induced apoptosis [5,8,9]. In a paradigm involving short ischaemic exposures, intracerebral injection of CsA ameliorated ischaemic brain damage in hyperglycaemic rats [10,11]. CsA also ameliorated ischaemic brain damage in hypoglycaemic cell death [10–12]. In cell-free model systems of apoptosis, pro-oxidants or receptor-mediated activation of interleukin-converting-enzyme-like enzymes may induce an mPT, liberating mitochondrial components (cytochrome c, apoptosis-inducing factor) that activate nuclear chromatin condensation [4,13,14]. In these models and in toxicity experiments on hepatocytes, protection by a variety of pharmacological agents known to prevent mPT induction has implicated the mPT as an intermediate step in both necrosis and apoptosis [15–17].

The vast majority of our knowledge of the mPT stems from tissues other than brain. In liver and heart mitochondria, accumulation of excess matrix calcium combined with phosphate or an oxidative event leads to the opening of a non-specific permeability in the inner mitochondrial membrane that is referred to as the mPT [6]. mPT induction is modulated by the mitochondrial transmembrane potential ($\Delta \Psi_m$), matrix pH, Mg$^{2+}$, free fatty acids, the redox status of mitochondrial protein thiols and the surface potential generated by the largely anionic phospholipids of the inner mitochondrial membrane [6]. Pharmacological inhibition of the mPT can be accomplished with the immunosuppressant CsA and some of its analogues, adenine nucleotides and the adenine-nucleotide-transporter inhibitor bongkrekic acid [6].

Understanding the conditions for mPT induction in brain mitochondria becomes essential for delineating those pathophysiological conditions where mitochondrial dysfunction determines survival. Neuronal mitochondria are intimately involved in buffering cytosolic calcium loads imposed by physiological and pathophysiological stimuli [18–21]. Experiments combining cytosolic calcium indicator dyes with specific inhibitors of mitochondrial function have demonstrated that intracellular calcium levels increase when sequestered mitochondrial calcium pools are released into the cytosol following mitochondrial de-energization [5,18–20,22–26]. Responses to neuronal depolarization and short glutamate applications similarly elevate both cytosolic and subsequently mitochondrial calcium [18,19,21,22,24,27,28]. With longer exposure to higher levels of glutamate, the mitochondrial calcium loading is postulated to reach quite high levels, sufficient to induce an mPT [5]. To characterize the mPT of brain mitochondria, two preparations were used. Fluorescent dyes were
applied to cultured hippocampal neurons or cortical astrocytes to study mitochondrial responses to physiological challenges in intact cells. In subsequent experiments, isolated CNS mitochondria were prepared to examine biophysical and pharmacological properties of the brain mPT.

Astrocyte mitochondria were visualized in situ with the mitochondrial dye JC-1 (5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolocarbocyanine) and examined under a conventional microscope. In cultured cortical astrocytes, cytosolic and presumably mitochondrial calcium levels were increased abruptly when 100 μM Ca^{2+}-containing medium was applied after permeabilization of the plasma membrane with digitonin or application of the calcium ionophore, 4Br-A23187 in Mg^{2+}-containing saline. Astrocyte mitochondria, normally appearing as vermiform structures radiating away from the soma, changed shape when the medium was changed to one containing elevated calcium. First, mitochondria appeared blebbed, like ‘beads on a string’ and then proceeded to round up into large, swollen structures. Subsequent removal of Ca^{2+} by EGTA reversed the morphological changes. Mitochondrial swelling was also induced in the absence of divalent cations by 2.5 mM phosphate in digitonin-permeabilized astrocytes. Ca^{2+}-induced mitochondrial shape changes were inhibited by Mg^{2+}, ADP and combinations of CsA and the phospholipase A2 inhibitor, butacaine. Application of Ruthenium Red (RR), an inhibitor of the Ca^{2+} uniporter, and/or CGP37157, an inhibitor of the Na^{+}/Ca^{2+} exchanger, to digitonin-permeabilized astrocytes prevented calcium-triggered mitochondrial swelling, indicating that calcium cycling contributed to mPT induction. Subsequent application of ionophore produced mitochondrial swelling and mPT induction, independent of calcium cycling [29]. Thus the morphological changes in astrocyte mitochondria resemble mPT-associated mitochondrial swelling in terms of induction and inhibition [29].

Comparable experiments were performed on cultured hippocampal neurons labelled with JC-1 examined on a confocal microscope or with fura-2 acetoxymethyl ester and rhodamine-1,2,3 (R123) examined with time-lapse microspectrofluorimetry on a conventional microscope [30,31]. Introduction of 10–100 μM calcium after pretreatment with 4Br-A23187 in Mg^{2+}-containing medium caused increases in cytosolic calcium followed closely by mitochondrial depolarization. For low external calcium concentrations, the ionophore-introduced calcium was not always sufficient to cause immediate mitochondrial shape changes. Morphological alterations of the mitochondria were only associated with secondary step increases in cytosolic calcium as mitochondria released calcium into the cytosol. Additional mitochondrial depolarization accompanied the secondary calcium increases, consistent with mPT induction. For higher applied calcium levels, mitochondria rapidly became greatly distorted or rounded following the initially high depolarization accompanying calcium influx [30]. Thus, similar to astrocytes, neuronal mitochondria could undergo apparent swelling following large increases in cytosolic calcium and mitochondrial depolarization.

When neurotoxic levels of glutamate were applied to hippocampal cultures, neuronal mitochondria responded with a progressive change in
Cytosolic calcium increases were followed by increases in the R123 signal. R123 fluorescence dequenches as mitochondria depolarize [32]. The observation that mitochondria depolarize is consistent with mitochondrial sequestration of the cytosolic calcium. Accompanying the depolarization, a subtle increase in diameter of many mitochondria was observed (Figure 1b). As mitochondria remained depolarized, the dye slowly redistributed into the cytoplasm, increasing the background cytoplasmic fluorescence and decreasing the R123 intensity in mitochondria. In about a third of the neurons, a secondary increase in the partially recovered cytoplasmic calcium level was observed. With time, mitochondrial morphology became increasingly distorted as the mitochondria assumed a spherical shape (Figure 1c). This progression of morphological changes required 5–30 min after the initial glutamate-induced increases in cytosolic calcium. Glutamate-induced changes in neuronal mitochondrial morphology were prevented by substitution of Ba^{2+} for external Ca^{2+} [30]. In many experiments CsA prevented glutamate-induced mitochondrial swelling, but the results were variable [30,31].
In both sets of experiments, the CsA-sensitive, calcium-induced changes in mitochondrial morphology suggested that an mPT may have occurred. In the majority of neurons, mitochondrial exposure to high levels of calcium may have led directly to a permeability transition. These were the cells in which ionophore- or glutamate-induced abrupt increases in cytosolic calcium and mitochondrial depolarization were rapidly followed by morphologically altered mitochondria. In neurons with lower initial calcium increases and less mitochondrial depolarization, mitochondrial calcium cycling may have been occurring without initial mPT induction. Morphological changes were only observed in these cells after secondary increases in cytosolic calcium and mitochondrial depolarization. This is consistent with a late induction of the mPT, releasing mitochondrial calcium into the cytosol and depolarizing mitochondria. Thus both ionophore- and glutamate-induced mPT-like phenomena in the neuronal cultures could be triggered by activation of continuous calcium cycling as well as rapid accumulation of calcium into the matrix space [30].

A puzzling aspect of mPT behaviour in CNS mitochondria and astrocytes was the relatively low potency of CsA inhibition [29]. This is consistent with the dearth of comprehensive reports concerning CsA’s ability to prevent neuronal death over a broad range of ischaemic and injurious conditions. Neurotoxicity experiments in cultures have only reported protection from a single dose of CsA against a single dose of excitotoxin [5,8,9]. Neuroprotection \textit{in vivo} has only been reported from paradigms involving severely altered glucose levels [10–12]. Thus there is reason to believe that either CsA is ineffective under some conditions or that the mPT is involved only in certain cell-death pathways. Indeed, in our hippocampal cultures, 1 μM CsA failed to protect against glutamate excitotoxicity over a wide range of glutamate concentrations. Statistically significant neuroprotection by CsA was only observed against 500 μM glutamate challenges. Subsequent studies have focused upon delineating conditions for obtaining CsA antagonism of mPT activity in isolated brain mitochondria [33].

As a multiconductance channel, the mPT can be manifested in both low- and high-conductance states [34,35]. The high-conductance state of the mPT is typically monitored with light-absorbance decreases of mitochondrial suspensions following sucrose accumulation and consequent mitochondrial swelling [6,36]. Electron microscopic examination showed that calcium-induced decreases in absorbance of isolated brain mitochondria corresponded to increases in mitochondrial diameter, loss of the orthodox conformation and prominent swelling of mitochondria (Figure 2). In initial experiments, CsA only partially prevented calcium-induced absorbance decreases [29]. Pairwise combinations of low concentrations of CsA, adenine nucleotides and Mg$^{2+}$ inhibited mPT induction by low calcium concentrations. In subsequent experiments, CsA could fully prevent absorbance decreases produced by up to 50 μM calcium. Higher calcium loads produced swelling that was unaffected by the presence of CsA. Thus the effectiveness of CsA may be limited to conditions that produce submaximal induction of the mPT in brain mitochondria [33].
To study the early events in mPT formation, we sought a method to examine the mPT in its low-conductance state. In healthy, respiring liver or heart mitochondria large calcium loads are required to initiate mPT induction and mitochondrial swelling [37]. Initially mitochondria depolarize in response to such calcium loads but are often capable of recovering their membrane potential as the accumulated calcium is sequestered [37]. Upon massive calcium accumulation, the mPT opens, depolarizing mitochondria and releasing this calcium [6,37,38]. However, in many experiments, activation of H+/H11001 and K+/H11001 fluxes and Mg2+/H11001 or glutathione release have all been observed to precede mitochondrial swelling [39–45]. Early increases of permeability to H+ or K+ may occur through a low-conductance manifestation of the mPT. In actively respiring mitochondria, ion leakage can be compensated for by enhanced proton extrusion by proton pumps of the electron-transport chain. We reasoned that in mitochondria with partly suppressed respiratory activity, the initial increase of membrane permeability might be easier to observe. Partial suppression of respiratory activity of CNS mitochondria was achieved using 3 mM succinate as an oxidative substrate without rotenone. Under these conditions, oxaloacetate accumulated and succinate dehydrogenase was partially inhibited. Mitochondria respired, but not at the optimal levels sustained by a combination of both glutamate and succinate.

The hypothesized initial permeability would not manifest itself through massive mitochondrial swelling but could be monitored by following ΔΨm.

Figure 2 Mitochondrial swelling corresponds to absorbance decreases induced by calcium. Electron micrographs of purified, isolated brain mitochondria after exposure to 100 μM calcium (b) and under control conditions (a). Mitochondria were incubated in medium containing 215 mM mannitol, 50 mM sucrose, 3 mM KH2PO4, 10 mM succinate, 3 mM glutamate and 10 mM Hepes, pH 7.4. Following absorbance measurements (inset, traces a and b), mitochondria were pelleted, fixed and embedded for electron microscopy. Scale bar, 200 nm.
with tetrphenylphosphonium (TPP\(^+\)) and a TPP\(^+\)-sensitive electrode. Indeed, in sucrose mannitol medium with 3 mM succinate, only minor mitochondrial swelling was observed in response to Ca\(^{2+}\). In comparable medium with 3 mM succinate and 3 mM glutamate, maximal swelling occurred in response to 300 \(\mu\)M Ca\(^{2+}\). Addition of 50–100 \(\mu\)M calcium to suspensions of 2 mg/ml brain mitochondria energized by succinate produced a step change in \(\Delta\Psi_m\) measured with the TPP\(^+\) electrode. The degree of depolarization was dependent upon the calcium concentration. Participation of the mPT in the depolarization was determined by subsequent addition of 1 \(\mu\)M CsA or 100 \(\mu\)M ADP plus 1 \(\mu\)M oligomycin. Either of these mPT inhibitory treatments repolarized mitochondria depolarized by 50 \(\mu\)M calcium. Oligomycin alone did not repolarize mitochondria. CGP 37157, an inhibitor of the Na\(^+\)/Ca\(^{2+}\) exchanger, did not repolarize \(\Delta\Psi_m\), ruling out Ca\(^{2+}\) cycling through the Ca\(^{2+}\) unipporter and the Na\(^+\)/Ca\(^{2+}\) exchanger as a cause of the depolarization. Carboxyatractylate did not prevent repolarization by ADP plus oligomycin, indicating that their action was not mediated by the adenine nucleotide translocator. ADP plus oligomycin repolarized mitochondria depolarized by 100 \(\mu\)M calcium but the effects of CsA were variable, reminiscent of its effects in whole cells and in the absorbance assay [29,46].

To verify that the partial depolarization induced by calcium indeed represented opening of a low-conductance pathway and not a manifestation of calcium cycling, parallel measurements were made with a Ca\(^{2+}\)-selective electrode in the external solution [46]. Mitochondria readily sequestered Ca\(^{2+}\) when presented with 25 \(\mu\)M Ca\(^{2+}\) challenges. The progressive failure to fully sequester repeated small pulses can be explained by mitochondrial depolarization due to mPT induction [37,47,48]. Pretreatment of mitochondria with 1 \(\mu\)M RR prevented calcium accumulation. As expected, RR addition after calcium accumulation unmasked a slow efflux of accumulated calcium. Mitochondria challenged with 100 \(\mu\)M Ca\(^{2+}\) failed to accumulate observable Ca\(^{2+}\). Addition of RR produced no Ca\(^{2+}\) efflux. In these conditions, the limit of resolution of the Ca\(^{2+}\) electrode was 1.5 \(\mu\)M. Calcium cycling was unlikely because depolarization suppressed Ca\(^{2+}\) influx and the absence of a Ca\(^{2+}\) gradient prevented Ca\(^{2+}\) efflux. CsA in combination with ADP plus oligomycin did repolarize mitochondria under these conditions, indicating that an mPT had been induced. Matrix calcium accumulation did not occur during the Ca\(^{2+}\)-induced depolarization but did resume upon repolarization by either CsA or ADP plus oligomycin. Without initial Ca\(^{2+}\) movement into mitochondria, the observed depolarization must be produced by influx of another cation, possibly H\(^+\). Thus elevated extramitochondrial calcium may induce a low-conductance pathway permeable to H\(^+\), which depolarized mitochondria in an ADP- and CsA-sensitive manner [46]. A role for external Ca\(^{2+}\) in mPT induction has recently been proposed [49,50].

Pharmacological inhibitors were sought to repolarize mitochondria after this conductance was activated. ADP plus oligomycin fully repolarized mitochondria depolarized by 25–100 \(\mu\)M calcium. Surprisingly, 1 \(\mu\)M RR nearly completely repolarized mitochondria depolarized by 50 \(\mu\)M Ca\(^{2+}\), identical to the actions of 1 \(\mu\)M CsA. CsA added after RR as well as RR added after CsA
had no further effect. At higher Ca\(^{2+}\) concentrations, neither RR nor CsA alone were effective. Repolarization only occurred in the presence of both RR and CsA, independent of the order of addition. Sphingosine, a modulator of surface potential, did not mimic the effects of RR, ruling out surface-charge screening as a mechanism for the RR repolarization. Thus following Ca\(^{2+}\) challenge, the Ca\(^{2+}\) uniporter may be reversibly converted into a CsA-sensitive conduction pathway, depolarizing brain mitochondria. This pathway was activated independently of matrix calcium accumulation. Because this pathway has a pharmacological profile comparable with the mPT, it may represent the mPT pore in a low-conductance state [46].

The variable effectiveness of CsA was analysed by varying the concentrations of other known modulators of CsA inhibition of the mPT [33]. High doses of Ca\(^{2+}\), omission of external Mg\(^{2+}\), low doses of palmitic acid or very low doses of protonophore exacerbated Ca\(^{2+}\)-induced depolarizations and eliminated CsA repolarizations. Pretreatment with bovine serum albumin to absorb free fatty acids restored CsA inhibition. In all of these experiments, independent of the specific modulator, CsA failed to repolarize mitochondria that were depolarized beyond some threshold. This common threshold suggested that inhibition of the mPT by CsA may be voltage-dependent. Thus in brain mitochondria, CsA may only be able to close the mPT pore in its low-conductance state under conditions of partial depolarization [33].

These results obtained with the isolated mitochondria may explain the variability observed in the neuroprotection offered by CsA [29,30]. At the lower concentrations of glutamate, the associated calcium influx may not be sufficient to activate the mPT. For the highest glutamate challenges producing the largest increases in internal calcium concentration, the high-conductance mPT becomes activated and mitochondria depolarize into the range where CsA becomes ineffective. CsA is neuroprotective only against intermediate glutamate exposures that produce mitochondrial calcium loads sufficient to activate the mPT, yet result in a limited amount of mitochondrial depolarization. Variations in the concentrations of Mg\(^{2+}\), ADP and free fatty acids in situ modulate the mitochondrial response to calcium, indirectly altering CsA’s ability to prevent mPT induction or to close an open mPT pore following glutamate challenges [33].

What emerges from these investigations of the mPT in both isolated mitochondria and intact neurons and astrocytes is a picture of the CNS mPT as a multistage event. Calcium challenges may actually lead to opening of several non-specific pathways with different conductances. Initially, excess cytosolic calcium may promote transformation of the Ca\(^{2+}\) uniporter into an H\(^{+}\) channel, possibly permeable to other monovalent ions, depolarizing \(\Delta V_m\) and triggering low-amplitude mitochondrial swelling. For moderate depolarizations, CsA can effectively close the low-conductance pore. The Ca\(^{2+}\) uniporter antagonist, RR, acts synergistically with CsA to close this low-conductance pore over a broader range of conditions. In intact astrocytes and neurons, opening of this low-conductance mPT may correspond to the initial intermediate changes in mitochondrial shape. With increasing exposure to Ca\(^{2+}\), additional proteins in the inner mitochondrial membrane may become affected,
including the adenine nucleotide translocator, opening a high-conductance pathway. This pathway is insensitive to RR inhibition but remains sensitive to adenine nucleotides and, perhaps to some extent, CsA.

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References